ABSTRACTS

SEVENTH JOINT LEPROSY RESEARCH CONFERENCE

Nippon Toshi Center Chiyoda-ku, Tokyo, Japan November 8-10, 1972

U.S.-Japan Cooperative Medical Science Program Program of Leprosy Conference

INTRODUCTORY REMARKS

I would like to express my thanks and pleasure. I wish to extend my hearty welcome to all of you present here on this occasion.

Now as you know, the U.S. and Japan Leprosy Panels first met in Honolulu in October, 1965. At that first joint conference we talked about future problems and came to an agreement that the U.S. and Japan should continue the following research studies: cultivation of leprosy bacillus, experimental transmission to animals, a search for better drugs, studies of prophylaxis, and immunology of leprosy, respectively. Since then many valuable results have been obtained, as you already know.

Five full years have passed since the start of the U.S.-Japan Cooperative Medical Science Program. Therefore, it was suggested that thereafter each Panel should discuss its own future plans. For this reason the Parasitic Diseases Panel and the Leprosy Panel opened the 6th U.S.-Japan Cooperative Conference in Bethesda last year. During that conference the matter was reviewed in detail by both U.S. and Japan Committees and the results were reported at the 8th meeting of the U.S.-Japan Cooperative Medical Science Committee held in Tokyo this summer, 1972. The conclusion reached was that both Panels should be continued hereafter. This was pleasant news to both of us.

We are privileged to have 15 participants from the United States and Korea and 32 participants from Japan and, in addition, 15 members of the Japanese Leprosy Association joined as observers. There will be 25 papers presented and after each a discussion will follow.

Studies concerning cultivation of leprosy bacillus, immunology of leprosy, experimental leprosy, and leprosy drugs, and in addition some papers on clinical trials, are expected to be presented at this conference.

The 10th International Leprosy Congress will be held in Bergen, Norway, in August 1973. This is a memorable year for us because of the discovery of the leprosy bacillus. We will recollect and be stimulated by the great work of Armauer Hansen done a century ago. I wish that the present conference may become an even more profitable one with even livelier discussions than those we have had in the past.

Thank you.

YOSHIO YOSHIE, Chairman Japan Leprosy Panel

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Program of the Seventh Joint Leprosy Research Conference

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Sasaki, N., Kawatsu, K. and Sushida, K. Histopathological studies on the effect of ¹³¹I in infection of murine and human leprosy.

First experiments on ¹³¹I were carried out with murine leprosy in dd-strain mice. The premise was that destruction of the thyroid gland causes hypofunction of the reticuloendothelial system (RES) and lowering of immunologic ability, and that consequently, the resistance of the intact body is reduced.

Changes in the endocrine organs were seen in mice given a large quantity of ¹³¹I. Progression of murine leprosy lesions were observed. Methods of administration of ¹³¹I were investigated in order to accelerate the development of murine leprosy lesions more effectively.

It was found that the most suitable dose of ¹³¹I was 100 μ c. One or two injections before and/or after inoculation were more effective than only one injection before inoculation of the *M. lepraemurium*.

It was found that murine leprosy lesions were more progressive and the bacilli multiplied more extensively as a result of the injections. The hypophysis, especially the anterior lobe, became more hypertrophic (three to five times normal weight). This change was paralleled by the degree of thyroid gland destruction and by the amount of pigment absorption as seen in a skin diffusion test with neutral red performed for the purpose of investigating the mobilization of the RES.

Accordingly, we understand that progression of murine lepromas occurs because of hypofunction of the RES followed by imbalance of the endocrine system. Application of the findings to animal infection with M. leprae indicates that the use of ¹³¹I only, is inadequate. Therefore, combinations of immunosuppressive agents, such as anti-thymocyte γ -globulin and/or cortisone are being used in addition to ¹³¹I.

Sushida, K. Inoculation tests using leprosy bacilli in the testis of mice previously injected with sodium iodine (¹³¹I).

Sodium iodine (¹³¹I) has been injected into the mice for the purpose of diminishing their natural resistance to human leprosy. The authors have previously (1970) reported that the average body temperature of the ¹³¹I-mice was decreased, and the function of the reticuloendothelial system (RES) lessened, as shown by the clearance test and by histopathologic examination.

The leprosy bacilli (nine clinical strains were employed) were injected into the testis of the mice. Acid-fast bacilli taken from these primary infected ¹³¹I-mice were transplanted into the second generation ¹³¹I-mice. This paper contains: 1) the results of the transmission of leprosy bacilli into the ¹³¹I-mice; 2) the relationship between the infection with leprosy bacilli in such ¹³¹I-mice and their body temperature; 3) the effect of various doses of ¹³¹I; and 4) the effect of the source of the bacilli (several lepromas from different patients). The mice were injected subcutaneously with ¹³¹I, ranging from 25 µc to 300 µc per mouse. After about one month, the leprosy bacilli were inoculated into the testis. Acid-fast bacilli in stamp-smear samples taken from the testis were examined microscopically nearly ten months after the inoculation. Cells having abundant acidfast bacilli were observed to form so-called 'globi" (+G), which were found often in the capsule of the testis during histologic examination.

Acid-fast bacilli (+G) in the testis of ¹³¹I-mice occurred in 17.7% (21/119), as contrasted with 1.4% (1/69) in control mice having no ¹³¹I. The result of the second transmission into the next group of 131Imice was that about 3% (2/65) were positive. The body temperature was carefully measured rectally with a thermister probe on the day before the inoculation of the leprosy bacilli and then once a month for ten months. Sixty-seven percent of the positive (+G) mice (8/12) showed a reduced temperature (less than 36.4°C). Seventy-seven percent of the negative (-)mice (42/54) showed a higher temperature, over 37.0°C. In about 90% of mice having the higher temperature, acid-fast bacilli were not found. Acid-fast bacilli (+G), however, were not present in any large amount in mice having a lower body temperature. The effect produced by 131Idoses ranging from 25 μ c to 300 μ c was found not to vary significantly. However, nine strains from different leproma-materials had been used for inoculation in this examination. It seems that the difference between the strains used in this examination was more important than the difference in ¹³¹I-dose. Three strains (LL11, LL28 and LL21) produced more succesful results than the other six strains. These three leproma-materials contained rod and segmented forms of bacilli, and had not been subject to treatment with chemotherapy, but were sometimes clinically exacerbated.

Fieldsteel, A. H. Further studies on Mycobacterium leprae infection in immunosuppressed Lewis rats.

Maximum levels of *M. leprae* after foot pad inoculation of intact Lewis rats average approximately 2×10^6 , compared with less than 10^5 after testes inoculation.

Neonatal thymectomy with no further treatment rarely results in wasting disease and does not perceptibly alter the life span of the animal, yet it results in a dramatic alteration in susceptibility to M. leprae infection. Bacillary levels of 108 or greater were reached 12 months after foot pad inoculation and 3 \times 10 8 or greater after testes inoculation. These levels were maintained for at least an additional 2 months, but appeared to be falling off by the 16th month after infection. Although the numbers of M. leprae in thymectomized rats treated with antithymocytic serum (ATS) were not significantly greater at 12 months than they were in those without ATS treatment, the levels remained high and in some instances have continued to increase by the 19th month after infection. Also, in the ATS-treated groups there has been spread of acid-fast bacilli to uninoculated sites (front feet and ears). To date, the greatest number of M. leprae, 2×10^{9} , has been found 17 months post-inoculation in the feet and testes of a neonatally thymectomized rat irradiated with 925 r and infused with syngeneic bone marrow.

Additional experiments are under way to determine the effects on *M. leprae* infection of neonatal thymectomy plus single or multiple doses of sublethal radiation. In one experiment as little as 450 r given to neonatally thymectomized rats four months after infection appeared to be effective. Ten months after inoculation with 2×10^4 *M. leprae*, the testes of one rat yielded 9×10^8 acid-fast bacilli. By the 14th month, the

level is still high (5×10^8) in this group. Similar results were obtained in rats given 350 r, one and four months after inoculation.

Although optimum methods of immunosuppression in addition to neonatal thymectomy have yet to be determined, it is apparent from the data obtained to date that all methods investigated resulted in consistently higher levels of *M. leprae* in testes than in foot pads. [Supported by the U.S.-Japan Cooperative Medical Science Program, Grant AI-08417]

Evans, M. J. and Levy, L. Early response of the mouse foot pad to *Mycobacterium leprae*.

The purpose of these experiments was to study the early response of the mouse foot pad to *M. leprae.* To accomplish this, mice were inoculated in both foot pads with large and small numbers of organisms. The animals were sacrificed at intervals from 2 hours to 27 days after inoculation.

Foot pads from some mice were prepared for microscopy and the other used to determine how much of the original inoculum could be recovered. The microscopic results, which utilized normal BALB/c and thymectomized-irradiated B6C3F1 mice, showed that the tissue responded first with an influx of polymorphonuclear cells and later lymphocytes and monocytes. The latter formed a diffuse infiltrate in the tissues. Under conditions where growth normally occurred (normal BALB/c mice receiving a small inoculum, and thymectomizedirradiated B6C3F1 mice receiving large or small inocula) the mononuclear cell infiltrate did not persist. The organisms were found within phagocytic cells and the interstitial space. They were always contained within a phagosome and often fused with lysosomes. Most of the organisms appeared to be degenerating at all of the times studied.

Studies to determine the number of organisms which could be recovered from the foot pad after inoculation showed there was a large loss of inoculum from the foot pad in all of the experiments. At two hours after inoculation about 30% of the organisms could be recovered from the foot pad. About the same number or less were recovered at later times. The mean percentage of organisms recovered was 19.7 with a range of 4.8% to 39.1%. These results indicated that most of the organisms leave the foot pad shortly after inoculation and those that remain are contained in inflammatory cells.

Okamura, K. Macrophage migration inhibition test made with the lymphocytes of guinea pigs sensitized to BCG and human leprosy bacilli.

The lymphocytes of animals that have been sensitized to develop delayed type allergy, or in a much broader sense, those of animals that have acquired cellular immunity, are known to produce different kinds of mediators when they are in contact with the respective antigen, and it is said that the production of macrophage inhibition factor runs in parallel to the development of delayed type allergy.

Lymphocytes obtained from the peripheral blood of leprosy patients, after mixing with either Dharmendra antigen or old tuberculin *in vitro*, were used in migration inhibition tests with macrophages derived from the peritoneum of normal guinea pigs. Macrophage migration was inhibited when the lymphocytes of normal individuals and tuberculoid type patients, positive by both lepromin and tuberculin skin tests, were used. On the other hand, no inhibition was seen when the lymphocytes of lepromatous patients, who were negative by both lepromin and tuberculin skin tests, were used.

In the present study, similar tests were repeated utilizing the lymphocytes of guinea pigs sensitized to BCG and human leprosy bacilli. In the control study made with the lymphocytes of normal guinea pigs negative in both lepromin and tuberculin reactions, no inhibition of macrophage migration was observed against Dharmendra antigen or tuberculin. When the lymphocytes of BCG sensitized guinea pigs were used after mixing with tuberculin at a concentration of 1:100 or 1:200, the macrophage migration inhibition was positive. In contrast, the inhibition of macrophage migration was negative after mixing with Dharmendra antigen even at a concentration of 1:5. On the other hand, when the lymphocytes of guinea pigs sensitized to human leprosy bacilli were used after mixing with Dharmendra antigen 1:1 or tuberculin 1:100, the inhibition of macrophage migration was positive to a certain degree in both cases. Accordingly, it was found that the inhibition of macrophage migration by the lymphocytes of lepromatous patients even after mixing with Dharmendra antigen *in vitro* is of a degree comparable to that of normal guinea pigs.

Morrison, N. E., Congdon, C. C. and Collins, F. M. Multiplication of BCG in the normal versus the T-cell deficient mouse.

The thymectomized, irradiated and bonemarrow-reconstituted mouse shows impaired cell-mediated immunity due to deficiency of thymus-derived small lymphocytes or T-cells. The adoptive transfer of specific antimycobacterial immunity is accomplished by theta-antigen-bearing immunocompetent thymocytes. No evidence is at present available to indicate a mycobacterial infection immunity role for the, bone marrow derived lymphocyte or Bcell. This report is concerned with the multiplication of BCG, an otherwise attenuated organism for the normal mouse which undergoes excessive multiplication in the T-cell deficient mouse with resultant death of the animal.

Animals were inoculated intravenously and the multiplication of BCG in lungs, liver, spleen and nodes was followed by plate counting with time. Pulse labeling of DNA in host cells by ³H-thymidine incorporation was used to follow kinetics of cellular proliferation during the immune response. Measurement was also made of tissue sensitization to tuberculin PPD by measuring delayed-type hypersensitivity reactions in the foot pad.

It was found that the initial multiplication rate of BCG in the T-cell deficient animal was similar to the normal animal; however, a higher peak number of organisms occurred in the lungs, liver, or spleen before an immune response caused a decrease in numbers. Increased antigen trapping, particularly in the lungs and liver of the T-cell deficient animal, likewise contributed to the increased numbers of organisms. Following the initial impact of the immune response a decrease in multiplying organisms was seen in both types of animals. However, the T-cell deficient animal was unable to maintain an adequately regulated state of cell-mediated immune response and an explosive burst of BCG division took place, particularly in the lungs. The increasing BCG multiplication in the lungs led to a chronic loss of pulmonary function associated with body weight loss and ultimate death of the animal. Autopsied lungs showed bilateral pulmonary consolidation, loss of water buoyancy and absence of macroscopic tubercle formation.

Since the absence of an adequately regulated immune response appears to be due to the low number of residual T-cells present in the deficient animal, it is apparent that a critical number of T-cells is required, in relation to antigen challenge, for a regulated response to occur.

Bullock, W. E. Disturbance of T-lymphocyte circulation by granulomatous infection.

The circulation of thoracic duct lymphocytes (TDL) from normal Lewis rats was traced through lymphoid tissues of rats infected with Mycobacterium lepraemurium and the results suggest impaired TDL recirculation in rats with granulomatous infection. A mean of 1.3 x 10⁶ uridine-³Hlabeled TDL/g body weight was given intravenously to four matched pairs of infected and normal rats. Lymphocytes were then collected from thoracic duct fistulas (TDF) every eight hours for three days. The mean peak (16-24 hours) lymphocyte output (LO) of infected rats was decreased $(17.7 \times 10^{6}/hr)$ as compared with normals $(22.0 \times 10^6/hr)$ and was below normal at all other time intervals. By radioautography, the ratio of labeled lymphocyte output (LLO) from infected versus normal rats at 24 hours was 0.56. Four pairs of rats were depleted of lymphocytes by TDF three

days before intravenous injection of labeled TDL. Lymph was collected every 4 hours thereafter for 48 hours. The mean LO of normals was 5.4 x 106/hr at 0 hour with a peak of 17.4 x 106/hr at 16 hours. LO of infected rats at 0 hours was 4.4 x 106/hr and increased only to 7.1 x 106/hr at 16 hours. The ratio of LLO from infected versus normal rats at 16 hours was 0.41. Two pairs of lymphocyte-depleted rats were sacrificed at 12 and 24 hours respectively after injection of ⁵¹chromium-labeled TDL. Mean radioactivity/100 mg of infected versus normal spleen at 12 and 24 hours was 28.8 and 38.6%, respectively. Radioactivity of infected lymph nodes was 41.1 and 35.4% of normal at these times. These findings demonstrate a mechanism by which granulomatous involvement of lymphoid tissue may impair the delayed immune response by interference with Tlymphocyte recirculation. (Research supported by NIH Grant AI-10094)

Abe, M., Minagawa, F. and Yoshino, Y. Indirect fluorescent antibody test for detection and identification of *M. leprae* and corresponding antibodies.

Serological identification of M. leprae became possible, as reported previously, by the use of rabbit fluorescent antibodies against a protein antigen in leprosy nodules. However, this method was unsatisfactory for practical use because a large quantity of fresh leproma was necessary for the preparation of the rabbit antiserum and the standardization of fluorescent antibodies was not always easy in all laboratories. In order to overcome these difficulties and to find our specific antibodies against M. leprae in the serum of leprosy patients, an indirect method was attempted, using a commercial product of antihuman globulin fluorescent antibody which was more readily standardized than the antinodule extract fluorescent antibody.

The technic of the test closely resembles the FTA-ABS test, a serodiagnostic test for syphilis. The sera to be tested were previously absorbed with cardiolipin, lecithin and polysaccharide of tubercle bacilli (an antigen for Middlebrook-Dubos' hemagglutination) and then reacted with the smears of M. leprae. Apparent fluorescence of the bacilli caused by the serum diluted to 1:40 or more and by the fluorescent antibody in optimum dilution was judged to be positive. Among 87 specimens of sera from patients with lepromatous leprosy, 77 cases (88.5%) showed positive reaction, whereas only 7 (33.3%) of 21 patients with tuberculoid leprosy were positive. The test was negative in 50 specimens of sera from healthy persons. A comparison of antibody titers before and after the absorption of the sera from lepromatous patients was performed with the smears of M. leprae, M. lepraemurium, M. tuberculosis and BCG. The antibody titers against M. leprae were not significantly reduced by the absorption, whereas that against the other mycobacteria distinctly dropped and sometimes became negative after the same procedure. No significant difference in the titer was observed among the smears of M. leprae obtained from different patients and from the foot pad of a mouse. However, the titer was greatly reduced by the procedures used in concentration of bacilli, such as trypsin digestion, acetic acid and chloroform-ether extractions.

Using fluorescent antibodies monospecific to IgG, IgM and IgA, respectively, we examined the class of immunoglobulins to which the antibodies combining specifically to *M. leprae* belong. The results indicated that IgG and IgM antibodies were present in various ratios of titers and that no IgA antibody could be detected in the sera hitherto examined.

Mori, T. A common antigen in human finger bone and Mycobacterium bovis (BCG).

Innami (La Lepro 37 [1968] 331-339) reported that a common antigen among leprosy bacilli, *Mycobacterium bovis* (BCG) and human finger bone was demonstrated by Ouchterlony's immunodiffusion technic. At the U.S.-Japan Leprosy Panel Conference in 1970, Mori and Innami reported an immune cross-reaction among leprosy bacilli, *M. bovis* (BCG), human finger bone, and human cartilage. It is a wellknown fact that sometimes degradation of finger bone is seen in lepromatous leprosy patients, and aseptic vertebral caries are

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also seen in tuberculosis patients. Moreover, the cause of rat adjuvant diseases remains obscure. An immune cross-reaction between acid-fast bacilli and bone is suspected to be a cause of these diseases. In the present study, characteristics of the common antigen and the serum fraction of the antibody will be discussed.

Methods. For anti-BCG serum, a New Zealand white rabbit was immunized by the usual method using heat-killed BCG bacilli mixed with Freund's incomplete adjuvant. For the water extract of BCG, a water suspension of BCG live cells was sonicated for two hours, and a clear extract was obtained by an ultracentrifugation. For the water extract of bone, a bone was stripped from soft connective tissue and crushed in a steel mortar. The water suspension of bone powder was sonicated repeatedly. The supernatant was concentrated by evaporation, and a clear extract was obtained by ultracentrifugation. For the alkali hydrolysate of BCG and bone, the residue of the above extraction was suspended in 0.5 NaOH solution and hydrolysed for two days at room temperature. The supernatant of the hydrolysate was dialysed for three days against running tap water and concentrated by evaporation. A clear extract was obtained by ultracentrifugation. In Ouchterlony's immunodiffusion method the formation of precipitation line was difficult in an agar plate so acrylamide gel was used. A suitable vessel for making an immunodiffusion plate was devised with acryl resin.

Result. Anti-BCG rabbit serum, which produced a precipitation to the alkali hydrolysate of human finger bone by ring-test to a dilution of 1:8 was used. Sheep red blood cells, sensitized with the alkali hydrolysate of human finger bone, were agglutinated by anti-BCG serum diluted 1:8. Anti-BCG serum formed a common precipitation line against alkali hydrolysate of BCG and human finger bone by Ouchterlony's immunodiffusion technic. Since the formation of the precipitation line was stable to heating the antigens, the active component of the antigens was thought to be a polysaccharide moiety. Azuma reported that arabino-galactan and arabino-mannan were the main components of polysaccharide antigen of acid-fast bacilli. However pure arabino-galactan and arabinomannan donated by Azuma did not make a common precipitation line with an alkali hydrolysate of human finger bone when the anti-BCG serum was used. In addition to these findings, alkali hydrolysate of rat limb bone and BCG formed a common precipitation line against the anti-BCG serum.

Discussion. One must recognize that sometimes a lipid fraction will make a precipitation resembling an antigen-antibody reaction. Nevertheless, the result of the hemoagglutination test means that some fraction of the alkali hydrolysate of human finger bone binds to surface of sheep red blood cells, and some components of anti-BCG serum (not determined yet to be immunoglobulin) bind to the sensitized red blood cell. It is not yet concluded that the binding reactions observed are related to the cause of diseases mentioned in the preface. The characteristics of the common antigen and serum fraction of anti-BCG serum must be established.

Gelber, R. H., Epstein, W. V., Fasal, P. and Drutz, D. J. Erythema nodosum leprosum: An immune complex disorder.

Erythema nodosum leprosum (ENL) is a clinical syndrome that may include erythematous nodular skin eruptions, fever, arthritis, lymphadenitis, painful neuritis, orchitis, uveitis, and nephritis. Histopathologically, ENL is characterized by vasculitis with infiltration of polymorphonuclear leukocytes, a picture not encountered elsewhere in leprosy. The protean nature of tissue involvement and the presence of vasculitis have led to the comparison of this syndrome with auto-immune or collagenvascular diseases, particularly systemic lupus erythematosus (SLE).

Recently a gel precipitin reaction utilizing the CIQ component of complement (the site through which the first component of complement combines with gamma globulin or specific antibody) has been shown to react with preformed immune complexes and, also, has proved useful in demonstrating the presence of similar material in the immunologically active state of SLE. This precipitin reaction is positive in the majority of sera from hypocomplementemic patients with active lupus nephritis, but negative with sera from quiescent normocomplomentemic SLE patients and the vast majority of patients hospitalized with other disorders.

Studies were performed seeking circulating immune complexes in the sera of leprosy patients. Ten of 18 serum specimens in 6 of 11 ENL patients were positive for "CIQ precipitins." In contrast, only one of 15 sera from 14 patients with uncomplicated lepromatous leprosy produced a precipitin line with CIQ. In borderline and tuberculoid leprosy sera, "CIQ precipitins" were only rarely present. In the few instances when serial sera were examined before, during, and after ENL, "CIQ precipitins" were temporally related to the ENL reaction.

Although various abnormal serological features have been associated with leprosy, such as cryoglobulins, antinuclear factors, rheumatoid factors, and thyroglobulin antibodies, their presence has no clear relationship to the occurrence of ENL. These results indicate that circulating material exhibiting behavior consistent with immune complexes is present in the sera of patients at the time of ENL, bears a particular relationship to ENL, and may well be etiologically related to the ENL process.

Weiser, R. S. and Han, S. H. The granulomatous response of leprosy patients to killed BCG and *M. leprae*.

The study was predicated on the postulate that the immunological defect in lepromatous leprosy (LL) lies in a specific lack of capacity to react to M. leprae with an allergic granulomatous response. Since the V defect could involve specific incompetence of lymphocytes to respond to some antigen(s) of M. leprae and/or some macrophage defect, it was thought that a study of the granulomatous response of leprosy patients to heat-killed BCG and M. leprae at the histologic and ultrastructural levels might provide a clue to the nature of the immunological defect in LL. Each of 6 well-nourished male volunteers (ages 30 to 50 years) with polar tuberculoid and 11 with polar LL (Ridley and Jopling) were

injected at two sites with 0.1 ml of lepromin (Wade method) and with autoclaved BCG (grown on Dubos' medium) given into lesion-free skin of the upper arm. (Tuberculin tests conducted earlier showed that all subjects were tuberculin-positive except two lepromatous patients.) At intervals thereafter, appropriate biopsy specimens were taken from injection sites under local anesthesia and processed for histologic and EM study. Within three to four weeks all six tuberculoid patients responded with typical lepromin granulomas (five of which measured 5 mm or more in diameter) composed of epitheliod cells, lymphocytes and giant cells. In contrast, 8 of the 11 lepromatous patients remained negative; the other 3 developed tiny papules less than 3 mm in diameter which disappeared after two weeks. Biopsy specimens taken from injection sites at the fourth week showed essentially normal histology. All 17 patients developed typical granulomas to BCG (none were less than 5 mm in diameter and most were larger) consisting of epitheliod cells, many lymphocytes, and a few giant cells. BCG granulomas in the tuberculoid patients were a little larger than those of lepromatous patients but the differences were not significant. Ultrastructural studies on biopsy specimens taken at intervals from 48 hours to several weeks after injection have not been completed. (Supported by the United States-Japan Cooperative Medical Science Program, NIH Grant AI 08211; and a grant from the National Science Council, Taipei, Taiwan, Republic of China.)

Tsutsumi, S., Nakamura, K. and Ozawa, T. Several problems experienced during the screening of various series of compounds by mouse foot pad method.

Preparation of the substances to be tested. Several sulfones (SF) such as adamantane-(1)- or 5-nitropyridyl-(2)-derivatives of DDS and of $4-NH_2\emptyset SO_2\emptyset NO_2-4'$ (I) were prepared and tested in the first series.

Some alkylated DDS derivatives (C_n : 1,4-6) were synthesized and tested in the second series. The long heating of 4-ClØSO₂ØNH₂-4' (II) with a mixture (HeNH₂+Et₃N+Cu/DEG) in a glass tube produced 4-HeNHØSO2ØNH2-4' (MHeDDS) in a good yield. The similar substitution could not be realized in the cases of RNH₂+4-ClØSO₂ØNO₂-4' (III) and Bu₂NH+ (II) MBu- and MAmDDS (polyalkyl-free, tested by mass-spectrography) were isolated after direct alkylation of DDS in an acidic medium, though the latter yield was very poor. The reaction of (III) and RNH₂+Cu/HCONMe₂, followed by the reduction of the product, yielded MMe₂DDS. By the reaction of (1) and BuBr/BuOH in a sealed tube, MBu₂DDS was obtained. Similarly sym.-TEuDDS was obtained from DDS, and sym.-DBuDDS from mono-acyl-DDS. The bromination of MBu- and MHeDDS yielded the corresponding 4-bromides, and that of MADDS 2-bromide. The labeling of MRDDS and MADDS by ³H₂-reduction of the respective bromides was completed and that of MR₂DDS is in progress. Several A-, G-, and S-conjugates of MRDDS were synthesized.

The synthesis of numerous derivatives of 1,4-naphthoquinone (NQ) is in progress and some of them are being tested in the fourth series. The substitution of the various amines including SA-type drugs for one chlorine of 2,3-di-ClNQ followed by the further introduction of OK, SR, or SØCl-p (X) to another residual chlorine yielded 2-amino-3-XNQ. The chemical structures of these compounds bear a resemblance closer to those of coenzymes Q, than to 2-amino-3-ClNQ (Prescott). Also, 2,3-di-XNQ was prepared, and in one case (X=SR) it converted into 2-amino-3-XNQ after heating with the corresponding amine.

Screening of the substances synthesized or those kindly supplied by others. The experiments consisted of four series, and the methods can be classified into two: first, the coarse but rapid method (A); next, the more precise one (B). By method A, numerous compounds could be tested rapidly within one series, and the individual deviation of bacillary counts could also be tested. The use of SPF mice markedly diminished the deviation. By method B, the compounds already selected were tested under the set of three dosages. The changes of MI values were also examined simultaneously. The first series, tested by A: twenty-eight compounds were tested. Six SF, 5-Ac-8-OHquinoline, the three derivatives of leucoM, Ac-SPM, and the three compounds (Saltron, 1,7-di-MeO-phenazine, and 4-ClØNHCSC-CØ) among seven retested ones showed a lowering of BC. However, one questionable result was the delay of the bacterial growth, seen only in the untreated group.

The second series, tested by B: MBuDDS showed inhibition even at 0.0001%, while DBu- and TBuDDS were ineffective even at 0.01%. Several compounds retested again inhibited growth to a certain extent only at 0.01%. The main questions were the MI values at 30 weeks, and the difference in the bacterial count levels between A and B.

The third series, tested by A and B: all of DDC (Prabhakaran), the five compounds relating to DDC, MBu₂DDS, and DDS were ineffective even at 0.01%, when dosed during the log phase. Thus, the test of the other eight compounds failed.

On the basis of the results obtained, the problems of the animal experiments will be discussed.

Peters, J. H., Gordon, G. R., Levy, L. Jacobson, R. R. Enna, C. D. and Kirchheimer, W. F. Acetylation characteristics and the metabolic disposition of dapsone in patients with dapsone-resistant leprosy.

Earlier, we showed that the well-established genetic polymorphism for the acetylation of drugs such as isoniazid and sulfamethazine (SMZ) also applies to dapsone (DDS) in American (Gelber *et al*, Clin. Pharmacol. Ther. **12** [1971] 225) and Philippine subjects (Peters *et al*, Amer. J. Trop. Med. Hyg. **21** [1972] 450). The same conclusion was reached in subsequent studies in Indian and African subjects (Peters *et al*, Internat. J. Leprosy **40** [1972] 221-222).

More recently, studies on the acetylation characteristics and the metabolic disposition of DDS have been completed in patients harboring DDS-resistant *M. leprae.* Tests of acetylator phenotype with SMZ in 18 such patients revealed an unusual acetylator distribution—12 rapid, 5 intermediate and 1 slow—in comparison with the distribution of these phenotypes in African, Indian, or Philippine subjects or patients

Population group ^a	Acetylator	% Acetylation	DDS, plasma	
	phenotype ^a	SMZ, plasma	(Mean & range)	
African (21)	Rapid (9)	72, 56-87	32, 25-41	
	Slow (12)	28, 16-38	15, 10-21	
Indian (50)	Rapid (19)	77, 64-92	31, 24-43	
	Intermediate (6)	46, 40-50	16, 13-19	
	Slow (25)	29, 20-36	14, 8-25	
Philippine (32)	Rapid (23)	72, 59-86	37, 27-52	
	Slow (9)	22, 19-31	21, 12-27	
DDS-Resistant (18)	Rapid (12)	75, 55-96	34, 15-50	
	Intermediate (5)	48, 45-49	29, 18-43	
	Slow (1)	36	27	

TABLE 1. The distribution of acetylator phenotypes in various populations receiving 10 mg SMZ/KG or 50 mg DDS orally.

a The number of subjects in the groups are indicated in parenthesis.

TABLE 2. Comparisons of the mean half-times of disappearance of DDS and MADDS from the plasma of various populations receiving 50 mg DDS orally.

	Half-time of disappearance from plasma (hr) ^a						
Population group	DDS			MADDS			
	No.	Mean	S.E.	No.	Mean	S.E.	
African	21	28	1	21	31	2	
Indian	47	28	1	43	28	1	
Philippine	39	29	1	35	30	1	
DDS-resistant	18	20 ^b	2	18	22 ^b	2	

a Derived from 8-, 24-, and 48-hour levels; only decay curves whose regression lines yielded $R \ge 0.9000$ were considered to yield valid $T_{1/2}$ values. ^b Significantly lower (P < 0.01) than any other group.

(Table 1). Acetylation of SMZ and DDS was directly related in these 18 patients. Thus, this group also had an unusually high number of rapid acetylators of DDS. In addition, four other DDS-resistant patients, not yet tested with SMZ, exhibited high capacities for the acetylation of DDS (range, 34-55%), which also characterizes them as rapid acetylators. Thus, of a total of 22 patients tested with DDS, 16 (73%) were rapid acetylators, 5 (23%) were intermediate, and 1 (5%) was a slow acetylator.

In earlier studies (loc cit.), no relationship was found between acetylator phenotype of individuals and the half-time of disappearance (T ½) from the plasma of DDS, or its major circulatory metabolite, monoacetyldapsone (MADDS). Current studies of the T ½ of DDS and MADDS in the 18 DDS-resistant patients yielded the

mean values shown in the last line of Table 2. The T ½ values for both DDS and MADDS in these patients were significantly less than in any other population group studied previously.

These results suggest causal relationships between the occurrence of DDS-resistance and the rapid acetylator phenotype and an accelerated clearance of DDS and MADDS from the plasma. (Supported in part by the U.S.-Japan Cooperative Medical Science Program administered by NIAID, Grants AI-07801 and 08214, and Contract NIH 70-2283, NIH)

Hazama, S. Therapeutic trial on the effect of rifampicin (RFP) combined with the standard DDS therapy for lepromatous leprosy. (Six months observation.)

The Cooperative Research Team for Chemotherapy of Leprosy among eight Japanese National Leprosaria has been conducting a research trial on the effect of RFP in leprosy since autumn, 1971. Herewith we present the results acquired during the past six months. Prior to the start of the research, full discussion was carried out among us on case selection, standardization of administration, and methods of clinical description and examination.

We selected only pure lepromatous patients for the trial, dividing them into five groups: Group A, untreated cases; Group B, relapsed cases; Group C-1, cases that had not recovered after five or more years of regular treatment; Group C-2, cases that had worsened after one year's regular treatment; Group D, cases with impending eye complication and threatening blindness.

For drug administration, we adopted two different regimens. One consisted of 450 mg RFP per day six times a week, the other of 450 mg per day two times a week; each was combined with standard DDS therapy. As a general rule, in examining the effect of a drug, it is best to have the drug dispensed separately, since independent usage of this drug accelerates the appearance of drug-resistant bacilli within a relatively short period. Thus we had to resort to an alternative therapy combined with DDS.

The number of cases was as follows (total for the group, number treated by two-day method, and number treated by six-day method, respectively): Group A 2, 2, 0; Group B 10, 10, 0; Group C-1, 42, 27, 15; Group C-2, 3, 1, 2; and Group D 4, 3, 16 with a grand total of 61.

The judgment on six months' treatment with RFP and DDS is as follows. Among 61 cases, 56 had a favorable result, and 5 had an excellent clinical response. As to Group C-1, those who had not recovered after lengthy treatment with DDS or other drugs, 38 of 42 have thus far showed a good result. Besides those responding favorably, two showed no response and three showed some apparent reaction in the way of red patches, but we are unable to make a decision on their response until we have an opportunity to observe their future course.

No remarkable fall of the Bacillary In-

dex was observed in this period; a decrease in Morphological Index was clearly seen in three months in almost all cases. No important difference between the two-day and six-day method could be detected. No side effect was seen in this period, but 35 of 61 cases had *erythema nodosum leprosum* to a certain extent.

In view of all these observations we have concluded that this drug is very effective in leprosy treatment. [Hazama, S., Secretary, Cooperative Research Team for Chemotherapy of Leprosy among Japanese National Leprosaria. The director of this team is Dr. Yoshikazu Yajima, Tama Zenshoen. This team consists of the concerned members of the following leprosaria and institutes: Matsugaoka Hoyoen, Kuriu Rakusenen, Tama Zenshoen, Suruga Leprosarium, Nagashima Aiseien, Kikuchi Keifuen, Hoshizuka Keiaien and National Institute for Leprosy Research.]

Shepard, C. C., Levy, L. and Fasal, P. \checkmark Rifampin and *M. leprae*.

The action of rifampin on M. leprae is being studied in mice and in man. The pharmacokinetics of the drug are similar in the two species, and antibacterial effects are similar with the same dosage per kilogram. The chief feature is rapid bacterial killing in both species.

In mice even single doses, given by gavage, are effective. The minimum single dose exerting a detectable bactericidal effect has varied from 10 to 30 mg/kg in different experiments. Doses in the range of 25-50mg/ kg have usually eliminated the infection (which probably contained about 10³ viable bacilli at the time of dosing). An experiment was carried out to see the effect of spacing; the same total amount of drug was given in a single dose and also in doses spread out over three weeks; either once a day, three times a week, or once a week. At a total of 30 mg/kg, the antibacterial effect of the various schedules was roughly equivalent. A total of 100 mg/kg eliminated the infection with all schedules. As the background for human trials of a combination of rifampin and acedapsone (DADDS), attempts were made to learn the minimal dose of rifampin that, when given at 11-

40, 4

week intervals, would prevent increase of M. leprae. Results from several experiments indicate that single doses of 10 and 15 mg/ kg do not kill enough M. leprae to prevent their replacement in 11 weeks through multiplication of survivors, but a dose of 25 mg/kg does. The effect of two doses given 11 weeks apart was also tested; the results confirmed the impression gained with single doses. Several experiments have confirmed the finding that rifampin's activity is not blocked by dapsone, even when dapsone is given ahead of time to induce bacteriostasis before rifampin is administered. Whether there is any synergism between the drugs is not yet clear.

In studies of rifampin in man (lepromatous patients), the viability of M. leprae has been tested by the inoculation of mice with bacilli from skin punch biopsy specimens removed from patients at suitable intervals, from the same lesion when possible. On a dosage of 600 mg/day, viability was no longer detectable by the time the first specimen was taken after the start of treatment; 7 days after the start in ten patients, 4 days in one, and 14 days in one.

To have as much bactericidal effect in all patients treated, dapsone must, in contrast, be given more than 100 days, clofazimine (B663) 150 days, and acedapsone more than 300 days.

McRae, D. H., Russell, D. A., Vincin, D. R. and Shepard, C. C. Progress report on the chemotherapeutic trial of acedapsone (DADDS) in the Karimui, New Guinea.

Leprosy is highly prevalent in the people of the Karimui, a region of difficult access. All patients with leprosy are being treated with injections of acedapsone every 75 days. With the exception of the two patients discussed below, the clinical response of the more than 430 patients who have been treated more than four years has been satisfactory, and the bacteriological response of the patients with bacilliferous lepromatous leprosy has been satisfactory also. Of these lepromatous patients, 28 were previously untreated and had enough bacilli in their skin smears to allow monitoring of their response with measurements of the solid ratio (MI). The Bacterial Indices (BI's) in these lepromatous patients have decreased regularly, and eight of them had negative skin smears at four years. The solid ratios dropped to baseline level in the first year, and can now be determined in only a few patients because of the low BI's. Two lepromatous patients had received prior dapsone therapy and their response was satisfactory also.

The two exceptional cases are being studied more completely. One, a 30-year old female, responded normally for the first two years, but at three and four years was found to have some solid bacilli in her smears. Her BI ceased to respond. Clinically she showed some remaining disease activity. Her ability to absorb and deacetylate DADDS was normal, as shown by the presence of 22 ng DDS/ml serum 75 days after the previous DADDS injection. A skin biopsy specimen was obtained and shipped to Atlanta so the bacilli could be inoculated into mice for tests of sulfone resistance. The results indicate complete susceptibility of bacilli to dapsone at the minimal effective dosage, 0.0001%, a dosage that produces about 10 ng DDS/ml serum. The strain is being passed to allow confirmation of this surprising result.

Another patient was found to have a few solid bacilli in his smears four years after the start of treatment. His clinical condition had become nearly normal. Further details are not yet available.

A collaborative effort of the U.S. Leprosy Panel and the Leonard Wood Memorial. Spaced clofazimine therapy of lepromatous leprosy.

Several lines of evidence suggest that clofazimine (B663) is stored in the tissues of man and experimental animals. It appeared likely that the storage of the drug in the tissues might permit intermittentlyadministered B663 to exert a continuous effect against *Mycobacterium leprae*. Also, it appeared possible that larger doses of B663 administered less frequently might result in more rapid storage of the drug, and, therefore, a more rapid therapeutic effect. The spaced administration of B663 would be more convenient than daily administration, and more reliable as well, if the ad-

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ministration could be sufficiently infrequent to enable the supervision of each dose at a treatment facility or by a traveling paramedical worker. To test these possibilities, a trial of several intermittent B663 regimens was undertaken at the Eversley Childs Sanitarium, Cebu, the Philippines.

Five B663 regimens were compared: I. 200 mg six days per week; II. 100 mg three days per week; III. 300 mg once weekly; IV. 600 mg once every two weeks; and V. 600 mg on each of two consecutive days every four weeks. Regimen I is the standard regimen. Regimen II was expected to be fully efficacious, on the basis of the pilot trial of 100 mg twice weekly reported by Waters (Internat. J. Leprosy 36 [1968] 391), but less effective than the standard regimen. The intermittent regimens (III-V) were designed to deliver the same average dosage of B663 as regimen II. Forty-eight patient volunteers with previously untreated lepromatous leprosy were admitted to the sanitarium and assigned randomly to one of the five regimens. The response to treatment was measured primarily by means of mouse foot pad inoculation of the M. leprae recovered from skin biopsy specimens obtained before treatment and after two, four, and six months of treatment. Mice were inoculated in Cebu; portions of the same skin biopsy specimens were shipped by air on wet ice to San Francisco, where duplicate inoculations were performed for 90% of the specimens. Infectivity of M. leprae for the mouse was measured as previously described; loss of infectivity is interpreted as a decrease in the proportion of viable M. leprae in the standard inoculum.

The results of the inoculation of mice with M. *leprae* recovered from 192 skin biopsy specimens are presented in the ac-

companying table. A "+" specimen is one which yields definite evidence of multiplication of the organisms in the mouse foot pad. A " \pm " specimen is one which contains only a small proportion of viable M. leprae, that is, organisms are found in a monthly foot pad section, but subsequent harvests fail to reveal evidence of multiplication of M. leprae. A "-" specimen is one which yields no evidence of multiplication of organisms in the mouse foot pad. These results show that regimens I and II are more effective than regimens IV and V; the effectiveness of regimen III falls between that of the less and that of the more widelyspaced regimens.

Thus, contrary to our expectations, B663 treatment of lepromatous leprosy was found to be more efficacious when the drug was administered more rather than less frequently. The spaced administration of B663 was therapeutically effective, however, and may be useful in combination with some other intermittently-administered antimicrobial. [The trial protocol was developed by a Chemotherapy Committee, which also supervised the conduct of the trial. Members of the Committee were C. H. Binford, R. S. Guinto, L. Levy, C. C. Shepard and J. G. Tolentino. Other participants in this investigation were R. Abalos, E. dela Cruz, T. T. Fajardo, Jr., J. N. Rodriguez and G. P. Walsh. The trial was carried out at the Eversley Childs Sanitarium, Cebu, with the assistance of Dr. A. P. Perez, Director of the Sanitarium, and of the Government of the Philippines.]

Hirai, M., Sasaki, N. and Namba, M. Leprous bulbar palsy.

Although a considerable number of reports of leprous bulbar palsy have appeared

Regimen #	Pre- Rx	After 2 months	After 4 months	After 6 months		
	+ ± -	+ ± -	+ ± -	+ ± -		
I	9	4 1 4	3 6	2 6		
II	11	7 1 2	1 2 8	1 9		
III	10	9 1	2 1 7	1 1 8		
IV	8	7 1	3 2 3	2 4 2		
v	10	9 1	3 2 5	3 4 3		

Results of mouse inoculation.

in the past, little is known about its pathology. We want to report the pathology briefly and to prevent the repetition of such sad progress.

This is the newest case in our institution. The patient was a 48 year-old female, who was admitted here four years ago for a foot-drop operation. On admission there was no skin eruption at all. The only noteworthy symptoms were anesthesia in the distal parts of four extremities with loss of bilateral thenar and hypothenar eminences and slight enlargement of the left great auricular nerve and of both peroneal nerves without tenderness. The lepromin test was positive in both early and late readings. Skin smears were negative for acid-fast bacilli. The diagnosis was, borderline leprosy.

Manifestation of the reaction. Acute reaction appeared abruptly after three years without apparent exciting cause. Symptoms were: diffuse red swelling of whole face with relatively clear cut margins, annular red infiltration of the whole body akin to the reactivated tuberculoid eruptions. Other characteristic symptoms were severe headache, nausea, dysarthric speech, hoarse voice, diffuse salivation, tachycardia and difficulty of swallowing. These symptoms continued for the 48 days before her death.

Pathologic findings. The main lesion was located in the medulla oblongata in the right median lateral side at the center of the nucleus ambiguus. There were no remarkable changes macroscopically, except slight edematous swelling. Microscopically, there were a few localized minimal epithelioid cell granulomas, lymphocytic infiltration around the vessels and in the Virchow-Robin's space, multiplication of the capillaries in the peripheral zone of the granulomatous foci, and the occasional appearance of a giant cell in a vessel wall. However, no leprosy bacilli were observed anywhere. Degeneration of nerve cells in these areas, that is, change of shape of cells, of the nucleus, granular proliferation, vacuolation of the cytoplasm and irregularity of nerve fibers, and proliferation of microglia cells were visible. In addition, a predominant lymphocytic aggregation in peripheral nerves was observed. There was no infiltration in the vagus nerve, although there were slight vacuolar changes of nerve fibers.

Matsuo, Y. Cultivation of *Mycobacterium lepraemurium* in mouse foot pad cell cultures.

One of the most important problems in cell culture of Mycobacterium lepraemurium may be how to adjust the relationship between the rate of multiplication of the organism and that of the cell. The cells usually multiply faster than the bacteria, and, therefore, the number of bacteria per cell decreases on every occasion of cell division. Furthermore, prior to sufficient multiplication of the bacteria, contact inhibition of cells occurs and cells, especially those heavily loaded with bacteria, rapidly break down. This leads to diluting out of bacteria from the culture. Unsatisfactory results in cell cultures of this particular organism may be partly due to the facts mentioned here.

Special attention has been given to maintaining the infected mouse foot pad cells for longer periods. The infected cells are incubated at 30°C rather than at 37°C. As soon as a monolayer growth of the cells is obtained, concentration of serum in the culture medium is reduced from 10% to 2%. At the appropriate time of cultivation, the infected cells are suspended in fresh culture medium containing 10% serum two times as great as the preceding culture. One-half of the suspension is transferred to a new culture flask of the same size. An appropriate portion of the remaining half is used for the counting of acid-fast bacilli and the rest is transferred to Leighton tubes to observe the intracellular appearance of multiplication of the organism.

Serial increase in the number of acidfast bacilli with successful sub-cultures has been maintained. There has been a cumulative bacterial increase of 1.34×10^8 -fold for 760 days, 2.75×10^8 -fold for 746 days and 1.4×10^4 -fold for 436 days so far. This represents an overall generation time of 28.1, 28.0 and 31.6 days, respectively.

All attempts to grow the acid-fast bacilli in cultures on artificial culture media have failed. The capability of the organisms to produce typical lesions in mice has been well maintained even after two years of cultivation.

Ogawa, T. and Hiraki, M. Attempt to cultivate Mycobacterium lepraemurium in vitro.

Aim. On the hypothesis that a heavy inoculation of M. *lepraemurium* on an egg yolk medium might produce gross visible growth of the organisms, a series of cultural experiments were undertaken.

Methods. Two strains of M. lepraemurium (Hawaii and Keishicho) were used. Mice, dd-N strain, were inoculated intravenously or subcutaneously with bacterial suspension prepared from the infected tissue and sacrificed at monthly intervals. The organs, including superficial lymph nodes and subcutaneous lepromata, were removed aseptically, ground in a mortar, and inoculated, with or without decontamination treatment, on the 1% egg yolk and the 1% Ogawa egg medium. Each medium was dispensed in 5-ml amounts in 18 by 170 mm tubes and inspissated in slanted position at 90°C for 1 hour. The tubes were incubated at 37°C for over three months and observed for the development of gross colonies. An appearance of mycobacterial, slow growing, buff-colored, rough colonies on the egg yolk medium but not on the egg medium was taken to indicate a positive culture.

Primary isolation. None of the specimens derived from mice infected 1-4 months previously proved culture-positive. On the other hand, a number of the specimens from mice sacrificed 5-11 months after injection were culture-positive. Of 469 specimens from mice inoculated with the Hawaiian strain 46 (9.8%) were positive, and of 63 specimens from mice with the Keishicho strain 25 (40%) were positive. Sometimes, rapid growing mycobacteria were also isolated.

Characteristics of the isolates. The development of colony growth of acid-fast bacilli could be confirmed only on the egg yolk medium after 2-3 months' incubation. Ordinary culture media favorable for the growth of the tubercle bacillus do not support their growth, nor does the egg yolk medium deprived of glycerol.

No visible growth has been obtained by the use of the bacterial suspension prepared from the *in vitro* culture of the bacillus. Niacin formation is negative, and catalase activity is weakly positive, as in the case of the tubercle bacillus.

Subcultivation. Serial subcultures on the 1% egg yolk medium have been successful. A portion of the growth of the preceding culture was transferred by a loop lightly onto the middle of the surface of medium. An increase in amount of the growth was always determined by comparing growth obtained on the egg yolk slant with that on the egg slant (the negative control). At present, the isolate from the mice with the Hawaiian strain is now in the 16th transfer and that from the mice with the Keishicho strain in the 10th transfer.

Reproduction test. Mice, dd-N strain, were inoculated with a graded dose of bacilli. These inocula, which were prepared from their respective 6th and 2nd subcultures, ranged from 0.1 to 0.001 mg and were given subcutaneously and intravenously. The animals were sacrificed 3-11 months later. Their organs were examined macroscopically, histologically (in a small number), and culturally. The findings thus obtained indicate that the infection was reproduced with each of the doses employed.

Conclusions. It seems likely that our aim was achieved. We regard the two isolates of acid-fast bacilli as the Hawaiian and the Keishicho strains respectively.

Nakamura, M. Quantitative multiplication of *M. lepraemurium* in cell free medium.

In a previous communication (LSM Memo-309, and J. Gen. Microbiol., in press), it was shown that the growth of *M. leprae-murium* could be certainly seen in the medium, referred to as NC medium, which contained α -ketoglutaric acid and cytochrome C in Kirchner medium. Multiplication of the bacilli was recognized by morphological observation of a smear on a glass slide on which the organism was cultivated and by quantitatively counting the increase of bacilli.

This paper will report a culture medium more suitable for remarkable multiplication of the bacilli than the NC medium. Estimation of the growth of bacilli is by the method mentioned above. The cell-free culture medium used here is NC medium improved by addition of hemin and 1-cysteine.

The culture medium, designated NC-5, is composed of a basal medium together with additives. The basal medium, which is referred to as the EK medium, i.e. enriched Kirchner medium, is composed of the original Kirchner medium containing 0.005% calcium pantothenate and glucose and pyruvate at the final concentration of 0.25%, respectively. As additives, α -ketoglutaric acid 0.1%, cytochrome C 0.01%, hemin 4 μ g/ml, 1-cysteine 0.01%, and goat serum 10%, respectively, at final concentrations, were added to the EK medium.

When the bacilli of the Hawaiian strain of M. lepraemurium were smeared on a silicon-coated glass slide, or a suspension of the bacilli was inoculated in the NC-5 medium and cultivated at 30°C, the bacilli gradually elongated and began to multiply after approximately ten days of incubation. Thereafter, the number of bacilli quantitatively increased and reached about 50 times as much as the initial value, after 60 days' cultivation. These bacilli maintained pathogenicity for mice characteristic of murine leprosy. No growth and no pathogenicity could be demonstrated for bacilli cultivated in EK medium under the same conditions used for the NC-5 medium.

It is concluded that the growth of bacilli seen in the NC-5 medium was unequivocally none other than *M. lepraemurium* rather than other mycobacteria.

In another experiment, a trial cultivation of *M. leprae* was performed under the same conditions as those employed for *M. lepraemurium.* There were no morphologic changes in the bacilli and no multiplication of *M. leprae* occurred.

(At the time of going to press the following elaboration concerning the NC-5 medium was received from the author, together with the statement that a further presentation of this medium is to appear in the January issue of the "Proceedings of the Japan Academy."—Editor.)

Basal medium (Enriched Kirchner [EK] medium) was composed of: KH_2PO_4 , 6.0/

gm: Na₂HPO₄.12H₂O, 7.0/gm; sodium citrate, 3.7/gm; sodium glutamate, 15.0/gm; MgSO₄.7H₂O, 9.0/gm; glucose, 4.0/gm; sodium pyruvate, 5.0/gm; glycerol, 30.0/ml; distilled water added to 1,000/ml, adjusted with NaOH to pH 7.0.

Five volumes of the basal medium and one tenth volume of 0.5% (w/v) calcium pantothenate aqueous solution were mixed, and then the mixture was sterilized by autoclaving at 115°C for 20 minutes.

Additives: The following additives were aseptically added to the basal medium.

- 2) 2% (w/v) α -ketoglutaric
- acid solution 0.5 vol 3) 0.1% (w/v) cytochrome C aqueous solution 1 vol
- 4) Hemin solution 0.5 vol
- 5) 0.3% (w/v) 1-cysteine HCl . 0.2 vol (or 0.1% 1-cysteine) 0.5 vol

After mixing well, each 7 ml of the culture medium prepared was aseptically distributed into a sterilized test tube (10.5 cm \times 1.3 cm), and then fitted with sterile rubber stoppers. The materials from 2) to 5) were sterilized by filtration through Millipore filters. Hemin solution was prepared according to the method of Biberstein and Gills. In brief, the stock hemin solution was prepared by dissolving 80 mg in 2 ml of triethanolamine and making 100 times more dilution in distilled water.

Nakayama, T., Endo, H., Hirata, T. and Yamada, M. Studies on the cultivation of *M. leprae*.

. During attempts to culture leprosy bacilli, it was not uncommon to observe some colony growths. Most were merely "contaminants," but there was a specific group of acid-fast bacilli (e.g., KN-1, KN-2, KN-3,, KN-7, KN-8, KN-9, etc), which showed a remarkable pleomorphism depending on changes in nutritional environments.

With a working hypothesis that these strains might be "variants" and have some metabolic relation to leprosy bacilli, trials were made to separate a growth promoting factor from these strains. KN-1, KN-2, and KN-3 factors were extracted from the respective cultures by hot alcohol and purified by taking advantage of their solubilities in organic solvents. The combination of KN-1, KN-2 and KN-3 factors produced acceleration of growth of **M. leprae** (Nakayama, T., Internat. J. Leprosy **36** [1968] 502).

There was, however, a barrier to dealing with the identification problem in that the growth of KN-1, KN-2 and KN-3 strains were not extensive and they were occasionally unstable. Therefore, it was difficult to obtain sufficient amounts of growth factors for use in detailed experiments. Finally, KN-1, KN-2 and KN-3 strains were abandoned, and since then the KN-7, KN-8 and KN-9 strains have been employed because of their luxuriant growths. The conditions under which they would grow stably and yield good amounts of growth factors were found after a long series of investigations. The most suitable media contained about 70 ingredients.

KN-7, KN-8 and KN-9 bacilli formed colonies on each medium after four, ten, seven days at 32°C, 20°C and 27°C, and the relevant cultures were yellow, red and brown, respectively. They were all acidfast, but morphologically quite different from usual mycobacteria. KN-7, KN-8 and KN-9 factors were extracted and refined.

After inoculating leprosy bacilli on the synthetic medium together with added KN-7, KN-8 and KN-9 factors and incubating at approximately 27° C for two weeks, smooth yellow-white colonies grew. These colonies were transplantable on the special medium used, but they did not grow on conventional media. The colony obtained was organized, with bacterial forms and homogeneous matrix substances. Each bacterial form was not distinguishable from *M. leprae*.

Dhople, A. M. and Hanks, J. H. The energetics (ATP) of *Mycobacterium lepraemurium* in diffusion chambers incubated *in vitro* and *in vivo*.

ATP is the source of biologic energy and is assayable in picogram amounts. This criterion of growth potential has now been refined to a degree which is adequate to evaluate claims of limited microscopic growth of *Mlm* (*Mycobacterium lepraemurium*) outside of host cells. Employing dilute suspensions of *Mlm* sealed in the diffusion chambers used by Ito and Rightsel, two such claims have been examined. In cooperation with Dr. Oiwa, the first systems comprised Ito-type chambers incubated *in vitro*. The second system consisted of chambers which Dr. Rightsel had incubated in the peritoneal cavities of mice. Microscopic counts and ATP determinations were conducted to learn whether either of the reports on extracellular growth of *Mlm* could be subtantiated.

In the Oiwa experiments, the chambers in 15 x 100 mm test tubes at 37° were supplied routinely with 2 ml of Oiwa medium (supplemented base, 10% bovine serum and 10% filtered mouse brain extract). Controls included: a) the bacteria centrifuged against the bottom of 10 mm test tubes, with media renewed by methods that did not disturb the bacteria; b) the basal medium only; c) mouse brain extracts not filtered and mouse brain extracts pasteurized; d) the foregoing four media not renewed; and e) heat killed bacteria. Results were assessed by pooling the contents of three chambers or tubes at each sampling period.

Since the results in the different experimental conditions and media agreed closely, averages of all variables were combined. After 17 days the bacterial counts were 72% of the original, while the ATP per cell had fallen to 10% of the original. Equivalence was observed between diffusion chambers and test tubes and between the basal medium and supplementation with the various mouse brain extracts. Renewals of media three times a week did not sustain the energetics of the bacilli.

The chambers supplied by Dr. Rightsel have been maintained in the mouse peritoneal cavity for 50 days. The bacterial counts increased 3.4-fold and the ATP levels 2.5fold. After correcting for the decreased length of the incubated bacilli, the actual increase in biomass was 2.7-fold. The fact that the energetics of the bacilli had been expanded in proportion to the increase in biomass indicates that the organisms were capable of further growth in the environment devised by Rightsel. Insofar as it is known, this represents the first validation of the extracellular growth of an "intracellular parasite." [Supported by the U.S.-Japan Cooperative Medical Science Program administered by NIAID, Grant AI08866, NIH]

Kusaka, T., Matsuki, G., Nakagawa, H., Kashiwabara, Y. and Nakamura, K. Quantitative determination of decanoate uptake, in vitro, in Mycobacterium lepraemurium and its application to Mycobacterium leprae.

It can be clearly demonstrated by gas chromatography that Mycobacterium lepraemurium contains a special pattern of fatty acids which is known to be characteristic of many strains of mycobacteria. Accordingly, it appears reasonable to suppose that this organism may be capable of synthesizing these fatty acids, at least a part of them, by itself. From our results obtained by enzymatic experiments during past years, however, it has been shown that this organism was almost incapable in vitro of synthesizing long carbon chain fatty acids from acetate or malonate, but fairly capable of elongating some medium length fatty acids, such as decanoate or octanoate, to form several kinds of long fatty acids. It may be speculated, therefore, that these medium length fatty acids would be taken up directly by the organism from the host cells. In order to confirm this assumption, M. lepraemurium, purified aseptically from mice, were incubated at 37°C in Kirchner's medium containing 10% bovine serum with a 1-14C-decanoate. This incubation system has been found to be adequate to maintain, for a time, the virulence of M. lepraemurium in mice, by Prof. M. Nakamura.

In consequence, incorporations of radioactivity from 1-¹⁴C-decanoate into *M. lepraemurium* could be consistently observed and these incorporations were shown to be a biological function of this organism, because a) much more radioactivity was obtained from native cells than heat-killed (55°C, 45 min.) cells, b) various radioactive lipid components (tri-, di-glycerides and cardiolipin etc.) could be detected in a chloroform-methanol extract of the radioactive cells, and c) several antibiotics and respiratory inhibitors showed inhibitory action against the incorporation of radioactivity when the cells were preincubated with these reagents. Incorporation of other ¹⁴Clabeled fatty acids, ranging from C_2 to C_{18} , into M. lepraemurium were also examined by the same system as above, and the results showed that, among fatty acids from C2 to C₁₀, 1-14C-decanoate was incorporated most actively, 1-14C-octanoate was next in activity, whereas 1-14C-acetate and 2-14C-malonate were found to be only slightly incorporated into the cells. Incorporation of fatty acids from C_{12} to C_{18} could not be precisely compared with each other, since the relatively high radioactivity found with heat-killed cells (probably because of absorption) disturbed the quantitative determination of the incorporation of these fatty acids. It is noteworthy, however, that the predominant uptake of decanoate and the negligible uptake of acetate or malonate by M. lepraemurium correspond very closely to the biosynthetic activity of long fatty acids by this organism, as already mentioned.

The method used for determining fatty acid uptake, in vitro, in M. lepraemurium was then applied to M. leprae. Cells of M. *leprae* can be purified from human leproma by essentially the same centrifugation method as that used for M. lepraemurium, except that an aliquot of heat-killed cells of M. lepraemurium was added as a carrier to the homogenate of leproma before centrifugation. So far, in the few tests completed, the radioactivity of native cells of M. leprae has been found to be always much higher than that of heat-killed (55°C, 45 min.) cells, when the cells were incubated with a 1-14C-decanoate. Analysis of radioactive lipids in a chloroform-methanol extract of the radioactive native cells is now being carried out.

Mifuchi, I. and Saito, K. Shift of tricarboxylic acid cycle enzymes in induced respiratory-impaired mutants of Mycobacterium smegmatis.

The energy yielding pathway of mycobacteria depends mainly on respiratory systems because of the absence of anaerobic glycolysis. The noncultivable state of leprosy bacilli seems to depend on minimal capacity of respiration. It has been observed that *M. lepraemurium*, separated from infected tissues, does not exhibit the cytochrome bands which are readily detectable in cultivable mycobacteria. This seems to indicate that leprosy bacilli have genetically lost the adaptive production of respiratory systems. If we are able to obtain a clearer understanding of the defect in the respiratory systems of mycobacteria, it may be possible to find some approach to the puzzling problem of cultivating leprosy bacilli.

From this point of view, we are studying the alteration of respiratory systems in respiration-impaired mutants of M. smegmatis which were induced by N-methyl-N'nitroso-N-nitrosoguanidine. The weakly impaired mutants showed about 25% decrease of respiration in comparison to the wild strain, and the strongly impaired mutants exhibited about 70% decrease of respiration. The cytochrome pattern of intact cells of respiratory-impaired mutants was measured by the opal-glass method. The weakly impaired mutants did not show a significant difference in cytochrome pattern in comparison to the wild strain. In the case of the strongly impaired mutants, however, it was found that the cytochrome content of the a₃ region was clearly reduced.

Activities of TCA cycle enzymes in these mutants were investigated comparatively. With an impairment of respiration, TCA cycle enzymes showed a tendency to increased activity. When the respiratory impairment was more intensive, the aconitase and isocitrate dehydrogenase had significantly reduced activities, whereas activities of succinate dehydrogenase, fumarase, malate dehydrogenase and α -ketoglutarate dehydrogenase had increasing activities.

The results seem to suggest that a shift in the TCA cycle may occur in mycobacteria with impairment of respiration. With more intensive respiratory impairment, a break in the TCA cycle seems to occur at the initiation portion of the cycle. The fact is of interest considering the observation of Dr. Mori, Osaka University in Japan, that *M. lepraemurium* does not exhibit the complete TCA cycle. Nishiura, M. Three-dimensional observation of leprosy lesions by freeze etching technic.

Three-dimensional ultrastructures of leprosy lesions are now being studied by the freeze-etching technic in our laboratory. In the present study, structures of lepromatous lesions have been studied by this technic.

Interesting findings of this study were as follows:

1. Intracytoplasmic foamy structure (socalled lipid droplets inside the lepra cell) always shows a homogeneous appearance and never shows the lamellar structures of neutral fat droplets.

2. Each band structure of a leprosy bacillus is composed of two strings attached together side by side. This band structure is located in some bacilli on the outermost surface of the cell wall, but in other bacilli it is embedded in the cell wall.

3. Around each foamy structure, there is always a limiting membrane which combines a group of small droplets together to form a large ball in the cytoplasm.

4. When lepra cells are broken, many foamy structures are thrown out of the lepra cell and they float in the extracellular fluid.

5. Extracellular leprosy bacilli were frequently found which were thrown out of the lepra cell together with foamy structures.

6. The three-dimensional ultrastructure of the ENL lesion is now also being studied and the findings will be reported in the conference.

Peters, J. H., Gordon, G. R., Murray, J. F. Jr., Fieldsteel, A. H. and Levy, L. Minimal inhibitory concentration of dapsone for *M. leprae* in rats. (Abstract read by title)

Previously, we reported on the metabolic disposition of dapsone (DDS) in normal å and \Im Lewis rats (Murray *et al*, Proc. West. Pharmacol. Soc. **15** [1972] 100) and in å Lewis rats inoculated with *M. leprae* (Gordon *et al.* Internat. J. Leprosy **40** [1972] 220-221). From knowledge of plasma levels of DDS obtained in short-term

	×.			Nu	fast bacilli	bacilli seen ^b	
Days post	Diet level of DDS	Plasma level (ng/ml)		In 60 fields examined			Calculated
inocu- lation				Left	Right	Total	mean/pad
	lation	(10 ⁻⁵ gm%) ^a	DDS	MADDS	pad	pad	
	NA KOOMACHINA		Male rats	¢	57 8061 6 804 6 804	6.5.8 A.A.	
120	0			120	54	174	2.85
140	0	< 0.5	< 0.5	46	36	82	1.30
	1.5	< 0.5	< 0.5	20	38	58	0.85
	5.0	1.5	< 0.5	7	1	8	0.13
	15	4.0	1.0	0	3	3	0.05
	50	11.0	3.0	2	0	2	0.03
202	1.5	—		30	53	83	1.28
	5.0	· · · · ·		0	15	15	< 0.24
	15			1	4	5	0.07
	50			1	0	ĩ	< 0.03
240	1.5			22	37	59	0.94
	5.0	_		4	10	14	0.23
	15	_		17	10	27	0.42
	50			9	5	14	0.23
260	0			36	120	156	2.49
				s ^e	narananana atata atata	1 171 172	
120	0	—	_	19	34	53	0.86
141	0			60	70	130	2.16
	0.5	0.9	0.3	19	42	61	1.00
	1.5	1.8	0.9	2	3	5	0.08
	5.0	3.0	1.2	ī	0	1	0.02
	15	12.9	5.3	õ	Õ	õ	0.00
199	0.5			59	72	131	2.22
	1.5			31	96	127	2.12
	5.0			1	0	1	0.02
	15			0	0	õ	0.00
239	0		-	29	35	64	1.32
	0.5			16	26	42	0.89
	1.5	—		2	14	16	0.30
	5.0	—	<u></u>	17	9	26	0.57
	15			0	3	3	0.07

Relationship among dietary level of DDS, plasma levels of DDS and MADDS, and multiplication of M. leprae in foot pads of male and female Lewis rats.

^a Animals were fed diets containing DDS from day 0 through day 140 or 141; diet free of DDS was fed for the remainder of the experiment.

^b Each rat was inoculated with 5 \times 10³ M. leprae/hind foot pad.

e Drug levels and number of bacilli are the results from one animal at each diet level.

feeding experiments, we designed diets for rats inoculated with *M. leprae* to obtain plasma levels bracketing the known minimal inhibitory concentration (MIC) of DDS for *M. leprae* in mice (≤ 10 ng/ml).

Twenty 3 and 20 9 Lewis rats were inoculated with 5 x 10³ *M. leprae*/hind foot pad, and groups of four rats were immediately fed diets containing from 0 to 15 \times 10⁻⁵ gm% DDS (9) or from 0 to 50 \times 100⁻⁵ gm% DDS (3). At 60 and 90 days we determined levels of DDS and monoacetyldapsone (MADDS) in plasma derived from blood obtained by cardiac puncture without sacrificing the animals. Counts of acidfast bacilli in the foot pads were made on day 120 in single control animals and on days 140 or 141 in one rat on each diet. Plasma levels of DDS and MADDS were also determined. After days 140 or 141, all animals were fed drug-free diet for the rest of the experiment; bacillary counts were made on days 199 or 202 and 239 or 240 in these rats and on day 260 in the control ϑ rat.

The number of acid-fast bacilli in the foot pads and the plasma levels of DDS and MADDS are shown in the table. The drug levels found on day 140 or 141 were similar to those found at 60 and 90 days for the respective sexes receiving the same diets and were nearly identical to levels found in noninfected rats of both sexes during earlier short-term feeding studies. The diet level of DDS required to obtain the same plasma level in both sexes was found to be three times higher in δ than in 9 rats, consistent with our earlier observations of a sex difference in DDS disposition in this species. The MIC of DDS for M. leprae was essentially the same in both sexes of Lewis rats-1.5 to 4.0 ng/ml in 3, and 1.8 to 3.0 ng/ml in 9 rats. Both ranges are similar to the estimated MIC of DDS for M. leprae in mice. The increase in number of acid-fast bacilli in all animals between day 140 or 141 and day 239 or 240 suggests that the DDS in the diets was exerting a bacteriostatic rather than a bactericidal action.-[Supported in part by the U.S.-Japan Cooperative Medical Science Program administered by NIAID, Grants R22-AI-07801, -08214, and -08417, NIH]