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TITLE OF THESIS.....IMMUNOLOGICAL STUDIES ON MYCOBACTERIUM LEPRAE MURIUM.....

.....

UNIVERSITY.....UNIVERSITY OF ALBERTA.....

DEGREE FOR WHICH THESIS WAS PRESENTED.....M. Sc.....

YEAR THIS DEGREE GRANTED.....1973.....

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THE UNIVERSITY OF ALBERTA

IMMUNOLOGICAL STUDIES ON MYCOBACTERIUM LEPRAEMURIUM

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF MEDICAL BACTERIOLOGY

EDMONTON, ALBERTA

SPRING, 1973

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Immunological Studies on Mycobacterium lepraemurium" submitted by Janice Elaine Trylinski in partial fulfilment of the requirements for the degree of Master of Science.

R.L.S. Whitelaw
Supervisor

Date March 27, 1973

ABSTRACT

The passive transfer of sensitivity to murine lepromin was investigated in guinea pigs. The Hong Kong strain of M. lepraemurium was used to infect strain 2 and strain 13 guinea pigs. Syngeneic unsensitized animals were given intravenous injections of M. lepraemurium-sensitized, live spleen cell suspensions. Control groups were given no cells, heat-killed cells (60°C for 30 minutes) or frozen-thawed cells. The animals were then skin-tested with murine lepromin with and without phenol and tissue suspensions. It was found that both live and heat-killed spleen cell suspensions but not frozen-thawed cells transferred the ability to form a granulomatous response to murine lepromin. On the basis of this evidence, the response appeared to be a delayed type of hypersensitivity. Work on the attempted transfer of lepromin sensitivity in rats confirmed earlier findings that lepromin sensitivity in rats cannot be determined by intradermal skin tests.

Lepromin purification was carried out by sequentially digesting heated, ground, epidermis-free murine lepromas with DNAase, RNAse, and pronase, followed by sonication for 20 minutes and differential centrifugation. The supernatant was termed partially purified murine lepromin and did not elicit a specific skin reaction in guinea pigs. A method for the characterization of cell-free mycobacterial extracts by polyacrylamide gel electrophoresis was developed.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. R.L.S. Whitehouse for his encouragement and guidance during my graduate program and his advice in the preparation of this thesis.

Appreciation is also expressed to Dr. P.C. Wong for her conscientious efforts and supervision of the project during her stay in this department.

To all members of the Department of Medical Bacteriology I extend my thanks for their suggestions, encouragement and help throughout my graduate program. Thanks are also expressed to Miss C. Harpe and Mrs. E. Davies for their expert typing of this thesis and to Mrs. M. Petley-Jones for preparation of the photographs.

I would also like to thank my family for their constant encouragement during my graduate program.

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LIST OF ABBREVIATIONS

BCG	-Bacillus Calmette-Guérin, an attenuated strain of *Mycobacterium tuberculosis*.
DNCB	-2,4-dinitrochlorobenzene.
HL-A	-histocompatibility.
MEM	-minimal Eagle's medium.
MIF	-migration inhibitory factor.
OT	-Old Tuberculin.
PHA	-phytohemagglutinin.
Poly INb	-a polymer of D-arabofuranose and D-galactopyranose in a molar ratio of 3:1.
PPD	-purified protein derivative.

INTRODUCTION

Leprosy is a disease of man caused by Mycobacterium leprae. Evidence that leprosy or a leprosy-like disease has afflicted mankind for several thousand years is presented in the sacred writings of several cultures (Cochrane 1964). During the Middle Ages leprosy was endemic in much of Europe and as late as the nineteenth century it was a public health problem in Norway. A Norwegian, Gerhard Armauer Hansen is generally credited with the discovery of the leprosy bacillus in 1873. The human leprosy bacillus was the first pathogenic bacterium to be described and associated with a disease of man (Hanks 1951a). Despite its antiquity, leprosy remains a health problem, for it is estimated that there are approximately ten million people in tropical and semitropical regions suffering from leprosy (WHO 1965).

M. leprae is an obligate intracellular parasite which causes a chronic disease. The incubation period is usually three to five years although it may be as short as a year or as long as twenty years. The disease is mildly contagious and clinically presents two forms, the lepromatous and the tuberculoid form. The lepromatous form is the severe form where the patient has little or no defense against the organism, and his tissues are packed with acid-fast bacilli. If the disease is of a number of years duration it may have caused disfiguration of the patient. Patients with lepromatous leprosy are usually lepromin negative, that is, they exhibit no delayed hypersensitivity to lepromin, an antigen containing killed M. leprae. Tuberculoid leprosy is a less severe form of the disease. It is usually associated with a

good prognosis and good resistance in the patient. The lepromin test is usually positive in the tuberculoid form. There are many gradations of the disease between the two polar forms, the lepromatous and the tuberculoid.

Many factors contribute to the prognosis of a patient with leprosy; climate, nutrition, general well being, age and heredity are all considered important. Treatment of leprosy is usually performed in special clinics and is varied. For those still treatable the sulfone drugs may be used. For those no longer curable, good hygiene and the refinements of physiotherapy may help to ease their suffering.

The prime obstacle in finding a treatment for leprosy is the fact that the causative organism of leprosy cannot be successfully cultivated on artificial media and it is only with limited success that it can grow and divide in tissue culture and laboratory animals. The human leprosy bacillus has a generation time of 12 to 13 days in the mouse footpad, this is the longest generation time for any known bacterium. Leprosy research is fraught with enormous difficulties because of the inability of M. leprae to grow and divide in artificial media. M. leprae is accepted as the cause of leprosy although Koch's postulates are still unproven with respect to leprosy.

In the present situation the immunological processes occurring in human leprosy are difficult to study because of the length of time required to develop the disease and because patients are invariably given some type of drug therapy which may interfere with the immune response. An animal model might facilitate the study of immune processes in leprosy so the investigation of the course of murine

leprosy in laboratory animals seemed logical. The use of a model in disease processes is now well established and has been successfully used in the study of malaria and helminth infections. The use of rat leprosy as a model for human leprosy has been suggested by other workers in the field (Carpenter 1951 and Tepper 1971). M. lepraemurium was chosen for the present work because of its similarity both morphologically and culturally to the human leprosy bacillus. In addition, M. lepraemurium can be kept reasonably well in experimental animals, an important consideration in this part of the world where human leprosy is rare.

Rats are susceptible to murine leprosy but they are unsuitable for immunological work because they do not exhibit any dermal sensitivity to murine lepromin even when they are sensitized (Wallace 1958a). Although mice are susceptible to murine leprosy they too do not exhibit a dermal response to murine lepromin when sensitized. Goihman-Yahr et al. (1969a) were able to achieve passive transfer of hypersensitivity to human lepromin in guinea pigs so it was felt that guinea pigs might provide a good experimental model. In studying the immune response of laboratory animals to infection with M. lepraemurium the present work followed several main approaches: the investigation of cellular immunity, the investigation of humoral immunity, the investigation of the immunogenicity of treated M. lepraemurium suspensions and the investigation of polyacrylamide gel electrophoresis characterization of cell-free mycobacterial extracts for possible application to disc immunoelectrophoresis. It was felt that by exploring these areas an insight into the immunological processes in the infected animal might be gained.

REVIEW OF LITERATURE

Bacteriology of Rat Leprosy

Rat leprosy, a disease of rats caused by Mycobacterium lepraemurium, was first reported by Stefansky in 1903. While working on the plague in Odessa, Stefansky observed a disease of brown rats which closely resembled human leprosy. The lesions of the rats contained large numbers of intracellular, acid-fast bacilli morphologically similar to those found in human leprosy lesions. In the same year a similar organism was independently reported by Dean in London (Dean 1905). Rat leprosy has been found in every country investigated with the exception of the Danish Antilles (Lowe 1937). The highest incidence of occurrence of rat leprosy is in the northern hemisphere and its distribution is independent of the epidemiology of human leprosy. Rat leprosy was originally found in the brown rat. However, rat leprosy has been reported in other species of rat, namely Rattus rattus, Rattus rattus alexandrinus, Rattus rattus diardii and Rattus concolor and in mice (Mus musculus) (Lowe 1937). Rat leprosy is not infectious for humans.[1]

The murine leprosy bacillus or Stefansky's bacillus is a member of the genus Mycobacterium of the family Mycobacteriaceae of the order Actinomycetales. Mycobacteria are spherical to rod-shaped, aerobic, mesophilic, gram-positive bacteria. Mycobacteria are noted for their

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[1]Marchoux (1922) described a patient who appeared to be suffering from rat leprosy. This is the only case recorded in the literature.

ability to resist decolorization with acidic organic solvents and are termed acid-fast as a result of this characteristic. This ability is due to their lipid-rich cell walls. Mycobacteria have a spectrum of habitats which ranges from soil and water to the human body and can be divided into three main groups; the saprophytes existing in nature, the potential pathogens and the pathogens. The nonparasitic soil and water forms are probably concerned with the degradation of lipids in nature. The group of mycobacteria of uncertain parasitic and pathogenic potential for humans are referred to as atypical, anonymous or unclassified mycobacteria. Over the past twenty years these organisms of low pathogenic potential have been found causing or have been associated with tuberculosis-like diseases in man. Runyon (Timpe and Runyon 1954 and Runyon 1965) has classified these organisms into four groups on the basis of pigment production and growth rate. Runyon's first group are the photochromogens which form pigment on exposure to light, eg. M. kansasii, which also happens to be pathogenic for man. The second group, the scotochromogens, form pigment in light or in the dark, on all types of media at all stages of growth. M. flavescens (not an official name) is a scotochromogen not known to be associated with pathogenicity for humans. Group three, the nonchromogens, are a group of heterogeneous slow growers containing both pathogenic and nonpathogenic bacteria. The Battey-avium complex is a pathogenic member of the third group. The fourth group known as the rapid-growers, is mainly composed of saprophytes with the exception of M. fortuitum which is pathogenic for man. Other workers have used biochemical tests such as the niacin test, nitrate reduction, the catalase test, Tween 80 hydrolysis, the aryl sulfatase test and tellurite reduction

(Somers and Russel 1967);pyrolysis-gas-liquid chromatography (Reiner and Kubica 1969); acrylamide gel electrophoresis (Wright and Mallman 1966 and Affronti et al. 1972) and base ratio analysis (Gross et al. 1970 and Slosárek 1970) to try to work out a system of relationships between members of Runyon's grouping and M. tuberculosis. Among the pathogenic mycobacteria there are some such as M. tuberculosis and M. bovis which are highly pathogenic but are not obligate parasites while others such as M. leprae and M. lepraemurium are not extremely pathogenic but may at present be considered as obligate parasites as no way has yet been found of growing them successfully under completely in vitro conditions. Recently (Dept. Nat. Health and Welfare 1972) the existence of an acid-fast bacterium causing a leprosy-like disease in northern Manitoba has been reported. This leads one to speculate on the possibility of pathogenic parasitic forms of mycobacteria arising from the saprophytic soil form by mutation as has been discussed before with reference to M. tuberculosis (Hobby 1967). So far no attempt has been made to work out a possible relationship between M. tuberculosis and M. leprae or M. leprae and M. lepraemurium.

The rat leprosy bacillus averages 1.5 um in length, 0.3 to 0.5 um in width and has rounded ends. Inside host cells the bacilli are distributed at random and arrange themselves around the nucleus; by contrast human leprosy bacilli are found in cigar-like bundles and displace the nucleus (Wilson and Miles 1964). M. lepraemurium like M. leprae is a parasite of mesodermal cells which lives on the same substances as the host cell but not at the expense of the host cell. The rat leprosy bacillus is weakly gram positive and often appears

granular when stained. M. lepraemurium stains well with the Ziehl-Neelsen and auramine-rhodamine methods. Marchoux and Sorel (1912a) reported that the organism resists decolorization as well as or better than the human leprosy bacillus; 10% nitric acid, 25% sulphuric acid, and 3% hydrochloric acid in alcohol did not decolorize it quickly. Lowe (1937) reported the organisms resistant to decolorization with 5% sulphuric acid and 90% alcohol.

The structure of M. lepraemurium has been examined with the electron microscope since the late 1950s. Chapman et al. (1959) examined M. lepraemurium-infected mouse spleen and estimated the bacterial cells walls to be 150A° thick. They also reported the appearance of low density areas with granular threads of denser material assumed to be nuclear inclusions, membrane limited inclusions and low density areas different from the nuclear areas. Rees et al. (1960) reported the appearance of denser bodies in an essentially uniform cytoplasm in the rat leprosy bacillus. They believed that with the use of the electron microscope they could distinguish between normal and degenerate (dead) forms of the bacillus. Imaeda and Ogura (1963) showed good sections of larger electron dense bodies in M. lepraemurium but did not comment on them. In 1969 Okada examined the sedimentation coefficients of ribosomes of murine leprosy bacilli and found them to be 100 S, 70 to 80 S, 50 S and 30 S, and in addition 20 to 23 S particles were also found. Negatively-stained ribosomes were observed with the electron microscope and some 100 S particles were found to be composed of two particles. Higher magnification of 50 S ribosomes showed particulate subunits and filamentous structures. The amount of ribosomal material in murine leprosy bacilli was found

to be less than that found in E. coli. For 1 g of bacilli (dry weight), E. coli yielded 306.6 mg of ribosomal material and M. lepraemurium 2.6 mg. No further work on the ribosomal content of M. lepraemurium has been reported.

Whitehouse et al. (1971) have examined the rat leprosy bacillus with the use of the electron microscope and have reported the existence of two types of inclusions. One type is a small, electron-dense particle which confirms Chapman's earlier observation. These particles have a mean diameter of 30 nm (range 10 to 60 nm), occur in clusters and are thought to contain metaphosphates. The other types of inclusion is larger and less electron dense. This type has a mean diameter of 175 nm, is a fine granular homogeneous mass and is presumed to be partly lipid. They also found elaborate membrane systems in the cytoplasm. In some cases these systems were shown to be invaginations of the cell membranes.

Metabolism of Rat Leprosy

The rat leprosy bacillus cannot be grown successfully in vitro, consequently it is necessary to maintain the bacilli by serial passage in animals. This requirement has lead to study of the viability and metabolism of the organism. The viability of the rat leprosy bacillus has been studied extensively. M. lepraemurium retains its viability well in liquid media and freeze-drying of M. lepraemurium in saline and other solutions has been found to be an excellent method for maintaining viability (Sato and Nishimura 1967). Nakayama and Hayashi (1958) found that 30 minutes of sonication (at 9.5 KC, 200 W) destroyed M. lepraemurium cells. Sato and Nishimura (1967) found that M. lepraemurium loses its activity after 2 to 5 hours of sonication

using the Kubo Ultrasonic apparatus and is partially destroyed in 10 to 30 minutes. Toda (as quoted by Sato and Nishimura) found the organisms were killed by heating at 100°C for 2 minutes or at 56°C for 30 minutes. Marchoux and Sorel (1912a) state that heating at 60°C for 5 minutes does not kill the organism but it is killed when exposed for 15 minutes. Muir and Henderson (as quoted by Lowe 1937) reported complete death after 25 minutes at 60°C.

The metabolism of murine leprosy bacilli has been studied since 1938 when Prudhomme (as quoted by Hanks 1951b) showed that o-cresol-indol-2,6-dichlorophenol could be reduced by washed suspensions of M. lepraemurium after incubation for one or more days. Reduction did not occur if the M. leparaemurium suspension had been damaged by heat or chemical agents. Using a method based on the reduction of tetrazolium violet, Hanks (1951b,c) was able to measure the transfer capacity of murine leprosy bacilli under anaerobic conditions in the absence of substrate. Since mycobacteria are aerobic and preferentially transfer hydrogen through their cytochrome system, strict anaerobic conditions are required for hydrogen transfer to an artificial acceptor system. Hanks found that the hydrogen transfer capacity of incubated suspensions was an indication of the relative levels of viability which existed among aliquots of a suspension.

Gray (1952) found that M. lepraemurium from rat testicles showed endogenous respiration. In a 4 to 5 hour test period the bacilli were unable to oxidize 50 simple susbstrates including the intermediates of glycolysis, members of the tricarboxylic acid cycle, and amino acids and substances used by other mycobacteria as energy or cofactor sources.

Heat stable yeast and liver fractions stimulated endogenous respiration but failed to induce oxidation of substrates. The constant response of M. lepraemurium to yeast and liver extracts suggested that the extracts were replacing certain low molecular weight cofactors lost in washing. Endogenous respiration of the bacilli was damaged by anaerobic storage and it is thought that the damage occurs in the terminal respiratory system.

Kusaka (1958a) found that heated rat liver mitochondria extract stimulated succinate oxidation of the bacilli under aerobic and anaerobic conditions. In addition the murine leprosy bacilli were found to oxidize L-glutamate, L-glycine and palmitate under anaerobic conditions. While examining the endogenous metabolism of murine leprosy bacilli under anaerobic conditions Kusaka (1958b) found that it was stimulated by the addition of pyridine co-enzymes. Flavin co-enzymes and pyridine co-enzymes seemed to stimulate succinate oxidation by the bacilli. Succinoxidase, glucose-6-phosphate dehydrogenase, and aldolase were found in the bacillary extract albeit the enzyme activities were low.

Tamemasa and Tsutsumi (1958) tested the dehyrogenase activities of M. lepraemurium from subcutaneous rat tissue (under anaerobic conditions) on a total of more than 80 compounds including sugars, amino acids, alcohols, aldehydes, and amines, using tetrazolium violet as the hydrogen acceptor. O-aminophenol, indol, skatole and some of the higher fatty acids such as lauric and myristic acid were the only compounds utilized. They found that M. tuberculosis (H37Rv) and BCG also dehydrogenate o-aminophenol, fatty acids and indole. This may indicate that M. lepraemurium and M. tuberculosis have similar

nutritional requirements and possible similar metabolic cycles. It is possible that our inability to grow M. leprae and M. lepraemurium rests on the fact that their nutritional requirements have not been met.

Growth of Rat Leprosy in vitro and in Tissue Culture

Since the discovery of M. lepraemurium in 1903 many attempts have been made to grow the bacillus in vitro. Wolbach (1914) reviewed the literature from 1903 to 1914 and concluded that the rat leprosy bacillus had not been cultured in vitro. Many attempts were made between 1914 and 1937 when Lowe reviewed reports of successful cultivation of M. lepraemurium. Lowe failed to grow the rat leprosy bacillus in vitro following the methods of Clegg, Bayon, Walker, Sweeny, Uchida, Ota and Asami, Cilento and North, Lowenstein, Soule and McKinely and McKinely and Verder. From the description given it seems likely that there was at least one or more contaminant for every report of successful growth.

In 1960 Hart and Valentine reported the elongation of M. lepraemurium in a cell free medium. In 1963 in a more complete report they obtained doubling of the bacilli in the generation time observed in host cells and quadrupling before the bacilli became degenerate two months after the start of the work. The pH range for optimum elongation is rather slim :pH 6.0 to 6.4 and there was almost no elongation of pH 7.2.

In 1970 Wong et al. reported elongation and branching of M. lepraemurium when kept in a Hart and Valentine's medium supplemented with 0.5% Tween 80 and catalase and incubated in air plus 5% carbon dioxide.

In 1970 Nakamura et al. reported on the effects of the depth of culture medium on the elongation of M. lepraemurium in a modified Hart-Valentine medium. Fresh M. lepraemurium used shortly after harvest elongated consistently. When stored for 50 days (at -20°C) M. lepraemurium elongated when inoculated into large volumes of medium and when stored for 86 days no elongation occurred. When fresh material was inoculated into 12 ml of medium an average of 2.2 um to 6.2 um of elongation was seen. In 6 ml of medium the average elongation was 3.5 um. In general the greatest elongation occurred at pH 6.0.

The first successful report of growth of M. lepraemurium in vivo was made by Zinsser and Carey in 1912. They observed multiplication of the bacilli in two of a number of young rat spleen explants infected with M. lepraemurium in vitro and incubated for two weeks. In 1937 Lowe reported no definite increase in the number of murine leprosy bacilli in rat tissue infected in vivo and kept alive for two weeks.

In 1957 Wong reported limited multiplication of M. lepraemurium in M. lepraemurium-infected spleen explants observed for two months. In 1958 Rees and Wong reported an increase in numbers of cells incubated without the addition of drugs and a relatively stable number of acid-fast bacilli in streptomycin/isoniazid treated cell cultures. Wallace et al. (1958b) reported significant multiplication of M. lepraemurium in in vivo infected explanted mouse spleen cells and in vitro infected hydrocortisone-treated L strain mouse fibrocytes. The multiplication rate during an 8 to 10 day period was similar to the maximal rate observed in susceptible animals.

In 1958 Garbutt announced multiplication of M. lepraemurium in

drug free cultures of 14pf fibrocytes. Increases of 5.5, 4.1, and 3.1 times in bacterial population were obtained in a forty day time period. This represented a significantly greater increase in multiplication than those obtained in spleen cultures or in hydrocortisone treated L cells. In a more complete report in 1962 Garbutt et al. reported more continuous intracellular growth of M. lepraemurium by repeatedly subculturing the infected cells. Multiplication of the bacteria was maintained only when a high proportion (50-75%) of the infected cells were transferred at each subculture. One experiment, continued for 156 days, showed an increase in bacilli equivalent to eight generations and the bacilli were still infective for animals.

Meanwhile Chang (1961) obtained growth of M. lepraemurium in in vitro infected mouse macrophages. In a more complete report (1967) Chang et al. state that the optimal mouse macrophage maintenance medium was composed of 40% horse serum, 50% NCTC 109 and 10% of a 1:5 dilution of beef embryo extracts supplemented with liver extract and ferric nitrate. Multiplication of the bacilli was observed in 1 week and maximal growth in 6 to 7 weeks. Subcultures achieved rapid and uniform growth. The bacilli retained their infectivity for mice and showed no growth in bacteriological media. Recently Rightsel (1971) was successful in growing M. lepraemurium in cell-impermeable diffusion chambers in animals. Chambers kept in a susceptible host showed better growth than those in a nonsusceptible host such as a guinea pig.

In electron-microscope studies of rat fibroblasts infected with M. lepraemurium Brown and Draper (1970) noticed that the bacteria were

often surrounded by lysosomal material. In electron-microscope studies on liver and spleen of M. lepraemurium infected mice Draper and Rees (1970) observed fibrillar structures closely associated with the cell wall of M. lepraemurium. The possibility that such structures might resemble Wax D of M. bovis or might be an inert mechanical protective device for M. lepraemurium was discussed.

Transmission and Pathology of Rat Leprosy

In rats or mice the natural disease is first characterized by lymphadenopathy of the inguinal, axillary and cervical regions (Marchoux and Sorel 1912a) and then by involvement of the skin and underlying musculature. The glandular or early form affects the lymph nodes so that they become enlarged and firm. The lymph nodes show large accumulations of acid-fast bacilli and multinucleate giant cells may also be present. In the musculocutaneous form the lesions frequently undergo superficial necrosis and ulceration and alopecia may also be present. In the dermis histiocytes may advance from the subcutis into the adjacent musculature infiltrating the muscle bundles and causing pressure atrophy. Dissemination of the bacilli to the spleen and liver is infrequent.

Natural transmission of the disease in rats is thought to occur through the skin by bites, scrapes and scratches.

Marchoux et al. (1935) investigated the infection of rats with M. lepraemurium via the eye and found that it is possible to infect rats by putting a drop of an emulsion rich in M. lepraemurium on the eye. A chancre did not form at the point of inoculation. Marchoux et al. found that the infection of the eyeball which frequently occurs

in human leprosy is not the immediate result of infection but the consequence of a long standing infection.

Marchoux and Chorine (1938) found that 5 rat leprosy bacilli injected subcutaneously into a rat would cause leproma formation in ten months and that lepromas so formed were identical to those formed in 4 to 5 months by the injection of large numbers of rat leprosy bacilli. Hanks and Backerman (1950) studied tissue sites favourable for the development of murine leprosy transmitted experimentally in rats and mice and found that the testis was the best site, followed by the subcutis, the cutis, the eye, and the brain. They also found that the "Swiss" mouse strain was more susceptible to infection than the Wistar rat strain. Badger and Fite (1940) examined the virulence of three strains of rat leprosy, the Hawaiian, the Florida, and the San Francisco strains. The disease produced by these strains were grossly similar. However the Hawaiian strain appeared to have a predilection for subcutaneous tissue. Appearance of gross lesions following inoculation with M. lepraemurium was used as the criterion for virulence. Lesions consistently appeared earlier with the Hawaiian strain and the Florida strain than with the San Francisco strain. Further evidence suggests that the Hawaiian strain is more virulent than the Florida strain.

Rat leprosy has been transmitted to monkeys. The guinea pig and the mongoose have no native resistance to rat leprosy but develop a high degree of resistance following infection. Balfour-Jones (1937) transmitted rat leprosy to the golden hamster (Cricetus auratus) by subcutaneous and intraperitoneal injections. The lesions were most

often found in the liver and spleen, occasionally the kidneys and lungs were involved and interstitial nephritis was noted.

The Immunological Relationship Between Human and Rat Leprosy

The pathology of human and murine leprosy is quite different since human leprosy has a predilection for the peripheral nerves and the skin, whereas rat leprosy involves the superficial lymph nodes and only rarely infects the nerves. However the immunological processes occurring in both diseases may not be dissimilar and both cases will be discussed.

The immunology of leprosy has been studied since the early part of this century when Mitsuda skin-tested lepers with a suspension of autoclaved M. leprae in human tissue but the immunological basis of the disease spectrum seen in leprosy has not yet been elucidated. Research on leprosy is hampered by the lack of a specific antigen due to the fact that M. leprae cannot yet be grown in vitro. The antigen presently used is called lepromin and consists of a partially purified suspension of heat-killed M. leprae in human or animal tissue. Lepromin has been purified by extraction with chloroform and ether but most leprologists prefer to use the crude lepromin (Turk 1971).

Suspensions of lepromin are standardized to contain approximately 1.6×10^8 acid-fast bacilli/ml (Turk 1971) and 0.1 ml of the suspension is injected intradermally into the forearm. The skin reaction to lepromin is biphasic. The first phase is a typical delayed-type hypersensitivity reaction called the Fernandez reaction and is read between 24 and 48 hours. The second phase is known as the Mitsuda reaction; it appears between the second and fourth weeks and is

usually read at three weeks. The reaction is an indurated skin nodule and is considered positive if it is greater than 4 mm in diameter. The nodule will ulcerate in strong reactions. Responses to both the initial and late reactions are considered specific for antigens of M. leprae and are parallel in most patients. Most leprologists use the late reaction as an indication of lepromin positivity. The lepromin skin test may be difficult to interpret in normal subjects because of the contaminating tissue elements, the cross reactivity of M. leprae with other mycobacteria and the immunogenic potential of the intradermal injection (Shephard and Saitz 1967). The lepromin reaction can not be used as an index of infection with M. leprae. However the ability of a patient to respond to lepromin with the late or Mitsuda reaction parallels the patients' ability to eliminate M. leprae from the body. The reason for this anergy has not yet been elucidated. A patient who is lepromin negative does not have the capacity to eliminate M. leprae from the body and it is quite likely that the patient would develop the lepromatous form of leprosy.

The ability of leprosy patients to produce a cell-mediated immune response has been studied quite intensely in the last few years. Patients with lepromatous leprosy appear to be less capable than patients with tuberculoid leprosy to respond to chemical skin testing agents. Waldorf (1966) found that patients with lepromatous leprosy did not respond to 2,4-dinitrochlorobenzene (DNCB) a specific contact allergen which sensitized 95% of the controls. Bullock (1968a) found that only 23 of 54 patients with lepromatous leprosy responded to picryl chloride which sensitized 28 out of 30 controls. More recently Saha and Mittal (1971) skin tested leprous patients with tuberculin, DNCB and allogenic

lymphocytes. Their results indicated that leprosy is associated with depression of the delayed allergic response, and the depression is more severe in lepromatous cases than in tuberculoid cases. The dose and potency of the antigen were found to be important in inducing a delayed-type response. Lymphocyte transformation in cultures is considered an *in vitro* reflection of immunological capacity and has been associated with delayed-type hypersensitivity and homograft histoincompatibility. The transformation of small lymphocytes into large blast-like cells capable of mitosis can be induced by stimulants such as phytohemagglutin (PHA) and streptolysin O in lymphocytes from normal persons. Other stimulants such as tuberculin will only stimulate lymphocytes of sensitized persons. Rodriguez *et al.* (1968) found that 32% of lepromatous patients studied had lymphocyte reactivity to PHA known as blast formation. Dierks and Shepard (1968) found that patients with active lepromatous leprosy had markedly depressed lymphocyte response to PHA and mycobacterial antigens. Sheagren *et al.* (1969) found patients with lepromatous leprosy had impairment of blast formation when streptolysin O was used as a stimulating agent. There was no difference however in the mean of the percent of transformed cells in the control group and the lepromatous group when the lymphocytes were stimulated with PHA. Wong *et al.* (1971) measured the effect of PHA on the incorporation of radioactive thymidine and uridine in lymphocytes from patients with leprosy. They found that there was a depression in the response of untreated lepromatous cases of leprosy, and to a lesser degree in cases of tuberculoid leprosy.

Rodriguez et al. (1969) were able to transfer lepromin reactivity to lepromatous patients using leukocytes from sensitized donors. Of 13 lepromatous patients (all previously tuberculin, lepromin and histoplasmin negative) 4 responded with a positive Fernandez reaction, three to tuberculin and one to both antigens. In 2 patients the reactivity persisted almost a year. This suggests that the cell-mediated immune deficiency present in leprosy patients could be a primary or central failure since the efferent arc of the immune response is still capable of action.

Godal et al. (1971) studied the blastogenic response of leukocyte culture from patients with leprosy and concluded that lepromatous patients lack circulating lymphocytes responding to M. leprae. Turk and Waters (1971) examined the histology of lymph nodes from persons with leprosy. Lymph nodes from patients with lepromatous leprosy had paracortical areas infiltrated with undifferentiated cells of the histiocyte-macrophage series which failed to eliminate mycobacteria. As resistance to infection was increased the histiocytes became epithelioid and small lymphocytes appeared in the paracortical areas. In polar tuberculoid leprosy the paracortical areas of the lymph nodes were well developed and populated with lymphocytes and immunoblasts.

Recently Bullock et al. (1972) were able to transfer lepromin sensitivity to 6 of 9 lepromatous patients using sensitized leukocytes or transfer factor from sensitized leukocytes. This partial success in transferring cell-mediated immunity to lepromatous patients may reflect a deficiency of their thymus-dependent-lymphocyte function.

These discrepancies in lymphocyte response of lepromatous patients

were not satisfactorily explained until Bullock and Fasal (1971) found that the type of serum used in which to incubate the lymphocytes affected their response. Bullock and Fasal measured PHA and antigen-induced DNA synthesis in leukocyte cultures of patients with leprosy, using tritiated thymidine incorporation as a measure of DNA synthesis. They found a factor inhibitory to antigen-induced DNA synthesis by sensitized leukocytes in the plasma of some patients with lepromatous leprosy. The depressor factor was non-dialyzable, stable after prolonged storage at 20°C, resistant to heating at 56°C and its activity was lost in low dilutions. The authors suggested that the in vitro demonstration of impaired cellular immune response may reflect a primary cellular defect, the depressive effect of a humoral factor, or both. Nelson et al. (1971) studied PHA-induced lymphocyte transformation in leprosy and found that depressive humoral factors are substantially important in influencing lymphocyte behaviour.

Katz et al. (1971) found that lymphocytes from patients with lepromatous leprosy failed to produce or produced only small amounts of a migration inhibitory factor (MIF) to lepromin in vitro while lymphocytes of patients with tuberculoid leprosy responded with greater MIF production. Under certain in vitro conditions sensitized lymphocytes upon interaction with antigen, elaborate a migration inhibitory factor which will inhibit migration of normal macrophages. MIF production is immunologically specific, and a direct correlation between the result of this test and the delayed-type hypersensitive skin reaction are thought to exist. Katz's results lend support to this theory.

Patients with tuberculoidleprosy have been found to have normal immunoglobulin levels while patients with lepromatous leprosy have raised immunoglobulin levels. Turk (1971) reported on three studies of patients with lepromatous leprosy. In all three studies IgG and IgA levels were above normal. Sheagren et al. (1969) found that leprosy patients without erythema nodosum leprosum had normal complement levels. Rees et al. (1965) found that sera from all patients with lepromatous leprosy in his study contained antibodies which reacted strongly causing precipitation in gel in a dilution of between 1/80 and 1/100 with cultural filtrates of M. tuberculosis. Antibodies were also detected in lepromatous lepers against M. leprae and other mycobacteria which cross reacted, e.g. M. balnei, M. marianum and M. phlei. Norlin et al. (1966) found precipitins against antigens from a number of common mycobacteria including M. kansasii and M. smegmatis in the sera of all 19 lepromatous patients studied, in half of the bacillary positive tuberculoid leprosy patients and not in the sera of 22 bacillary negative patients.

The bacillary load in leprosy patients and the amount of antibody present at any time to mycobacterial antigens seem to be directly related. Rees et al. (1965) and Norlin et al. (1966) found antimycobacterial antibodies only in patients with M. leprae bacilli in their tissues. Moreover Rees observed a fall in the titer over a period of 24 months of antileprosy treatment while the bacillary load was falling.

From recent work on leprosy it seems likely that patients with lepromatous leprosy have a defect in their cell-mediated immune apparatus. This defect would account for loss of capacity to sensitize patients to express delayed-type hypersensitivity responses. The

immunotherapy given by Bullock (1972) appears to restore lepromin sensitivity to previously anergic patients with lepromatous leprosy. This seems to suggest that these patients have some capacity to react to M. leprae antigens with a cell-mediated response. Therefore the defect might lie in antigen recognition or in antigen processing. Turk's work with lymph nodes suggests that lepromatous patients have a deficiency of small lymphocytes in the paracortical regions of the lymph nodes. However the depressive effect of humoral factors from lepromatous patients in lymphocyte blast formation has been noted, as well as the increased levels of antibody in patients with lepromatous leprosy. It appears that humoral antibody formation is altered in lepromatous leprosy and this suggests either derepression of antibody forming cells or perhaps the loss of control of antibody forming cells because of a thymus-derived lymphocyte deficiency.

One of the more interesting theories on susceptibility to leprosy is that of genetic predisposition. The fact that lepromatous leprosy appears to affect certain families, that it occurs more often in males than in females (the male-to-female ratio from 1.6/1 to 2/1) and that it seems to occur in no more than 2% of the population (usually 0.5 to 1.0%) regardless of the living conditions is suggestive of genetic predisposition to leprosy (WHO 1966). In view of the evidence presented on the immunological defects of patients with leprosy it would be reasonable to suggest a genetically determined lack of immunological competence. Recently the relationship between the presence of histocompatibility antigens and disease states such as Hodgkin's disease and multiple myeloma has been under exploration. Histocompatibility or transplantation antigens (termed HL-A antigens) are genetically

determined antigens on the cell surface which play an important role in human allograft rejection. The genes that determine the response to transplanted tissue are termed histocompatibility or HL-A genes. In man this gene has many alleles and these alleles have been found to have an unexpected distribution in some disease states compared to a normal population. The W18 (4c') allele has been found to have a significantly higher frequency in patients with Hodgkin's disease (Bertrams 1972). Diseases most likely to reveal HL-A correlations are those involving autoimmunity or those of suspected viral etiology (Finkelstein 1972). Since leprosy is a disease which is associated with an upset of the immune response system it would be interesting to investigate the HL-A types of patients with leprosy. It is possible that the immune response to leprosy is associated with the HL-A region of the human genome.

The immunology of rat leprosy has been studied since 1908 when Wherry did a very crude agglutination test using 24 hour old serum from 3 cases of human leprosy as the source of antibody and centrifuged, washed suspensions of rat leprosy bacilli as the antigen. There was no agglutination in any sera after one hour at room temperature and only one when examined 24 hours later. The author states that valid criticism of this experiment can be made and that it does not prove any relationship between human and rat leprosy. In 1909 Wherry attempted to vaccinate rats against rat leprosy using a suspension of dead bacilli given after the infecting dose of live M. lepraemurium. This program failed to protect the rats from infection. However, two rats which received the vaccination 21 and 7 days before the infecting dose showed marked delay of the disease when compared to the controls. Wherry also

reported that chloroform treatment of aqueous emulsions of rat leprosy bacilli freed the murine leprosy bacilli from the tissue elements.

In 1941 using a sonicated preparation of murine lepromin to skin test lepers Kitano et al. found that the sonicated preparation induced a reaction but that it was much weaker than the unsonicated preparation. The intensity of the reaction produced was unrelated to the clinical status of the patient and the reaction of the patient to the Mitsuda type human lepromin.

Markianos (as quoted by Lowe 1937) gave rats preliminary injections with defatted M. lepraemurium and found marked retardation of the development of the disease after subsequent inoculation. Lowe (1937 unpublished) found no evidence of the production of immunity in rats injected with heat-killed bacteria.

Muir and Henderson (as quoted by Lowe 1937) could not produce immunity in BCG-injected rats subsequently inoculated with murine leprosy bacilli. They also fed experimentally infected rats with decomposed protein diets deficient in vitamins A and B and noted no increase in the rate of the development of the disease.

Wallace (1958a) claimed to have transferred sensitivity to M. lepraemurium to guinea pigs using systemic injections of spleen homogenate or peritoneal exudate cells from sensitized rats. He also claimed that intracutaneous injections of unsensitized cells suspended in Old Tuberculin (OT) or M. lepraemurium antigen transferred responses to OT as well as M. lepraemurium antigen. The ability to transfer sensitivity was destroyed by heating the sensitized cells at 56°C for 30 minutes. Cellular reactivity of M. lepraemurium-doubly challenged

rats was greater than that of M. lepraemurium-singly challenged animals. This finding was in keeping with the relative resistance of the two groups of animals.

It was demonstrated by Rees et al. (1962 and 1965) that in tissue culture intracellular M. lepraemurium cells release a soluble polysaccharide antigen that can readily escape from the host cells into the culture medium. This polysaccharide reacted strongly with rabbit-anti-M. lepraemurium serum and less strongly with rabbit anti-M. tuberculosis serum (Rees et al. 1962). This observation could account for the presence of precipitating antibodies in the sera of patients with lepromatous leprosy and the finding that antibodies in the sera of patients with lepromatous leprosy react predominantly with mycobacterial polysaccharide (Rees et al. 1965).

Reyes-Gomez et al. (1968) isolated an antigenic polysaccharide from M. lepraemurium-infected tissue. The polysaccharide was subjected to agar precipitation in Ouchterlony's agar plate and gave three bands with serum from a lepromatous patient. One band appeared to be immunologically identical to Pol INb (a polymer of D-arabofuranose and D-galactopyranose in a molar ration of 3:1) and the other two bands showed partial identity with Poly INb. Poly INb is a group specific polysaccharide present in mycobacteria, Nocardia, and tissues rich in M. leprae.

Glasgow and Bullock (1972) recently found that M. lepraemurium-infected CF_1 mice had impaired interferon production in response to Chikungunya virus but that interferon production to the synthetic polynucleotide, polyinosinic: polycytidylic acid, was normal and was

enhanced following E. coli OIII B_4 exposure. It is suggested that intracellular infection with M. lepraemurium may have a significant role in determining host reactions to subsequent viral infections.

Ptak et al. (1970) found impairment of cell-mediated immune responses to skin sensitizers and homografts, but no detectable impairment of humoral antibody function in mice with murine leprosy. The suppression of cell-mediated immunity was not complete since the mice could reject skin homografts and respond to higher doses of oxazolone. The M. lepraemurium-infected mice showed histological changes in the thymus and lymph nodes. In the thymus there was a progressive depletion of lymphoid cells with replacement by macrophages, many of which contained bacilli. The lymph nodes showed depletion of paracortical immunoblasts and accumulation of macrophages. The authors believe the defect in cell-mediated immunity to be secondary to massive infection with M. lepraemurium.

At present the knowledge of the immunological processes in the animal body after infection with M. lepraemurium is slight. Some animals such as the mongoose and guinea pig are resistant to the disease while other animals such as the rat and mouse appear to be almost invariably susceptible to experimental infection. It is generally thought that protection to murine leprosy is a cellular mechanism. In the human situation cellular immunity is important as a protective device although the effects of humoral immunity complicate the total immune response. Thus one can conjecture that the inability of rats and mice to resist rat leprosy is due to a failure of their cell-mediated immune system. However if murine leprosy parallels

human leprosy then one would also expect a high level of antibody in lepromatous rats. The possibility exists that M. lepraemurium modifies the system in such a way as to prevent the recognition of the bacteria as foreign.

MATERIALS AND METHODS

1. Reagents

All reagents and materials used were of reagent grade and were obtained from commercial suppliers.

Intermediate test strength tuberculin (purified protein derivative) was obtained from Parke-Davis.

Freund's Complete Adjuvant and Freund's Incomplete Adjuvant were obtained from Difco Laboratories.

B grade Pronase (activity 45,000 P.U.K./g) was obtained from Calbiochem. Deoxyribonuclease (B grade from pancreas, typical activity 42,480 Dornase units/mg) was also obtained from Calbiochem.

Ribonuclease (bovine pancreas type, activity 75 Kunitz Units/mg) was obtained from Sigma Chemical Company.

2. Organisms and Growth Conditions

The Hong Kong strain of *Mycobacterium lepraemurium* was kept in male albino rats. Young (two to three month old) unsensitized male albino rats were given 0.5 ml of a heavy suspension of *M. lepraemurium* in 1% albumin saline which contained 2.6×10^9 to 2.6×10^{10} bacilli/ml, subcutaneously into the left flank. The rats were observed for lepromas at monthly intervals. Leproma formation began at three to four months and was allowed to continue until the lump was approximately 5 cm in diameter; this usually happened at about six months after infection. The lepromas were harvested aseptically before they broke open and became contaminated. A suspension of *M. lepraemurium* was

prepared following the standard technique (Wong 1957) and the appropriate dilution of M. lepraemurium was injected into unsensitized male albino rats.

M. smegmatis TMC 1515 was provided by the U.S.-Japan Cooperative Medical Science Program-NIAID from the Trudeau Inst. Inc., Saranac Lake, New York. Stock cultures were maintained at -4°C and the organisms grown on Trypticase Soy agar.

3. Counting of M. lepraemurium

The method used to count the number of organisms in a suspension of M. lepaemurium is a modification of the pinhead method of Hanks (1964). The modified method used auramine-rhodamine (Truant 1962) for staining the organisms which were suspended in 1% albumin formalin saline. The rat leprosy bacilli to be counted were suspended in 1% albumin formalin saline and stamped onto glass slides using a standardized pinhead. The spots were air dried and formalin fixed. The spots were stained for 15 to 20 minutes at room temperature using Truant's auramine-rhodamine stain which is composed of the following:

auramine O C I 4100	1.5 g
rhodamine B C I 749	0.75 g
glycerol	75.0 ml
phenol	10.0 ml
distilled water	50.0 ml

The dyes were combined with the phenol and 25 ml of the water and mixed well. The water and glycerol were added and mixed well using a magnetic stirrer. The solution was clarified by filtration through glass wool. The spots were rinsed gently with tap water, decolorized

with acid alcohol (0.5% HCl in 70% ethanol) for 2 to 3 minutes, rinsed with tap water and air dried.

The slides were first examined under visible light and only spots with perfect edges were chosen for counting. An eyepiece which gave a square field and a 40 X objective were used for examining the slides using a fluorescent microscope with an ultraviolet light source. By using a standard technique it was possible to convert the number of organisms observed per microscope field to the concentration of organisms in the original sample.

4. Antigen Preparation

A. Mitsuda Type Murine Lepromin

In preparation of the Mitsuda type murine lepromin it is preferable to use lepromas from several animals. This lessens the chance of exclusion of important antigenic materials which may be absent in one leproma but present in another. Each leproma was swabbed and the swab used to inoculate blood agar plates incubated aerobically and anaerobically at 37°C for twenty-four hours. Absence of growth after twenty-four hours was taken to mean absence of contamination. Material from each leproma was also examined by a Ziehl-Neelsen and fluorochrome stain (Truant 1962) for the presence of acid-fast or fluorescent bacilli. Only growths with acid-fast bacilli present were used.

Contaminant-free lepromas from male albino rats infected with M. lepraemurium were trimmed of excess fat, hair and connective tissue. The lepromas were weighed and the weights recorded. The

lepromas were autoclaved at 121°C for 15 minutes and then ground in a mortar with normal saline and strained through sterile gauze to remove the larger tissue elements. The remaining suspension was adjusted so that it contained 1 g of original tissue per 10 ml of saline. The suspension was then dispensed in small amounts and 33% phenol was added to some of the aliquots to give a concentration of 0.5% phenol. The suspensions were re-autoclaved at 121°C for 15 minutes. Thus there were two types of murine lepromin: one containing 0.5% phenol and one without phenol.

The lepromin suspension was counted by the modified pinhead method and contained approximately 7×10^7 bacilli per ml.

B. Partially Purified Murine Lepromin

Four lepromas from standard laboratory strain male albino rats infected with the Hong Kong strain of M. lepraemurium and two lepromas from standard laboratory strain male albino rats infected with the Hawaiian strain of M. lepraemurium were treated as usual to prepare a suspension of M. lepraemurium.

A partially purified murine lepromin was prepared by sequentially digesting heated, ground, epidermis-free murine lepromin with DNAse, RNAse, and pronase following the method of Goihman-Yahr et al. (1969a) with some modifications. The modifications consisted of subjecting the murine lepromin suspension to short bursts of sonication (two minutes) to free the bacilli from the tissues, and in using phosphate buffered saline pH 7.2 with adjustment to the optimal pH of the enzyme being used with NaOH or HCl and the addition of $CaCl_2$ and $MgCl_2$ as required. The suspension was not filtered through Millipore membranes or

sterilized with ethylene oxide.

The treated murine lepromin was sonicated for 20 minutes using a medium sized probe on the Bronwill Biosonik III and spun at 10,000 x g in a Sorvall RC2-B for one hour and the supernatant collected. The supernatant was centrifuged at 144,000 x g in a Beckman L2-65B ultracentrifuge for one hour. The protein content of the final supernatant was determined by Lowry's (1951) method using crystalline bovine serum albumin as the reference protein. The final supernatant was called partially purified murine lepromin and was used to skin test guinea pigs.

C. Normal Rat Tissue Antigen

Several grams of normal rat tissue taken from the left flank area of two normal male albino rats were treated as if theycontained M. lepraemurium. A tissue suspension was prepared following the steps outlined in "Preparation of Mitsuda Type Murine Lepromin". The tissue preparations were divided into two and phenol added to one of them as outlined above.

D. Rat Leprosy Bacilli Extract

The preparation of the murine leprosy antigen followed the method outlined by Middlebrook and Dubos (1948) using a M. lepraemurium suspension (prepared as outlined earlier) as the antigen source.

5. Sensitization of Animals

A. For Lepromin Sensitivity Skin Tests

Five normal stock guinea pigs were injected intraperitoneally with 1 ml of a mixture of equal parts by volume of complete Freund's adjuvant and live M. lepraemurium (2 to 4 x 10^9 bacilli/ml). The guinea pigs were boosted with 0.5 ml of the same suspension one month later. Five other normal stock guinea pigs were treated by the same method except that incomplete Freund's adjuvant was used.

Two normal stock rats were each injected intraperitoneally with 0.5 ml of a mixture of equal parts of live M. lepraemurium (2 to 4 x 10^9 bacilli/ml) and complete Freund's adjuvant. One month after the initial sensitization the animals were given a booster dose of 0.25 ml of the same mixture. One week after the booster dose the animals were skin tested with lepromin without phenol and tuberculin both in the footpad and flank. The tuberculin was purified protein derivative (PPD) from Parke-Davis and was given in intermediate strength (0.0002 mg/0.1 ml).

B. For Preliminary Cell Transfer Work in Guinea Pigs

Five male strain 13 guinea pigs were each inoculated with 0.8 ml of a mixture of equal volumes of live M. lepraemurium and incomplete Freund's adjuvant. Each guinea pig received 2.3 x 10^6 bacilli. One month later two of the animals were skin tested with lepromin without phenol. Two days after the skin test the animals were killed and the spleen cells were used for cell transfer.

C. For Cell Transfer in Guinea Pigs and Rats

Five female strain 13 guinea pigs were each given 2 ml of a mixture of equal volumes of live M. lepraemurium suspended in incomplete Freund's adjuvant, intraperitoneally. Each guinea pig received approximately 1.77×10^8 bacilli. Six weeks later the animals were skin tested for sensitivity to murine lepromin and the results recorded.

Two male strain 2 guinea pigs each received a total of 1 ml of a mixture of equal parts by volume of live M. lepraemurium (1.2×10^9 bacilli) suspension and incomplete Freund's adjuvant. As much of this volume as possible was injected intradermally into the four footpads and the remainder injected intraperitoneally. Three other male strain 2 guinea pigs were injected as above except the total number of bacilli that each animal received was 1.2×10^8. One month later each guinea pig received a booster dose of 0.1 ml of murine lepromin without phenol into the top left flank. One month after the booster dose the sensitivity to the booster dose was recorded. One week later one of the sensitized guinea pigs was sacrificed and the region of its skin reaction and spleen were processed for histological examination.

Ten Fischer strain 344 rats were each injected intraperitoneally with 1 ml of a mixture containing equal parts by volume of incomplete Freund's adjuvant and live M. lepraemurium (each animal received a total of 1.76×10^9 bacilli).

D. For Serology and Partially Purified Murine Lepromin Skin Testing

A male albino rat and two normal stock guinea pigs were sensitized with live M. lepraemurium for future use in antibody testing. The rat was injected subcutaneously in the flank with 0.25 ml of a suspension of rat leproma containing 5×10^8 to 1×10^9 live M. lepraemurium. The rat developed a small, unbroken leproma indicating that it had rat leprosy but was not yet severely ill.

Two normal stock guinea pigs were injected intraperitoneally with 1 ml of a mixture of equal parts by volume of M. lepraemurium (2 to 4×10^9 organisms/ml) and complete Freund's adjuvant. Another guinea pig was treated identically except incomplete Freund's was used in place of the complete form. The guinea pigs were given booster injections one month later with 0.5 ml of live M. lepraemurium (2 to 4×10^9 organisms/ml) and the appropriate adjuvant mixed as above. The guinea pigs were bled two weeks after the booster dose.

The animals used in skin testing were sensitized indirectly. One strain 13 guinea pig was given 1×10^8 heat-treated (at 60°C for 30 minutes) M. lepraemurium-sensitized syngeneic spleen cells intracardially and skin tested five months later. Two strain 13 guinea pigs were each given 1.5×10^6 M. lepraemurium-sensitized syngeneic spleen cells intracardially and were also skin tested five months later.

6. Skin Testing

A. Skin Testing of Normal Stock Guinea Pigs

Two of the guinea pigs treated with M. lepraemurium and complete Freund's adjuvant were skin tested one week after the booster dose

(or five weeks after the initial sensitization) with 0.1 ml of lepromin in the flank and footpad and 0.1 ml of tuberculin (PPD) (intermediate test strength, 0.0002 mg/0.1 ml) in the other flank and footpad. Two other of the M. lepraemurium-complete Freund's adjuvant-treated guinea pigs were skin tested two months after the booster dose (or three months after the initial sensitization) following the above procedure. Two of the M. lepraemurium-incomplete Freund's adjuvant-treated guinea pigs were skin tested one week after their booster dose (or five weeks after the initial sensitization) following the above procedure.

B. Cell Transfer Skin Tests

The skin tests were performed by intradermal injection of 0.1 ml of the antigen into the guinea pigs. The reactions were observed 24 hours later and monitored at approximately daily intervals for one month and the diameters of induration were recorded. The cell transfer donors were skin tested with the Mitsuda type murine lepromin without phenol. The recipients of the cell transfers were skin tested with both types of murine lepromin and both types of normal rat tissue antigen. The diameter of induration obtained with normal rat tissue antigen was subtracted from the murine lepromin antigen reading and the difference was noted as the "corrected reading". The same was done for the phenol-containing antigens.

C. Partially Purified Murine Lepromin Skin Testing

The animals were skin tested intradermally with 0.1 ml of the partially purified murine lepromin suspension containing 100 ug of protein per ml, into the center of the back. Three sensitized strain 13 guinea pigs, one unsensitized strain 13 guinea pig and two unsensi-

tized strain 2 guinea pigs were skin tested and the reactions were followed for ten days.

7. Cell Transfer

A. Spleen Cell Preparation

The spleens were removed aseptically from the guinea pigs and the fat and connective tissue removed. They were then minced in minimal Eagle's medium (MEM) supplemented with 10% fetal calf serum. The pieces of spleen were mashed through a sterile sieve using a sterile glass syringe plunger. The suspension was transferred to a sterile glass test-tube and kept on ice for 10 minutes to sediment the larger tissue particles. The cell suspension was removed and centrifuged at medium speed on a clinical centrifuge for 10 minutes. The cell pellet was resuspended in a small amount of MEM with 10% fetal calf serum (approximately 0.5 ml per spleen). A small aliquot of cells was removed for counting. The aliquot of the spleen cell suspension was resuspended in 3% acetic acid to lyse the red blood cells. Dilutions of the aliquot were counted using a haemocytometer. The eosin exclusion test for viability was carried out to determine the viability of the strain 13 guinea pig cells to be transferred and again the cells were counted using a haemocytometer.

B. Peritoneal Cell Preparation

Twenty to twenty-five mls of Hanks Basal Salt Suspension was injected intraperitoneally into each animal a few minutes before sacrificing them. The peritoneal fluid was removed as aseptically as possible and any exudate containing a visible trace of blood was

discarded. The exudates were pooled, washed three times with fresh Hank's solution and twice with sterile saline. The cells were resuspended in MEM plus 10% fetal calf serum. An aliquot of cells was diluted in 3% acetic acid and counted using a haemocytometer.

C. Preliminary Cell Transfer Work

Two male strain 13 guinea pigs were each given intracardially 0.1 ml of the sensitized spleen cell suspension (1.5×10^7 cells) prepared as outlined in the methods section on cell transfer. One month later the animals were skin tested with tuberculin (of strength previously mentioned), lepromin with phenol and lepromin without phenol.

D. Cell Transfers in Strain 13 and Strain 2 Guinea Pigs

Three strain 13 guinea pigs each received 1×10^8 live M. lepraemurium-sensitized syngeneic spleen cellsintracardially. Three other strain 13 guinea pigs each received 1×10^8 heat-treated (at 60°C for 30 minutes) sensitized syngeneic spleen cells. The guinea pigs were skin tested six days after transfer with murine lepromin with and without phenol and normal rat tissue antigen with and without phenol, and their reactions were followed for over a month. In addition, four unsensitized strain 13 guinea pigs were skin tested with the same antigen and their reactions were also monitored.

Four strain 2 guinea pigs each received an intracardiac injection of 1.15×10^8 M. lepraemurium-sensitized syngeneic spleen cells. Three strain 2 guinea pigs each received 1.15×10^8 M. lepraemurium-sensitized syngeneic spleen cells which were heat-treated (60°C for 30 minutes). Killing of the spleen cells was assessed by the eosin exclusion test for viability. Two strain 2 guinea pigs each received 1.15×10^8

frozen-thawed cells as above. The guinea pigs which had received the transferred material were skin tested with murine lepromin with and without phenol, two days after receiving the cells. Five days after the skin tests the reactions were monitored. Five strain 2 guinea pigs which had received no injections were used as control. These were skin tested with the same antigens and their reactions monitored one day after skin testing for one month.

E. Cell Transfers in Rats

One month after sensitization the rats were sacrificed and their spleens taken for transfer. Three of the rats were used for peritoneal washings. The transfers were done in syngeneic animals. Four rats received 1.2×10^8 peritoneal cells. Four other rats received 5.3×10^7 spleen cells and four others received 1.0×10^8 spleen cells. All cells were given intracardially. There were three control groups. Two rats received 0.5 ml of incomplete Freund's adjuvant, two rats received 0.5 ml of complete Freund's adjuvant and two rats were uninoculated. All animals were skin tested with murine lepromin, murine lepromin with phenol, tissue antigen and tissue antigen with phenol. The test group was skin tested at different times after cell transfer and the reactions were monitored at daily intervals for one month. One of each of the adjuvant-inoculated rats was skin tested one and one and one half months after inoculation and one of each ,two months after inoculation. One rat in each of the cell dosage groups was skin tested one week after transfer and the remainder one month after transfer.

8. Serology

A. Haemagglutination

The haemagglutination test was carried out on the sera of the M. lepraemurium-sensitized rat and the M. lepraemurium and adjuvant-sensitized guinea pigs following the method of Middlebrook and Dubos (1948).

B. Immunodiffusion

The sera of the rat and guinea pigs used in the haemagglutination test were assayed for precipitating antibodies using a modification of Crowle's (1958) slide diffusion test. The modification consisted of the incorporation of 0.8% sodium barbital (pH 7.4) and 0.01% thimerosal (Merthiolate) in the agar gel and the use of plastic squares in which the distance between the centers of the central and peripheral wells is 4.5 instead of 4.0 mm.

The sera were used undiluted, 1/2, 1/5, and 1/10 concentrations and the antigens, used undiluted were tuberculin (intermediate test strength) and murine lepromin without phenol.

9. Histology

Three strain 13 guinea pigs which were sensitized by cell transfer were sacrificed two to two and one half months after skin test. The lesions produced in response to lepromin and lepromin with phenol were excised and fixed in 10% buffered formalin. Paraffin sections (5 µm thick) of the spleen were stained by the modified Zeehl-Neelsen method for M. leprae and skin sections were stained with hematoxylin

and eosin and examined microscopically.

One strain 2 guinea pig which was sensitized with live M. lepraemurium, skin tested with murine lepromin without phenol two months after sensitization, was sacrificed one month after skin testing. The lesions which formed in response to the murine lepromin and the spleen were excised, fixed in 10% buffered formalin and treated as above.

Five strain 2 guinea pigs which were sensitized by cell transfer were sacrificed 5 weeks after skin testing. The lesions which formed in response to the lepromin with phenol and the spleens were excised, fixed in 10% formalin and treated as above.

10. Test for Tissue-Induced Leproma Formation

The guinea pigs which received live M. lepraemurium and adjuvant used for preliminary work in lepromin sensitivity testing were also used as cell donors to test for tissue-induced leproma formation. The livers and spleens were removed from the animals, kept separate and treated as follows. The tissue was minced in 1% albumin saline, the resulting suspension centrifuged at 500 rpm for 1 minute on a Servall RC-2 centrifuge using an SS-34 rotor. The supernatant was centrifuged at 10,000 rpm for 30 minutes using an SS-34 rotor. The sediment was resuspended in 2.5 ml of 1% albumin saline and examined for acid-fast bacilli using the Ziehl-Neelsen stain.

The tissue suspensions were used to inoculate animals. Two normal stock rats were injected subcutaneously with 0.5 ml of the tissue suspension from the guinea pig infected with M. lepraemurium and complete Freund's adjuvant. Six normal stock mice were each given 0.2 ml of the same tissue suspension intraperitoneally. Two normal

stock rats and six normal stock mice were treated identically except that the tissue suspension from the guinea pig infected with M. lepraemurium and incomplete Freund's adjuvant was used. The animals were examined for leproma formation at monthly intervals for one year.

II. Preparation of Mycobacterial Extracts

A. Phenol-Acetic Acid-Water Preparation of Mycobacteria

Trypticase soy agar plates of M. smegmatis, a member of Runyon's group IV, were heat-killed at 80°C for one hour, harvested aseptically and washed three times in 0.25 M NaCl. The sediment was suspended in 0.25 M NaCl (0.4 g/ml of 0.25 M NaCl) and the mycobacterial extract prepared following the method of Razin et al. (1967) with modifications. The modifications consisted of sonicating the phenol-acetic acid-water-(2:1:0.5 w/v/v) treated cell suspension (two volumes of phenol-acetic acid-water to one volume of cell suspension) for 10 minutes using the medium probe of a Bronwill Biosonik III keeping the suspension in an ice bath; and centrifuging the sonicate for 30 minutes at 30,000 x g instead of 15 minutes at the same speed. The concentration of protein in solution was determined by following the method of Lowry et al. (1951) using crystalline bovine serum albumin as the reference serum.

B. Preparation of Mycobacterial Extract in Phosphate Buffer

Trypticase soy agar plates of M. smegmatis were heat-killed at 80°C for one hour, harvested aseptically and washed three times with sterile phosphate buffered saline pH 7.2. The sediment was resuspended in phosphate buffered saline (0.4 g of sediment/ml of saline) and sonicated for 60 minutes with the medium probe of a

Bronwill Biosonik III while maintained in an ice bath. The suspension was centrifuged at 15,000 rpm on a Sorvall RC2-B centrifuge for two hours using an SS-34 rotor. The concentration of protein in solution was determined using Lowry's method.

C. Preparation of Mycobacterial Proteins Using Acetone

The mycobacterial extract prepared in phosphate buffered saline was diluted with five to ten volumes of reagent grade acetone and evaporated at room temperature. The sediment was resuspended in buffer (to the original volume) and centrifuged at 15,000 to 18,000 x g for one hour to sediment extraneous material. The supernatant was termed treated-extract and the protein content was determined using Lowry's method.

12. Polyacrylamide (Disc) Gel Electrophoresis

A. Polyacrylamide Gel Electrophoresis of Phenol-Acetic Acid-Water-Prepared Mycobacterial Extract

Polyacrylamide gel electrophoresis was carried out in 6 mm x 7 cm glass tubes following the method of Takayama et al. (1966). The gel system consisted of 7.5% acrylamide, 35% acetic acid and 5M urea. The tank buffer was 10% acetic acid. From 0.2 to 0.5 ml of sample was layered over the top of the gels. Electrophoresis was carried out at 4°C for one hour with a constant current of 5mA per tube, and auramine-rhodamine was used as a tracker dye. The gels were stained by placing them in a 1% (w/v) solution of Amido black in 7% acetic acid for one hour. Destaining was done either electrophoretically or by diffusion in 7% acetic acid.

B. Polyacrylamide Gel Electrophoresis of Phosphate Buffer Prepared Mycobacterial Extract

Polyacrylamide gel electrophoresis of mycobacterial cell extract was carried out in 6 mm x 7 cm or 6 mm x 12 cm glass tubes in a 7% polyacrylamide gel made up following the method of Davis (1964). The tank buffer was tris-glycine pH 8.3 diluted 1:10 following the method of Affronti (personal communication) and a constant current of 5 mA per tube was used. From 0.2 to 0.5 ml of sample material containing about 300 ug of protein in 20% sucrose was layered over the top of the gels. Electrophoresis was allowed to proceed until the bromphenol blue tracking dye reached a point 1 cm from the bottom of the tube. The differential stains for protein used were Amido black and Coomassie Blue. The gels to be stained by Amido black were fixed and stained simultaneously by placing them in a 1% (w/v) solution of Amido black in 7% acetic acid for one hour. When the Coomassie Blue stain was used the gels were first fixed in 10% trichloracetic acid overnight. The gels were then washed once with water, immersed in a 0.25% aqueous solution of the dye for three to four hours at room temperature, rinsed again with water and destained. Destaining was carried out either electrophoretically or by diffusion in 7% acetic acid. The gels stained by both methods were stored in 7% acetic acid. Bands were still visible after storage for more than one year.

RESULTS AND DISCUSSION

1. Skin Testing

The preliminary work on murine lepromin skin testing indicated that the type of Freund's adjuvant used was unimportant in inducing a delayed-type hypersensitivity response to murine lepromin in guinea pigs, as shown in Tables I and II. It was decided that incomplete Freund's adjuvant would be used in subsequent animal sensitization in an attempt to keep the system as simple as possible.

The guinea pigs did not appear to be as sensitive to murine lepromin three months after initial sensitization as the group tested six weeks after sensitization, as shown in Table III. Subsequently it was decided to use guinea pigs for cell transfer as close to six weeks after their initial sensitization as possible. The M. lepraemurium-sensitized strain 13 guinea pig response to murine lepromin six weeks after the initial sensitization was similar to that of the normal stock guinea pigs sensitized and skin-tested in a similar manner, as shown in Tables IV and I.

The results of skin testing with partially purified murine lepromin are not very revealing. The unsensitized strain 2 and strain 13 guinea pigs showed a transient induration which appeared one day after skin testing and then disappeared, as shown in Table V. The erythema they exhibited lasted two days before disappearing. This suggests that the purified lepromin had constituents which cause a nonspecific inflammatory reaction in unsensitized guinea pigs. The

TABLE I

RESPONSE OF STOCK GUINEA PIGS SENSITIZED[a] WITH M. LEPRAEMURIUM AND INCOMPLETE FREUND'S ADJUVANT TO MURINE LEPROMIN WITHOUT PHENOL AND TUBERCULIN[b] 24 HOURS AFTER SKIN TESTING

	mm of induration in response to murine Lepromin without Phenol		mm of induration in response to tuberculin	
	In flank[c]	In footpad	In flank	In footpad
Guinea pig 1	10	-[d]	-	-
Guina pig 2	10	-	-	-

a The animals were sensitized with 0.5 ml of a mixture of equal volumes of live M. lepraemurium (2 to 4 x 10^9 organisms/ml) and incomplete Freund's adjuvant 6 weeks before skin testing. They were boosted with 0.5 ml of the same suspension 4 weeks after the initial sensitization.

b Intermediate test strength (0.0002/0.1 ml) tuberculin (PPD) was used.

c Animals were skin tested with 0.1 ml of each antigen both in the flank and footpad.

d No response.

TABLE II

RESPONSE OF STOCK GUINEA PIGS SENSITIZED[a] WITH M. LEPRAEMURIUM AND COMPLETE FREUND'S ADJUVANT TO MURINE LEPROMIN WITHOUT PHENOL AND TUBERCULIN[b] 24 HOURS AFTER SKIN TESTING

	mm of induration in response to murine Lepromin without Phenol		mm of induration in response to tuberculin	
	in flank[c]		In flank	In footpad
Guinea pig 1	8.5	-[d]	-	-
Guinea pig 2	9.0	-	-	-

[a] Six weeks before skin testing the animals were given 1 ml of a suspension of equal volumes of live M. lepraemurium (2 to 4 x 10^9 organisms/ml) and complete Freund's adjuvant intraperitoneally. The animals were boosted with 0.5 ml of the same suspension 1 week before skin testing.

[b] Intermediate test strength (0.0002 mg/0.1 ml) tuberculin (PPD) was used.

[c] Animals were skin tested with 0.1 ml of each antigen both in the flank and footpad.

[d] No response.

TABLE III

RESPONSE OF STOCK GUINEA PIGS SENSITIZED[a] WITH M. LEPRAEMURIUM AND COMPLETE FREUND'S ADJUVANT TO MURINE LEPROMIN WITHOUT PHENOL AND TUBERCULIN[b]

	Days after skin test	Response to murine Lepromin without Phenol[c]		Response to tuberculin	
		In flank[d]	In footpad	In flank	In footpad
Guinea pig 3	1	-[e]	-	-	-
	2	3	-	-	-
Guinea pig 4	1	6	-	-	-
	2	3	-	-	-
	12	3	-	-	-

a The animals were sensitized intraperitoneally with 1 ml of a mixture of equal volumes of live M. lepraemurium (2 to 4 x 10^9 organisms/ml) and complete Freund's adjuvant and boosted 5 weeks after sensitization with 0.5 ml of a subcutaneous inoculation of the same mixture. The animals were skin tested 3 months after the initial sensitization.

b Intermediate test strength tuberculin (0.0002 mg/0.1 ml) tuberculin (PPD) was used.

c Results are given in mm of induration.

d Animals were skin tested with 0.1 ml of each antigen in both the flank and footpad.

e No response.

TABLE IV

RESPONSE OF SENSITIZED[a] STRAIN 13 GUINEA PIGS TO MURINE LEPROMIN WITHOUT PHENOL

Days after skin test	Response to murine Lepromin without Phenol[b]
1	7.6
2	5.6
3	4.0
6	2.6
7	2.6

[a] Six weeks before skin testing guinea pigs were given intraperitoneal injections of 2 ml of a mixture of equal volumes of incomplete Freund's adjuvant and live M. lepraemurium (1.77×10^8 bacilli/animal).

[b] Results are given in mm of induration and are the average of readings from 5 guinea pigs.

TABLE V

RESPONSE OF UNSENSITIZED[a] GUINEA PIGS TO PARTIALLY PURIFIED MURINE LEPROMIN

Days after skin testing	Guinea pig					
	A		B		C	
	Ind[b]	Er[c]	Ind	Er	Ind	Er
1	2	6	2	5	2	10
2	-[d]	-	-	5	-	5
3	-	-	-	-	-	5
6	-	-	-	-	-	-
7	-	-	-	-	-	-
8	-	-	-	-	-	-
9	-	-	-	-	-	-
10	-	-	-	-	-	-

[a] Guinea pigs A and B were unsensitized strain 13 animals and guinea pig C was an unsensitized strain 2 animal.

[b] Results are given in mm of induration.

[c] Results are given in mm of erythema.

[d] No response.

sensitized guinea pigs showed essentially the same type of response as the unsensitized control, a transient induration and a longer erythema, as shown in Table VI. This suggests that the guinea pigs were no longer sensitive or that if they were still sensitive to M. lepraemurium that the sensitivity was not being detected by this lepromin preparation.

The response of the M. lepraemurium-sensitized rats to lepromin and tuberculin was read one day after the skin test. Both animals showed no response to tuberculin in either the footpad or the flank and no response to lepromin in the footpad. However, there was a response of 6 mm to lepromin in the flank. Since the result was unusual it was decided to attempt a cell transfer.

2. Cell Transfer

Figures 1 and 2 indicate that there is almost no difference in the response of strain 13 guinea pigs to murine lepromin with phenol and murine lepromin without phenol. Unsensitized animals did not respond to any of the test antigens used. It was surprising to find that guinea pigs receiving live cells and guinea pigs receiving heat-killed cells gave a virtually identical response in terms of magnitude and character. Although the guinea pigs were skin tested six days after cell transfer the response did not appear until about two weeks after skin testing.

The reaction of the strain 2 guinea pigs used as the cell donors in the passive transfer of hypersensitivity, to murine lepromin without phenol was recorded one month after skin test (or booster dose) and is the average response of four guinea pigs. The average

TABLE VI

RESPONSE OF SENSITIZED[a] STRAIN 13 GUINEA PIGS
TO PARTIALLY PURIFIED MURINE LEPROMIN

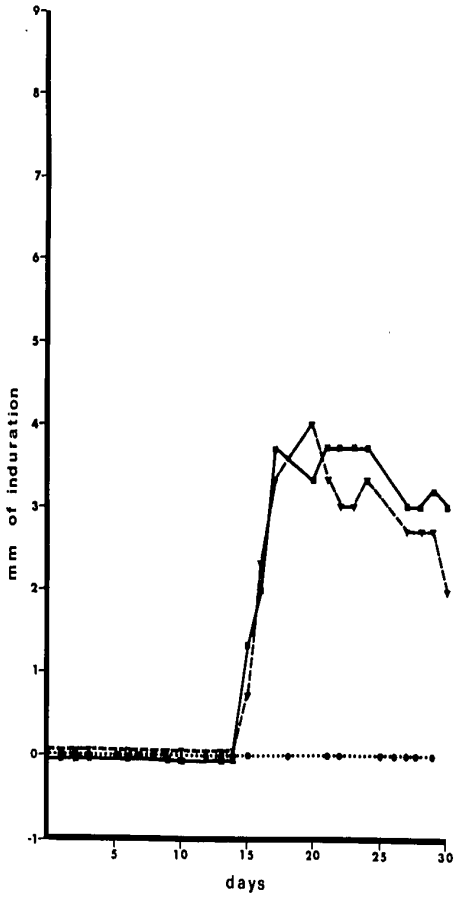
Days after skin test	GUINEA PIG					
	D		E		F	
	Ind[b]	Er[c]	Ind	Er	Ind	Er
1	2	10	2	10	2	10
2	-[d]	5	-	5	-	5
3	-	5	-	-	-	-
6	-	-	-	-	-	-
7	-	-	-	-	-	-
8	-	-	-	-	-	-
9	-	-	-	-	-	-
10	-	-	-	-	-	-

[a] Guinea pig D was given 1 x 10^8 heat-killed M. lepraemurium-sensitized spleen cells intracardially 5 months before skin testing. Guinea pigs E and F were given 1.5 x 10^6 M. lepraemurium-sensitized live spleen cells 7 months before skin testing.

[b] Results are given in mm of induration.

[c] Results are given in mm of erythema.

[d] No response.


mm of induration
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days

FIGURE 1

RESPONSE OF STRAIN 13 GUINEA PIGS TO MURINE LEPROMIN WITHOUT PHENOL

Three guinea pigs each received 1 x 10^8 live, syngeneic, M. lepraemurium-sensitized spleen cells intracardially. Three other guinea pigs each received 1 x 10^8 heat-treated (at 60°C for 30 minutes) syngeneic, M. lepraemurium-sensitized spleen cells also intracardially. Both groups were skin tested six days (time 0) after cell transfer. Four unsensitized strain 13 guinea pigs were also skin tested as a control. Values shown are the arithmetic means of the differences between the diameter of induration (mm) at the site injected with test lepromin and the diameter of induration at the site injected with the appropriate tissue control.

Live cell recipients

Heat-treated cell recipients

Unsensitized animals

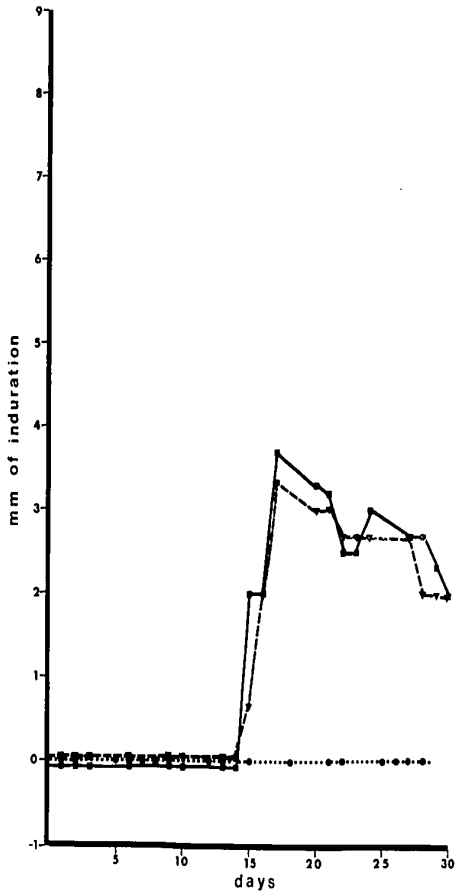
mm of induration
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FIGURE 2

RESPONSE OF STRAIN 13 GUINEA PIGS TO MURINE LEPROMIN WITH PHENOL

Three guinea pigs each received 1 x 10^8 live, syngeneic, M. lepraemurium-sensitized spleen cells intracardially. Three other guinea pigs each received 1 x 10^8 heat-treated (at 60°C for 30 minutes) syngeneic, M. lepraemurium-sensitized spleen cells also intracardially. Both groups were skin tested six days after (time 0) after cell transfer. Four unsensitized strain 13 guinea pigs were also skin tested as a control values shown are the arithmetic means of the differences between the diameter of induration (mm) at the site injected with test lepromin and the diameter of induration at the site injected with the appropriate tissue control.

Live cell recipients

Heat-treated cell recipients

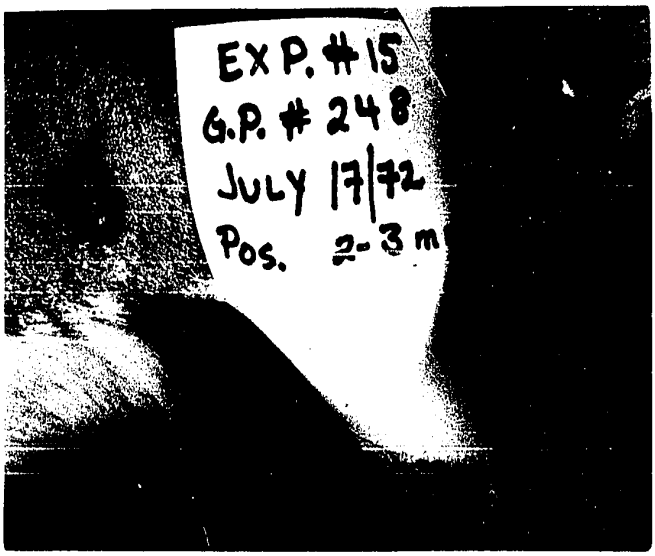
Unsensitized animals

diameter of induration was 3.25 mm. An example of the skin reaction is shown in Figure 3.

Strain 2 guinea pigs did not react in the same way to cell transfer as did the strain 13 guinea pigs. The unsensitized strain 2 guinea pigs responded to the test antigens, as shown in Figures 4 and 5. The response of the unsensitized animals to lepromin without phenol was greater than the response to lepromin with phenol. It is not certain if this is an important difference. The response of the unsensitized group to both lepromin with and without phenol remained below that of the live and heat-treated response curves and the frozen-thawed cell recipients gave a response similar to the unsensitized animals. The live cell recipient and the heat-killed cell recipient response to murine lepromin without phenol was initially greater than the response of the unsensitized and frozen-thawed cell recipients but after about 20 days the magnitudes of the responses were similar.

Figures 6 and 7 are included to indicate the within treatment variance in the cell transfer experiments. The range from minus to plus one standard deviation contains about two thirds of all possible points. Similar standard deviations obtained from the remaining data in Figures 1,2,4, and 5, are shown in the Appendix.

None of the control animals in the cell transfer experiment in rats showed any response to the skin test antigens at any time. Neither the peritoneal cell recipients nor the spleen cell recipients (both low and high dose) showed any response to the skin test antigens during the test period. Preliminary work on rat sensitivity to murine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JULY 17/72
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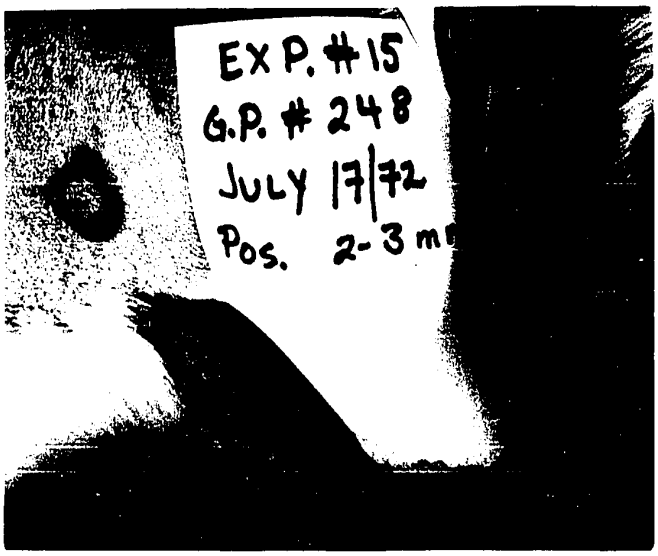
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JULY 17/72
Pos. 2-3 m

FIGURE 3

STRAIN 2 GUINEA PIG RESPONSE TO MURINE LEPROMIN

The response to murine lepromin without phenol of a strain 2 guinea pig sensitized with live M. lepraemurium and incomplete Freund's adjuvant was recorded one month after skin test.

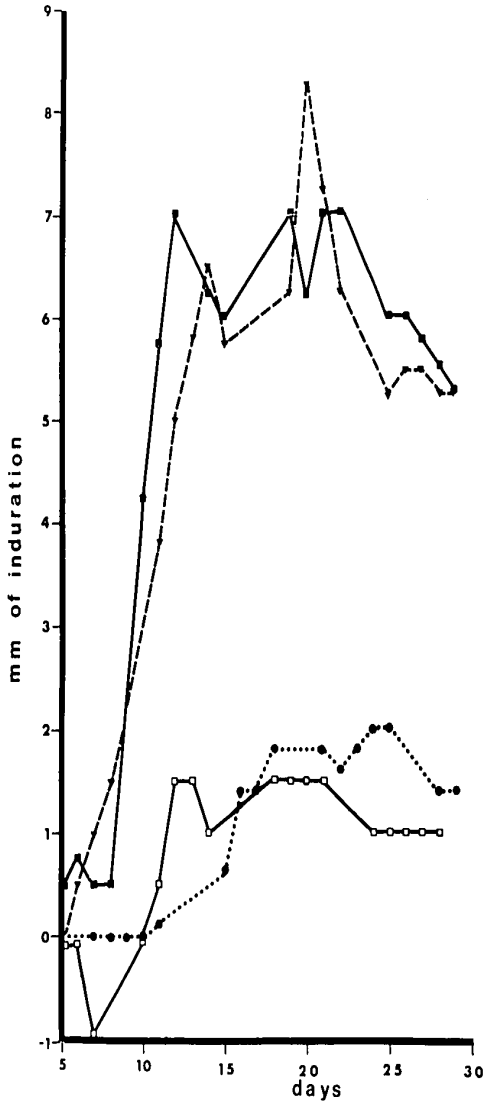
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FIGURE 4

RESPONSE OF STRAIN 2 GUINEA PIGS TO MURINE LEPROMIN WITHOUT PHENOL

Four guinea pigs each received 1 x 10^8 live, syngeneic, M. lepraemurium-sensitized spleen cells intracardially. Four other guinea pigs each received 1 x 10^8 heat-treated (at 60°C for 30 minutes) syngeneic, M. lepraemurium-sensitized spleen cells intracardially. Two other guinea pigs each received 1 x 10^8 frozen-thawed, syngeneic, M. lepraemurium-sensitized spleen cells intracardially. Five unsensitized strain 2 guinea pigs were also skin tested as a control. All three groups of cell recipients were skin tested two days (time 0) after cell transfer. Values shown are the arithmetic means of the differences between the diameter of induration (mm) at the site injected with test lepromin and the diameter of induration at the site injected with the appropriate tissue control.

Live cell recipients

Heat-treated cell recipients

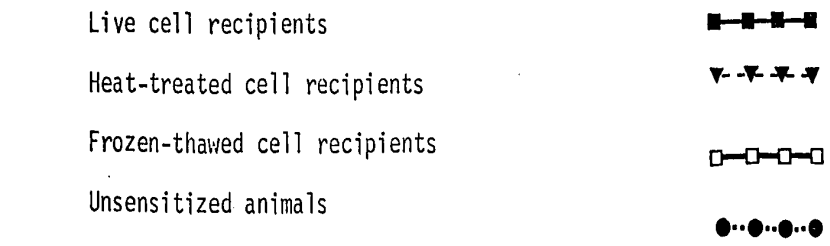
Frozen-thawed cell recipients

Unsensitized animals

FIGURE 4

RESPONSE OF STRAIN 2 GUINEA PIGS TO MURINE LEPROMIN WITHOUT PHENOL

Four guinea pigs each received 1 x 10^8 live, syngeneic, M. lepraemurium-sensitized spleen cells intracardially. Four other guinea pigs each received 1 x 10^8 heat-treated (at 60°C for 30 minutes) syngeneic, M. lepraemurium-sensitized spleen cells intracardially. Two other guinea pigs each received 1 x 10^8 frozen-thawed, syngeneic, M. lepraemurium-sensitized spleen cells intracardially. Five unsensitized strain 2 guinea pigs were also skin tested as a control. All three groups of cell recipients were skin tested two days (time 0) after cell transfer. Values shown are the arithmetic means of the differences between the diameter of induration (mm) at the site injected with test lepromin and the diameter of induration at the site injected with the appropriate tissue control.



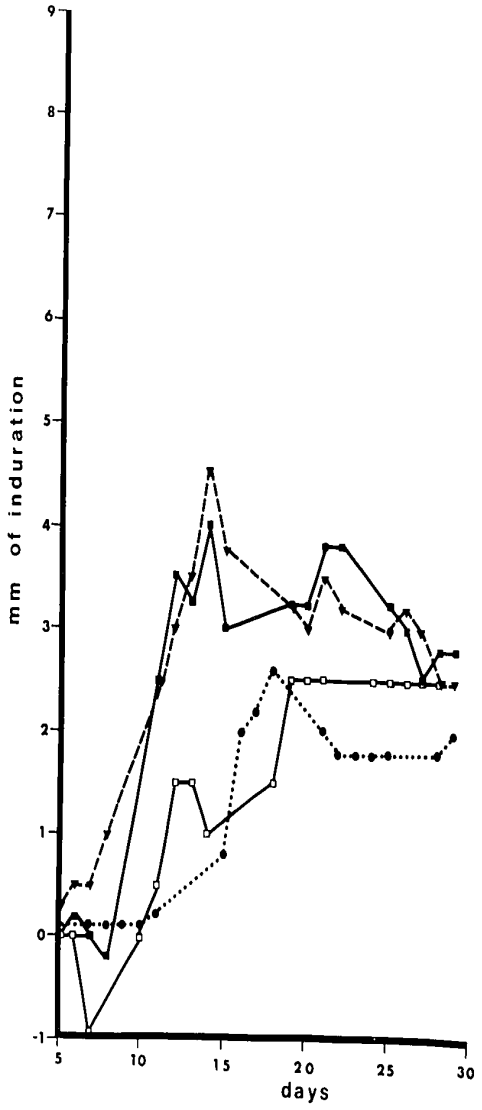

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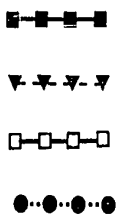
FIGURE 5

RESPONSE OF STRAIN 2 GUINEA PIGS TO MURINE LEPROMIN WITH PHENOL

Four guinea pigs each received 1 x 10^8 live, syngeneic, M. lepraemurium-sensitized spleen cells intracardially. Four other guinea pigs each received 1 x 10^8 heat-treated (at 60°C for 30 minutes) syngeneic, M. lepraemurium-sensitized spleen cells intracardially. Two other guinea pigs each received 1 x 10^8 frozen-thawed, syngeneic, M. lepraemurium-sensitized spleen cells intracardially. Five unsensitized strain 2 guinea pigs were also skin tested as a control. All three groups of cell recipients were skin tested two days (time 0) after cell transfer. Values shown are the arithmetic means of the differences between the diameter of induration (mm) at the site injected with test lepromin and the diameter of induration at the site injected with the appropriate tissue control.



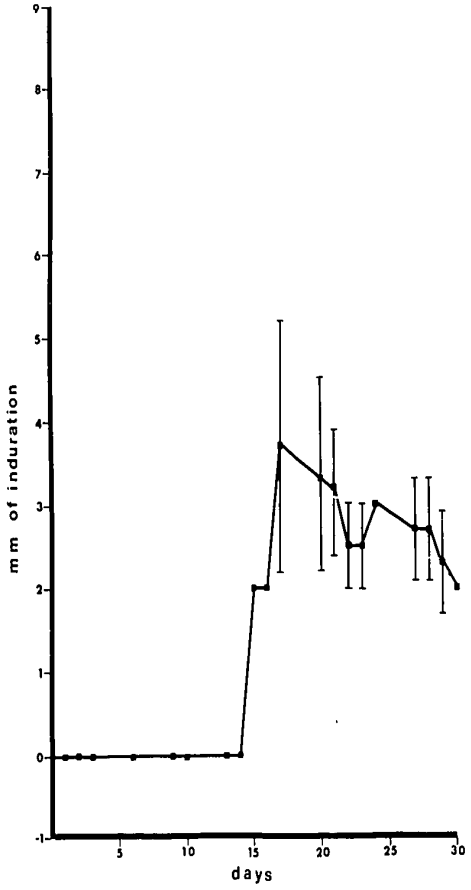

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FIGURE 6

RESPONSE OF STRAIN 13 GUINEA PIGS SENSITIZED WITH LIVE SPLEEN CELLS TO MURINE LEPROMIN WITHOUT PHENOL

Three strain 13 guinea pigs each received 1 x 10^8 live, syngeneic, M. lepraemurium-sensitized spleen cells intracardially. Values shown are the arithmetic means of differences between the diameter of induration at the site injected with test lepromin and the diameter of induration at the site injected with the appropriate control (±1 standard deviation).

FIGURE 6

RESPONSE OF STRAIN 13 GUINEA PIGS SENSITIZED WITH LIVE SPLEEN CELLS TO MURINE LEPROMIN WITHOUT PHENOL

Three strain 13 guinea pigs each received 1 x 10^8 live, syngeneic, M. lepraemurium-sensitized spleen cells intracardially. Values shown are the arithmetic means of differences between the diameter of induration at the site injected with test lepromin and the diameter of induration at the site injected with the appropriate control (±1 standard deviation).

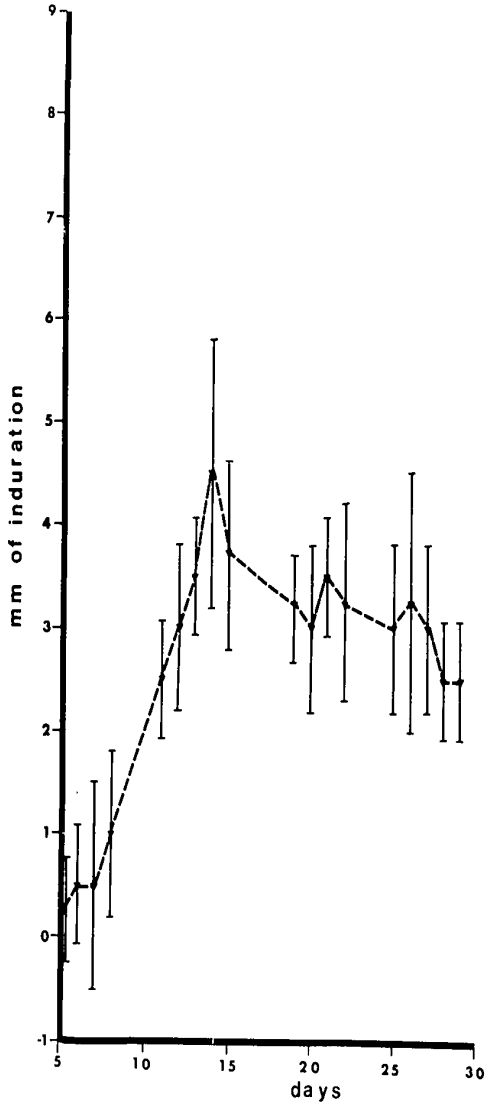
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FIGURE 7

RESPONSE TO STRAIN 2 GUINEA PIGS SENSITIZED WITH HEAT-KILLED SPLEEN CELLS TO MURINE LEPROMIN WITH PHENOL

Four strain 2 guinea pigs each received 1 x 10^8 heat-treated, syngeneic, M. lepraemurium-sensitized spleen cells intracardially. Values shown are the arithmetic means of differences between the diameter of induration at the site injected with test lepromin and the diameter of induration at the site injected with the appropriate tissue control (±1 standard deviation).

lepromin indicated that the rats were lepromin sensitive in contrast to earlier work (Wallace 1958a) that rats were not lepromin sensitive. Subsequently the lepromin was tested for sterility and it was found to be contaminated with a gram-positive chromogenic bacillus. However the cell transfer work had already been initiated so it was decided to follow it through. The later work with sterile lepromin with and without phenol confirmed earlier findings that lepromin sensitivity in the rat cannot be determined by intradermal lepromin skin tests.

3. Serology

The sera and controls did not exhibit haemagglutination at a dilution of 1/10. Haemagglutinating antibodies in a M. lepraemurium infected rat and in M. lepraemurium and adjuvant infected guinea pigs were not demonstrated by this test. The type of adjuvant used in infected guinea pigs did not influence haemagglutinins in guinea pig sera.

The sera tested with tuberculin were completely negative for precipitating antibodies. The sera tested with murine lepromin without phenol did not show any precipitation but did show a fuzzy, grey line.

Although neither haemagglutinating antibodies nor precipitating antibodies could be detected it is not possible to say that the animals were not producing antibody. Although the study was of a preliminary nature it must be admitted that the animal sample was very small and that the tests themselves lacked sensitivity. However there are other greater difficulties.The antigen used is necessarily only

partially purified at best and this causes problems not only in sensitizing the animals but in assaying the humoral response.

4. Histology

Spleen sections stained by the modified Ziehl-Neelsen method from three strain 13 guinea pigs sensitized by cell transfer, five strain 2 guinea pigs sensitized by cell transfer and one strain 2 guinea pig sensitized by live M. lepraemurium showed no acid-fast bacilli present.

The tissue sections from the same animals all showed the same type of focal inflammatory response similar to the histological picture in tuberculin sensitivity. The sections showed varying degrees of the common morphological characteristics seen in the lesions of delayed hypersensitivity.

5. Tissue-Induced Leproma Formation

No acid-fast bacilli were observed in the tissue suspensions from both complete and incomplete Freund's adjuvant and M. lepraemurium infected guinea pigs when examined by the Ziehl-Neelsen method. None of the four rats or twelve mice inoculated with the tissue suspensions had shown any leproma formation when the experiment was terminated one year later. This evidence strongly suggests the idea that M. lepraemurium are not being transferred to the test animals in the sensitized cell suspensions. If the sensitized cell suspensions contained even a small number of live M. lepraemurium they would have had time to induce leproma formation within a year according to Marchoux's (1938) work on experimental infection caused by small

numbers of bacilli.

6. Polyacrylamide (Disc) Gel Electrophoresis

Zone electrophoresis in polyacrylamide gel (polyacrylamide gel electrophoresis) is a gentle, high resolution method for fractionation and physical-chemical studies of molecules on the basis of size, conformation and net charge (Chrambach 1971). The mycobacterial extract prepared using phenol-acetic acid-water gave bands of protein when separated by electrophoresis following Takayama's method (1966). However, the bands did not show good separation; after one hour of electrophoresis all bands remained in the top third of the gel. The method of extraction and electrophoresis was very harsh and would not lend itself to immunoelectrophoresis (Catsimpoolas et al. 1968). For immunoelectrophoresis the integrity of the proteins is important. The harsh treatment of the proteins most probably would denature them. It was decided to try a gentler extraction and electrophoresis method, a 7% gel and a pH 8.3 tris-glycine buffer.

Initially 6 mm x 7 cm glass tubes were used to prepare the gels following the Davis method (1964). This gave moderately good separation as shown in Figure 8. However most of the bands were still in the top half of the gel by the time the bromphenol blue tracker dye had migrated through the gels. It was decided to try a longer gel using 6 mm x 12 cm glass tubes. The results are shown in Figure 9.

The use of long gels increased the resolution of the bands. This was not without hazard, for frequently material would not separate properly. After staining the gel a top disc was visible and

FIGURE 8

ELECTROPHORESIS OF M. SMEGMATIS EXTRACT

Samples of M. smegmatis extract containing 300 µg of protein were subjected to polyacrylamide gel electrophoresis on short (6 mm x 7 cm) gels, as described in Materials and Methods. This gel was stained with Amido black.

FIGURE 9

ELECTROPHORESIS OF *M. SMEGMATIS* EXTRACT ON LONG GELS

Samples of *M. smegmatis* extract containing about 300 µg of protein were subjected to polyacrylamide gel electrophoresis on long (6 mm x 12 cm) gels. This gel was stained with Amido black.

from this disc a long funnel-shaped tail extended into the gel. This was probably due to the presence of glycoprotein. This difficulty was overcome by using a simple acetone treatment of the mycobacterial extract which did not interfere with band formation but allowed proper electrophoresis, as shown in Figure 10. Amido black and Coomassie Brilliant Blue stained the gels quickly and easily.

The 60 minute sonication time used to break the cells became tedious so investigations on the minimum amount of time required to break the cells were undertaken. Samples were sonicated for 0,5,10,15, 20,40,50, and 60 minutes, negatively stained with potassium phosphotungstate and examined with the electron microscope. At 0 minutes of sonication the cells were intact and appeared normal. At 5 minutes of sonication the cells were intact although some cellular debris was seen. At 10 minutes of sonication most of the cells were still intact although the occasional cell was ruptured. At 20 minutes of sonication only the occasional cell (ruptured or intact) was seen. At 40, 50 and 60 minutes mostly cellular debris was seen with the occasional ruptured cell present. On the basis of this work it was decided to sonicate cells for 20 minutes.

Nakayama and Hayashi (1958) monitored the sonic disruption of M. lepraemurium with the electron microscope. They sonicated M. lepraemurium with a sonic wave generator (9.5 KC,200 W) and found that 30 minutes of sonication disrupted the cells. Recently Affronti et al. (1972) reported sonication of mycobacterial cells for 20 minutes in preparation of a cell free extract for polyacrylamide gel electrophoresis. Therefore, the treatment used is in general aggree-

FIGURE 10

ELECTROPHORESIS OF ACETONE-TREATED M. SMEGMATIS EXTRACT

Samples of acetone-treated M. smegmatis extract containing about 300 µg of protein were subjected to polyacrylamide gel electrophoresis on long (6 mm x 12 cm) gels. This gel was stained with Coomassie Blue.

ment with the treatments given by others.

Polyacrylamide gel electrophoresis showed promise as an experimental method for the comparison of mycobacteria on the basis of protein patterns but time did not permit its application. Although the time for preparation of material and electrophoresis itself is relatively short, the time required to obtain an adequate amount of M. lepraemurium is the limiting factor since this organism grows only in vivo and has a long generation time.

GENERAL DISCUSSION

Strain 2, strain 13, and normal stock guinea pigs were established as a model for the study of delayed-type hypersensitivity to murine lepromin. Normal stock guinea pigs actively sensitized with M. lepraemurium exhibited a larger skin reaction (read at 24 hours) when skin tested six weeks after sensitization than when skin tested twelve weeks after sensitization. It appears that guinea pigs lose their sensitivity to M. lepraemurium with time.

Cell transfer work in rats was unsuccessful. Neither the control group (which received no cells) nor the peritoneal or spleen cells recipients (both high and low dose) showed any response to the skin test antigens at any time during the test period. The importance of sterility in murine lepromin preparations cannot be overemphasized in view of the encouraging response obtained in preliminary work on rats in which the murine lepromin was subsequently found to be contaminated with a gram-positive organism.

The rat is incapable of displaying the Mitsuda type reaction to M. lepraemurium but Wallace (1958a) claimed that this is not the result of the inactivity of leukocytes of such animals. Wallace claims to have transferred reactivity to M. lepraemurium to normal guinea pigs by mixing exudate cells of murine leprosy infected rats with murine lepromin. Systemic transfers were very weak but local transfers of cells plus antigen injected intradermally appeared successful. However the reactions were not specific since they were

also obtained by mixing the murine lepromin sensitive cells with Old Tuberculin. It is not certain if local deposition of cells plus antigen is a useful indicator of hypersensitivity (Bloom and Chase 1967). Although Wallace's work is interesting it would be unwise to put much faith in interspecies transfers.

The inability of the M. lepraemurium-infected rat to form a skin reaction to an intradermal challenge of murine lepromin is a limiting factor in the study of the disease. It would be more informative if one could follow the skin reaction in an animal capable of developing the disease than in an animal which does not develop the disease.

Although the sera from the guinea pigs and the rat did not exhibit haemagglutination or precipitation at a dilution of 1/10 this work is of a preliminary nature and a larger study with more controls is necessary for a more accurate view. The appearance of antibodies to M. lepraemurium in the infected rat and guinea pig cannot be ruled out. Haemagglutination and precipitation are not sensitive tests and may not be detecting antibody present at a low level. The use of a more sensitive method for assaying humoral antibody response such as radio-immuno precipitation might detect small amounts of antibody. However the limitations of murine lepromin as an antigen are obvious and conclusive serology would require a more purified, more specific antigen. Sophisticated immunological techniques cannot be successfully applied when the antigen is so crude.

The results obtained from the work with partially purified murine lepromin were inconclusive. Either the guinea pigs were no longer sensitive to M. lepraemurium or if they were sensitive their

sensitivity could no longer be detected. It is possible that the antigenic fragments responsible for inducing the delayed type of response were destroyed in the purification or that they were not present in large enough quantities. In view of that fact that only immunogenic particle will elicit a delayed type of hypersensitivity response, future work on purification of murine lepromin should test its immunogenic ability.

The antigenic properties of M. lepraemurium and its ability to induce a humoral response in laboratory animals has not been thoroughly investigated. There is some discussion in the literature on the ability of M. lepraemurium to induce antibody formation in rats. Using the Middlebrook-Dubos haemagglutination reaction with an aqueous extract of M. lepraemurium as the antigen, Yamada (as quoted by Sato and Nishimura 1967) reported a higher rate of positivity than that obtained using Old Tuberculin as the antigen. Using the Middlebrook-Dubos test Nishimura (Sato and Nishimura 1967) reported that 2 of 10 rats with leprosy had antibodies to an aqueous defatted murine leprosy bacillus extract while only 1 of 10 rats of the same group had antibodies to M. tuberculosis. Nishimura and Kono (Sato and Nishimura 1967) found that the serum gamma-globulin level in murine leprosy infected rats is almost the same as in the normal healthy animal which suggests that antibody present in the infected rats must be present at a low level. The humoral response of the guinea pig to M. lepraemurium has not been studied before. In view of the resistance of guinea pigs to infection with M. lepraemurium it would be reasonable to expect some type of humoral response which might aid in protecting the animal.

The present work showed that transfer of sensitivity to murine lepromin in strain 2 and strain 13 guinea pigs by systemic injections of M. lepraemurium-sensitized live spleen cells and heat-killed spleen cells was possible. It was not possible to transfer sensitivity to strain 2 guinea pigs using frozen-thawed cells. The strain 13 guinea pigs were skin-tested with murine lepromin with and without phenol six days after cell transfer. There was no response until about 15 days (or 21 days after cell transfer) when a peak response of about 4 mm in duration was reached by both live cell recipients and heat-killed cell recipients but not by the control group which received no cells. The peak response persisted for about one week, then it declined to around 3 mm where it remained for another week. Essentially the same response was seen to murine lepromin both with and without phenol.

The strain 2 guinea pigs were skin tested with murine lepromin with and without phenol two days after cell transfer. The live cell recipients showed a maximum response to murine lepromin without phenol of about 7 mm around ten days after skin testing (twelve days after cell transfer). This peak response persisted for about two weeks and then declined to around 6 mm for another week. The frozen-thawed cell recipients gave a response of about 1.5 mm twelve days after skin test. This response persisted for about two weeks and then declined to 1 mm. The control group (given no cells) showed a near peak response of 1.8 mm at 18 days, this persisted for about two weeks. It is unfortunate that the control group began to give a response but since it was much lower than the response of the live or heat-killed cell recipients and appeared much later it was thought to be due to

some nonspecific irritation.

The response of the live and heat-killed cell recipient strain 2 guinea pigs to murine lepromin with phenol was much lower than the response to lepromin without phenol. The significance of this difference is not clear. A peak response of about 4 mm of induration was reached at 14 days after skin testing, persisted for about a week and then declined to about 3 mm. The frozen-thawed cell recipients began to respond at about 10 days, and the response reached a peak of 2.5 mm around 20 days where it remained until the end of the test period. The control group (unsensitized animals) began to react around 15 days, reached a maximum of 2.6 mm at 21 days and declined to about 2 mm where it remained until the end of the test period. Since the response of the unsensitized animals differed in both character and magnitude to that of the live and heat-killed cell recipients it was felt that the responses of the control animals were not significant.

The magnitude of the strain 2 guinea pig response to murine lepromin without phenol was much greater than the strain 2 guinea pig response to murine lepromin with phenol or the strain 13 guinea pig response to murine lepromin with phenol or murine lepromin without phenol. The response of the strain 13 guinea pigs to murine with phenol was of the same magnitude as the strain 2 guinea pig response to murine lepromin with phenol or without phenol. In strain 2 guinea pigs the peak response of the live and heat-killed cell recipients occurred at 15 days after skin-testing. In strain 13 guinea pigs the peak response of the live and heat-killed cell recipients occurred at 10 days. As mentioned earlier the strain 2 guinea pigs were skin tested two

days after cell transfer. It may be that the transferred cells are more capable of initiating an immune response to murine lepromin two days after cell transfer than six days after cell transfer. This difference may also represent the capacity of strain 2 guinea pigs to respond to murine lepromin more quickly than strain 13 guinea pigs.

In the present work the response of the heat-killed cell recipients to murine lepromin may reflect some peculiarity of the antigen used. To rule out this possibility the work should be repeated when a purified murine lepromin becomes available. However the response of the heat-killed cell recipients to murine lepromin may also reflect the potential of sensitized heat-killed cells to provide information to live unsensitized cells enabling them to respond to murine lepromin.

The transfer of sensitivity to murine lepromin by both live and heat-killed spleen cells appears to be a characteristic of these guinea pigs systems. It was surprising that heat-killed spleen cells should transfer sensitivity to murine lepromin and that frozen-thawed cells should not. Since strain 2 guinea pigs are not sensitized by frozen-thawed cells it seems that heat-killing at 60°C for 30 minutes does not destroy the factor(s) responsible for transfer of sensitivity but that freezing and thawing was able to destroy this factor(s). Since freezing and thawing is unlikely to destroy M. lepraemurium the fact that frozen-thawed cells did not elicit a response in the recipients suggests that M. lepraemurium cells or their antigens are not being transferred in the spleen cell suspension.

The spleen sections from eight of the guinea pigs sensitized by cell transfer were stained by the modified Zeehl-Neelsen method

and showed no acid-fast bacilli; tissue sections of the skin-test sites of the same animals showed the characteristics of a delayed-type hypersensitive response. Thus the histological work detected no gross deviations from the expected picture and lends support to the cell transfer theory. Spleen cells from guinea pigs inoculated with M. lepraemurium were injected subcutaneously into rats and mice. The fact that no leproma-formation was observed even one year after inoculation is evidence that viable organisms were not being transferred with the spleen cells, but that the sensitivity developed by cell transfer recipients is due to the passive transfer of sensitized spleen cells.

Bloom and Chase (1967) have thoroughly reviewed the literature on the delayed-type hypersensitivity with living and killed cells and subcellular fractions. Lawrence (1970) and others have shown that in man long lasting sensitivity to a variety of antigens may be transferred by cell extracts containing polypeptide/polynucleotide of low molecular weight which is termed transfer factor. The existence of such a factor in animal systems has not been conclusively demonstrated (Bloom and Chase 1967). Although doubt has been cast on the validity of transfer of hypersensitivity in animals with killed cells or subcellular fractions further work must be done before the possibility may be discarded.

Goihman-Yahr et al. (1969) reported transfer of sensitivity to human lepromin with phenol in strain 2 and strain 13 guinea pigs by systemic injections of sensitized viable lymphoid cells but not by heat-killed cells or serum. However, in their work strain 2 guinea pigs which received heat-killed cells had a peak response of 4 mm to

human lepromin whereas live-cell recipients had a peak response of only 1 mm more than this. The response of the heat-killed cell recipients then decreased to the zero level. The strain 13 response is more clear cut since the heat-killed cell recipients had a negligible response. The discrepancy of the response of heat-killed cell recipients to human lepromin is difficult to explain but the apparent potential of the heat-killed cells to induce a delayed-type hypersensitivity should be further explored. In Goihman-Yahr's work both strains of animal appeared to be equally sensitive to human lepromin. By contrast the present work showedthat strain 2 guinea pigs were more sensitive to murine lepromin skin tests than strain 13 guinea pigs.

In later work Goihman-Yahr et al. (1969b) found that treatment of guinea pigs with the immunosuppressant methotrexate depresses responsiveness to human lepromin. Methotrexate prevents the induction of reactivity which leads to granuloma formation on exposure to lepromin but has no detectable effect upon an established reactive state. This is further evidence to support the idea that human lepromin reactivity in the guinea pig is a type of hypersensitivity to M. leprae.

Cellular passive transfer of sensitivity by lymphoid cells has served as the principal criterion for identifying a given immunological process as the cellular type. It appears that human and murine lepromin sensitivities are delayed or cellular hypersensitivities since they satisfy the criterion of passive transfer by lymphoid cells.

CONCLUSIONS

It is possible to transfer sensitivity to murine lepromin (as assayed by intradermal skin tests) from M. lepraemurium-sensitized guinea pigs to unsensitized syngeneic guinea pigs using live sensitized spleen cells. After receipt of the live cells the animals developed a granulomatous response to murine lepromin. The reactivity of guinea pigs to murine lepromin is of the delayed (or cellular) type as judged by passive transfer of this sensitivity to normal animals by viable lymphoid cells. Heat-killed spleen cells also transferred this sensitivity but frozen-thawed cells did not. The significance of this finding is not clear. No lepromas were induced in rats or mice by injection of tissue material from M. lepraemurium-infected guinea pigs, which strongly suggests that cells of M. lepraemurium are not being transferred to the test animals in the sensitized cell suspension. It is not possible to determine sensitivity to M. lepraemurium in rats by intradermal skin tests with murine lepromin. In addition, polyacrylamide (disc) gel electrophoresis of cell free mycobacterial extracts showed promise as a method for comparing mycobacteria.

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APPENDIX

TABLE VII

RESPONSE TO ANTIGENS BY STRAIN 13 GUINEA PIGS INJECTED WITH LIVE SPLEEN CELLS[a]

Days after skin test	Response to Lepromin without Phenol[b]	Standard deviation	Response to Lepromin with Phenol	Standard deviation
1	-[c]	-	-	-
2	-	-	-	-
3	-	-	-	-
6	-	-	-	-
9	-	-	-	-
10	-	-	-	-
13	-	-	-	-
14	-	-	-	-
15	1.3	1.2	2.0	0
16	2.0	0	2.0	0
17	3.7	1.5	3.7	1.5
20	3.3	1.2	3.3	1.2
21	3.7	0.6	3.2	0.8
22	3.7	0.6	2.5	0.5
23	3.7	0.6	2.5	0.5
24	3.7	1.2	3.0	0
27	3.0	0	2.7	0.6
28	3.0	0	2.7	0.6
29	2.7	0.6	2.3	0.6
30	3.0	0	2.0	0

[a] Guinea pigs received 1 x 10^8 syngeneic live sensitized spleen cells via the intracardiac route and were skin tested 6 days after transfer.

[b] Results are given in mm of induration and are the average of corrected readings from 3 guinea pigs.

[c] No response.

TABLE VIII

RESPONSE TO ANTIGENS BY STRAIN 13 GUINEA PIGS INJECTED WITH HEAT-TREATED SPLEEN CELLS[a]

Days after skin test	Response to Lepromin without Phenol[b]	Standard deviation	Response to Lepromin with Phenol	Standard deviation
1	-[c]	-	-	-
2	-	-	-	-
3	-	-	-	-
6	-	-	-	-
9	-	-	-	-
10	-	-	-	-
13	-	-	-	-
14	-	-	-	-
15	0.7	1.2	0.7	1.2
16	2.3	0.6	2.0	0
17	3.3	1.5	3.3	1.5
20	4.0	1.0	3.0	1.0
21	3.3	0.6	3.0	1.0
22	3.0	1.0	2.7	1.2
23	3.0	1.0	2.7	1.2
24	3.3	1.2	2.7	1.2
27	2.7	0.6	2.7	0.6
28	2.7	0.6	2.0	1.7
29	2.7	0.6	2.0	1.7
30	2.0	1.7	2.0	1.7

[a] Guinea pigs received 1×10^8 syngeneic heat-treated sensitized spleen cells via the intracardiac route and were skin tested 6 days after transfer

[b] Results are given in mm of induration (diameter was measured) and are the average of corrected readings from 3 guinea pigs.

[c] No response.

TABLE IX

RESPONSE TO ANTIGENS BY STRAIN 2 GUINEA PIGS INJECTED WITH LIVE SPLEEN CELLS[a]

Days after skin test	Response to Lepromin without Phenol[b]	Standard deviation	Response to Lepromin with Phenol	Standard deviation
5	0.5	1.2	-[c]	-
6	0.8	1.0	0.2	0.5
7	0.5	1.0	0	1.2
8	0.5	0.6	- 0.2	0.5
11	4.2	1.0	2.5	0.6
12	5.8	1.0	3.5	1.0
13	7.0	0.8	3.3	0.5
14	6.2	1.0	4.0	1.4
15	6.0	1.2	3.0	0.8
19	7.0	0.8	3.2	0.5
20	6.2	0.5	3.2	1.2
21	7.0	0.8	3.8	1.0
22	7.0	0.8	3.8	1.2
25	6.0	0.8	3.2	1.5
26	6.0	0.8	3.0	0.8
27	5.8	1.0	2.5	0.6
28	5.5	0.6	2.8	1.2
29	5.3	1.0	2.8	1.2

a Guinea pigs received 1 x 10^8 syngeneic live sensitized spleen cells via the intracardiac route and were skin tested 2 days after transfer.

b Results were given in mm of induration and are the average of corrected readings from 4 guinea pigs.

c No response.

TABLE X

RESPONSE TO ANTIGENS BY STRAIN 2 GUINEA PIGS INJECTED WITH HEAT-TREATED SPLEEN CELLS[a]

Days after skin test	Response to Lepromin without Phenol[b]	Standard deviation	Response to Lepromin with Phenol	Standard deviation
5	-[c]	-	0.2	0.5
6	0.5	0.6	0.5	0.6
7	1.0	1.4	0.5	1.0
8	1.5	1.0	1.0	0.8
11	3.8	1.0	2.5	0.6
12	5.0	0.8	3.0	0.8
13	5.8	0.5	3.5	0.6
14	6.5	1.0	4.5	1.3
15	5.8	1.0	3.8	1.0
19	6.2	2.2	3.2	0.5
20	8.2	1.7	3.0	0.8
21	7.2	1.7	3.5	0.6
22	6.2	1.7	3.2	1.0
25	5.2	2.1	3	0.8
26	5.5	2.1	3.2	1.3
27	5.5	2.1	3	0.8
28	5.2	1.7	2.5	0.6
29	5.2	1.7	2.5	0.6

[a] Guinea pigs received 1 x 10^8 syngeneic heat-treated sensitized spleen cells via the intracardiac route and were skin tested 2 days after transfer.

[b] Results are given in mm of induration and are the average of corrected readings from 4 guinea pigs.

[c] No response.

TABLE XI

RESPONSE TO ANTIGENS BY STRAIN 2 GUINEA PIGS INJECTED WITH FROZEN-THAWED SPLEEN CELLS[a]

Days after skin test	Response to Lepromin without Phenol[b]	Standard deviation	Response to Lepromin with Phenol	Standard deviation
4	-[c]	-	-	-
5	-	-	-	-
6	-	-	-	-
7	- 1.0	1.4	- 1.0	1.4
10	-	-	-	-
11	0.5	0.7	0.5	0.7
12	1.5	0.7	1.5	0.7
13	1.5	0.7	1.5	0.7
14	1.0	0	1.0	0
18	1.5	0.7	1.5	0.7
19	1.5	0.7	2.5	0.7
20	1.5	0.7	2.5	0.7
21	1.5	0.7	2.5	0.7
24	1.0	0	2.5	0.7
25	1.0	0	2.5	0.7
26	1.0	0	2.5	0.7
27	1.0	0.7	2.5	0.7
28	1.0	0.7	2.5	0.7

[a] Guinea pigs received 1 x 10^8 syngeneic frozen-thawed sensitized spleen cells via the intracardiac route and were skin tested 2 days after transfer.

[b] Results are given in mm of induration and are the average of corrected readings from 2 guinea pigs.

[c] No response.

TABLE XII

RESPONSE TO ANTIGENS BY STRAIN 2 GUINEA PIGS

Days after skin test	Response to Lepromin without Phenol	Standard deviation	Response to Lepromin with Phenol	Standard deviation
4	- [c]	-	0.1	0.2
7	-	-	0.1	0.2
8	-	-	0.1	0.2
9	-	-	0.1	0.2
10	-	-	0.1	0.2
11	0.1	0.2	0.1	0.3
15	0.6	0.5	0.2	0.8
16	1.4	1.3	0.8	1.9
17	1.4	1.3	2.0	2.0
18	1.8	1.8	2.2	2.4
21	1.8	1.8	2.6	1.9
22	1.6	1.5	2.0	1.6
23	1.8	1.8	1.8	1.6
24	2.0	1.9	1.8	1.6
25	2.0	1.9	1.8	1.6
28	1.4	1.5	1.8	1.6
29	1.4	1.3	2.0	1.9

[a] Guinea pigs received no injections of cells.

[b] Results are given in mm of induration and are the average of corrected readings from 5 guinea pigs.

[c] No response.

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