Experimental Leprosy in Three Species of Monkeys

Abstract. *Eleven mangabey monkeys inoculated with* Mycobacterium leprae *developed lepromatous-type leprosy. Nine of the mangabeys were inoculated with* M. leprae *isolated from a mangabey with naturally acquired lepromatous leprosy. Immune function was depressed in some of these animals after dissemination of the disease. Two mangabeys developed lepromatous leprosy after inoculation with human* M. leprae *passaged in an armadillo. Three rhesus and three African green monkeys inoculated with mangabey-derived* M. leprae *also developed lepromatous leprosy. Mangabeys may be the first reported nonhuman primate model for the study of leprosy. Rhesus and African green monkeys may also prove to be reproducibly susceptible to the disease.*

Discovery of naturally acquired leprosy in a sooty mangabey monkey (*Cercocebus atys*) (*1, 2*) has prompted us to reevaluate the susceptibility of nonhuman primates to the disease. The nine-banded armadillo (*Dasypus novemcinctus*) has been, to our knowledge, the only previously reported immunologically intact species that develops lepromatous-type leprosy (LL) after inoculation with *Mycobacterium leprae*. Unlike leprosy in man, the disease is fatal to most armadillos within 36 months after inoculation (*3, 4*). Other species develop either a limited infection (*5*) or must be immunologically compromised to permit dissemination (*6–8*). We now report the successful transmission of leprosy to three monkey species: sooty mangabeys, rhesus monkeys (*Macaca mulatta*), and African green monkeys (*Cercopithecus aethiops*). Attempts to transmit leprosy to the squirrel monkey (*Saimiri sciureus*) were not successful.

Sources of inocula were either a naturally infected mangabey or armadillos previously infected with *M. leprae* from patients. Inocula were prepared by homogenization and differential centrifugation of tissue; bacilli were counted with standard methods (*9*).

Monkeys were sedated with ketamine hydrochloride (10 mg/kg). Biopsy specimens were processed with standard histological techniques and the Fite-Faraco method for staining *M. leprae*. Viability of inocula was confirmed by growth in mouse footpads and armadillos.

Mangabey monkeys. Four mangabeys were inoculated with *M. leprae* intravenously and at several sites intradermally. Each of two animals received a total of 2.7×10^9 *M. leprae* isolated from the mangabey with naturally acquired leprosy. By all available criteria this organism is identical to *M. leprae* isolated from lepromatous patients and infected armadillos (*1, 2, 10, 11*). Two other mangabeys were each inoculated with a total of 3.1×10^{10} *M. leprae* isolated from untreated LL patients and passaged in a nine-banded armadillo. All four mangabeys eventually developed LL or near-LL according to the Ridley-Jopling classification of leprosy (*12*).

Both mangabeys inoculated with *M. leprae* of mangabey origin developed lesions after 4 to 6 months at inoculation sites on the ears. By 10 months both animals had lesions on the scrotum, an uninoculated site. Histopathological examination of biopsy specimens revealed borderline lepromatous (BL) disease in one animal and less severe borderline (BB) disease in the other. In the ensuing months the infection in the latter animal progressed rapidly, and by 24 months after inoculation the disease was similar in both monkeys (Figs. 1 and 2). By 29 months nasal secretions from both animals contained large numbers of *M. leprae* and there was progressive dissemination to numerous sites on the face, ears, and extremities. The disease in both animals was then BL-LL histopathologically. There were many vacuolated histiocytes containing large numbers of acid-fast bacilli (AFB). Studies of blood lymphocytes before infection and at intervals thereafter revealed, in both animals, a decline in the mitogenic responsiveness of these cells to concanavalin A, phytohemagglutinin, and pokeweed mitogen as dissemination progressed (*13*). Crossed immunoelectrophoresis studies showed antibody levels against mycobacterial antigens 2, 5, and 7 in a pattern similar to that seen in LL patients and experimentally infected armadillos (*2, 14, 15*).

One monkey with disseminated LL died 46 months after inoculation upon being anesthetized for biopsies. The examination revealed gross and histopathological changes similar to those induced by LL in humans. Unlike leprosy in armadillos, in which there is extensive involvement of the liver, spleen, and other internal organs, the disease in the mangabey was confined to the skin, peripheral nerves, nasal mucosa, eyes, testes, and peripheral lymph nodes, with minimal involvement of the liver and spleen (*16*).

The two mangabeys inoculated with *M. leprae* of human origin developed lesions at inoculation sites on the face and ears 4 to 6 months after inoculation. One animal developed a more severe infection initially, but eventually both animals had lepromatous disease. At 45 months there were large lepromatous nodules confined to the dermal inoculation sites on the face and ears of both animals. Unlike the two mangabeys inoculated with the mangabey isolate, these animals have not, at this writing, developed lesions in uninoculated areas. Many AFB have been demonstrated repeatedly in nasal secretions from both animals, however, indicating that the infection has disseminated.

Rhesus monkeys. Two rhesus monkeys, a male and female, were inoculated with *M. leprae* isolated from the naturally infected mangabey. The male was inoculated intradermally and intravenously; the female only intradermally.

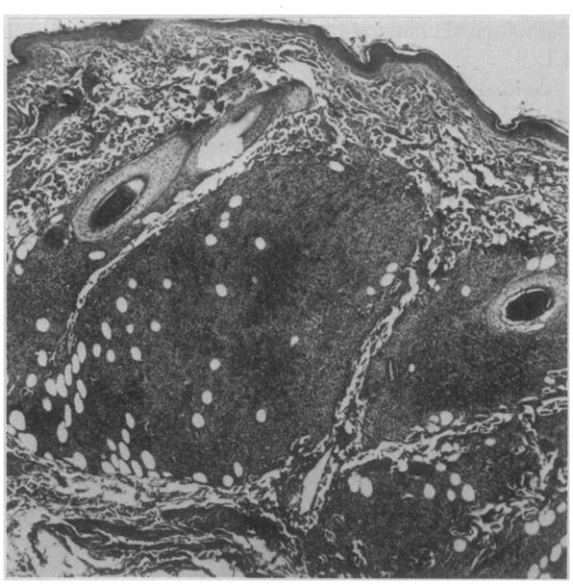

Fig. 1. Section of skin from infiltrated lesion on leg of mangabey monkey inoculated with *M. leprae* 17 months earlier. There is extensive cellular infiltration of the dermis and subcutis. Stain, hematoxylin and eosin; magnification, ×40.

Fourteen months later the male developed large nodular lesions on the scrotum, an uninouclated site. There were large erythematous plaques at inoculation sites on the nose, with dissemination to uninoculated sites on the face and extremities. Skin sections contained few lymphocytes and many well-stained AFB in foamy histocytes; nerves in the dermis were extensively involved and the disease was classified as BL-LL. At 23 months the lesions had regressed and tissue sections contained more lymphocytes and fewer well-stained AFB. However, after 25 months the disease was exacerbated, with reactivation of old lesions and appearance of new ones. At this time skin biopsies contained large numbers of well-stained AFB in histiocytes and few lymphocytes, and the disease was classified as LL. Serological studies revealed an increase in antibody to mycobacterial antigens 2, 5, and 7 similar to that seen in the two mangabeys infected with the mangabey isolate.

The female rhesus monkey inoculated intradermally but not intravenously had no evidence of infection by 42 months; its serum contained no antibodies to mycobacterial antigens 2, 5, and 7 (*14*).

African green monkeys. The same suspension of mangabey *M. leprae* that was given to the rhesus was used to inoculate three female African green monkeys intradermally and intravenously. Each became infected by 19 months. Two animals had small but discrete lesions at inoculation sites on the ears; thickening of the ears of the third animal was noted at 22 months. By 22 months, nasal washings from all three animals contained 10^5 to 10^6 AFB per milliliter. Growth patterns of these organisms in the footpads of mice and all other tests confirmed their identification as *M. leprae*. Histopathological changes were those seen in LL.

Squirrel monkeys. Three adult female squirrel monkeys were inoculated intradermally and intravenously with the same mangabey-derived *M. leprae* suspension that produced disease in African green and rhesus monkeys. No disease was apparent at 42 months.

Of the species studied, mangabeys are the most promising animal model for studies of experimental leprosy. In addition to the four mangabeys described, in more recent studies with mangabey inoculum 5 of 8 mangabeys developed disseminated leprosy by 12 months and 2 of 11 other mangabeys developed leprosy by 6 months. These results confirm the susceptibility of this species to the disease. Immunologic changes in infected mangabeys are similar to those seen in lepromatous patients (*13*).

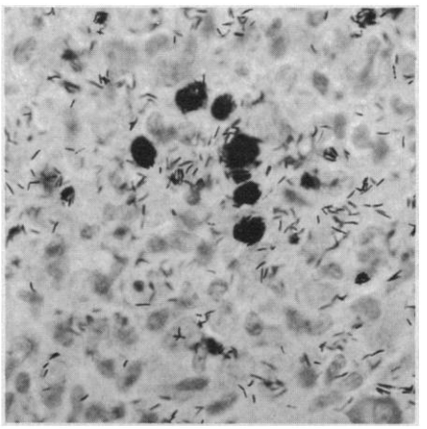

Fig. 2. Detail of section shown in Fig. 1. There are numerous acid-fast bacilli, singly and in clusters (black bodies), in the histiocytes. Stain, Fite-Faraco; magnification, ×630.

Mangabeys breed in captivity, as demonstrated by the colony maintained by the Yerkes Regional Primate Research Center, Atlanta. The colony has continued to grow while providing more than 20 animals for our leprosy studies during the past 4 years. Hence the limited supply of mangabeys need not continue to be a problem. The mangabey life-span in captivity of more than 20 years makes possible the long-term observations needed in studying chemotherapeutic and immunotherapeutic approaches to leprosy.

The transmission of leprosy to one of two rhesus monkeys suggests that this species may also provide a useful laboratory model for leprosy. Although both rhesus monkeys were inoculated intradermally, only the animal that was also inoculated intravenously developed disease. We do not have enough information to determine whether the intravenous injection of *M. leprae* was crucial to the development of the infection. In a later experiment two male and two female rhesus monkeys were inoculated by combined intravenous and intracutaneous routes with approximately 1×10^9 *M. leprae* of mangabey origin. The two males have developed leprous infiltrates in the skin and nasal mucosa; the females have shown no signs of leprosy in almost 2 years since inoculation. Further experiments with larger numbers of animals may indicate whether sex has any influence on the susceptibility of monkeys to experimental infection by *M. leprae*.

All three of the African green monkeys (females) inoculated with the mangabey isolate developed leprosy, but much more slowly than mangabeys. Rhesus and African green monkeys inoculated with armadillo-passaged *M. leprae* of human origin have not developed lesions in 24 to 31 months since inoculation. The enhanced virulence of mangabey *M. leprae* suggests that this strain has adapted to monkeys, particularly mangabeys.

Earlier attempts to infect nonhuman primates were, with rare exceptions, unsuccessful (*15, 17, 18*). Possible explanations for these failures are that the inocula used contained too few viable *M. leprae*, that the routes of inoculation were inappropriate, and, in some cases, that the period of observation after inoculation was too short. Our successful transmission of leprosy to three species suggests a potential zoonotic reservoir for *M. leprae* in monkeys (*10*).

Availability of nonhuman primate models represents an important advancement in the study of experimental leprosy. Because monkeys are phylogenetically close to man, any information on the pathogenesis of the disease obtained in these species should be more applicable to man than that obtained from other species. Use of the mangabey model should make it possible to ascertain the modes of leprosy transmission, possible pathogenic immune mechanisms in leprosy, and the feasibility of developing a protective vaccine against this disease. Mangabeys will also be useful in studies of drug therapy and reconstructive surgery.

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The Role of Hemoglobin Denaturation and Band 3 Clustering in Red Blood Cell Aging

Abstract. *As hemoglobin begins to denature, it forms hemichromes that cross-link the major erythrocyte membrane–spanning protein, band 3, into clusters. These clusters provide the recognition site for antibodies directed against senescent cells. These antibodies bind to the aged red cell and trigger its removal from circulation.*

The homeostasis of the circulatory system depends on the removal of aged and damaged cells from the blood (*1*). Autologous antibodies [immunoglobulin G (IgG)] present in healthy human serum selectively bind to senescent red blood cells (RBC's) and initiate the removal of the senescent cells by phagocytes and macrophages (*2–6*). The age-specific antigen on senescent RBC's is the predominant membrane protein, band 3 (*4, 7–9*), but since band 3 is present on both young and old cells, the molecular change in band 3 that marks the erythrocyte as "aged" is unclear. Three hypotheses have been offered to explain this change in band 3: (i) aged band 3 is cleaved or covalently modified, (ii) a cryptic antigenic site on band 3 is exposed on aging, or (iii) the lateral distribution of band 3 in the membrane in aged cells is altered (*6, 9*). We now report that as hemoglobin begins to denature, it cross-links band 3 into aggregates, and these cross-linked aggregates constitute the antigenic determinant recognized by the antibodies to senescent cells. Thus, RBC's are removed from circulation and destroyed when they begin to falter in their primary function of O_2 transport.

The initial stages of hemoglobin denaturation involve the formation of hemichromes, which remain oligomeric but do not carry oxygen because of a conformational change in the vicinity of the heme (*10, 11*). We reported earlier that these hemichromes display a high affinity for the cytoplasmic domain of the membrane-spanning protein, band 3 (*10*). The association between hemichromes and the cytoplasmic domain of band 3 does not terminate with a single biomolecular complex but propagates with a 2.5:1 (hemoglobin to band 3) stoichiometry indefinitely into a tightly aggregated copolymer (*10*). Since a change in the distribution of band 3 in the membrane has been postulated as a possible indicator of erythrocyte senescence (*9*), we decided to investigate whether the clustering of band 3 by copolymerization with denatured hemoglobin might promote autologous antibody binding to whole RBC's.

Hemichromes were artificially induced in normal RBC's by treatment of the cells with low concentrations of phenylhydrazine, a reagent that catalyzes the denaturation of hemoglobin (*11, 12*). This drug-induced denaturation appears to produce an alteration in hemoglobin similar to that of normal hemoglobin denaturation in vivo (*11, 12*). After treatment with phenylhydrazine, the RBC's were washed, incubated in whole human serum to allow autologous IgG binding, rewashed to remove unbound IgG, and analyzed for adhering IgG by measuring the radioactivity of tightly absorbed ^{125}I-labeled protein A. Figure 1a shows that phenylhydrazine-promoted hemoglobin denaturation does, in fact, lead to enhanced binding of IgG to erythrocytes. The autologous IgG obtained by stripping the treated RBC's with 0.1*M* glycine buffer (*p*H 3) cross-reacted with intact band 3 in Western blots (*13*) of erythrocyte membranes, but not with the isolated cytoplasmic domain of band 3 prepared as described elsewhere (*14*) (not shown). This indicates, as recently demonstrated by Kay (*15*), that the bound IgG was directed against a determinant in the integral or external part of the band 3 molecule. With 16 m*M* phen-

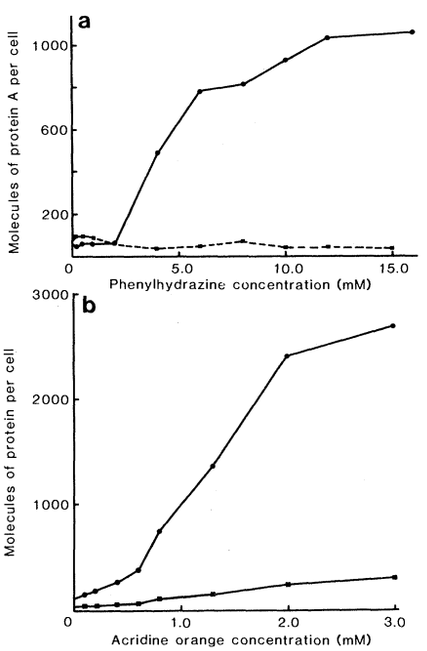

Fig. 1. (a) Binding of IgG to phenylhydrazine-treated cells. Whole blood was separated into fractions by the method of Murphy (*23*), and the upper (youngest or least dense) 10 percent was collected and washed. Portions of these cells were suspended at 5 percent hematocrit in phosphate-buffered saline (PBS), *p*H 7.4, 10 m*M* glucose, and 2 m*M* adenosine (buffer A) containing the indicated amounts of phenylhydrazine, incubated for 2 hours at 37°C, and then pelleted and washed three times in 20 volumes of buffer A. The cells were suspended at 5 percent hematocrit in the donor's own serum (●) or in incubated buffer A as a control (■) for 3 hours at room temperature, and then pelleted and washed five times with 40 volumes of buffer A, the last wash containing bovine serum albumin (1 mg/ml) (buffer B). The IgG-coated cells were then incubated at 4 percent hematocrit in buffer B containing ^{125}I-labeled protein A (approximately 0.4 μg/ml) for 1 hour at room temperature. Cells were then pelleted and washed six times with 40 volumes of buffer B, and the amount of radioactive label was determined. (b) Binding of IgG to acridine orange–treated cells. Red cells were separated as in (a), washed three times in 20 m*M* sodium phosphate and 125 m*M* NaCl (PBS), *p*H 7.3, and suspended at 5 percent hematocrit in PBS, *p*H 7.3, containing the indicated concentrations of acridine orange. Each sample was then incubated for 0.5 hour at room temperature, pelleted, and suspended either in the donor's own serum containing the same concentration of acridine orange (●) or in PBS containing acridine orange as a control (■). After 2 hours of incubation at room temperature, the cells were pelleted, washed five times in 20 volumes of PBS plus the desired concentration of acridine orange, and suspended at 4.5 percent hematocrit in the same acridine orange buffer containing ^{125}I-labeled protein A (approximately 0.23 μg/ml). Incubation then continued for 1 hour at room temperature, after which the cells were pelleted and washed five times with 20 volumes of the acridine orange buffer, and the amount of radioactive label was determined. Acridine orange was kept in all solutions, since band 3 clustering rapidly reversed when it was removed.