

FORMATION OF ANTIBODY AGAINST *MYCOBACTERIUM LEPRAE* ANTIGEN 7 IN ARMADILLOS

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ONE of the primary restrictions in leprosy research has been the limited amount of *Mycobacterium leprae* available. *M. leprae* has not yet been acceptably grown *in vitro* and, until the discovery of the susceptibility of the nine-banded armadillo (*Dasypus novemcinctus*) to experimental infection with *M. leprae* (Kirchheimer and Storrs, 1971), it was not possible to obtain large numbers of leprosy bacilli for experimental purposes.

Now with the relatively large amounts of *M. leprae* available, new information on the antigenic structure and other characteristics of armadillo-grown *M. leprae* has been obtained. However, for optimal use of the armadillo as a source of *M. leprae* more information is needed about several features of the infection in this animal. As only 40–60% of armadillos develop disseminated disease, even after intravenous inoculation with $1-10 \times 10^7$ *M. leprae* (Storrs *et al.*, 1974; Rees, 1976; Kirchheimer and Sanchez, 1977), it would be an advantage for the economical production of large amounts of infected tissues, if it were possible to identify at an early stage those animals developing a progressive infection. More information is also needed about the optimal time to harvest the bacteria from the infected armadillos. Thus, early harvest might provide a greater proportion of viable organisms in an active growth phase and containing the maximum of antigenic components though at the expense of a reduced yield of bacteria, while late harvest from armadillos with very advanced systemic infection might be more valuable for other purposes.

Recently it has been found that a proportion of nine-banded armadillos from the wild habitat are already infected with a mycobacterium that is, so far, indistinguishable from *M. leprae* (Walsh *et al.*, 1975; Rees, 1976; Binford *et al.*, 1977; Walsh *et al.*, 1977) and it is evident that animals selected for experimental infection with *M. leprae* must be shown to be free from previous mycobacterial infection. The development of tests to detect previous infection in armadillos before inoculation with *M. leprae* is, therefore, of high priority. Harboe *et al.* (1977a) developed a sensitive radioimmunoassay for the demonstration of antibodies against BCG antigen 60, which occur with high frequency in both lepromatous and tuberculoid leprosy (Harboe *et al.*, 1977b). A cross-reacting antigen is present in many mycobacterial species, including *M. leprae*,

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in which it is termed *M. leprae* antigen 7 (Harboe *et al.*, 1977c). Melsom *et al.* (1978) described a radioimmunoassay for the specific demonstration and quantitation of antibodies against *M. leprae* antigen 7. The purpose of the present investigation was to study the concentration of antibodies to *M. leprae* antigen 7 in several different groups of armadillos to determine whether such an assay would be of value in selecting animals for inoculation, in predicting the outcome of the experimental infection, and in obtaining information on the optimal time of harvest.

MATERIALS AND METHODS

Antibodies against M. leprae antigen 7 were demonstrated by methods based on previous experience with purification and labelling of BCG antigen 60 (Harboe *et al.*, 1977a and b). Briefly, *M. leprae* (A/10 preparation) was provided by the IMMLEP Programme of the World Health Organisation as freeze-dried bacilli purified from the liver of an infected armadillo by Draper's procedure (Draper, 1976). *M. leprae* (60 mg dry weight) was suspended in 10 ml of 0.1M phosphate buffer, pH 7.4, and sonicated on ice for 20 min. with a Branson B12 sonifier at 80 watts (Branson Sonic Power Co., Danbury, Conn., USA). After centrifugation for 15 min. at 20 000 *g* at 4°C, the supernatant fluid was collected to serve as unlabelled, crude *M. leprae* antigen. An aliquot of this preparation was labelled with ¹²⁵I by electrolytic iodination as described previously (Harboe *et al.*, 1977a; Melsom *et al.*, 1978). Free iodide and non-covalently bound iodide were removed by extensive dialysis against phosphate buffered saline at 4°C, and the iodinated preparation was diluted in immunoassay buffer and stored at -25°C until used. *M. leprae* antigen 7 has a particular affinity for iodine during electrolytic iodination and is selectively labelled. The resulting preparation was filtered through a Sepharose 4B column, and the fractions corresponding to the void volume were pooled. As described previously (Melsom *et al.*, 1978) this material contained labelled *M. leprae* antigen 7 and provided a monospecific radioimmunoassay for antibodies against this antigen of *M. leprae*.

Radioimmunoassay (RIA). The procedure previously described (Harboe *et al.*, 1977a and b) for the assay of antibodies against BCG antigen 60, was used. The technique is based on the separation of antibody-bound labelled antigen from free antigen by the use of protein A-containing staphylococci which serve as a solid phase and have a strong capacity to bind IgG antibodies (Jonsson and Kronvall, 1974), including IgG from armadillos (Kronvall *et al.*, 1970). Briefly, each tube contained 100 µl of the appropriate serum dilution and 100 µl of labelled *M. leprae* antigen 7, giving about 20 000 counts per 400 s. The mixtures were incubated for 30 min. at room temperature before the addition of 2 ml of a 1% suspension of formalinised staphylococci (Cowan I strain; NCTC85308). All dilutions of unlabelled and labelled proteins and of sera were made in RIA buffer of the following composition: 0.1M phosphate buffered saline, pH 7.4, containing 0.03M sodium azide, 0.001M EDTA and 0.2% human serum albumin (Reinst, Behringwerke, Marburg Lahn, Germany). After the reagents had been mixed, the tubes were centrifuged at 15 000 *g* for 20 min. the supernate was removed and the radioactivity in the bacterial pellet determined. All values are given as the mean of two tests. Where appropriate, they are given as radioactivity bound to staphylococci, expressed as the percentage of maximal binding activity by a reference serum pool containing four sera from patients with lepromatous leprosy selected for their strong antibody activity against antigen 7 of *M. leprae*. To establish maximal binding activity, this serum pool was tested at dilutions of 10, 20, 40 and 80. All these dilutions showed the same binding activity, indicating an antibody excess.

Crossed immunoelectrophoresis (CIE) with intermediate gel was used to demonstrate antibodies of other specificities against *M. leprae* antigens in armadillo sera. The procedure and the numbering of antigenic components of *M. leprae* has been described previously (Harboe *et al.*, 1977c; Melsom *et al.*, 1978).

Armadillo sera. Animals were caught in the wild habitat in Louisiana, USA, allowed to

adapt to captivity, tested for mycobacterial infection, quarantined, inoculated with *M. leprae*, sampled, and tested as outlined in protocol No. 1/75 describing the "Supply of *M. leprae* for IMMLEP programme" (World Health Organisation, 1976).

Sera were available from 21 normal armadillos, which were examined clinically and bacteriologically. Ear-skin scrapes, ear-clip tissue, buffy-coat preparations from peripheral blood, and nasal smears were examined for acid-fast bacilli; ear-clip tissue was also examined histologically. The normal animals gave negative results in all these tests.

Sera were obtained from seven animals with evidence of mycobacterial infection when caught in the wild habitat. These animals had nodular lesions containing non-cultivable acid-fast bacilli that are, so far, indistinguishable from *M. leprae* (Rees, 1976; Binford *et al.*, 1977; Walsh *et al.*, 1977). Infection was graded as in the experimentally inoculated animals.

Sera were also obtained from 20 armadillos experimentally inoculated with *M. leprae*. The animals were inoculated intravenously with 10^8 acid-fast bacilli derived from homogenates of lepromatous nodules from three untreated patients with lepromatous leprosy. The animals were killed and the bacilli harvested 24 months later or at other times, as indicated. From 17 animals with evidence of established mycobacterial infection, studies of the bacilli obtained indicated that their properties corresponded to the typical properties of *M. leprae*, again as outlined in IMMLEP protocol No. 1/75 (World Health Organisation, 1976).

Two sera were obtained from armadillos that had developed ulcers with extensive necrosis at the inoculation sites 9 weeks after intradermal inoculation with *Mycobacterium ulcerans* on the medial side of both thighs.

Human sera. The lepromatous serum pool consisted of a mixture of sera from 43 patients clinically and histopathologically classified according to the extended scale of Ridley and Jopling (1966) as LL, LI and BL leprosy.

The normal human serum pool consisted of sera from 25 healthy Norwegian medical students vaccinated with BCG.

M. leprae infection in armadillos was graded as follows. + = No gross skin lesions; bacterial counts of 10^6 – 10^8 per g of tissue; lymph nodes not notably enlarged; mild infection confirmed by histopathological examination. ++ = A few skin lesions; bacterial counts of 10^8 – 10^9 per g of tissue; strikingly enlarged lymph nodes; dissemination of disease to major organs confirmed by histopathological examination. +++ = Multiple gross lesions; bacterial counts of 10^9 per g of tissue or more; strikingly enlarged lymph nodes with many cutaneous lepromata; dense disseminated infection confirmed by histopathological examination.

RESULTS

Antibody against M. leprae antigen 7 in normal armadillos

The results of RIA for antibody against *M. leprae* antigen 7 in four serum samples are shown in fig. 1. The normal human-serum pool, obtained from healthy individuals living in a leprosy non-endemic area, showed strong binding of *M. leprae* antigen 7 at a dilution of 1 in 100. Similar antibody activity with the cross-reacting BCG antigen 60 has been described previously (Harboe *et al.*, 1977b) in the IgG fraction of normal human serum and was considered to be the result of antibody formation against this antigen after exposure to cross-reacting antigens in environmental mycobacteria. There was still significant antibody activity in normal human serum at a dilution of 1 in 1000. The findings with the normal armadillo-serum pool were similar, with strong antibody activity at a dilution of 1 in 100 and much lower activity at a dilution of 1 in 1000. At both these dilutions, the activity was lower in the armadillo serum pool than in the human serum pool. Strong antibody activity against

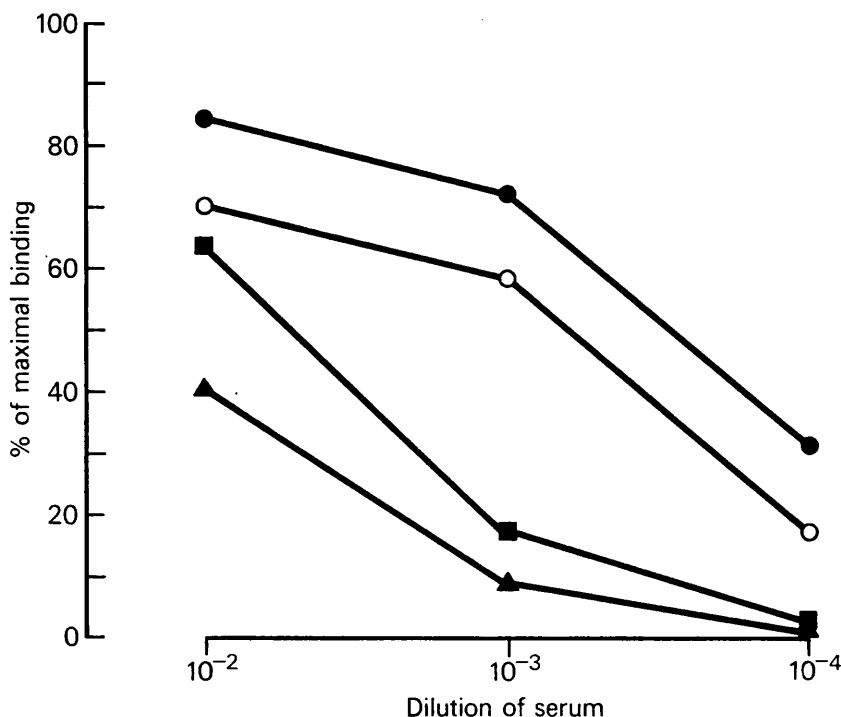


FIG. 1.—Radioimmunoassay for antibody activity against *M. leprae* antigen 7 in four serum samples. ● = Lepromatous serum pool; ○ = serum from armadillo no. 12; ■ = normal human-serum pool; ▲ = normal armadillo-serum pool. "Percentage of maximal binding" is defined in *Materials and methods*.

labelled *M. leprae* antigen 7 was demonstrated in the lepromatous serum pool, the titre being 10^6 . Fig. 1 also shows the results with serum from armadillo no. 12, an animal with severe systemic mycobacterial infection resulting from inoculation with *M. leprae*. The activity was considerably higher than that of the normal armadillo-serum pool.

When sera from different armadillos are to be compared for antibody content, various principles of analysis may be used (Harboe *et al.*, 1977b). Based on previous experience and the results of experiments such as those illustrated in fig. 1, it was decided to test all sera at a single dilution. A dilution of 1 in 1000 was selected as it gave the greatest differences between individual sera. Samples from a series of animals were tested simultaneously against the same labelled *M. leprae* antigen-7 preparation, and the antibody activity was recorded as the percentage of maximal binding activity by the reference serum pool.

The antibody activity in various serum samples is shown in fig. 2. The lepromatous serum pool showed an antibody activity corresponding to 73% of the maximum when diluted 1 in 1000, while sera from 10 Norwegian medical students showed low but significant antibody activity, with a median value of 22.5%. The median value of 21 sera from normal armadillos was 6%, and all

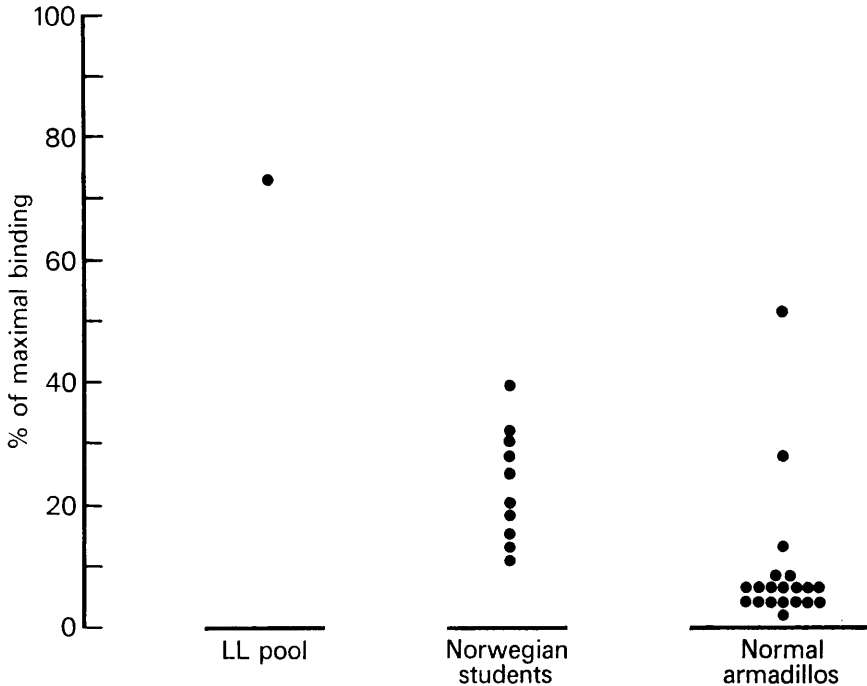


FIG. 2.—Radioimmunoassay for antibody activity against *M. leprae* antigen 7 in serum samples of different origin. Each point represents one serum tested at a dilution of 1 in 1000. LL = lepromatous serum.

but two of these sera showed less than 15% of maximal antibody activity when diluted 1 in 1000. One normal armadillo serum had 28% antibody activity, while another had 51% (armadillo no. 334). Further examination of the clinical records and histological preparations from the latter animal revealed no evidence of mycobacterial infection.

Antibody against M. leprae antigen 7 in infected armadillos

Fig. 3 shows the results of RIA for antibody activity in the serum of seven armadillos, infected with *M. leprae*, in the serum pool from normal armadillos, and in the serum of an eight-banded armadillo (*Dasypus sabanicola*). There was increased antibody activity against *M. leprae* antigen 7 in at least some of the armadillos that had developed systemic infection after inoculation with *M. leprae*.

The antibody activity in all sera from armadillos with evidence of mycobacterial infection is shown in fig. 4. Some of the sera from animals infected intravenously with *M. leprae* showed strong antibody activity. There was a striking variation in antibody content and an apparent lack of correlation of antibody with the degree of infection. Moreover, there was no correlation with duration of infection in animals tested 8–12 months after inoculation. Not all of the animals with established infection had significantly increased antibody to *M. leprae* antigen 7 antibodies, as compared with normal armadillos. Why

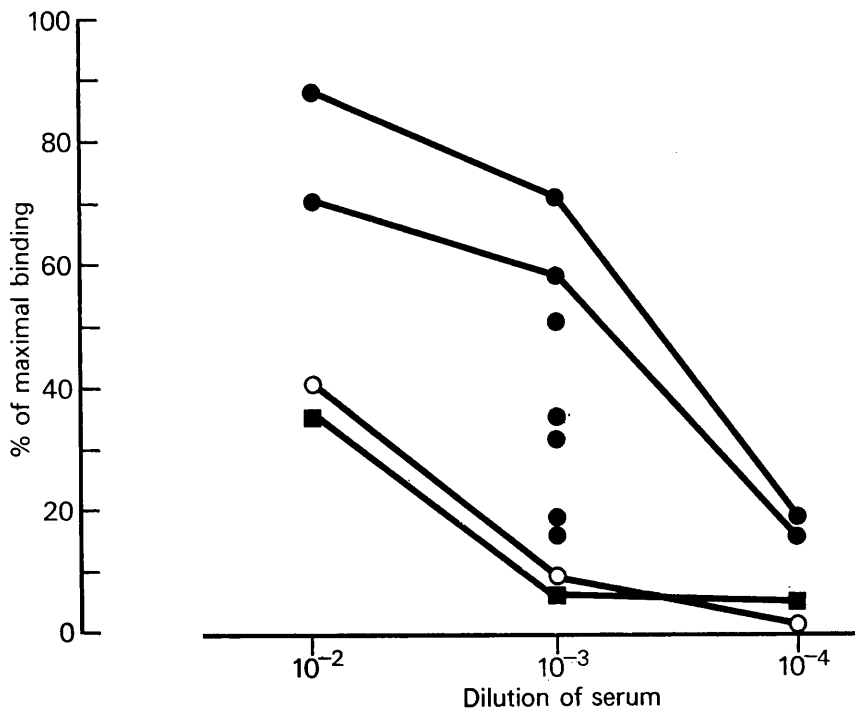


FIG. 3.—Radioimmunoassay for antibody activity against *M. leprae* antigen 7 in seven sera from armadillos with systemic *M. leprae* infection (●), in a serum pool from normal armadillos (○), and in serum from a single eight-banded armadillo (■). For simplicity, only data obtained at a dilution of 1 in 1000 are shown for five of the sera.

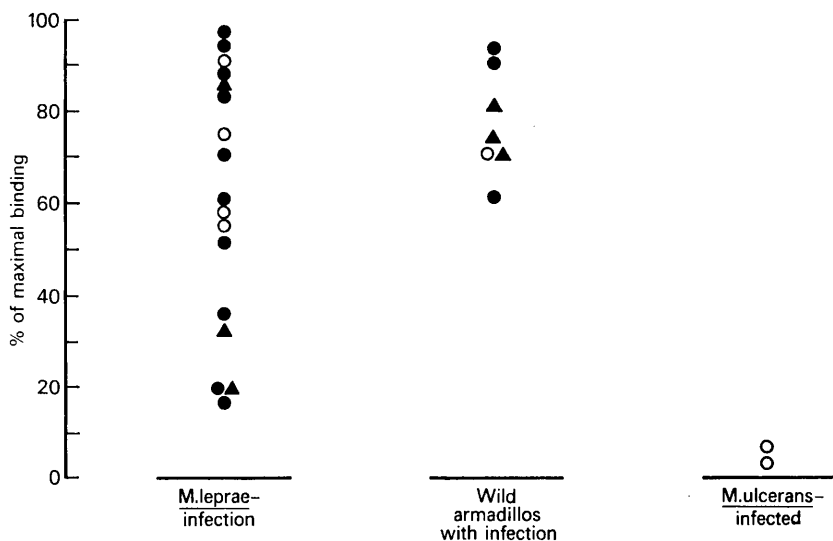


FIG. 4.—Radioimmunoassay for antibody activity against *M. leprae* antigen 7 in armadillos with mycobacterial infection. Each point represents one serum tested at a dilution of 1 in 1000. ● = Infection graded +++; ○ = infection graded ++; ▲ = infection graded + (see *Materials and Methods*).

some of the infected armadillos with established *M. leprae* infection did not contain more antibody is not clear, but 14 (82%) of the 17 armadillos with established infection had significant antibody activity, i.e., showed more than 30% of maximal binding. Seven animals with existing mycobacterial infection at the time of capture showed considerable antibody activity in the assay; again, there was no definite relationship between antibody activity and the degree of infection. Two animals with *M. ulcerans* infection had very low antibody activity against *M. leprae* antigen 7.

The results obtained from a group of seven animals inoculated 24 months previously with 10^8 *M. leprae* from a single batch of bacilli are shown in fig. 5. Three of the animals lacked signs of active infection and reacted negatively, whereas four animals with signs of infection of various grades gave positive results in the assay.

Demonstration of antibody other than that against antigen 7 in M. leprae infected armadillos

Sera from five armadillos with systemic *M. leprae* infection after intravenous inoculation were selected, by reason of their strong antibody activity against

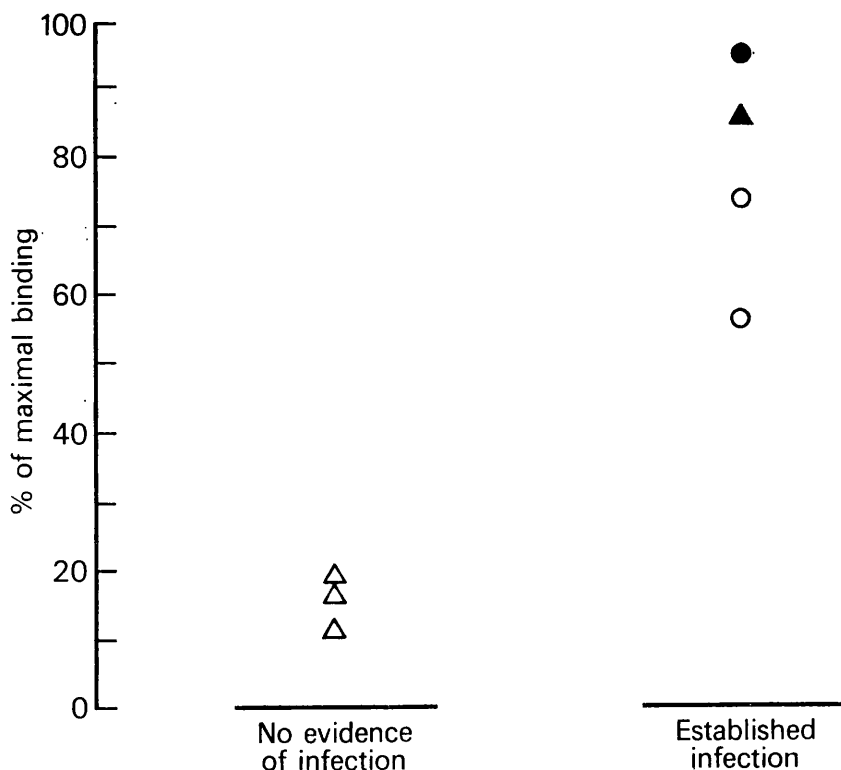


FIG. 5.—Radioimmunoassay for anti-*M. leprae* antigen 7 activity in seven armadillos inoculated 24 months previously with 1×10^8 *M. leprae* from the same batch; △ No evidence of infection at autopsy; ● = infection graded +++; ○ = infection graded ++; ▲ = infection graded +.

M. leprae antigen 7 in the RIA, and then tested by CIE with intermediate gel. All sera contained antibody against *M. leprae* antigen 5 and four contained antibody against antigen 2. Antibodies against *M. leprae* antigens 7, 5 and 2 are the ones most frequently present in patients with lepromatous leprosy (Harboe *et al.*, 1977c).

The results of CIE are shown in fig. 6. The retention of antigen 7 at the bottom of the intermediate gel in the CIE test plate was due to strong antibody activity against this component in the serum of armadillo no. 65, an animal with systemic *M. leprae* infection. The positions of antigens 5 and 2 were significantly lower in the test plate than in the control plate, and the tails of the precipitates were turned inwards (arrows). In addition, antigen 8 was lower in the test plate and the precipitate extended into the intermediate gel. Antigen 3 was somewhat difficult to define in the control plate, as the line was partly hidden behind the strong precipitates of antigens 5 and 2. Antibody against antigen 3 in the serum of armadillo no. 65 retained the antigen in the intermediate gel, where it was easy to see as it now lay free of the other antigens. It was concluded that armadillo serum no. 65 contained antibodies against *M. leprae* antigens 2, 3, 5 and 7 and against an additional antigen not precipitated by the rabbit anti-*M. leprae* antibody in the top gel. This extra precipitate is indicated with an asterisk in fig. 6.

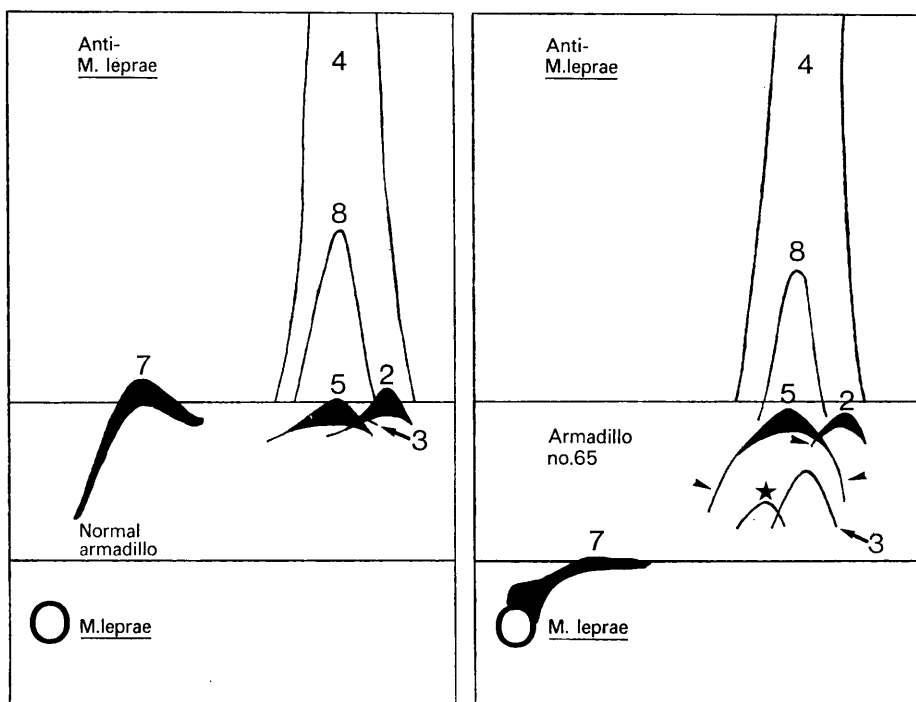


FIG. 6. (left and right)—Crossed immunoelectrophoresis. Control plate (left) with normal armadillo serum in the intermediate gel. Test plate (right) with serum from an armadillo (no. 75), with systemic *M. leprae* infection, in the intermediate gel. The arrows and the asterisk are explained in the text.

DISCUSSION

Ulrich *et al.* (1976) reported that the eight-banded armadillo has a typical mammalian distribution of lymphoid cells in thymus, spleen, lymph nodes and blood. Sensitisation with ovalbumin resulted in the production of precipitating antibodies that induced strong Arthus reactions, and the animals developed strong cell-mediated immune responses to 2:4-dinitro-chlorobenzene (DNCB) and *Mycobacterium tuberculosis*. There is, however, only limited information on the immune response of the nine-banded armadillo inoculated with *M. leprae*. The present experiments showed that the RIA developed for the measurement of antibodies against *M. leprae* antigen 7 in man (Melsom *et al.*, 1978) could be applied to the armadillo and that there was a distinct humoral response against this antigen in most of the *M. leprae*-inoculated armadillos that developed systemic mycobacterial infection. CIE with armadillo serum in the intermediate gel showed that *M. leprae*-inoculated armadillos also produced antibodies against other components of *M. leprae*.

The antibody against *M. leprae* antigen 7 in normal armadillo serum is probably due to exposure to cross-reacting antigens in environmental mycobacteria; an analogous state of affairs occurs in man (Harboe *et al.*, 1977b). Except in one instance, the activity was lower in normal armadillo sera than in 10 Norwegian medical students vaccinated with BCG. The explanation for this aberrant behaviour is not known. Sera from a larger group of normal armadillos must be examined in order to obtain a better estimate of the frequency with which normal armadillo sera have high antibody activity against *M. leprae* antigen 7. Additional information about the specificity of the antibodies in the exceptional sera from normal armadillos will be sought with another RIA developed to study antibodies reacting with antigenic determinants highly specific for *M. leprae* (Harboe *et al.*, 1978). In this test the specificity of anti-mycobacterial antibodies can be characterised with great sensitivity in experiments involving stepwise absorption with ultrasonicates prepared from different species of mycobacteria.

The reason for the striking variation in antibody production against antigen 7 among 17 armadillos that developed systemic mycobacterial infection after inoculation with *M. leprae* is unknown, but the data indicate that there was no correlation between antibody content and degree of infection (fig. 4). Similar observations have been made previously in patients with lepromatous leprosy (Harboe *et al.*, 1977b; Melsom *et al.*, 1978). The test for antibody activity in sera should, however, be a sensitive indicator of successful inoculation of armadillos with *M. leprae*, as 14 (82%) of the 17 inoculated armadillos with established infection had significantly raised antibody activity. All of the seven armadillos with mycobacterial infection at the time of capture had strong antibody activity against *M. leprae* antigen 7. Thus, the assay also appears to be valuable for the selection of animals suitable for inoculation with *M. leprae*.

Serial samples taken at two-monthly intervals from a group of *M. leprae*-inoculated armadillos are currently being screened for antibody against *M.*

leprae antigen 7. The present observations indicate that such longitudinal studies may provide new information on the development of systemic *M. leprae* infection in armadillos and on the accompanying immune response.

SUMMARY

A radioimmunoassay developed to measure antibody against *Mycobacterium leprae* antigen 7 in man was applied to the nine-banded armadillo (*Dasypus novemcinctus*). Normal armadillo sera had low but significant antibody activity in the test. Fourteen of 17 armadillos with systemic mycobacterial infection after inoculation with *M. leprae* showed increased antibody activity in the assay, and in some instances the activity was higher than in a pool of sera from patients with lepromatous leprosy. Crossed immunoelectrophoresis with armadillo serum in the intermediate gel revealed antibodies against five distinct antigenic components of *M. leprae*. Development of systemic mycobacterial infection after inoculation with *M. leprae* is thus associated with a distinct humoral immune response. The use of radioimmunoassay for selection of animals for inoculation and for following the development of the infection is discussed.

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