Clin. exp. Immunol. (1990) **79**, 397–402

Confirmation of a false-positive result associated with a competition inhibition assay used for detecting antibodies to a protein epitope of *Mycobacterium leprae*

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(Accepted for publication 9 October 1989)

SUMMARY

A competitive antibody-binding assay (CABA) was developed to detect antibodies in infected armadillos and leprosy patients which compete with an *M. leprae*-specific ^{125}I monoclonal antibody IIIE9 for the species-specific *M. leprae*-IIIE9 epitope on the 65-kD protein. The results suggest armadillos and leprosy patients produce antibodies that inhibit the binding of ^{125}I-IIIE9 monoclonal antibody to the IIIE9 epitope on crude, native 65-kD protein preparations. When purified, recombinant 65-kD protein was substituted for crude antigen, there was no evidence in the CABA of antibody to the IIIE9 epitope. False-positive results, possibly induced by steric hindrance, are likely to be associated with CABA which incorporate crude cell wall extracts as solid-phase antigen.

Keywords competitive antibody-binding assay IIIE9 epitope recombinant 65-kD protein *Mycobacterium leprae* armadillo

INTRODUCTION

Activation of the immune system during infection generally leads to the proliferation and clonal expansion of specific T and B lymphocytes producing, among other things, lymphokines capable of modulating the actions of other lymphocytes and accessory cells, as well as specific antibodies directed against antigenic determinants of the infectious organism. Serodiagnostic tests have been designed to take advantage of the presence of species-specific antibodies produced during the host's immune response and have provided powerful tools to diagnose and monitor infectious diseases.

Development of serological tests designed to detect a specific antibody population requires either the purified target antigen, in a form capable of binding population antibody present in test samples, or a second antibody preparation directed against species-specific antigenic determinants which can be used in a competitive antibody-binding assay (CABA). The CABA is particularly suited when purified antigen preparations from the infectious agent are not available as is the case with micro-organisms that are difficult to grow, such as *M. leprae*. The CABA has been employed to detect antibodies to specific protein antigenic determinants of *M. leprae* and *M. tuberculosis* (Hewitt *et al.*, 1982; Sinha *et al.*, 1983). In each of these assays monoclonal antibodies (MoAbs) were used as the competitive reagent and crude extracts of the mycobacteria were used as antigen.

The premise upon which mycobacterial CABA are currently founded is that labelled MoAbs directed against a defined epitope in crude antigen extracts would be blocked by antibody in serum of humans exposed to this infectious agent. A major advantage of this assay is that antibody, specific for a single epitope in serum which contains multiple specificity can be detected which mirrors that of the MoAb. An additional advantage is that specificity is not lost by utilizing crude antigen extracts.

Conversely, a disadvantage of CABA which uses a crude antigen preparation is the effect upon specific antibody binding (either labelled MoAb or test sera) by other serum antibodies which bind to antigenic determinants located in close proximity to the target epitope. This phenomenon, referred to as steric hindrance, may play a role in blocking the specific reaction which may lead to false-positive results. One approach by which to examine the role of steric hindrance and the degree to which it influences a given set of data is to compare results derived from CABA in which crude antigen is employed with findings in experiments where purified native protein or recombinant protein has been used as the antigen source. Unfortunately, in most situations purified native protein or recombinant protein containing the epitope of interest are not readily available. As a

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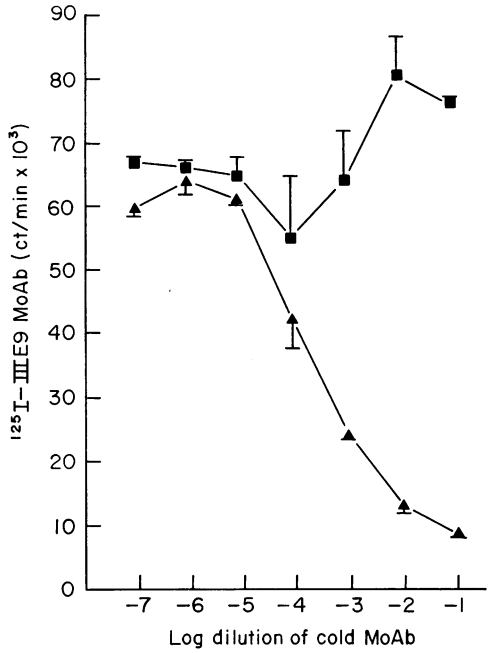

Fig. 1. Examination of the specificity for competitive antibody-binding assay (CABA) using 10 kp. Crude cell wall extract of *M. leprae* (10 kp) was diluted to contain 0·2 mg/ml in 0·05 M carbonate-bicarbonate (pH 9·2) coating buffer; 100 μl of the cell wall extract were added to each well of the microtitre plate. The effect of relevant (cold IIIE9; ▲) and irrelevant (cold D_2D_3; ■) monoclonal antibody (MoAb) on binding of ^{125}I-IIIE9 MoAb to the IIIE9 epitope was analyzed. Radiolabelled MoAb was used at a final dilution to contain 1×10^6 ct/min along with a serial dilution of cold MoAbs ranging from 10^{-1} to 10^{-7}.

result most investigators continue to use the CABA with crude antigen.

We were interested in evaluating the potential of armadillo antibodies directed to an *M. leprae*-specific epitope (IIIE9) on the 65-kD protein, for development of an assay which detects subclinical leprosy. In order to circumvent the laborious antigen purification process, and taking advantage of available MoAb to the IIIE9 epitope, we selected the CABA to analyse 175 serum samples for antibody to the IIIE9 epitope collected from 26 animals during the course of *M. leprae* infection. During the course of this study, recombinant 65-kD protein (r65-kD) became available and the specificity of our data was evaluated by comparison of results obtained from CABA developed with non-purified native antigen to that of CABA developed using purified r65-kD protein. The findings suggest that false-positive results due to steric hindrance can occur when crude cell wall extracts of *M. leprae* as the source of IIIE9 epitope are used in the CABA.

MATERIALS AND METHODS

Armadillo sera

Serum samples were collected from 26 nine-banded armadillos (*Dasypus novemcinctus*) prior to infection (day 0) and then at 3-month intervals for a period of 1–3 years. The conditions for adapting animals into the armadillo colony and their maintenance have been described previously (Vadiee *et al.*, 1988).

Patient sera

Serum samples taken from four leprosy patients were supplied by Dr W. R. Levis (Bayley Seton Hospital Medical College, New York, NY). Each serum sample had been identified as positive for antibody to the IIIE9 epitope in CABA using crude cell wall extract (personal communication).

Preparation of M. leprae *extracts*

Irradiated, lyophilized, *M. leprae* purified from lymph nodes of infected armadillos was provided by Dr P. Brennan (NIH contract AI-52582, Colorado State University). Bacilli (24 mg dry weight) were suspended in 3 ml of 0·01 M phosphate-buffered saline (PBS), pH 7·2. The organisms were disrupted by sonication for 30 min at 4°C at 130 W on a sonifier cell disrupter with a temperature control module (Model W1851, Heat System; Ultrasonics, North Tonawanda, NY). The sonicated material was sedimented at 10000 *g* for 10 min at 4°C. The supernatant fluid (10 ks) had a protein concentration of 7·15 mg/ml using protein estimation assay (Lowry *et al.*, 1951). The pellet (10 kp) was resuspended in 3 ml of 0·01 M PBS, pH 7·2, and was determined to have 8 mg protein/ml. Aliquots of the 10 kp were frozen at −20°C. The expression 10 kp and crude cell wall extract of *M. leprae* will be used interchangeably.

Preparation of r65-kD protein of M. leprae

The procedure used to clone and express the gene which encodes the 65-kD protein has been described (Meeker *et al.*, 1989). Crude protein extract prepared from recombinant *Escherichia coli* T65-3·6, expressing the 65-kD protein, were prepared by disrupting the bacterial suspension in a French Press. A soluble form of the r65-kD protein was prepared by resuspending the insoluble fraction of the bacterial lysate in PBS which contained 1% sodium dodecyl sulphate (SDS), 1 mM EDTA and 1 mM benzamidine HCl (pH 7·0), following centrifugation at 15000 *g* for 15 min. The antigen preparation was equilibrated by dialysis against 50 mM tris-HCl, 150 mM NaCl, 10 mM EDTA, pH 8·0. Purification of r65-kD from the crude soluble extract was accomplished using affinity chromatography with MoAb IIC8 (Gillis & Job, 1987).

Immunoblot

The immunoblot technique used to detect armadillo IgG antibodies to antigens of *M. leprae* was done as described previously (Vadiee *et al.*, 1988). Briefly, 800 μl which contained 0·66 mg/ml of purified r65-kD protein were mixed with 800 μl of 2 × buffer (0·1 M tris-HCl, 4% SDS, 10% 2-mercaptoethanol (2-Me), 0·1% glycerol, 0·025% bromophenol blue) boiled for 3 min and loaded (100 μl/well) onto a 12% polyacrylamide gel. The antigen was electrophoresed for 4 h at 45 mA and then transferred to BA85 nitrocellulose paper (NCP) (Schleicher & Schuell, Keene, NH) at constant voltage (10 V) overnight in a tris-glycine-methanol buffer, pH 8·0. The NCP was cut into strips, and each strip was incubated with a 1:30 dilution of the appropriate armadillo serum for 1 h at room temperature. Following three washes in 1% bovine serum albumin (BSA)–PBS, the strips were incubated with rabbit anti-armadillo IgG, gamma-chain specific at 1:100 dilution for 1 h. After washing in BSA–PBS, the strips were placed in a 1:500 dilution of goat anti-rabbit IgG, IgM, IgA-peroxidase conjugate for 1 h, followed by the addition of H_2O_2/horseradish peroxidase (HRP) (BioRad, Richmond, CA) for 10 min at room temperature.

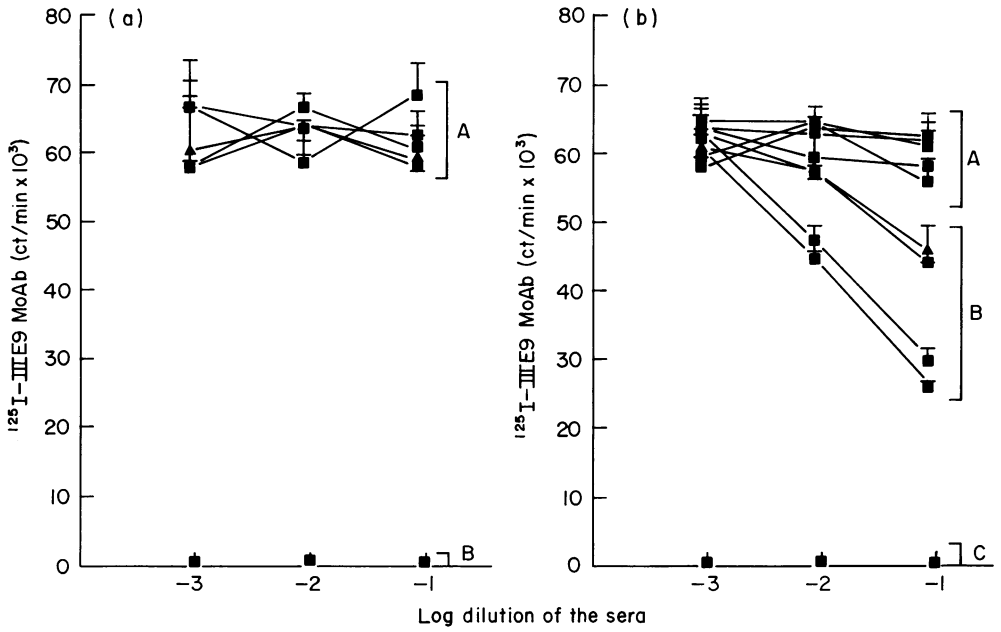

Fig. 2. (a) Titration of armadillo sera collected prior to the experimental inoculation with *M. leprae* (normal sera). Technical procedure for coating microtitre plates was the same as in Fig. 1. Armadillo sera at dilutions ranging from 10^{-1} to 10^{-3} were used. ^{125}I-IIIE9 was used at final dilution containing 1×10^6 ct/min. A, normal sera ($n=6$); B, non-specific binding (no antigen). (b) Titration of armadillo sera collected at 100 and 363 days post-inoculation with *M. leprae*. Technical procedure as in (a) A, 100 days post-inoculation; B, 363 days post-incubation; C, non-specific binding (no antigen).

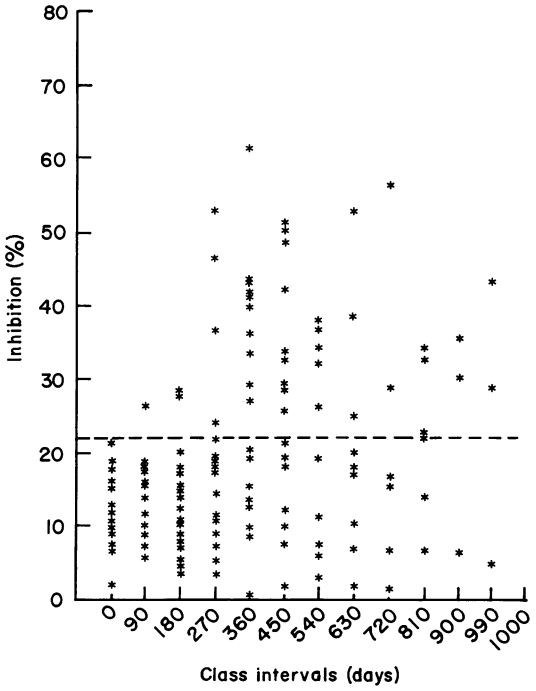

Fig. 3. Detection of armadillo antibodies directed to the *M. leprae*-specific epitope (IIIE9) of 65-kD protein prior to and during the course of experimental infection. Technical procedure for coating microtitre plates as in Fig. 1. Armadillo sera at final dilution of 1:30 and ^{125}I-IIIE9 at final dilution containing 1×10^6 ct/min were used. For determining a positive response a mean of the % inhibition of the normal sera (0 day)+2 s.d. (dashed line) was designated as baseline. A positive response was defined as $\geq 22\%$ inhibition.

Radiolabelling of MoAb IIIE9

Murine MoAb IIIE9 was purified from ascites by MAPS II affinity chromatography according to the manufacturer instructions (BioRad, Richmond, CA), and iodinated with carrier-free Na^{125}I (ICN Pharmaceuticals, Irvine, CA) by the chloramine T method (Greenwood, Hunter & Glover, 1963).

CABA

Immulon® I remove-a-well microtiter plates (Dynatech Laboratories, Va) were coated with 100 μl of *M. leprae* 10 kp (0·2 mg/ml) or with 100 μl of affinity-purified r65-kD protein at 0·2 μg/ml dissolved in 60 mM carbonate buffer, pH 9·2, as coating buffer. The assay was performed on these plates after one wash with 0·01 M PBS, pH 7·0, and a 30-min blocking step with 0·01 M PBS which contained 3% BSA. Briefly, 50 μl of iodinated MoAb IIIE9 at 1:50 dilution containing 2×10^6 ct/min and 50 μl of armadillo serum diluted 1:15 or 1:5 human serum were added to wells and incubated at 4°C overnight. Each well was washed six times with 0·01 M PBS, pH 7·0, (250 μl per well) to remove unbound components and then flicked dried. Individual wells were cut out from each plate and counted in a gamma counter (Model 4000, Beckman Instruments, Fullerton, CA) and the results were expressed as percent inhibition (using the following formula:

$$\% \text{ Inhibition} = 100 - \% \text{ Bound}$$

and

$$\% \text{ Bound} = 1 - \frac{\text{ct/min 100\% bound} - \text{ct/min test sample}}{\text{ct/min 100\% bound}} \times 100$$

where ct/min 100% bound = antigen + ^{125}I-IIIE9 MoAb. The 100% bound control was obtained from counts observed when ^{125}I-IIIE9 was added to antigen plates in the presence of 1%

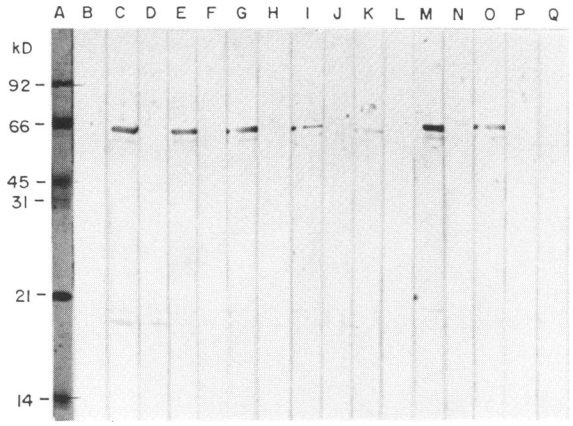

Fig. 4. Immunoblot to demonstrate the antibody binding of *M. leprae*-infected armadillos to the recombinant 65-kD (r65-kD) protein of *M. leprae*. Purified r65-kD protein (0·658 mg/ml) was analysed by immunoblotting. Nitrocellulose paper (NCP) was incubated with sera from normal or *M. leprae*-infected armadillos at 1:50 dilution, washed, and incubated with rabbit anti-armadillo gamma chain at 1:1000 dilution. The NCPs were then washed, and incubated with peroxidase-conjugated goat anti-rabbit IgG, IgM and IgA at 1:500 dilution. The NCP was washed and treated with dye-developing reagent (H_2O_2, 4-chloronaphthol). Lane A, low molecular weight protein standards: phosphorylase b (92-kD), BSA (66-kD), ovalbumin (45-kD), carbonic anhydrase (31-kD), soybean trypsin inhibitor (21-kD), lysozyme (14-kD); lane B and every other alternate lane, normal serum; lane C and every other alternate lane, serum from infected armadillos.

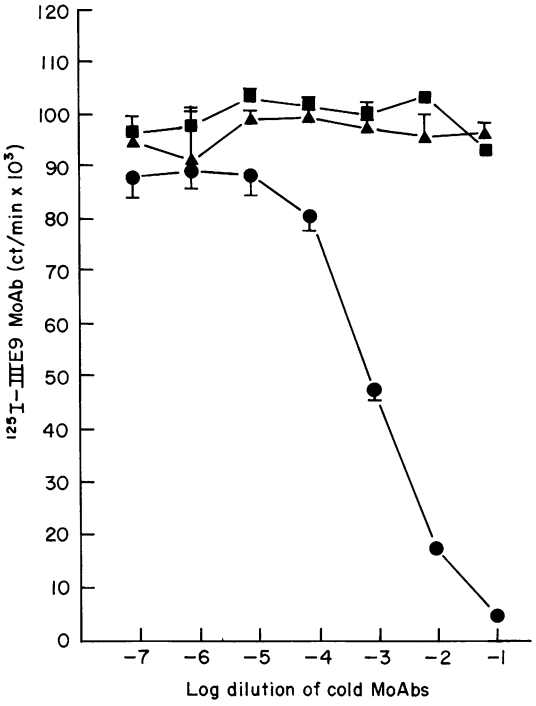

Fig. 5. Examination of the specificity for competitive antibody-binding assay (CABA) using r65-kD protein. Purified r65-kD protein of *M. leprae* was diluted to contain 0·2 μg/ml in 0·05 M carbonate-bicarbonate, pH 9·2, coating buffer; 100 ml of the diuted r65-kD were added to each well of the microtitre plate. The effect of relevant (cold IIIE9; ●) and irrelevant (cold D_2D_3 ▲; cold IIC8, ■ monoclonal antibodies (MoAbs) on binding of ^{125}I-IIIE9 MoAb to the IIIE9 epitope was analysed. Radiolabelled MoAb was used at a final dilution to contain 1×10^6 ct/min along with a serial dilution of cold MoAbs ranging from 10^{-1} to 10^{-7}.

BSA/PBS. All experimental values were corrected for background due to non-specific binding of reagents with uncoated polystyrene remove-a-well plates. Background counts did not exceed 500 ct/min.

RESULTS

Antibody competition analysis using M. leprae *10 kp*

A titration of ^{125}I-IIIE9 MoAb against crude cell wall antigen extract of *M. leprae* (10 kp) as the source of IIIE9 epitope was done. In this experiment ^{125}I-IIIE9 MoAb at 1×10^6 ct/min, and 10 kp at 0·2 mg/ml, was chosen in order to maximize the sensitivity with minimal reagent usage (data not shown). The specificity of the CABA was examined in a competition between labelled and non-labelled IIIE9 MoAb. An irrelevant MoAb D_2D_3 which recognizes a 28-kD protein of *M. leprae* was included as a control. Binding of ^{125}I-IIIE9 MoAb to the IIIE9 epitope in the crude cell wall extract increased as the concentration of cold IIIE9 MoAb was reduced (Fig. 1). In addition, irrelevant MoAb D_2D_3 did not interfere with the binding of ^{125}I-IIIE9 MoAb to the IIIE9 epitope.

Normal armadillo sera as well as sera collected at 100 and 363 days post-inoculation were titrated against *M. leprae* (Fig. 2a, b). Based upon titration findings of sera collected at 363 days post-inoculation, a 1:30 dilution was chosen for the CABA. When the optimal conditions were determined, the assay was performed for detection of armadillo antibodies to the *M. leprae*-specific epitope (IIIE9) on the 65-kD protein (Fig. 3). One-hundred and seventy-five serum samples taken from 26 armadillos were examined. The results are presented as % inhibition. To determine a positive response a mean of the % inhibition by the normal sera (0 day) + 2 s.d. was designated as baseline. Therefore, a positive response was defined as ≥22% inhibition. The sera from most animals were positive for antibodies to the IIIE9 epitope, primarily during the time when animals are burdened with disseminated bacilli (Fig. 3). The level of *M. leprae* dissemination was assessed on the presence of acid-fast bacilli in the buffy coat or in ear snip biopsies.

IgG response of M. leprae-*infected armadillos to r65-kD protein*

In order to determine whether armadillo antibodies directed to the native 65-kD protein also bind to r65-kD, serum samples from 16 armadillos were tested by Western blot using purified r65-kD as the antigen source. The serum samples tested were collected late in infection, after dissemination of *M. leprae* had occurred. Immunoreactivity was observed at the region of 65-kD in the sera of 15 of the 16 animals tested (Fig. 4). Pre-infection serum showed no evidence of anti-r65-kD activity.

Antibody competition analysis using affinity purified r65-kD protein

A preliminary titration indicated that ^{125}I-IIIE9 MoAb (1×10^6 ct/min) and purified r65-kD at 0·2 μg/ml were optimal for the assay (data not shown). When the specificity of the CABA, using purified r65-kD protein as the source of IIIE9 epitope was examined (Fig. 5), the binding characteristics were similar to those seen for native IIIE9 epitope (Fig. 1). Next, the CABA

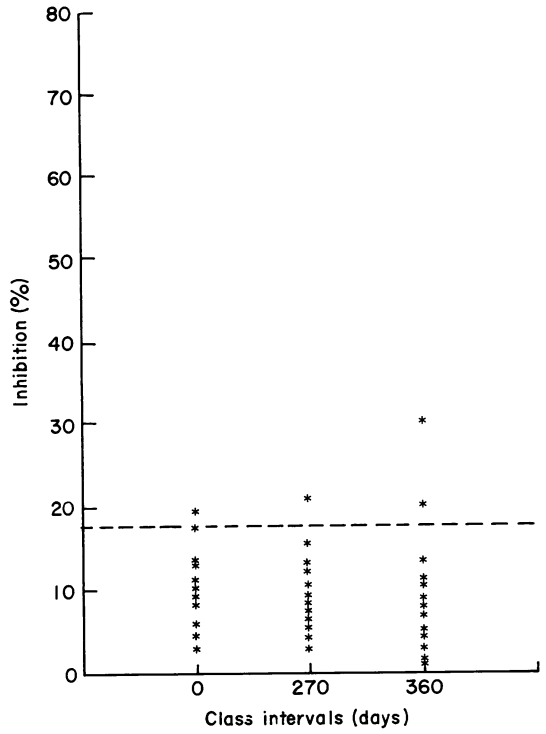

Fig. 6. Competitive antibody-binding assay(CABA) which incorporate purified r65-kD protein as the source of IIIE9 epitope. Technical procedure for coating microtitre plates as in Fig. 5. Armadillo sera at final dilution of 1:30 and ^{125}I-IIIE9 at final dilution containing 1×10^6 ct/min were used. For determining a positive response a mean of the % inhibition of the normal sera (0 day)+2 s.d. (dashed line) was designated as baseline. A positive response was defined as ≥18% inhibition.

Table 1. Comparison of a competitive antibody-binding assay (CABA) that incorporated crude cell wall extract or purified recombinant 65 kD protein as the source of the IIIE9 epitope

Serum from leprosy patients	Inhibition using crude cell wall extract (%)		Inhibition using purified recombinant 65-kD protein (%)
	A	B	B
99·29	43	49	0
98·28	24	27	0
67	31	51	0
72	54	58	0

A, Determined in the laboratory of Dr W. Levis.

B, Determined in the laboratory at the Gillis W. Long Hansen's Disease Center.

incorporating r65-kD protein was performed to assess the presence of the armadillo antibodies produced to the murine MoAb defined, *M. leprae*-specific epitope (IIIE9). Post-infection sera (270 and 360 days) were tested in this experiment because these sera produced positive responses in the assay in which crude cell wall extract was used as the source of 65-kD protein. In this experiment the results are presented as % inhibition and a positive response was defined as ≥18% inhibition (Fig. 6). Only four animals were positive for anti-IIIE9 activity. One animal showed low level of activity in the pre-infection serum group.

Analysis of leprosy patients sera for antibody to IIIE9 epitope in CABA

A preliminary titration of patient sera in the CABA in which crude cell wall extract was the source of IIIE9 epitope revealed that a serum dilution of 1:10 was optimal. Four serum samples, from different patients, were then tested by CABA against crude cell wall extract (at 0·2 mg/ml) or purified r65-kD protein (at 0·2 μ/ml). The results from CABA using crude cell wall extract were consistent with the findings reported by Dr W. R. Levis and associates (personal communication). When the same serum samples were analysed in CABA using purified r65-kD, no inhibition of binding of ^{125}I-IIIE9 MoAb to the IIIE9 epitope (Table 1) was observed.

DISCUSSION

Many of the antigenic determinants of mycobacteria are expressed by heterologous mycobacterial species; and as a result, antibodies to one mycobacterium species will often react with another species. Recently, protein antigens of *M. leprae* have been the focus of intensive study. Using MoAbs, it has been shown that certain protein epitopes of *M. leprae* are species-specific (Engers, Bloom & Godal, 1985). A cell-wall associated 65-kD protein molecule is an immunodominant component of *M. leprae*, *M. tuberculosis*, and BCG. The 65-kD molecule has one *M. leprae* species-specific epitope (IIIE9) and a minimum of 13 epitopes expressed by heterologous species (Buchanan *et al.*, 1987). The 65-kD protein has been shown to be immunogenic in both humans and mice. Antibody as well as T cell clones reactive to this molecule have been isolated from leprosy patients, their contacts, tuberculosis patients, and individuals responsive to purified protein derivative (Engers, 1985; Emmrich *et al.*, 1986; Oftung *et al.*, 1987). These findings have encouraged investigators to focus on this molecule as the target antigen for development of a serodiagnostic assay. The method used to detect antibody directed to a given epitope, located within a molecule, is competitive antibody binding assay (Daniel & Baum, 1968; Freedman, 1976; Morris *et al.*, 1979). Levis *et al.* (1986) isolated a crude cell wall extract of *M. leprae* as antigen in the CABA to assess leprosy patient serum for activity against the 65-kD IIIE9 epitope in the presence of ^{125}I MoAb. They reported that 19 out of 59 multi-bacillary patients had antibody directed to the *M. leprae*-specific epitope (IIIE9) on the 65-kD protein, while no antibody to this epitope was detected in the serum of 15 paucibacillary patients.

Since armadillos serve as the animal model for multi-bacillary leprosy (Job, Sanchez & Hastings, 1985), we tested armadillo sera for the presence of detectable antibody to the IIIE9 epitope. The CABA which utilized 10 kp, resulted in 60% inhibition in binding the ^{125}I-IIIE9 MoAb to the IIIE9 epitope, and was observed generally with serum collected 360–540 days post-infection. These findings suggest that the IIIE9 epitope is immunogenic in armadillos. Conversely, when purified r65-kD

protein was used in the CABA in place of crude antigen preparation, no antibody activity to this epitope was detected. However, immunoblot analysis of armadillo sera to r65-kD protein showed that the armadillo produces antibody (IgG class) to the 65-kD protein. It appears that armadillos produce antibody to the 65-kD protein, which is not directed toward or reactive with the IIIE9 epitope. Consequently, the lack of concordance between the two CABA may be due to steric hindrance caused by interaction of antibodies with heterologous specificity to epitopes other than IIIE9 present in the 10 kp antigen.

To substantiate this finding, we retested leprosy patients sera (supplied by Dr W. R. Levis) which was previously identified as positive for activity to the IIIE9 epitope when crude native antigen was used. In our laboratory these sera were also positive in CABA using crude cell wall extract, however, these sera samples were negative in the CABA using purified r65-kD protein.

In a time-course analysis of infected armadillo sera tested in an immunoblot assay using sonicated *M. leprae* as antigen source, we have observed that these animals produce elevated levels of antibody titres to *M. leprae* components with molecular weights which range from 21 to 45 kD. The sera of some animals recognize serologically larger repertoires of *M. leprae* components in the later stages of infection with *M. leprae* (personal observation). Immunoblot studies done with sera of human leprosy patients revealed that lepromatous leprosy patients produce antibody to various *M. leprae* components (Chakrabarty, Maire & Lambert, 1982). With these findings in mind it is believed that the lack of concordance between findings of CABA using crude cell wall extract *versus* those of CABA using purified r65-kD protein is most likely the result of steric hindrance induced by irrelevant antibody molecules present in polyclonal serum.

The IIIE9 epitope of *M. leprae* does not appear to be immunogenic in the armadillo or the leprosy patient. Although serum from greater numbers of leprosy patients must be analysed, the influence of steric hindrance must be considered when crude antigen preparations are employed especially with serum taken from patients with multi-bacillary leprosy, who produce antibody to a wide spectrum of components in the bacillus.

ACKNOWLEDGMENTS

This work was supported in part by grants from the American Leprosy Missions and the Hansen's Disease Foundation. We wish to express our appreciation to personnel of the Microbiology Research Department for making available the armadillo plasma samples. We are grateful to Mr Melvyn Morales for the computer analysis; Mr Sterling Christy for processing and storing the plasma samples; and to Ms Penne Cason for secretarial assistance. Our appreciation also goes to Dr R. S. Siebling of the Department of Microbiology at Louisiana State University for the critical review of this manuscript as well as thoughtful discussions.

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