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A Passive Hemagglutination Test for Leprosy Using a Synthetic Disaccharide Antigen[1]

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The fluorescent leprosy antibody absorption (FLA-ABS) test was the first successful method for the serodiagnosis of subclinical infection of leprosy ([1, 2]). Thereafter, improvements on reproducibility, sensitivity, and specificity have been made through the use of enzyme-linked immunosorbent assays (ELISA) with *Mycobacterium leprae*-specific antigens, e.g., phenolic glycolipid ([4, 8, 12]), synthetic disaccharide and trisaccharide ([5, 6]). Increased sensitivity is achieved through the ELISA, while the new generation of *M. leprae*-specific antigens enhance specificity. However, the ELISA is not entirely practical for field use. To make serodiagnosis of subclinical infection in leprosy available for evaluation in the largest number of contacts as early as possible, a simple, sensitive, specific, rapid and inexpensive test is needed. Bovine serum albumin conjugated to a synthetic disaccharide containing the specific antigenic determinants of the native phenolic glycolipid-I antigen of *M. leprae* ([6]) provided us with an excellent antigen for the sensitization of erythrocytes to make a sensitive and specific passive hemagglutination (PHA) test for the serodiagnosis of leprosy.

MATERIALS AND METHODS

Patients and controls. Sera for this study were collected from 62 leprosy cases, 6 contacts of multibacillary leprosy, 51 pulmonary tuberculosis cases, and 54 blood donors. All sera were collected, stored at −80°C, and tested within 1 year after collection. The leprosy sera were collected from the Rachaprachasamasai Institute in Thailand. Leprosy was classified using the Ridley-Jopling scale ([11]).

Passive hemagglutination test. The *M. leprae*-specific antigen used was a conjugate of disaccharide to bovine serum albumin prepared as described previously ([6]).

Sheep erythrocytes (SRBC) were collected in Alsever's solution, stored at 2–8°C for 2–8 days, washed three times in normal saline solution (NSS), and packed by centrifugation. SRBC were stabilized with aldehydes by the method described by Farshey ([9]). Briefly, an equal volume of a 4% SRBC in 0.15 M phosphate buffered saline (PBS), pH 7.2, was mixed with an equal volume of 2% pyruvic aldehyde in the same buffer, stirred at room temperature for 30 min, left overnight at 4°C, and washed three times in NSS. The washed SRBC were made into a 2.5% suspension in PBS, and mixed with an equal volume of tannic acid diluted 1:20,000 in the same buffer, incubated at 37°C for 30 min, washed three times in NSS, and made into a 4% suspension in PBS. One volume of 2% glutaraldehyde in PBS was added, and the mixture was rotated at room temperature for 2 hr, washed three times with NSS, and the treated SRBC were suitable for sensitization with antigen.

Sensitization of tanned and aldehyde-treated SRBC was made as follows: One tenth ml of packed SRBC was mixed with an optimal dilution of disaccharide conjugated to bovine serum albumin (BSA-DISAC) ([6]) in 0.1 M acetate buffer, pH 5, incubated at 37°C for 1 hr, washed three times in NSS, and resuspended as a 0.5% SRBC suspension in PBS containing 1% bovine

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serum albumin (BSA) and 0.1% sodium azide. The sensitized SRBC were now suitable for the PHA test for leprosy. The sensitized SRBC can be lyophilized for longer storage. Control SRBC were prepared by sensitizing SRBC with BSA using the method described in the sensitization with BSA-DISAC.

The optimal dilution of BSA-DISAC for the sensitization was determined by a checkerboard titration with negative and positive serum controls, and it was the highest dilution of BSA-DISAC which produced sensitized SRBC that yielded the highest titer with positive serum while maintaining a low titer with negative serum.

SRBC for the absorption of heterophile antibody were prepared by mixing washed and packed fresh SRBC (0.1 ml) with 0.1 M phosphate buffered saline, pH 7.2 (1.2 ml), and 0.2 ml of 2.5% glutaraldehyde. The mixture was rotated at room temperature for 1 hr, washed three times in NSS, and made into a 10% SRBC suspension in phosphate buffered saline, pH 7.2, containing 0.1% sodium azide.

The PHA test was performed by adding 0.025 ml of 0.5% sensitized SRBC to 0.025 ml of sera serially diluted from 1:32 to 1:1024 using PBS containing 0.5% BSA and 0.1% sodium azide as diluent. As a control, 0.025 ml of control SRBC was added to 0.025 ml of serum diluted 1:16 in the same diluent. The PHA titers were the highest serum dilution showing hemagglutination in the test well without hemagglutination in the control well. Serum showing hemagglutination in the control well was mixed with 15 volumes of 10% SRBC suspension, left at room temperature for 1 hr, and the supernatant retested.

RESULTS

The PHA titers in the leprosy and control groups are shown in The Table. PHA titers were higher in multibacillary leprosy and lower in paucibacillary leprosy. When a titer of $\geq$1:64 was considered positive, the test had a sensitivity of 92% in 38 cases of lepromatous leprosy, 25% in 24 cases of tuberculoid leprosy, and 33.3% in 6 contacts of multibacillary leprosy patients. It was also positive in 13.7% of 51 cases of pulmonary tuberculosis and 9.3% of 54 blood donors. When a tier of $\geq$1:128 was considered positive, the percentage of positives were: 84.2% in lepromatous and 16.7% in tuberculoid patients, 16.7% in contacts of multibacillary leprosy, 11.8% in pulmonary tuberculosis cases, and 3.7% in normals. The cutoff titer can be set at 1:64 or 1:128, depending upon the sensitivity and specificity needed.

DISCUSSION

When a PHA titer of $\geq$1:128 was considered positive, the sensitivity and specificity were strikingly similar to the ELISA for IgM antibody to the same BSA-DISAC antigen ([7]). The present test is positive in 84.2% of lepromatous and 16.7% of tuberculoid patients, 16.7% of contacts of multibacillary leprosy cases, 11.8% of tuberculosis patients, and 3.7% of normal controls. The positive results by ELISA using the same synthetic disaccharide were 85% in LL and BL patients, 14.8% in tuberculosis cases, and 3.6% in normals ([7]). The equal sensitivity of the PHA and ELISA could be due to the sensitivity of PHA to IgM antibody ([3]) which accounts for >90% of antibody to BSA-DISAC in LL patients ([7]). It also reflected good binding of the BSA-DISAC conjugate to SRBC, since both BSA and carbohydrate are very efficient in binding to SRBC. The similarity in the positive reactions in tuberculosis patients and normal controls, in the PHA and ELISA ([7]) using this antigen, reconfirm the need to synthesize antigen more specific to *M. leprae*. However, the ease with which a protein-disaccharide conjugate can be used for making two sensitive tests for leprosy points out that this approach is an excellent one for reaching an ideal serodiagnosis of leprosy.

The high efficiency in the antigen-coating process plus the sensitivity for IgM antibody make it possible for PHA to be used at a 1:128 serum dilution. PHA, when used at 1:128, is free from interference by heterophile antibody and other nonspecific factors.

The semiquantitative nature of PHA makes it less suitable for the follow-up of chemotherapy in which the decline in antibody can be demonstrated clearly by ELISA ([7, 12]). However, its sensitivity, simplicity, and rapidity could make PHA an

THE TABLE. *Percentages of positive PHA test at different cutoff titers.*

PHA titer cutoff	Percentages of positives				
	LL (N = 38)	TT (N = 24)	Contacts (N = 6)	Tuberculosis (N = 51)	Blood donors (N = 54)
≥1:64	92%	25%	33.3%	13.7%	9.3%
≥1:128	84.2%	16.7%	16.7%	11.8%	3.7%

excellent screening test, used alone or in combination with testing PHA-positive sera by a specific ELISA using phenolic glycolipid-I (PGL-I) or a synthetic antigen. This two-tier approach is more realistic until a test with a better combination of sensitivity and specificity becomes available. Using this test in contacts of multibacillary leprosy patients will identify 167 seropositives in 1000 contacts. The actual incidence of new cases among these contacts is approximately 10/1000 per year. If these cases are confined to the 167 seropositive contacts, then the rate of hit, i.e., finding new cases from among the seropositive contacts, will be approximately 1 in 17. The addition of a more specific ELISA may improve the rate of hit without the need to perform an ELISA in all 1000 contacts.

A PHA test using a synthetic antigen more specific for *M. leprae* could become a highly valuable tool for the serodiagnosis of subclinical infection in leprosy. PHA can be lyophilized and distributed for field use. It needs only the expense of microtiter equipment and instruction from a manual. A technician can easily test 200 specimens daily with reproducibility. The test does not require absorption, and the result is available in 1 hour.

The present test was recently evaluated in an international multilaboratory study using sera from many countries. It showed sensitivity and specificity comparable to the FLA-ABS test, and was found more sensitive than the ELISA using PGL-I, deacylated PGL-I, and synthetic disaccharide and trisaccharide. These results will be reported separately.

This PHA test for leprosy can readily undergo additional evaluation in other laboratories. A large quantity of reagent can be easily and cheaply prepared, lyophilized, and distributed to other countries. The prospect for worldwide application looks promising. Its rapidity, simplicity, and sensitivity alone make it highly qualified for large-scale and long-term applications in leprosy-endemic areas, which could lead to early detection of cases and improved control of leprosy.

SUMMARY

There is a need for a simple, sensitive, and specific test for the serodiagnosis of leprosy. A passive hemagglutination (PHA) test for leprosy was developed to meet these requirements. A synthetic disaccharide, conjugated to bovine serum albumin and specific for the phenolic glycolipid of *Mycobacterium leprae,* was sensitized to aldehyde preserved and tanned sheep erythrocytes (SRBC). The sensitized SRBC were used for testing sera from leprosy and tuberculosis cases and normal controls at 1:64 and 1:128 serum dilutions. It was found that if the hemagglutination reaction at ≥1:128 is considered positive, the test was positive in 84.2% of 38 cases of multibacillary leprosy, 16.7% of 24 cases of paucibacillary leprosy, 16.7% of 6 contacts of multibacillary leprosy, 11.8% of 51 cases of tuberculosis, and 3.7% of 54 blood donors. If the cutoff value used was 1:64, the test was more sensitive but less specific. The results are similar to that of an ELISA for IgM antibody to the same synthetic antigen. The present PHA test is simple and sensitive, but moderately specific. Its simplicity and sensitivity make it highly suitable for large-scale screening of contacts in leprosy-endemic areas.

RESUMEN

Puesto que existe la necesidad de contar con una prueba simple, sensible y específica para el serodiagnóstico de la lepra, se desarrolló una técnica de hemaglutinación pasiva utilizando un disacárido sintético con la especificidad del glicolípido fenólico del *Mycobacterium leprae* conjugado a albúmina bovina y acoplado a eritrocitos de carnero tratados con ácido tánico. Se probaron sueros de casos con lepra, con

tuberculosis y de controles sanos a las di luciones 1:64 y 1:128. La prueba resultó positiva (hemaglutinación a la di lución 1:128 o mayor) en el 84.2% de 38 casos con lepra multibacilar, en el 16.7% de 24 casos con lepra paucibacilar, en el 16.7% de 6 contactos de casos multibacilares, en el 11.8% de 51 casos con tuberculosis y en el 3.7% de 54 donadores de sangre. Tomando como límite de positividad la aglutinación a la di lución 1:64, la prueba fue más sensible pero menos específica. Los resultados son similares a los encontrados por ELISA (para anticuerpo IgM) usando el mismo antígeno sintético. La prueba de hemaglutinación pasiva descrita es simple y sensible pero moderadamente específica. Su simplicidad y sensibilidad la hacen particularmente útil en la exploración, a gran escala, de contractos en áreas endémicas de lepra.

RÉSUMÉ

Il serait nécessaire qu'une épreuve à la fois simple, sensible, et spécifique, soit disponible pour le diagnostic sérologique de la lèpre. Une épreuve d'hémaglutination passive (PHA) pour la lèpre, qui puisse répondre à ces critères, a été développée. Un disaccharide synthétique, conjugué à l'albumine du sérum bovin, spécifique pour le phénoglycolipide de *Mycobacterium leprae,* a été sensibilisé à une aldéhyde préservée et laquée avec des érythrocytes de mouton (SRBC). Les érythrocytes de mouton ont été essayés sur du sérum obtenu de malades atteints de lèpre et chez d'autres malades atteints de tuberculose, ainsi que chez des témoins normaux, à des dilutions de sérum allant de 1:64 à 1:128. On a constaté qu'avec un critère d'hémaglutination considéré comme positif à la valeur de $\geq$1:128, l'épreuve était positive chez 84,2% des 38 cas de lèpre multibacillaire, chez 16,7% des 24 cas de lèpre paucibacillaire, chez 16,7% parmi 6 contacts de lèpre multibacillaire, chez 11,8% de 51 cas de tuberculose et chez 3,7% de 54 donneurs de sang. Si le seuil utilisé est fixé à 1:64, l'épreuve était plus sensible mais moins spécifique. Les résultats sont semblables à ceux que l'on a observé avec un ELISA pour les anticorps IgM au même antigène synthétique. L'épreuve d'hémaglutination passive décrite ici est simple et sensible, mais seulement modérément spécifique. Sa simplicité et sa sensibilité la rendent tout à fait appropriée pour le criblage sur une grande échelle des contacts dans des régions endémiques pour la lèpre.

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