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EVALUATION OF ELISA AS A DIAGNOTIC TEST IN PULMONARY TUBERCULOSIS

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Summary: The reproducibility and ability of ELISA to discriminate patients with active disease from those infected were investigated.. The results obtained on 60 sputum-positive adult tuberculous patients and 60 adult control subjects showed groupwise discrimination. The anti-PPD antibodies (Ig G class) were significantly elevated in tuberculous patients as compared with control subjects. But because of the large day-to-day variation and considerable overlap in the distribution of antibody levels in tuberculous and control subjects, it is not possible to use ELISA as a diagnostic tool for adult pulmonary tuberculosis.

Introduction

Naturally occurring mycobacterial infections of both man and lower animals are associated with readily demonstrable circulating antibodies to mycobacterial antigens (Daniel and Braun 1968, Freedman et al 1966 and Minden & Farr 1969). Enzyme linked immunosorbent assay (ELISA) has recently been used to measure the serological response to a variety of. infectious agents (Akerlund et al 1969, Keven 1979 & Robins-Browue et al 1980). ELISA techniques are reported to be more sensitive, than agglutination, quantitative precipitation, passive hemagglutination, complement fixation and immuno-fluorescence tests (Wisdom 1976). Furthermore, the technique offers the potential of detecting immunoglobulin-class specific antibodies in unfractionated sera (Akerlund et al 1969 and Keven 1979).

This paper evaluates the potentiality of ELISA technique in measuring anti PPD antibodies for the diagnosis of pulmonary tuberculosis in adults, as any serological diagnosis will provide a major breakthrough in the control of tuberculosis.

Materials and Methods

A sample of serum was obtained from each of 60 sputum positive patients (47 males, 13 females; mean age 35 years) prior to start of treatment at the Tuberculosis Research Centre, Madras and from 60 blood bank donors (all males; mean age 28 years) at the Government General Hospital, Madras. In addition, for standardization of the test, separate sera samples were obtained from control subjects and patients. All sera were stored at –20°C in sterile condition until use.

Coating of microtitre plate (96 well U-shaped microtitre plates from Dynatech Laboratories Incorporated, Alexandaria, VA, USA) with antigen was carried out by dispensing 0.1 ml of PPD (5 µg/ml) (PPD-298 from Ministry of Agriculture, Fisheries & Food, Weybridge, England) into each well and sealing the plate with precut nylon sealing tape. The plate was incubated at 37°C for 3 hours and then stored at 4°C till use. The contents in the plate were washed in 3 changes of PBS-Tween-20. Next, 0.1 ml of serum dilution in PBS-Tween-20 was added and incubated for 30 minutes at 37°C. The plate was again washed as mentioned above and 0.1 ml of 1/1000 diluted antibody conjugate (Peroxidase-conjugated goat anti-human IgG, obtained from Cappel Laboratories, Inc., Cochranville, PA, USA) then added. Incubation (at 37°C for 30 minutes) and washings were carried as mentioned earlier. The substrate stock solution consisted of O-phenylenediamine at 2 mg/ml in methanol. The substrate working solution was prepared fresh before use by combining 1 ml substrate, 99 ml of deionised water and 0.1 ml of 3 % H_2O_2 ; 0.1 ml of the substrate was added to each well and the plates were incubated at room temperature in the dark. After 30 minutes of incubation, 50 µl of 8 NH_2So_2 was added to each well to arrest the reaction. The optical density of resultant colour was immediately read at 492 nm using Titertek Multiskan Vertical Photometer (Flow Laboratories, Mclean, VA, USA). All the sera samples were randomised and coded so that their identity was unknown at the time of setting up the test.

Results

Standardisation of ELISA

Eight samples of sera from tuberculous patients were set up, each in 8 two-fold serial dilutions. The relation between the optical density and the logarithm of reciprocal of serum dilution was found to be linear. The values of the correlation coefficient ranged from 0.95 to 0.98 for the different sera samples.

Tests were set up in six replicates independently by two laboratory staff on 4 samples of

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TABLE 1

Mean optical densities and standard deviation for 3 randomly divided sub-groups of sera from tuberculous patients and control subjects

Group	Day	No. of sera	Tuberculous patients	Day	No. of sera	Control subjects	P-Value*
Sub-group A	1	20	0.351 ± 0.159	1	20	0.190 ± 0.069	<0.001
Sub-group B	2	20	0.245 ± 0.108	2	20	0.129 ± 0.060	<0.001
sub-group c	3	20	0.255 ± 0.134	3	20	0.139 ± 0.070	0.001

* By Student's t–test

sera, each in 3 dilutions and on two occasions. There was a good agreement between the two sets of results (r=0.96).

Experiments were set up with the same set of specimens on 5 successive days, with 12 replicates on each day. The coefficient of replicate variation for within days was found to be between 3-6% and between days was found to vary between 10-15 %, when the mean optical density was 0.1 or above.

Comparison of tuberculous patients with control subjects

Sera from 60 sputum positive tuberculous patients and 60 control subjects (blood bank donors) were tested. The sera in each group of subjects were randomly divided into 3 equal sub-groups of 20 each. Each batch of tests included 20 sera from tuberculous patients and 20 control sera, and 6 reference sera, all tested in a single plate. The 46 sera were randomised and coded, and the tests were set up in duplicate, using 1/80 dilutions of the sera. It was found that the coefficient of day to day variation of the reference sera remained within 10-15%.

The mean optical densities and the standard deviations (for the different sub-groups) are shown in Table I. The mean value in the control subjects was significantly lower than the mean value in the tuberculous subjects on each day (occasion). There were substantial differences between the means of the randomly subdivided sub-groups within each group of subjects (Table I), indicating large day. to day variation. Detailed frequency distributions according to optical density in the tuberculous and the control subjects are presented in Table II. There does not appear to be any critical level which could be used for diagnostic purposes.

Discussion

Since the original description of ELISA by Engvall and Perlmann (Engvall and Perlmann, 1971), ELISA techniques have been extensively used to detect and quantitate immunoglobulins against infectious agents (Akerlund et al 1979), Keven 1979 and Robins-Browne et al 1980). In the present study, anti-PPD antibodies have been detected in human sera by employing ELISA technique. Preliminary experiments were undertaken to standardise the procedure and test the reproducibility. Even though within-day variations were considerably lower (3-6 %), day-to-day variation was found to be substantial (10-15 %). Irrespective of the day-to-day variation, on a given occasion, the tuberculous sera showed significantly higher levels of anti-PPD antibodies as compared to the control subjects. Similar observations were reported recently by Tandon and co-workers (Tandon et al 1980) and Grange and co-workers (Grange et al 1980) for anti-PPD antibodies and anti-BCG antibodies.

Tandon and co-workers (Tandon et al 1980) found that ELISA could discriminate tuberculous and control subjects group-wise and indicated its possible use as a diagnostic tool. Grange and co-workers (Grange et al 1980) reported that, while ELISA could discriminate. between tuberculous and control subjects. there was considerable overlapping in the levels of anti-BCG antibodies in the two groups. They made no recommendation regarding the use of ELISA as a diagnostic tool. Whereas Tandon and co-workers did not report on the day-to-day variation, Grange and co-workers reported

TABLE 2

Frequency distribution according to optical density in tuberculous patients and controls

Optical density	Tuberculous patients					Control subjects				
	A	B	C	All No	%	A	B	C	All No.	%
0.05–	0	0	3	3	**5**	2	7	7	16	**27**
0.10–	1	3	3	7	**12**	4	8	6	18	**30**
0.15–	2	6	2	10	**17**	3	2	4	9	**15**
0.20–	2	4	2	8	**13**	8	2	1	11	**18**
0.25–	3	2	3	8	**13**	2	1	1	4	**7**
0.30–	3	2	2	7	**12**	*1*	0	1	2	**3**
0.35–	3	0	2	5	**8**	0	0	0	0	**0**
0.40–	3	2	1	6	**10**	0	0	0	0	**0**
0.45–	3	1	2	6	**10**	0	0	0	0	**0**
Total	20	20	20	60	**100**	20	20	20	60	**100**

substantial day-to-day variation in their study in comparison with the variation in the replicates set up within a day. Our findings are in conformity with the two studies cited above with regard to group-wise discrimination. As regards the use of ELISA as a diagnostic tool, from Table II it is seen that because of the large day-to-day variation and considerable overlap in the distributions of tuberculous and control subjects, it is not possible to select a particular optical density level as a diagnostic criterion for tuberculosis! Thus we are led to conclude that, under the present conditions, ELISA has a very limited diagnostic value at least among adults, for the following reasons. Firstly there is a large day-to-day variation, as seen from the significant differences in the mean antibody levels of sub-groups within the same group of subjects. Further, inspite of carrying out the tests simultaneously in the same plate and on the same day, there was considerable overlap of the levels of antibodies in the tuberculous and the control subjects.

Mitchison and Tripathy, (Mitchison & Tripathy 1980) have reported that infection with M. tuberculosis and other environmental mycobacteria is very high in South India. Hence it can reasonably be assumed that a vast majority of the control subjects (males aged more than 20 years) would have been infected with tubercle bacilli. The likelihood of earlier BCG vaccination of control subjects producing higher antibody levels than that produced by natural infection is very low. It is not surprising that the control subjects show significant anti-PPD antibodies. The circulating antibodies are probably the result of subclinical infection with M. tuberculosis or other environmental mycobacteria. Because of its high sensitivity the ELISA test for anti-PPD antibody may not be able to discriminate between infected, apparently healthy subjects and patients with clinical tuberculosis in areas with high prevalence of infection with mycobacteria. Methods for the detection of circulating antigens and/or antibodies against species-specific antigens may prove more useful as diagnostic aids.

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