Immunochemical Characterisation of Mycobacterium Tuberculosis H37 Ra Antigen

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Introduction

Mycobacterium tuberculosis produces a great variety of antigenic products. Humans respond differently to various antigens of the tubercule bacillus. So single isolated antigen may be of limited value in diagnosis[1],[2]. Despite various immunodiagnostic techniques which have been used for the determination of mycobacterial antigen, no single test can be accepted as totally reliable both in sensitivity and specificity.

Many constituents of tubercle bacillus have been isolated and studied in a variety of ways. Most previous studies were focussed on the research for components having immunogenic activity. As a result many fractions have been isolated and partially defined with respect to physical structure and biological composition but a very few have been applied to the development of a diagnostic test to detect and measure humoral antibodies. Our approach was to isolate and purify the most immunogenic component from Mycobacterium tuberculosis sonicate antigen.

Materials and Methods

The Mycobacterial sonicate antigen was prepared from the cultures of M. tuberculosis H37 Ra by the method of Daniel *et al*[3]. 1 ml of the antigen (protein 4 mg/ml) was chromatographed on Sephacryl G-200 column (2.5 × 55 cm size) and the elution was done into 2 ml fractions with 0.05 M phosphate buffer containing 0.15 M NaCl, pH 7.2. The absorbance of the fractions at 280 nm and at 260 nm were measured with a Hitachi model 200-20 spectrophotometer. In order to ascertain the molecular wts of different fractions the column was calibrated with standard molecular weight markers, i.e., Bovine serum albumin (MW : 66,000) Egg albumin (MW : 45,000) Pepsin (MW : 34,000) and Lysozyme (MW : 14,300). The calibration plot of logarithm of molecular weight Vs elution volume is shown in Fig. 2. In order to determine the immunoreactivity of various purified fractions the ELISA was performed in microtitration plates using the method of Voller *et al*[4]. A set of known positive and negative sera and blanks were included with each set.

The elution profile of the sonicate antigen on sephacryl S-200 is presented in Fig. 1. Fractions corresponding to different peaks were pooled, concentrated and stored at - 20 c until further use. The carbohydrate[5] and protein[6] estimation were done. Nuleic acid determination were calculated from the optical density ratios at 280 nm and 260 nm. SDS-Poly acrylamide gel electrophoresis was carried out by the method of Laemmli[7] using slab gels in tris-glycine buffer. pH 8.3 at 2 mA per well. The gels were stained with silver stain[8].

Results

The elution profile of the sonicate antigen is presented in Fig. 1. Seven peaks were obtained. The immunogenicity of the various gel chromatographic fractions

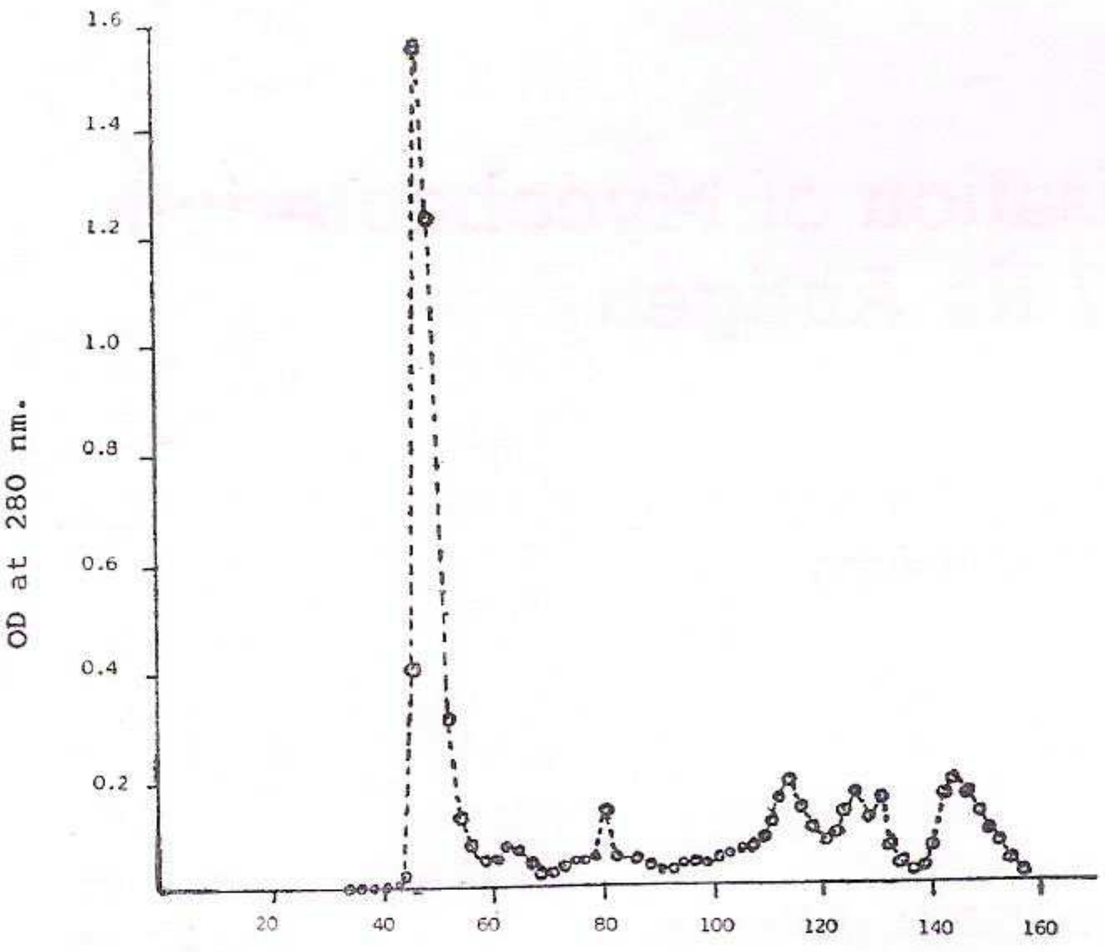

Fig.—1

Fractionation of Mycobacterial Sonicate Antigen on Sephacryl S-200 column (size 2.5 × 55 cm). The absorbance of each fraction (2 ml) at 280 nm. is shown. The contents under the indicated peaks were pooled to give Fr. 1 to 7. Void volume of the column was 48 ml. Discarded volume of the column was 36 ml.

were tested and fractions 10 to 15 and 21 to 25 which correspond to 2nd and 3rd peaks in the elution profile respectively were found to be immunogenic. The calibration plot of logarithm of molecular weight Vs elution volume is shown in Fig. 2. The approximate molecu-

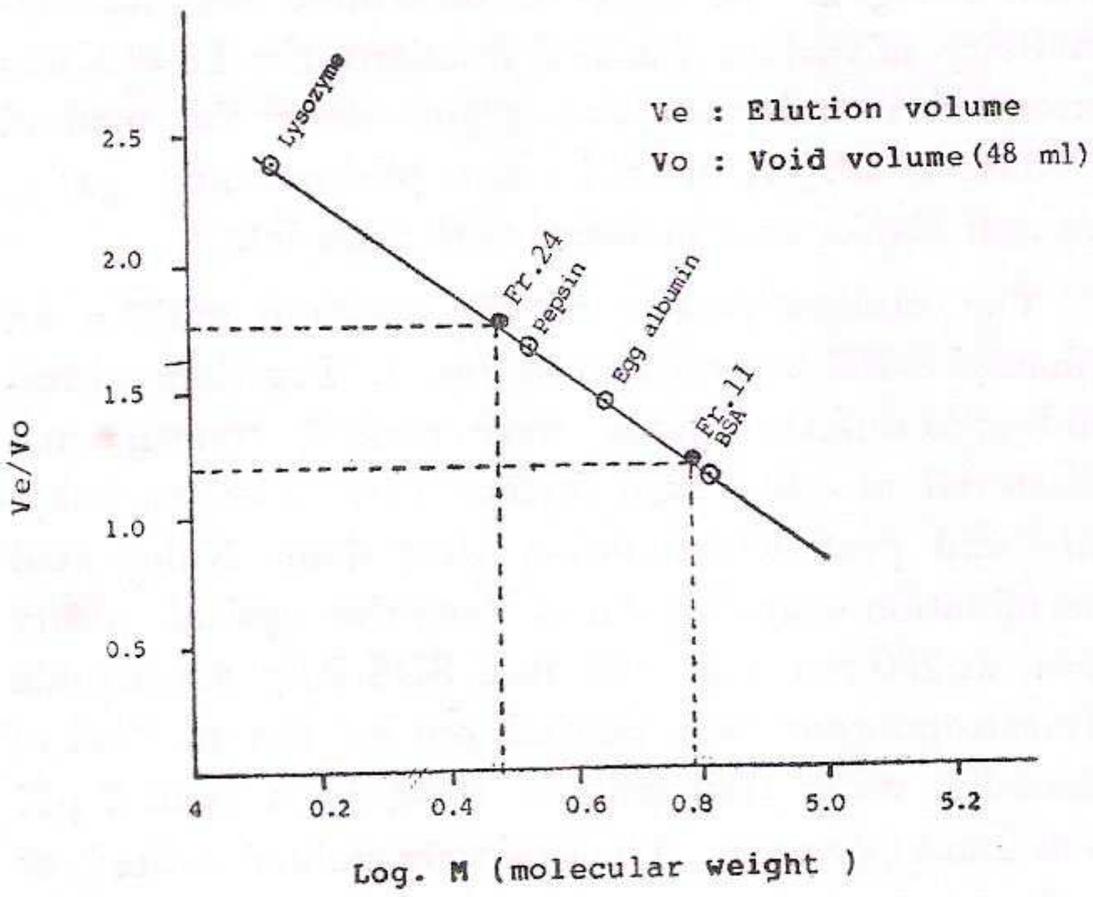

Fig.—2

Calibration curve for the determination of molecular weights of various fractions of Mycobacterial Sonicate Antigen. Most immunogenic fractions ie. Fr. 11 of 2nd peak and Fr. 24 of 3rd peak were having mol. wts. of 61,659 and 30,199 respectively.

lar weight of fractions in the 2nd and 3rd peaks were found to be 50,118 to 66,000 and 28,840 to 36,307 respectively. Fractions corresponding to different peaks were pooled and concentrated. The 2nd and 3rd peak fractions of the sonicate antigen were subjected to chemical analysis and details presented in the table No. 1. The fractions were found to be complex mixtures composed of protein, carbohydrate and nucleic acid.

Table I

Chemical analysis of pooled fractions (Fr. II & Fr. III) from various fractions of sonicate antigen.

Fractions	Protein ug/ml	Carbohydrate ug/ml	Ratio 280/260
Fr. II	156	143	0.685
Fr. III	173	196	0.731

Poly acrylamide gel electrophoresis pattern of the sonicate antigen showed many bands, suggesting that sonicate antigen is a complex mixture of various components. As the concentration of protein in Fr. II and Fr. III were very less the gel was stained with silver stain. Fr. II was found to have 3 bands and Fr. III showed only one band. The rf. values of the Fr. II were 0.46, 0.48 and 0.78 respectively. The rf. value of the Fr. III was 0.46.

Discussion

Seibert[9] in her attempt to purify isolated antigens from Mycobacteria by chemical fractionation, described four protein A.B.C. and D and two polysaccharides 1 and 2. Daniel and Affronti[10] by immuno electrophoresis demonstrated that protein A contain antigen 1,2,5, and 6 and probably also 4. Protein B was reported to contain antigen 1,2,5,6 and 7. Protein C was relatively inactive as a skin test antigen[11]. Protein D was not isolated and studied further. Both in vivo and vitro antigenicity and specificity were greatest in fractions containing antigen 5 and 6. Antigen 5 is sufficiantly pure so that only single band is recognised on acrylamide gel electrophoresis. It has a molecular weight of 28,500 to 35,000 and probably consists of single peptide chain[12]. Antigen 6 is the major constituents of Seiberts A protein with a molecular weight of 45,000 to 48,000 The relationship of molecular size to antigenicity of tuberculin components has been studied by Chapara and Baer[13] and Chaparas *et al*[14]. Complete antigene-

city was reported only in molecules of fairly large molecular size[15].

In our study elution profile of the sonicate antigen showed seven peaks. Immunogenecity of each fraction tested by ELISA test using patients sera showed fractions 10 to 15 and 21 to 25 to be most immunogenic and these fractions correspond with 2nd and 3rd peaks in the elution profile. 2nd peak fractions were found to have a molecular weight of 50,118 to 66,000 and 3rd peak fractions 29,000 to 36,000. Optical absorbance of the ELISA test were maximum in the fractions No. II of the 2nd peak and in the fraction No. 24 of the 3rd peak with a molecular weight of 61,659 and 30,199 respectively. When the optical absorbances of the ELISA test were plotted with the elution profile (Fig. 3) it was found that 2nd peak in the elution profile

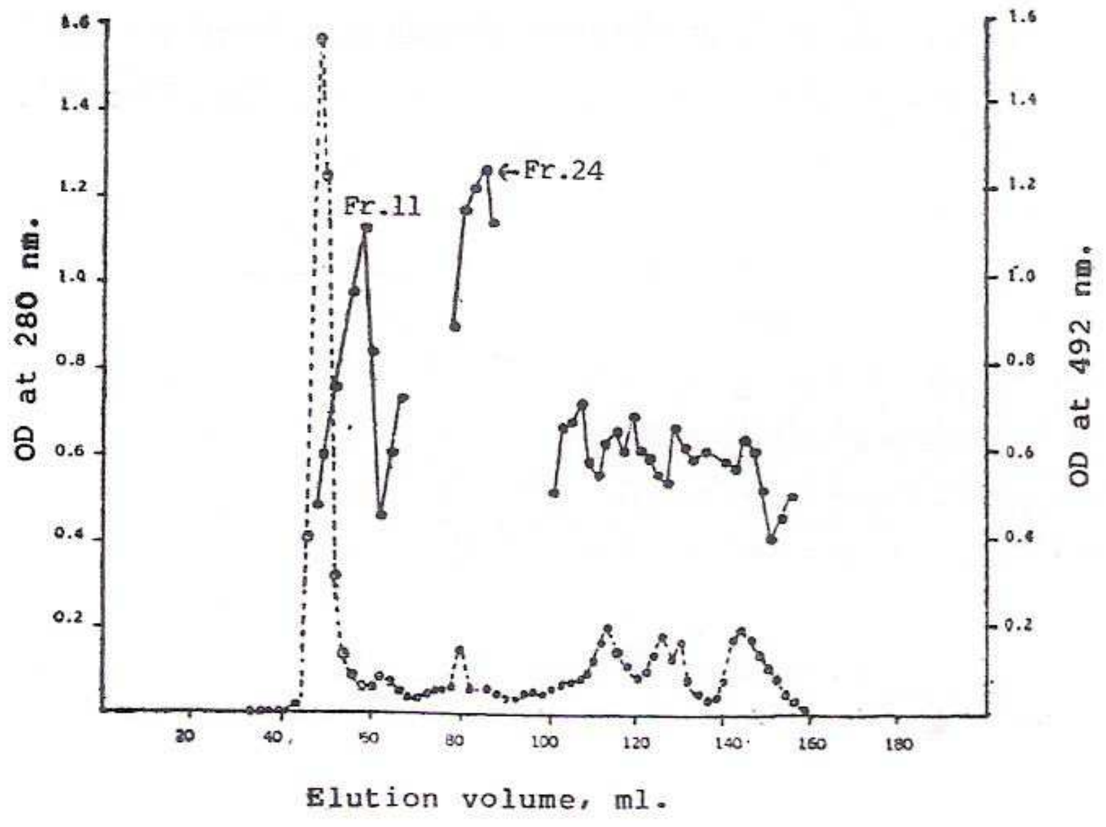

Fig.—3

Fractionation of Mycobacterial Sonicate Antigen on Sephacryl S-200 column (size 2.5 × 55 cm.). The absorbance of each fraction at 280 nm. is shown. The absorbance of each fraction with +ve sera at 492 nm. as determined by ELISA is also shown.

was composed of more than one component and 3rd peak, a single component. Polyacrylamide gel electrophoresis showed the 2nd peak fractions have 3 bands with rf. values 0.46, 0.48 and 0.78 respectively and 3rd peak fractions have a single band with rf. value of 0.46. These results suggest that the 2nd peak fractions were composed of 3 components and 3rd peak fractions a single component. Chemical analysis revealed that both the 2nd and 3rd peak fractions were composed of protein, carbohydrate and nucleic acid. Since peak 3 fractions gave a single band on SDS-PAGE with an approximate mole wt of 28,840 to 36,307, it is possible that it could be antigen 5. However further studies are needed to establish the relationship between them. From the analysis fraction No. 24 of the 3rd peak was found to have the maximum immunogenecity by the ELISA test and found to be mixture of protein, carbohydrate and nucleic acid. From the analysis 3rd peak fractions were found to be most immunogenic component which could be used for serodiagnosis of tuberculous meningitis.

Summary

Mycobacterial sonicate antigen was prepared and chromatographed on Sephacryl S-200 column. ELISA test with +ve pooled serum of tuberculous meningitis patients was performed and immunogenicity of the fractions were tested. Two fractions Fr. 2 and Fr. 3 were found to be most immunogenic. Mol. Wt. determination, chemical analysis and SDS-PAGE electrophoresis were done. Both fractions were found to be mixtures of protein, carbohydrate and nucleic acid. The Fr.3 with a mol. wt. range of 29,000 to 36,000 possibly could be antigen 5 and was found to be most immunogenic component.

References

1. J. M. Grange, T. Kardjiito-Serological tests for tuberculosis. Can the problem of low specificity be overcome ? *Int. J. Chest Dis. Allied Sciences*, **24** : 108-117, 1982.

2. J. Ivanyi, S. Sinha, R. Astan, D. Cussell, M. Keen V. Sengupta—Definition of species specific and cross reactive antigenic determinants of mycobacterium Laprae using monoclonal antibodies. *Clin. Exp. Immunol*, **52** : 528-536, 1983.

3. T. M. Dabuek, R. Good, B. V. Janicki—Immunoel ectrophoresis of mycobacterium tuberculous antigen. *Am. Rev. Resp. Diseases*, **112** : 639-644, 1975.

4. A. Voller, D. Bidwell, A. Bartlett-Enzyme, linked immunosorbernt assay. In : Manual of clinical immunology. N. R. Rose. Friedman Eds. (American Society for Microbiology, Washington, DC) p. 359, 1980.

5. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebis, F. Smith—Colorimetric method for determination of sugars and related substances. *Anal. Chem*, **28** : 350-356, 1956.

6. O. H. Lowry, N. J, Rose Brough, A. L. Farr, R. J. Randall—Protein measurement with folin phenol reagent—*J. Biol. Chem.*, **193** : 265-275, 1951.

7. U. K. Laemmli-cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227** : 680-685, 1970.

8. Morrissey-silver stain for protein in polyacrylamide gels. A modified procedure with enhanced uniform sensitivity. *Anal. Biochem*, **117** : 307-310, 1981.

9. F. B. Seibert, M. Stacey, P. W. Kent—An antigenic polysacharide, "Polysaccharide 11", isolation from tuberculin. *Biochem Biophys Acta*, **3** : 632-640, 1949.

10. T. M. Daniel, L. F. Affronti—Immunoelectrophoretic analysis of antigenic constituents of Seibert fractions of mycobacterial culture filtrates. Identification with the proposed United States-Japan reference nomenclature. *Am. Rev. Resp. Dis.*, **108** : 1244-1248, 1973.

11. H. M. Vandiviere, M. R. Vandiviere, F. B. Seibert —Comparison between purified individual tuberculo proteins and the international standard tuberculin, *J. Infect. Dis.*, **108** : 45-52, 1961.

12. T. M. Daniel—Soluble mycobacterial antigens. In: The mycobacteria : A source book (part A) Kubica G. P. and Wayne Eds. (Marcel Dekker Inc. New York and Basel) p 417-465, 1984.

13. S. D. Chparas, H. Baer—The immunology and chemistry of Tuberculin 111. Dialyzable BCG culture filtrate components obtained by sephadex chromatography and fractional dialysis. *Am. Rev. Resp. Dis.*, **90** : 87-96, 1964.

14. S. D. Chaparas, D. E. Thor, S. R. Hedrick—Comparison of lymphocyte transformation, inhibition of macrophage migration and skin tests using dialyzable and non dialyzable tuberculin fractions from mycobacterium bovis (BCG). *J. Immunol*, **107** : 149-153, 1971.

15. T. M. Daniel, B. W. Janicki—Mycobacterial antigens. A review of their isolation, chemistry and immunological properties. *Microbio. Rev.* **42** : 84-113, 1978.