PURIFICATION OF MYCOBACTERIAL ANTIGENS
FOR POTENTIAL USE IN THE
SEROLOGICAL DIAGNOSIS OF TUBERCULOSIS

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In describing progress in our efforts to develop a serological test for the diagnosis of tuberculosis, we include in this report results from the initial through the most recent protocols which we employed.

The progression of experimental procedures which we employed in the study include:

A. Fractionation of Mycobacterium tuberculosis H₃₇RV water soluble cell extracts for H₃₇RV species specific cell extract (CE) antigens.


In our initial studies with *Mycobacterium tuberculosis* H₃₇RV cell extracts (water soluble proteins), we developed an analytical gradient acrylamide gel electrophoresis-crossed line immunoelectrophoresis (AGAGE-CLIE) technique to compare the antigenic components of H₃₇RV with those in *Mycobacterium kansasii* CE. The AGAGE-CLIE was performed by resolving 200 μg of H₃₇RV CE (lot #1) in a discontinuous analytical polyacrylamide gradient (AGAGE column). The column gradient consisted of 5.0 cm of 12%, 1.5 cm of 7.5%, 1.0 cm of 4.75%, and 1.5 cm of 3.5% gel, and all gel columns had a tracking dye reference mark 1 cm from the anodic end (12% gel). One column was stained with Coomassie brilliant blue R 250(CBB) as a control for protein resolution, and an unstained duplicate was used for preparing the AGAGE-CLIE slide. The template (Fig. 1) for preparing the slide was placed under an 8x10 cm Gel Bond Film (Marine Colloids) coated with agarose (Marine Colloids). The column with the resolved H₃₇RV CE antigen was placed in a trough prepared in the agarose above the diagonal lines of region B (Fig. 1) so that the tracking dye in the AGAGE column superimposed the right margin of the Gel Bond Film. Three mgs of CE protein from *M. kansasii* (heterologous antigen) were included in region C, and the agarose matrix of region D contained 0.5 ml of homologous antiserum (goat anti-H₃₇RV Mars 002 lot 001). The AGAGE-CLIE slide was placed in a Gelman
electrophoresis chamber at 4°C, connected to barbitol buffer (pH 8.6) in reservoirs with paper wicks, and subjected to 10mA/slide in the direction of the arrows (Fig. 1) until the tracking dye was 0.5 cm from the anodic edge. After electrophoresis the slide was washed, dried, and stained with CBB.

The results from such a stained AGAGE-CLIE slide, in Fig. 2 suggested that, when compared with \( M. \) kansasii CE, 5 species specific antigens were indeed present in the \( H_37 \)RV CE. Antigens common to \( H_37 \)RV and \( M. \) kansasii formed precipitin lines of identity (LI) parallel to and above the \( M. \) kansasii matrix (Region C, Fig. 1). Five precipitin lines (N.I.) did not appear to be shared with \( M. \) kansasii in that they crossed lines of identity. As illustrated in the diagram of Fig. 3, relative mobility (RM) values of the specific antigens in the AGAGE column which correspond to precipitin arcs of non identity (N.I.) were determined by dividing the distance from the precipitin peak (vertical lines 1, 2, 3, 4, and 5, Fig. 3) to the right edge of the slide by the length of the unstained gel column between the tracking dye and the top of the 3.5% gel (8.0cm). RM values from lines 1, 2, 3, 4, and 5 were 0.26, 0.308, 0.33, 0.358, and 0.366 respectively, indicating that all five antigens were located in the 12% gel. The above RM values then were used as a guide to locate, fractionate, and enrich the \( H_37 \)RV specific antigens in preparative GAGE (PGAGE) columns (Fig. 4). It should be noted that, while the diameter of the AGAGE column and PGAGE columns are 0.5 and 5.0cm respectively, they both have identical discontinuous gradients. Thus, identical AGAGE and PGAGE gradients theoretically would provide the same relative mobility value of a selected antigen in either format and permit the use of AGAGE-CLIE results as a guide for fractionating PGAGE columns. For the fractionation effort, 25mgs of a selected lot (#2) of \( H_37 \)RV CE were added to each of 8 PGAGE columns which were subjected to 10mA/column at 4°C until the tracking reached the reference mark 1cm from the anodic end. When the tracking dye reached the reference mark, the preparative column was removed from the electrophoresis chamber and the 3.5% gel, 4.75% gel, and 1.3cm of the 7.5% gel were excised from the column. The remaining segment of the 7.5% gel and the 12% gel were placed in a gel slicer and sliced according to the template in Fig. 4. The first slice (5mm thick) was composed of 7mm of 7.5% and 3mm of 12% gel, and the remaining slices were 3mm segments of 12% gel. Identical slices from the eight columns
were placed in a screw-capped jar containing 40ml of distilled water, cut into small fragments, and placed on a reciprocating shaker to elute protein from the polyacrylamide fragments. Acrylamide fragments were separated from the solution by filtration through gauze and the eluate concentrated with an immersible CX-10 concentrator (Milipore Corp.). The concentrated eluates, i.e., fractions, were employed in an Ouchterlony procedure with both anti-M. kansasii and anti-H$_{37}$RV antiserum to provide serological evidence for common and specific antigens in each fraction (Fig. 5). The numbers 1-8 in Fig. 5 represent fractions added to the peripheral wells and 9 the location of antiserum in the center well of the Ouchterlony slide. Ouchterlony slides A and C are pictures of immunoprecipitates formed with CE fractions before absorption with anti-M. kansasii sepharose-coated beads. The anti-H$_{37}$RV antiserum and anti-M. kansasii antiserum used for the Ouchterlony are identified as 9-A and 9-C respectively in Fig. 5. While more immune-precipitate was formed between Fractions #1 through #8-A (Fig. 5) and the homologous antiserum (9-A, Fig. 5), antigens in each fraction (1-8C) formed precipitates (common antigens) with anti-M. kansasii (Fig. 5).

As a means of removing common antigens from specific H$_{37}$RV antigens, we considered the approach of immunosubtracting PGAGE fractions with heterologous antibody coated sepharose 4B beads. In order to prepare the antibody coated beads, immunoglobulin was fractionated from goat anti-M. kansasii antiserum by ammonium sulfate precipitation (3x), added to a G-200 sephadex column, and eluted with phosphate buffered saline. The globulin eluate was dialyzed with a pelicon system (Milipore Corp.), concentrated to 5.0mgs/ml with the immersible CX-10 concentrator (Milipore Corp.) and coupled to sepharose-4B beads (Sigma Chemical Corp.). For the purification of PGAGE Fraction #5, as an example, we combined 1ml of the fraction with 1ml of packed anti-M. kansasii coupled beads. After absorption (10x), Fraction #5 was placed in well 5-B and 5-D, anti-H$_{37}$RV in well 9-B, and anti-M. kansasii in well 9-D. Wells 1,2,3,4, 6,7, and 8 served as blank wells and were filled with phosphate buffered saline. Absorbed Fraction #5 was reactive with the homologous anti-H$_{37}$RV antiserum in 9-B but failed to react with anti-M. kansasii in 9-D (Fig. 5).
Fraction (#5) also was employed in an AGAGE-CLIE procedure (Fig. 6). The stained film of the AGAGE-CLIE slide with Fraction #5 (Region B) then was superimposed upon an AGAGE-CLIE film formed with the H$_{37}$RV crude CE (Region A) used for fractionation. As found in the montage of Fig. 6, the H$_{37}$RV specific antigens exhibited essentially the same RM values in Fraction #5 (Region B) as they did in the crude CE. Thus, the AGAGE-CLIE procedure appeared to be an effective and efficient format as a guide for fractionating preparative columns for specific selected CE components.

A-2 A Comparison of H$_{37}$RV with _M. Bovis_ (BCG 1011) CE for H$_{37}$RV specific antigens.

In view of the evidence in Fig. 5 that we could fractionate and purify water soluble CE H$_{37}$RV antigens (compared to _M. kansasii_ antigens), we proposed to compare H$_{37}$RV with _Mycobacterium bovis_ CE by the AGAGE-CLIE for H$_{37}$RV specific antigens. The AGAGE-CLIE was performed as described above except that _M. bovis_ CE replaced _M. kansasii_ CE in region C Fig. 1 as the heterologous antigen. Results from the AGAGE-CLIE slide in Fig. 7 and 8 suggested that when compared to _M. bovis_ CE antigens, one species specific antigen was present in the H$_{37}$RV CE. Figure 7 is a photograph of the top portion of the C region and the total D region of the AGAGE-CLIE slide. Here prominent lines of identity (L.I.) are observed well above and parallel to the C-region. A single rocket immunoelectrophoresis peak, i.e., a line of non-identity (N.I. see arrow in Fig. 7), and presumed to be a species specific immunoprecipitin band emerges near the C-region and crosses L.I. The small size of the N.I. precipitin line and the lack of staining intensity suggests that the specific antigen(s) is limited in number and concentration when compared to common antigens shared with BCG in H$_{37}$RV CE. In contrast, H$_{37}$RV specific antigens appear to be more numerous(s) and more highly concentrated when H$_{37}$RV CE are compared to _M. kansasii_ CE as heterologous antigens. The diagrammatic presentation in Fig. 8 was developed from the stained AGAGE-CLIE slide (Fig. 7) as a guide for fractionating preparative columns containing resolved H$_{37}$RV CE. The N.I. band emerges from the AGAGE column at a point 3.8cm, and has a center of concentration at 3.0cm from the column tracking dye or right edge of the slide (Fig. 8). For CE fractionation as before, 25mgs of H$_{37}$RV CE antigen
(lot #3) were added to each of four preparative columns which were subjected to 10 ma/column at 4°C until the tracking dye reached the reference mark 1cm from the anodic end. The fractionation of preparative columns was carried out according to the template found in Fig. 4 except that the first fraction cut at the 7.5%-12% gel interface was 7mm thick and the remaining slices prepared in the 12% gel were 5mm. After fractionation, CE protein was eluted from acrylamide and concentrated as previously described.

For purification of PGAGE fractions by absorption with anti-M. bovis coated sepharose-4B beads, Fractions #2 and #3 located nearest the center of the specific antigen (N.I.) concentration, as determined by the AGAGE-CLIE slide, were combined and treated as a single sample. Fractions #1 and #4 bracketing Fractions #2 and #3 were purified as individual samples. Fifty µl were removed from each fraction for analysis by polyacrylamide slab gel electrophoresis after the 3rd, 6th, and 9th absorption. These were loaded onto a one-dimensional slab gel for electrophoresis according to Table 1.

After electrophoresis at 30 ma for 3.5 hours, the slab gel was removed and stained by the Oakley Silver Stain Procedure. The results of the silver stain are found in Fig. 9 where discrete stained bands were observed at the top of the 10% gel in lane 2 which contained 15 µgrs of unabsorbed M. bovis CE. In the middle region of lane 2, staining intensity obscured protein bands that might have been formed during electrophoresis. The result of protein removal from M. bovis crude CE by immunosubtraction can be observed by comparing staining patterns in lanes 2-5. In lane 3, after the 3rd absorption of M. bovis crude CE, protein bands were not found at the top of the 10% gel and discrete bands were observed in the central region of the lane in contrast to lane 2. After the 6th absorption of M. bovis crude CE, the number and intensity of staining bands decreased in the center of lane 4, while discrete bands were not discernible in lane 5 after the 9th absorption. The middle region of lane 5 appears as a homogeneous stained smear, and might well reflect a low concentration of a given molecular species which would not have been identified by less sensitive detectors such as CBB R-250.
The sample, formed by pooling Fractions #2, #3, and Fraction #1 were recovered or fractionated from the 12% gel of PGAGE columns. When the unabsorbed and absorbed combined sample and Fraction #1 were applied to the slab gel (see Figure 9 and the diagram in Fig. 10) it is important to note:

1. The staining pattern of the unabsorbed combined sample (lane 6) was significantly different from the pattern developed in lane 10 with unabsorbed Fraction #1.

2. The molecular size of proteins in the pooled sample (Fig 2 and 3) on an average are smaller than proteins of unabsorbed Fraction #1.

3. Neither the unabsorbed pooled sample or Fraction #1 contained protein which remained at the top of the 10% gel during the interval of electrophoresis.

4. After the 9th absorption of the combined sample, residual protein was not detectable by the silver stain in lane 9.

5. After the 9th absorption, Fraction #1 gave a discrete band (a) in lane 13 to suggest that Fraction #1 contained an H₃₇RV specific CE antigen. Although bands labeled b in lane 13 were not found in the blank lane 14, they have been found in blank lanes of other slab gels to suggest that they might be artifacts of staining.

6. The slab gel capacity did not permit the inclusion of Fraction #4 in Figs. 9 and 10, however, after absorption (9x) H₃₇RV specific antigens were not detected in Fraction #4 by the silver stain to provide the type of result found in lane 9 with the absorbed pooled sample.

The above factors (Items 1-6) suggest that we were selectively fractionating H₃₇RV CE and not merely regenerating H₃₇RV CE by slicing and eluting preparative columns. Also, we significantly enriched and purified an H₃₇RV specific antigen(s) in Fraction #1 which was not found in closely associated preparative fractions. Some other comments about the slab gel analysis are provided and include:

1. The mixture of molecular weight markers applied to lanes 1 and 15 provided the expected skewed protein bands commonly found at the edges of slab gels. A diagrammatic representation of molecular weight markers, therefore, provided in Fig. 10 to permit the approximation of the molecular size of antigen(s) (30,000 Daltons) in Fraction #1 lane 13.
2. As previously mentioned AGAGE and PGAGE columns have identical discontinuous gradients, consequently the relative mobility of selected antigens should be identical or similar in AGAGE and preparative columns and is the basis of applying AGAGE-CLIE results as a guide for fractionating preparative columns.

3. In our past experience, while specific antigens identified by AGAGE-CLIE slides often are not located in the expected or theoretical position of PGAGE columns, they generally can be located within ±1 cm of the theoretical position (RM value). In the fractionation described above, the H₃₇RV specific antigen was expected in the combined sample (Fraction #2 and #3) but found in Fraction #1 approximately 0.5-1.0 cm from the expected location in the PGAGE column.

After purification by immunosubtraction (9x) and slab gel electrophoresis, the remaining 1 ml of Fraction #1 was used for analysis and compared with H₃₇RV crude CE by HPLC in Dr. Phil Hylemon's laboratory at the Medical College of Virginia, Richmond, Virginia. One ml of H₃₇RV crude CE containing 6.7 mg of protein was injected into a Beckman Model 341 Isocratic High Pressure Liquid Chromatograph with a Beckman DEAE column. The running buffer was a 20 mM solution of sodium phosphate at pH 6.0. The sample was eluted with a NaCl gradient ranging from 0 to 500 mM NaCl in 100 minutes, with a flow rate of 1 ml/min. The injection and elution of H₃₇RV crude CE was followed by the purified sample (Fraction #1). The elution profile for the CE is found in Fig. 11 where the ascending NaCl gradient ran for 49.32 minutes (Step 1). After the above interval, a 500 mM NaCl solution was used for elution to remove the remaining crude CE protein bound to the column without the benefit of gradient resolution (Step 2, Fig. 12). After the injection of purified Fraction #1, the ascending gradient ran for 38.94 minutes (Step 2) without any evidence of protein elution from the column. Shortly after the 39 minute interval, the 500 mM NaCl solution replaced the gradient (Step 3) for sample elution whereby protein was found in the eluate (Fig. 13). The column eluate (<300 µg/ml of protein) was dialyzed against physiological saline (0.9% NaCl), the volume reduced to 1.0 ml with the sequential application of the Millipore CX-10 and an Amicon concentrator. The concentrate which was reactive with anti-H₃₇RV as determined by the ELISA procedure was stored at -70°C.
The cells used to prepare CE (lot #3) for the above procedure and earlier fractionation efforts (lots #1 & 2) were grown on modified Proskauer Beck Broth (MPBB). In order to repeat and continue the fractionation described in A-2 above, it was necessary to prepare additional CE from cells grown on MPBB (MPBB-CE) (lots 4 & 5). When the new lots of CE were employed in the AGAGE-CLIE procedure, only lines of identity were detected as found in Fig. 14. Fig. 14 is a photograph of the D Region of an AGAGE-CLIE slide developed with H37RV (homologous antigen) and M. bovis BCG CE (heterologous antigen) for H37RV specific antigens. Lines of N.I. are absent in Fig. 14 in contrast to N.I. lines of Figs. 2, 3, 6, 7, and 8. To suggest that there were no H37RV specific CE antigens in lots 4 and 5 when compared to BCG CE as the heterologous antigen. Also enriched fractions from preparative columns were prepared with lots #4 and 5 H37RV CE. The unabsorbed and absorbed fractions were loaded onto a slab gel according to the protocol of Table #2. When these enriched fractions were absorbed with anti-M. bovis sepharose-coated beads, residual proteins were not detected in lanes #5, 7, 9, and 11 of the slab gel by the silver stain and reinforced the suggestion that H37RV specific antigens were not in lots #4 and 5 CE (Figs. 15 and 16). In addition to lots 4 and 5, we employed CE (lot 6) prepared from H37RV cells grown on Wong's broth (WB). When used in the AGAGE-CLIE procedure, lot 6 WB-CE like lots 4 and 5 which provided only lines of identity did not appear to contain H37RV specific antigens.

The AGAGE-CLIE procedures previously described in section A-1 and A-2 were performed with goat anti-H37RV Mars 002 lot 001 antiserum obtained from the Mycobacterial Culture Collection at the National Jewish Hospital (NJH), Denver, Colorado. Although lot 001 forms immunoprecipitates with CE antigens as found in the above immunoelectrophoretic results, information provided by the NJH states that a whole cell antigen (H37RV) was used as the immunostimulant for lot 001 production.

As an alternate source of an antiserum for our study, we prepared a CE antigen with H37RV cells grown on MPBB and WB. The MPBB-CE and WB-CE were used to prepare goat anti-H37RV MPBB-CE and anti-H37RV WB-CE. Also goat anti-H37RV CE (Erdman Strain) was provided by S.D. Chaparas. When lot 001,
and the above 3 anti-H$_{37}$RV CE antisera were employed in the AGAGE-CLIE, again, H$_{37}$RV specific antigens were not detected when BCG CE were used as a heterologous antigen.

B. A search for H$_{37}$RV specific antigens with absorbed antiserum.

The biohazzard laboratory where we developed H$_{37}$RV CE for fractionation was located on property owned by Norfolk General Hospital. According to the Eastern Virginia Medical School Administration, the hospital demanded that the biohazzard laboratory should be demolished. The process of demolition initiated in May 1984 interrupted our efforts to prepare CE which would contain a consistent source of H$_{37}$RV specific CE antigen(s). As a specific reagent, other than purified CE antigen(s) to provide evidence for mycobacterial infection, we considered the inherent potential of employing absorbed anti-H$_{37}$RV antiserum.

The fact that MARS 002 lot 001 antiserum, used for soluble antigen immunoprecipitation in the AGAGE-CLIE, was prepared against H$_{37}$RV whole cell antigens served as a stimulus for us to absorb lot 001 as a possible reagent specific for H$_{37}$RV antigens. The cell suspension prepared for antiserum absorption contained $10^8$ colony forming units/ml of BCG cells (M. bovis 1011). The cell suspension was killed with flowing steam at 80°C for 60 minutes. After heat inactivation, BCG cells were recovered from 5ml of suspension by centrifugation and resuspended in 5ml of lot 001 antiserum. The cells suspended in antiserum were placed on a rocking platform shaker (Hematek aliquot mixer) for 2 hours at 37°C for antibody absorption from the antiserum. After incubation, BCG cells were removed from the antiserum by centrifugation and the supernatant serum was transferred to another pellet of BCG cells for sequential absorption. An aliquot of antiserum was removed for ELISA analysis after the 4th and 7th absorption. The ELISA was performed according to the protocol in Fig. 17.

The ELISA titers found in Table 3 were obtained after lot 001 had been absorbed 4 times and in Table 4, 7 times with BCG whole cells. The absorbed antiserum had a higher ELISA titer against H$_{37}$RV whole cell antigen (WCA) than
against BCG-WCA (Tables 3 and 4). The differential titer of absorbed antiserum with H₃⁷RV and BCG-WCA suggests that H₃⁷RV has specific cell surface determinants not expressed by BCG.

The limited supply of lot 001 antiserum made it necessary for us to prepare additional anti-WCA antiserum. For antigen preparation, H₃⁷RV cells grown on MPBB and WB were killed with flowing steam at 80°C for 1 hour, washed 3x and adjusted to a density equivalent to a MacFarland Standard #1 in physiological saline with a 1:10,000 dilution of merthiolate as a preservative. In addition to H₃⁷RV, we prepared M. kansasii MPBB-WCA and BCG WB-WCA suspensions for antiserum production. The cell suspensions prepared from cells grown on MPBB and WB were used for the subcutaneous injection of goats until a maximum anti-WCA ELISA titer was obtained. The hyperimmune animals then were exsanguinated as a source of goat anti-H₃⁷RV MPBB-WCA, anti-H₃⁷RV WB-WCA, anti-BCG-WCA, and anti-M. kansasii MPBB-WCA antiserum. The volume of antiserum yield and respective ELISA titer with the homologous WCA was: goat anti-H₃⁷RV MPBB-WCA - vol. 770 ml and titer 1:100,000, anti-H₃⁷RV WB-WCA - vol. 1200 ml and titer 1:100,000, anti-BCG 1011 WB-WCA - vol. 700 ml and titer 1:100,000, and anti-M. kansasii MPBB-WCA - vol. 600 ml and titer 1:1,000,000.

The newly prepared goat anti-H₃⁷RV MPBB-WCA and WB-WCA, and an anti-H₃⁷RV culture filtrate (CF) (Mars 005 lot 002) antiserum were absorbed, in addition to lot 001, as a possible source of anti-H₃⁷RV specific antibodies. In the initial experiment (Tables 3 and 4) lot 001 antiserum was absorbed with 10⁸ CFU/ml of antiserum. In the second study, the antigen weight was increased to 600 mgs/5 ml of antiserum in an effort to increase absorbing efficiency. Although a differential titer was observed (Tables 5 and 6), when absorbed goat anti-H₃⁷RV MPBB-WCA, WB-WCA, and lot 001 antiserum were used against BCG and H₃⁷RV antigens in the ELISA, antiserum absorption with the increased cell weight did not improve the efficiency of antiserum absorption (compare Tables 2, 3, 4, and 5). As a result of the above data, we plan to continue the absorption studies with varied concentrations of the absorbing antigen in an attempt to prepare an H₃⁷RV specific antiserum as a reagent to detect circulating antigenemia in the tubercular patient.
When goat anti-\(H_{37}\text{RV}\) culture filtrate (CF) antiserum (Mars 005 lot 002) was absorbed by BCG-WCA, the evidence (Table 5) was not so clear that CF absorbed antiserum contained an \(H_{37}\text{RV}\) specific antibody when compared with lot 001, anti-\(H_{37}\text{RV}\) MPBB-WCA and anti-\(H_{37}\text{RV}\) WB-WCA absorbed antisera (Tables 3 through 6). Although surface determinants as well as water soluble mycobacterial proteins could accumulate in culture filtrate, as a result of their small molecular size and composition, the former components might not be immunogenic or serve as poor antigens so that an antiserum such as lot 002 has little or no anti-\(H_{37}\text{RV}\) specific surface determinant antibody.

C. Development of \(H_{37}\text{RV}\) specific monoclonal antibodies (MAB).

Results from the absorption studies in the foregoing section (B) stimulated us to undertake the development of anti-\(H_{37}\text{RV}\) specific monoclonal antibodies (MAB) against WCA. A panel of monoclonal antibodies could provide a specific reagent to detect mycobacterial disease while circumventing the need to prepare reagents by absorption as performed in Section B.

The fusion used to form the hybridoma for \(H_{37}\text{RV}\) MAB was and is currently being performed according to the protocol outlined in Fig. 18. The varied immunization protocols which we are using for \(H_{37}\text{RV}\) MAB production are found in Table 19. The first fusion for hybrid colonies was performed with NS1-P3X63 myeloma cells and spleen cells from Balb C mice immunized with a heat killed suspension of \(H_{37}\text{RV}\) cells grown on MPBB (Immunization Protocol #1 Fig. 19). Supernatant culture fluid aspirated from hybrid colonies then was screened against BCG (1011), \textit{M. kansasii} (12011), and \(H_{37}\text{RV}\) WCA with the ELISA procedure in Fig. 20. As a result of screening supernatant fluid from 152 colonies by the ELISA, we found supernatant fluid from one colony which contained mouse IgG anti-\(H_{37}\text{RV}\) MPBB-WCA which failed to react with BCG 1011 and \textit{M. kansasii} WCA (Table 7). The colony now is being cloned for cell expansion and mouse intraperitoneal MAB production. After purification from ascites fluid, the MAB can be used for the serological study of tuberculosis.

Spleen cell suspensions from mice immunized with \(H_{37}\text{RV}\) WB-WCA (Immunization Protocol #2) and \textit{M. kansasii} WCA (Immunization Protocol #3) are being
fused with NA1-P3X63 melanoma cells. Hybrid colonies resulting from the last fusion again will be screened for anti-H$_{37}$RV WB-WCA and anti-M. kansasii monoclonal antibodies. In addition to whole cell antigens, mice will be immunized with H$_{37}$RV culture filtrate (CF) and CE antigens obtained from NJH, Denver, Colorado. To prepare anti-water soluble protein MAB.

Summary

Initially, we used an immunoelectrophoresis (AGAGE-CLIE) procedure as a guide to selectively fractionate preparative acrylamide gel columns for Mycobacterium tuberculosis H$_{37}$RV specific antigens. Selected fractions then were absorbed with anti-M. kansasii antiserum to purify H$_{37}$RV specific antigens as compared to M. kansasii CE antigens.

Experience gained from the preliminary effort was employed to fractionate CE for H$_{37}$RV specific antigens when compared to M. bovis (1011) BCG CE antigens. While trace amounts of H$_{37}$RV specific antigen compared to BCG CE appeared to be purified in the first fractionation, we were unable to repeat the effort in subsequent fractionation attempts. In subsequent fractionation efforts, the concentration of H$_{37}$RV specific antigens (compared with BCG CE) might well have been below the sensitivity of detection by the AGAGE-CLIE. The demolition of our biohazzard laboratory interrupted efforts to prepare CE which would have a consistent source of H$_{37}$RV specific antigen(s) and adequate concentration for fractionation and purification.

As an alternative to CE fractionation, we absorbed goat anti-H$_{37}$RV whole cell antiserum with BCG whole cell antigen (WCA) in an attempt to prepare an H$_{37}$RV specific reagent for the serological study of tuberculosis. ELISA results with the absorbed antiserum suggest that, in comparison to BCG, H$_{37}$RV expresses specific antigens which might well be found as cell surface or outer membrane determinants. In addition, to absorbed antiserum, we are also preparing H$_{37}$RV specific monoclonal antibodies as a diagnostic serological reagent.
As a result of experience gained from this effort, we will propose the following collaborative study with Dr. Shriniwas at the all India Institute in New Delhi:

1. Provide Dr. Shriniwas with absorbed goat anti-$H_37RV$ MPBB and WB-WCA antiserum to confirm our observations and detect antigenemia and mycobacterial disease.

2. Provide Dr. Shiriniwas with MAB against WCA, CF and CE antigens to detect specific antigenemia in an attempt to identify active mycobacterial disease.

3. Prepare, at Eastern Virginia Medical School, $H_37RV$ monoclonal antibody against strains which are not identified by existing absorbed antiserum and monoclonal antibodies. The addition of new specific antibodies would expand the diagnostic value of the serological reagent.

5. Employ $H_37RV$ whole cell hydrolysates and lipid extracts in competitive binding ELISA reactions with absorbed antiserum and monoclonal antibody. The purification and identification of determinants in cell hydrolysates reactive with specific antiserum, could assist in the development of specific antigens to determine titers of specific $H_37RV$ antibodies in the tubercular patient.
Figures and tables are identified numerically with labels in the upper left corner on the reverse side of each figure or table. Titles and information about each figure and table are printed on paper following each figure or table.
Fig. 1.

Fig. 2.

Comparison of *M. Tuberculosis* H₃⁷RV with *M. kansasii* heterologous CE antigens by AGAGE-CLIE Procedure. H₃⁷RV antigens were resolved in AGAGE column as represented by stained column at the bottom of figure. Lines of identity (LI) reflect the reaction of common antigens in H₃⁷RV and heterologous CE with homologous anti-H₃⁷RV antiserum. The lines of non-identity (NI) with species specific antigens and antiserum.
A diagrammatic presentation of the results found in Fig. 2. TD = Tracking in AGAGE column. 1, 2, 3, 4, & 5 = Reactions of non-identity. D = Distance from Precipitin band (N.I.) 1. to tracking dye <-1->, <-2->, <-3->, and <-4-> = Regions of discontinuous polyacrylamide gradient occupied by 21.0%, 7.5%, 4.75%, and 3.5% gels respectively. C = Region of agarose matrix containing M. kansasii heterologous CE antigen. D = Region of agarose matrix containing anti-H$_{37}$RV homologous antiserum.
Fig. 4.

Template used for slicing PGAGE Column containing resolved *M. tuberculosis* H37RV CE. TD = position of tracking dye 3.5%, 4.75%, 7.0%, and 12.0% = Regions of 3.5, 4.75, 7.0, and 12.0% discontinuous polyacrylamide gradient. 1.] = Fraction 1 which was composed of 2 mm of 7.0% and 3 mm of 12.0% gel. 2.] through 13.] = Remaining gel slices cut from the 12% gel were consistently 3 mm. Identical slices from different PGAGE columns were combined for protein elution to prepare Fractions 1-13.
Fig. 5.

Photograph showing Ouchterlony results or immunoprecipitation reactions between Fractions 1-8 and anti-H$_{37}$RV antiserum and anti-M. kansasii antiserum.

A and C = Ouchterlony results developed with fractions before fractions were absorbed with anti-M. kansasii antiserum sepharose beads.
B and D = Ouchterlony results developed with Fractions after fractions were absorbed with anti-M. kansasii antibody coated sepharose beads.
A, 1-8 = H$_{37}$RV fractions (preabsorbed) 1-8 added to well 1-8, respectively.
A, 9 = Anti-H$_{37}$RV antiserum added to center well.
C, 1-8 = H$_{37}$RV fractions (preabsorbed) 1-8 added to well 1-8, respectively.
C, 9 = Anti-M. kansasii antiserum in center well
B, 1-4, 6-8 = Phosphate buffered saline added to wells 1-4 and 6-8.
B, 5 = H$_{37}$RV fraction (post absorbed) #5 added to well 5.
B, 9 = Anti-H$_{37}$RV antiserum added to center well.
D, 1-4, 6-8 = Phosphate buffered saline added to wells 1-4 and 6-8.
D, 5 = H$_{37}$RV fraction (post absorbed) #5 added to well 5.
D, 9 = Anti-M. kansasii antiserum added to center well.
Identification of *M. tuberculosis* H$_{37}$RV CE antigens and H$_{37}$RV specific antigens in fraction #5 when compared with *M. kansasii* CE as the heterologous antigen and anti-H$_{37}$RV as the heterologous antiserum.

A=D Region of AGAGE-CLIE slide developed with H$_{37}$RV crude CE (lot #2) homologous antigen, *M. kansasii* heterologous CE antigen, and anti-H$_{37}$RV homologous antiserum. L.I. = Line of identity. N.I. - line of non-identity.

B=D Region of AGAGE-CLIE slide developed with purified fraction #5 as the homologous antigen, *M. kansasii* CE as heterologous antigen, and anti-H$_{37}$RV homologous antiserum. B, 1 and 2 = Reactions of non-identity with purified Fraction #5.

AGAGE-CLIE slide A and B were combined to prepare the photographic montage.
Comparison of *M. Tuberculosis* $H_{37}$RV CE antigen with *M. bovis* (BCG TMC #1011) heterologous CE antigen with AGAGE-CLIE procedure $H_{37}$RV CE antigen was resolved in an AGAGE column as previously described. BCG CE antigen was incorporated in Region C (see Fig. 8) as the heterologous antigen and anti-$H_{37}$RV antiserum in Region D. The arrow in Fig. 7 identifies a single non-identity (N.I.) line.
Fig. 8.

Diagrammatic representation of results found in Fig. 7.
A = Agarose containing tracking dye well
B = AGAGE column with separated H$_{37}$RV CE antigen
C = Agarose with BCG (TMC 1011) CE heterologous antigen.
D = Agarose containing anti-H$_{37}$RV homologous antiserum Mars 002 lot 001.
T.D. = tracking dye
1 = 7.0, 4.75, and 3.5% gel regions of the AGAGE gradient.
N.I. = line of non-identity.
5 = distance from center of N.I. line to tracking dye (3.0cm)
L.I. - lines of identity
Fig. 9.

Photograph of a one-dimensional 10% polyacrylamide slab gel stained by the Oakley Silver stain. Sample addition to the different slab gel lanes is described in Table #1. PGAGE Fractions were prepared from lot #3 H$_{37}$RV crude CE antigens.
Molecular Weight Standards

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</tbody>
</table>
Fig. 10.

Diagrammatic presentation of photograph found in Fig. 9. The lane numbers are identified at the top of the figure and molecular weight markers in the column on the left side of figure. Sample addition to the lanes of the slab gel are found in Table 1. The sample (Fraction #1) added to lane #13 was absorbed 9X with anit-BCG sepharose beads, and in lane 13 = presumed $H_{37}$RV specific antigen (M.W. approx. 30,000). Although bands labeled b were not found in the blank lane 14, they have been found in blank lanes of other slab gels to suggest that they might be artifacts of staining.
Diagrammatic representation of HPLC elution profile developed with H₃₇RV crude CE (lot 3). Sample addition = 1.0 ml containing 6.7 mgs of H₃₇RV crude CE. Running buffer = 20mM NaPO₄, pH 6.0. Program developed for sample elution = NaCl linear elution gradient 0-500 mM for 100 minutes at 1.0 ml/minute. l = NaCl gradient was terminated after 59.32 minutes.
A continuation of elution initiated in Fig. 11. The NaCl gradient initiated for elution profile found in Fig. 11 was interrupted after 59.32 minutes and 500mM NaCl was started at 59.32 minutes to elute remaining H_{37}RV CE and wash HPLC column.
Diagrammatic representation of HPLC elution profile developed with H$_{37}$RV CE purified Fraction #1 (CE lot #3). Sample addition = 1.0 ml - protein concentration and limited volume of Fraction #1 did not permit protein determination by Lowery procedure. Running buffer = 20 mM NaPO$_4$, pH 6.0. Program developed for sample elution = NaCl linear elution gradient 0-500mM for 100 minutes at 1.0ml/minute.
1. Time = 21.71 minutes
2. Time = 38.94 minutes
3. Time = 42.04 minutes when the gradient was shifted to 500mM NaCl. Detector sensitivity increased to monitor Fraction #1 elution.
Fig. 14.

Comparison of *M. tuberculosis* H₃⁷RV CE antigen (CE lot #4) with BCG (TMC #1011) heterologous antigen by AGAGE-CLIE procedure. In order to develop AGAGE-CLIE slide, H₃⁷RV CE were resolved in AGAGE column, BCG CE placed in C region above template (Fig. 1), and anti-H₃⁷RV homologous antiserum added to region D. Fig. 14 is a photographic enlargement of the AGAGE-CLIE region D where lines of non-identity are not found.
Fig. 15.

Photograph of a one-dimensional 10% polyacrylamide slab gel stained by the Oakley silver stain. Fractions were prepared by fractionating PGAGE columns with H_{2}RV resolved lot #4 crude CE antigens. Sample or Fraction addition to the different slab gel lanes is described in Table #2.
Fig. 16.

A diagrammatic representation of lanes found in Figure 15. Protein band a found in Fig. 9 and 10 and presumed to be a H$_{37}$RV specific antigen is not found in Fig. 15 and 16 after fractions were absorbed with anti-BCG antiserum-coated sepharose beads.
ELISA PROTOCOL FOR WHOLE CELL ANTIGEN

Solutions:

Glycine buffer: 100 ml H$_2$O
0.751 g glycine
pH - 10.4 with 1M NaOH

Substrate solution: 100 cc glycine buffer
0.02033 g MgCl$_2$ (.001M)
0.15786 g p-nitrophenyl phosphate (.006M)

Wash Solution: .85% NaCl + .05% Tween 80
Use for all dilutions

Whole Cell Antigen - Washed heat killed cell suspensions adjusted to a
density equivalent to a MacFarland Standard #1.

Plating wells:
Whole cells - 160 ml of whole cell antigen (WCA) suspension in 0.1M
NaHCO$_3$ solution (with Na$_3$ as a preservative) were added to each
well.

1. Plate wells with WCA for 1 hour at 60°C.
2. Aspirate.
3. Add 50 microliters absolute methanol, incubate for 15 min. at room
temperature.
4. Discard methanol and plates are ready for use or refrigeration.
5. Wash wells 3 times and discard the wash solution.
6. Add unabsorbed or absorbed goat antiserum - 160 microliter to each well,
incubate at room temperature for 2 hours.
7. Wash wells 3 times and discard the wash solution.
8. Add anti-goat conjugate - 160 microliters at room temperature for 2 hours.
(Make a 1:100 dilution in 0.85% NaCl + .05% Tween 80).
9. Wash wells 3 times and discard the wash solution.
10. Add substrate, 160 microliters to each well and incubate at room
temperature for 30 minutes.
11. Add 3M NaOH, 40 microliters, to each well and shake the wells to mix
properly.
12. Read assay on the ELISA Dynatech Minireader 410 nm.
Fig. 17.

Outline of protocol used for ELISA with whole cell antigen (WCA) and absorbed WCA antiserum.
FUSION PROTOCOL

1. Remove spleens from 3-4 hyperimmune (IP)* Balb C mice after cervical dislocation.

2. Mince spleens in a petri dish containing DME-A* with a 30-mesh stainless steel screen and then pass cells through a series of needles.

3. Expose spleen cell suspension to \( \text{NH}_4\text{Cl} \) for 3 minutes for RBC lysis.

4. Wash spleen cells 3x with DME-A* and count viable cells.

Wash cells of Myeloma line NSI-P3X63 2X with DME-A and count viable cells.

5. Combine \( 10^8 \) spleen cells with \( 10^7 \) myeloma cells and gently add PEG. Dilute PEG with DME-A.

6. Centrifuge cell suspension and resuspend fused cells in 10ml of HAT*. Add 3.3 ml of suspension to each of 3 tubes containing 30 ml of HAT.

7. Plate wells by adding 0.2 ml of HAT cell suspension to the wells of a 96 well plate and incubate at 35°C.

8. Check 96 well plate for colonies and transfer colonies to 24 well plates containing HAT +5% FCS and incubate.

9. Screen colony supernatant culture fluid from 24 well plate against the desired antigen with an ELISA procedure.

* DME-A = Dulbecco Modified Eagle Medium + antibiotic

* HAT = Hyperanthine Aminopteria and thymidium medium.

* (IP) = See Table 7 for Balb C Mouse Immunization Protocol.
Fig. 18.

Fusion protocol for hybridoma and monoclonal antibody (MAB) development.
BALB C (Mouse) Immunization Protocol

1. Antigen - Heat killed cells adjusted to a density equal to MacFarland #1 standard - H$_{37}^{RV}$ MPBB-WCA.
   Injection - 3 mice - 0.2ml/mouse I.P. at 10 day intervals from 5/18/84 - 10/23/84.
   0.1 ml/mouse I.V (tail vein) 11/6/84.
   Fusion - 11/9/84.

2. Antigen - H$_{37}^{RV}$ WB-WCA-employed immunization protocol as described for Schedule #1.
   Fusion - 11/23/84

3. Antigen - M. kansasii MPBB-WCA-employed immunization protocol as described for Schedule #1.
   Fusion - 12/28/84

4. Antigen - H$_{37}^{RV}$ cells grown on H$_{37}^{RV}$-LJ-WCA Lowenstein-Jensen slants. Cells killed with flowing steam at 80°C for 1 hour. Washed with H$_{2}O$ and adjusted to a density = MacFarland #1 standard.
   Injection - 4 mice - 0.2 ml/mouse I.P. at 10 day intervals beginning 11/12/84.
   Fusion - After 3 months of immunization.

5. Antigen - H$_{37}^{RV}$ LJ-WCA. Cell suspension prepared as described in Protocol 4. Combined 1 ml of cell suspension with 1 ml of Freund's adjuvant.
   Injection - 4 mice - inject 0.1 ml of adjuvant-suspension subcutaneous. Subsequent injection. 0.1 ml cell suspension subcutaneous at 10 day intervals starting on 11/12/84.
   Fusion - After 3 months of immunization.
Fig. 19.

BALB-C (mouse) Immunization Protocol for the Hybridoma Procedure.
ELISA PROTOCOL FOR MONOCLONAL ANTIBODY

a. Coat 96-well plate with 160 ml of selected whole cell antigen suspension. Incubate at 60°C for 1 hour.

\[ \text{Ag} \]

b. Aspirate and wash with 160 ul of MeOH at room temperature for 15 minutes.

c. Add 160 ul of supernatant culture fluid and incubate at room temperature for 2 hours. Aspirate.

\[ \text{Ab} \]

\[ \text{monoclonal} \]

d. Add goat anti-mouse IgG alkaline phosphatase conjugate for 2 hours at room temperature. Aspirate.

\[ \text{anti-mouse} \]
\[ \text{IgG phosphatase conjugate} \]

\[ \text{PNPP} \]
\[ \text{(yellow)} \]

e. Add 160 ul of para-nitrophenyl phosphate (PNPP) for 30 minutes at room temperature.

\[ \text{PNPP} \]
\[ \text{PNPP (yellow)} \]

f. Record absorbance with the Dynatech Minireader (410 nm).
Fig. 20.

ELISA Protocol for Monoclonal Antibody Analysis. The composition of solutions used in MAB ELISA procedure is outlined in the WCA ELISA procedure.
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<th>Lane</th>
<th>Sample</th>
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<td>Molecular weight standard</td>
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<td>2</td>
<td><em>M. bovis</em> crude CE unabsorbed 50 ul</td>
</tr>
<tr>
<td>3</td>
<td><em>M. bovis</em> crude CE after 3 absorptions 50 ul</td>
</tr>
<tr>
<td>4</td>
<td><em>M. bovis</em> crude CE after 6 absorptions 50 ul</td>
</tr>
<tr>
<td>5</td>
<td><em>M. bovis</em> crude CE after 9 absorptions 50 ul</td>
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<td>Fraction #2, #3 unabsorbed (pooled sample) 50 ul</td>
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<td>7</td>
<td>Fraction #2, #3 after 3 absorptions (pooled sample) 50 ul</td>
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<tr>
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<td>Fraction #2, #3 after 6 absorptions (pooled sample) 50 ul</td>
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<td><strong>M. bovis</strong> crude CE after 10 absorptions 50 ul</td>
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<td>Fraction 6 after 10 absorptions 50 ul</td>
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## TABLE 2

SAMPLE ADDITION TO 10% POLYACRYLAMIDE SLAB GEL
Comparison of Reactivity of Absorbed Anti-H$_{37}$RV Antiserum* with *M. bovis* 1011 and H$_{37}$RV 102 whole cell antigens

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<td>$10^5$</td>
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<tr>
<td>Absorbance with H$_{37}$RV WCA in well</td>
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<td>1.44</td>
<td>0.24</td>
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<tr>
<td>Absorbance when mycobacterial WCA was omitted from well</td>
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Table 3.

Comparison of Reactivity of Absorbed Anti-H$_{37}$RV antiserum* with M. bovis 1011 and H$_{37}$RV 102 whole cell antigens. * = 5 ml of H$_{37}$RV antiserum Mars 002 lot 001 absorbed 4x with $10^8$ colony forming units/ml. M. bovis 1011 = BCG TMS #1011.
Comparison of Reactivity of Absorbed Anti-\(H_37\)RV Antiserum* with \(M.\ bovis\) 1011 and \(H_37\)RV 102 whole cell antigens (WCA)

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<tr>
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<td>1.06</td>
<td>0.35</td>
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<tr>
<td>Absorbance when mycobacterial WCA omitted from well</td>
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Table 4.

Comparison of Reactivity of Absorbed Anti-H₃RV Antiserum* with *M. bovis* 1011 and \( M. \) \( \text{H₃RV} \) 102 whole cell antigens (WCA). *\( *^{32} \) 5 ml of \( M. \) \( \text{H₃RV} \) antiserum Mars 002 lot 001 absorbed 7x with \( 10^8 \) colony forming units/ml. \( M. \) \( \text{bovis} \) 1011 = BCG TMS #1011.
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<tr>
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<td>7</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Absorbance with BCG WCA in well

| Absorbance | over | over | over | over | 0.73 | 0.76 | 0.35 | 0.35 | 0.53 | 0.67 | 0.37 | 0.0 |

Absorbance with H$_{37}$RV WCA in well

| Absorbance | 1.21 | 1.00 | 1.93 | 1.98 | 0.99 | 1.00 | 1.11 | 0.82 | 0.97 | 1.26 | 1.26 | 0.95 |

Absorbed Antiserum Lot 002

| Absorbance with BCG WCA in well | 1.17 | 0.93 | 1.34 | 1.91 | 0.55 | 0.57 | 0.18 | 0.30 | 0.40 | 0.65 | 0.42 | 0.66 |
| Absorbance with H$_{37}$RV WCA in well | 1.26 | 0.90 | 1.98 | 2.02 | 1.99 | 0.55 | 0.30 | 0.71 | 1.96 | over | 0.23 | 0.53 |
Table 5.
Comparison of Reactivity of Absorbed Antisera with *M. bovis* 1011 and *H_{37}RV* 102 whole cell antigens. Lot 001 = 5 ml of Mars 002 lot 001 anti-*H_{37}RV* absorbed 10x with 600 mgs of BCG cells. Antiserum was raised against *M. tuberculosis* *H_{37}RV* TMC #102 whole cell antigen (WCA). Lot 002 = 5 ml of Mars 005 lot 002 anti-*H_{37}RV* culture filtrate (CF) antiserum absorbed with 600 mgs of BCG cells.
### Comparison of Reactivity of Absorbed Antisera with M. bovis (1011) and H$_{37}$RV (102) Whole Cell Antigens (WCA)

#### Absorbed Antiserum-Goat Anti-H$_{37}$RV Whole Cell Antigen (WCA) MPBB

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</tr>
<tr>
<td><strong>Absorbance with BCG WCA in well</strong></td>
<td>0.25</td>
<td>0.23</td>
<td>1.63</td>
<td>1.64</td>
<td>0.0</td>
<td>0.0</td>
<td>0.16</td>
<td>0.18</td>
<td>0.04</td>
<td>0.0</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Absorbance with H$_{37}$RV WCA in well</strong></td>
<td>0.49</td>
<td>0.52</td>
<td>1.14</td>
<td>1.30</td>
<td>0.21</td>
<td>0.24</td>
<td>0.30</td>
<td>0.33</td>
<td>0.26</td>
<td>0.34</td>
<td>0.11</td>
<td>0.18</td>
</tr>
</tbody>
</table>

#### Absorbed Antiserum-Goat Anti-H$_{37}$RV WCA-WB

| **Absorbance with BCG WCA in well** | 0.76 | 0.67 | 1.95 | 2.23 | 0.18 | 0.18 | 0.03 | 0.03 | 0.0 | 0.07 | 0.0 | 0.05 |
| **Absorbance with H$_{37}$RV WCA in well** | 0.99 | 1.19 | 1.39 | 1.88 | 0.20 | 0.26 | 0.10 | 0.16 | 0.16 | 0.38 | 0.18 | 0.28 |
Table 6.

Comparison of Reactivity of Absorbed Antisera with M. bovis (1011) and H$_{37}$RV (102) whole cell antigens (WCA). Anti-H$_{37}$RV WCA MPBB = antiserum raised against a whole cell antigen where H$_{37}$RV cells were grown on modified Proskauer Beck broth (MPBB). Antiserum absorbed with 600 mgs of BCG cells. ELISA titer of unabsorbed antiserum = 1:100,000. Anti-H$_{37}$RV WCA-WB = antiserum raised against a whole cell antigen where H$_{37}$RV cells were grown on Wong's broth (WB). ELISA titer of unabsorbed antiserum = 1:100,000. Antiserum absorbed as described for H$_{37}$RV WCA MPBB.
Comparison of Reactivity of Anti-$H_{37}$RV MPBB-WCA Monoclonal Antibody with $H_{37}$RV MPBB-WCA, BCG-WCA, and $M. kansasii$ MPBB-WCA

<table>
<thead>
<tr>
<th>Culture Fluid from Plate : Row : Well</th>
<th>$H_{37}$RV MPBB-WCA</th>
<th>BCG WCA</th>
<th>$M. kansasii$ WCA</th>
<th>HAT Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 : C : 6</td>
<td>0.23</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Table 7.</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Comparison of Reactivity of Anti-H$<em>{37RV}$ MPBB-WCA Monoclonal Antibody with H$</em>{37RV}$ MPBB-WCA, BCG-WCA, and M. kansasii MPBB-WCA.</td>
<td></td>
<td></td>
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</tbody>
</table>