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EXTRACELLULAR PROTEINS OF TUBERCLE BACILLI
IV. α AND β ANTIGENS AS MAJOR EXTRACELLULAR PROTEIN PRODUCTS AND AS CELLULAR COMPONENTS OF A STRAIN (H37Rv) OF *MYCOBACTERIUM TUBERCULOSIS**

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SUMMARY The contents of two distinct protein antigens, α and β, in unheated culture filtrates of a virulent strain (H37Rv) of *Mycobacterium tuberculosis* and their association with the bacterial cell were studied. Quantitative precipitation employing specific anti-α and -β rabbit sera showed that the protein components responsible for α and β antigens comprised nearly 70 per cent of the total protein released by the strain H37Rv into the medium. Evidence is also presented indicating that both α and β antigens are present on/in the cells of the tubercle bacilli presumably as surface components.

INTRODUCTION

Unheated culture filtrates of a virulent strain (H37Rv) of *Mycobacterium tuberculosis* were previously shown (YONEDA and FUKUI, 1961 a,b) to contain two distinct protein components which were later designated as the α and β antigens on the basis of differences in their antigenic specificity (YONEDA, 1963; YONEDA and FUKUI, 1963). Since these antigens were subsequently isolated in a highly purified form (FUKUI and YONEDA, 1961), plans are now being made in our laboratory for the extensive studies of their immunogenic, allergenic, toxic and other relevant properties and also of their distribution in other mycobacteria.

However, before approaching these problems, the following problems should be solved; (1) whether α and β antigens are major protein products in the culture filtrate of strain H37Rv, and (2) whether these extracellular antigens are present on/in bacterial cell as cellular components. It was thought that the solution to these problems would provide fundamental information on the physiological profile of the α and β antigens.

Fractionation of these antigens suggested that they constituted a large part of the total protein released by the organism into the medium. Thus, from zone-electrophoretic dia-

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grams, the fraction responsible for these antigens was calculated to comprise about 80 per cent of the protein precipitable with 50 per cent saturation of ammonium sulfate at a concentration which precipitated the bulk of the protein (87.3 per cent) in unheated culture filtrates of strain H37Rv (YONEDA and FUKUI, 1961 a). However, the experiment was only roughly quantitative, and the actual contents of these antigens had to be determined on a quantitative immunochemical basis.

Recently anti-α and -β rabbit sera were obtained which seemed to contain no detectable amounts of precipitating antibodies reacting with any antigens in the crude protein fraction, other than α and β, and thus these antigens could be detected specifically both qualitatively and quantitatively. With these sera, attempts were therefore made to estimate the actual contents of α and β antigens in a crude culture filtrate of the strain H37Rv by means of quantitative precipitation and also to examine the association of these antigens with the bacterial cell by absorption and immunodiffusion procedures.

MATERIALS AND METHODS

1. *Organisms*

Mycobacterium tuberculosis strain H37Rv was from the stock collection of the Department of Tuberculosis Research of this Institute.

2. *Preparation of crude concentrates of unheated culture filtrates*

One loopful of strain H37Rv, cultivated at 37°C for two weeks on modified Sauton's medium (YONEDA and FUKUI, 1961 a), was inoculated into 80 ml volumes of medium in 300 ml Erlenmeyer flasks and incubated at 37°C for three weeks. After incubation, the bacilli were filtered off aseptically through Toyo No. 50 filter paper and then a Seitz filter in the cold without previous heating of the culture. To the final filtrates solid ammonium sulfate was added at 80 per cent saturation. The precipitate was collected by centrifugation, dissolved in M/15 phosphate buffer, pH 7.0, and dialyzed against the buffer and the concentrated solution, with a protein content of approximately 13 mg per ml, was used as the crude unheated culture filtrate.

3. *α and β preparations*

α and β, which were previously described as mtp-1 (or mtp-3) and mtp-4 respectively, were prepared from unheated culture filtrates of strain H37Rv by a combination of ammonium sulfate fractionation, zone-electrophoretic and chromatographic separations as described in detail previously (YONEDA and FUKUI, 1961 a, b; FUKUI and YONEDA, 1961).

4. *Preparation of anti-α and -β rabbit sera*

Anti-α and -β sera were prepared by immunizing rabbits with highly purified preparations of α and β antigens in incomplete Freund's adjuvants as described previously (YONEDA and FUKUI, 1961 a). In the present work, the first dose injected was 100 μg of protein per rabbit and the booster does was 50 μg. In Ouchterlony's agar plates, these antisera formed essentially a single precipitation line against concentrated crude culture filtrates of the strain H37Rv, indicating that they contain no detectable amounts of precipitating antibodies that react with the antigens in the crude protein fraction, other than α and β. The sera were heated at 56°C for 30 minutes before use.

5. *Preparation of bacterial cells in the logarithmic phase of growth*

One loopful of a culture of strain H37Rv, cultivated at 37°C for a week on a glycerol broth, was inoculated into 100 ml volumes of semi-synthetic medium in a 500 ml shaking flask containing four glass beads of 5 mm diameter and incubated at 37°C with shaking at 120 rpm on a rotary shaker for four days. At the end of the incubation, the optical density of the culture at 590 mμ (OD) in an Universal type Coleman spectrophotometer had usually reached 1 to 2, depending on the size of inoculum. The semi-synthetic medium contained (per liter): Bacto Casamino Acids (Difco, certified) 10 g, asparagine 1.0 g, KH_2PO_4 1.0 g, $Na_2HPO_4 \cdot 12H_2O$ 6.3 g, glycerine 30 ml, Tween 80 10 ml and Muller and Miller's II solution (MULLER and MILLER, 1941) 1.0 ml. Ten ml of the shaking culture was then transfered to 100 ml volumes of medium per flask and the diluted cultures were again incubated at 37°C with shaking on a rotary shaker as before. The growth, measured by the optical density, increased exponentially, doubling in about 20 hours and reaching an

OD of nearly 2. When the OD of the culture reached about 1, the cells were collected, washed three times by centrifugation at 12,000 rpm with M/15 phosphate buffered saline (pH 7.0) and the washed packed cells were employed as the material for absorption and disintegration.

6. *Disintegration of cells and preparation of subcellular fractions*

The thoroughly washed packed cells obtained from either shaking or still cultures were disintegrated as follows; 5 g of cells mixed with about 5 g of quartz powder (200 mesh) were ground in a mortar for about 10 minutes in the cold. The resulting muddy paste was then mixed with 3 volumes of Tris-HCl buffer (pH 7.8) containing 0.01 M $MgCl_2$ and 0.05 M KCl. The cell debris and quartz powder were removed by centrifugation at $5,000 \times g$ for 20 minutes. The turbid supernatant was then centrifuged at $14,500 \times g$ for 30 minutes in the cold and the resulting supernatant was further centrifuged in a Hitachi Model 40P ultracentrifuge at $105,000 \times g$ for 120 minutes.

7. *Quantitative precipitation*

The quantitative precipitation test was done as described by HEIDERBERGER and KENDALL (1935), employing α, β and a crude culture filtrate as antigens, and specific anti-α and -β rabbit sera as antibodies. The nitrogen contents of the specific precipitates were determined by a modified micro-Kjeldahl-Nessler method (YOKOI and AKASI, 1955).

8. *Immunodiffusion test*

The double diffusion precipitation method in agar described by OUCHTERLONY (1948) was employed with a slight modification. The results were usually examined after standing the agar plates at room temperature for three to four days.

RESULTS

1. *Determination of the contents of α and β in unheated culture filtrates of strain H37Rv*

a) Quantitative precipitation with anti-α rabbit serum

Increasing amounts of a preparation of α (from 8.0 to 47.0 μg N) and of unheated culture filtrates of H37Rv (from 20.6 to 110.0 μg N) in a final volume of 1 ml were placed in two series of centrifuge tubes. One ml of anti-α rabbit serum (Lot No. 2) was then added to each tube with thorough mixing. The mixtures were placed in a water bath at 37°C for 1 hour and then in the cold for one week. They were then centrifuged and the resulting supernatants were used for assay of the antigen and antibody remaining unprecipitated. The precipitates were washed three times with 2 ml of cold physiological saline and then their nitrogen contents were determined.

Fig. 1 summarizes the results obtained. It may be seen that, the nitrogen contents of the specific precipitates gave typical quantitative precipitation curves in both cases, each representing the pattern of a single antigen and antibody system. It will also be noted that the curve with the unheated culture filtrate superimposes that of the α preparation exactly.

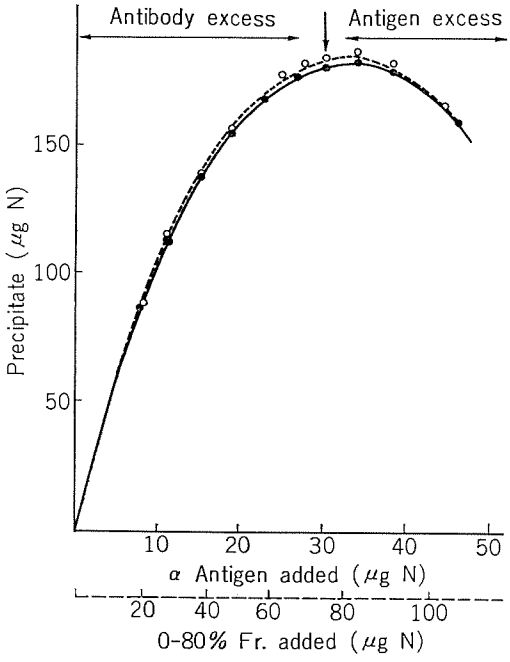

FIGURE 1 Quantitative precipitation reaction of α antigen and the 0–80% fraction with anti-α rabbit serum.
Arrow indicates equivalence point.

The α and its antibody remaining in the supernatants was examined by the ring test. None was detectable in the supernatant of tube 7 indicating that the antigen was equivalent to the antibody in this tube.

At this equivalence point, the amount of precipitate with unheated culture filtrate was identical, within the limits of experimental error, with that of α antigen, while the actual amount (76.0 μg N) of the crude fraction added was much more than that (31.2 μg N) of α in terms of protein nitrogen. Since a constant amount of specific antiserum was used in each case and since all the α antibodies were precipitated at the equivalence point, the quantity of α antigen in the culture filtrate could be calculated from the amount of both added and the amount of antigen precipitated at the equivalence point. It was calculated that about 41 per cent of the protein in the unheated culture filtrate of strain H37Rv was α antigen.

b) Quantitative precipitation with anti-β rabbit serum

The quantitative precipitation test was carried out as described above and results similar to those with α and its antiserum were obtained. In this case, the amounts of antigen added to the two series of 10 tubes were 9.2 to 92.3 μg N for β and 55 to 360 μg N for unheated culture filtrate. One ml of anti -β rabbits serum (Lot No. 3) was added to each tube.

As may be seen in Fig. 2, the precipitation curves obtained with β and with the crude culture filtrates, respectively, apparently represent single antigen and antibody systems and the two curves are essentially superimposable. At the equivalence point 58 μg N of the β preparation and 207 μg of the unheated culture filtrate were added. From these values, it can be calculated that about 28 per cent of the total protein nitrogen in the unheated culture filtrate was specifically precipitated with the anti-β rabbit serum.

2. *Absorption of α and β antibodies with washed intact bacterial cells*

Strain H37Rv grown either in still or in shake cultures was used as the source of the cells for absorption. When still cultures were used, organisms were grown at 37°C on a modified Sauton's medium for three weeks and when shaking cultures were used, cells were cultivated in a synthetic medium as described in Materials and Methods. After incubation, cultures were harvested and the bacterial cells were collected and washed thoroughly in phosphate buffered saline (pH 7.0) by centrifugation until no protein was detectable in the supernatant. The resulting washed packed cells were employed in the absorption tests. To 1 ml of the test serum prepared by mixing 0.6 ml of anti-α with 0.4 ml of anti-β sera, were added 0.2 g wet weight of washed packed cells. They were carefully suspended with a glass rod and then incubated at 37°C for 30 minutes with occasional stirring. After incubation, the suspension was centrifuged at 14,500 $\times$ g for 30 minutes and the resulting supernatant

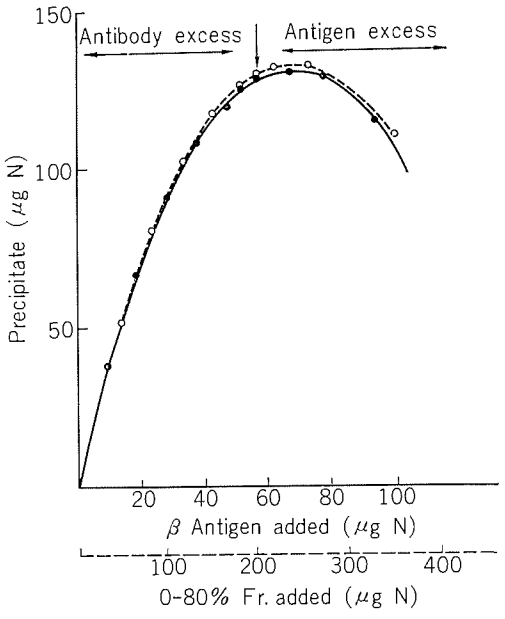

FIGURE 2 Quantitative precipitation reaction of β antigen and the 0–80% fraction with anti-β rabbit serum.
Arrow indicates equivalence point.

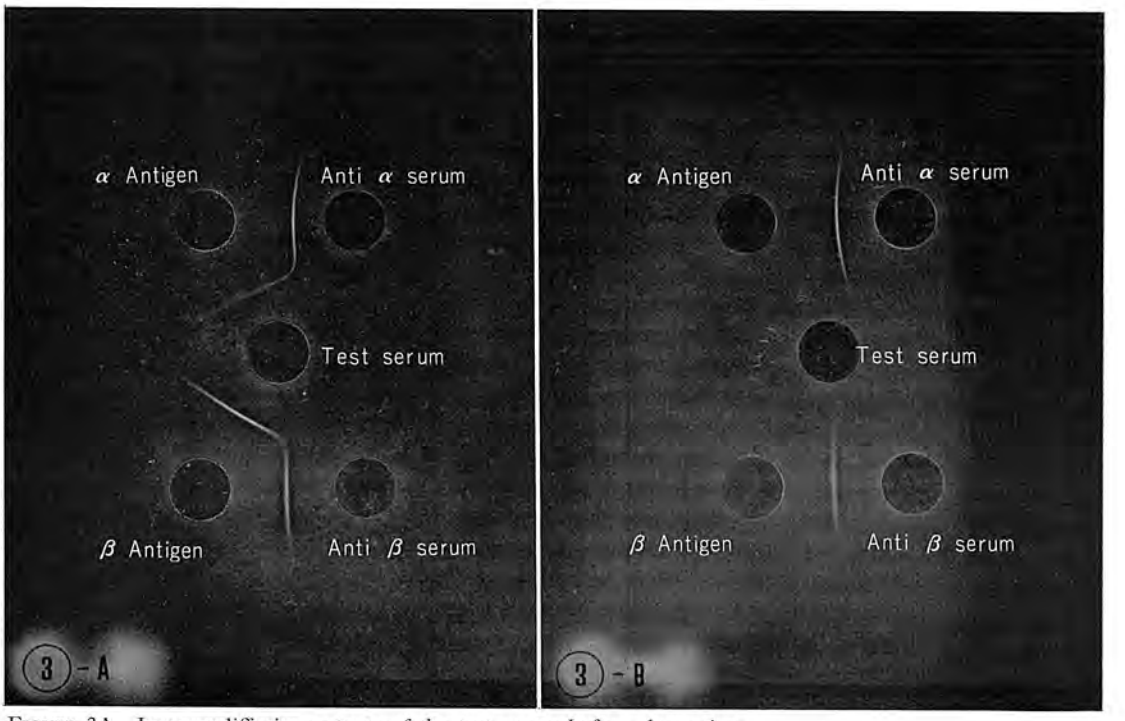

FIGURE 3A Immunodiffusion pattern of the test serum before absorption.
Test serum: A mixture of anti-α and -β rabbit sera
The protein concentration of both α and β antigens was 50 μg/ml.

FIGURE 3B Immunodiffusion pattern of the test serum after absorption.
Test serum: A mixture of anti-α and -β rabbit sera absorbed with washed intact cells of strain H37Rv
The protein concentration of both α and β antigens was 50 μg/ml.

was again subjected to absorption according to the procedure described above. The absorption was repeated three times in each case and the contents of antibodies in the supernatants obtained after each absorption were tested qualitatively by immunodiffusion against α and β.

Fig. 3A and 3B show the immunodiffusion precipitation patterns in Ouchterlony's agar plates of the test serum before and after the third absorption with washed, packed cells of strain H37Rv. In both cases, the test serum was put into the middle well. The concentration of α and β antigens employed as indicators was 50 μg protein per ml.

As may be seen from these Figures, a single precipitation line was formed between α or β and the original unabsorbed serum, and these lines each fused with the single line of these antigens with the respective indicator antiserum (Fig. 3A), while a precipitation line between α or β antigens and the absorbed serum was scarcely detectable (Fig. 3B). This clearly indicates that no detectable amounts of antibodies reacting with α and β antigens remain in the absorbed serum, suggesting the complete removal of these antibodies from the test serum by treatment with bacterial cells.

To see whether the absorption is specific for α or β antibodies, preparations of diphtheria horse antitoxin (100 units/ml) and of anti-bovine-serum-albumin rabbit serum were

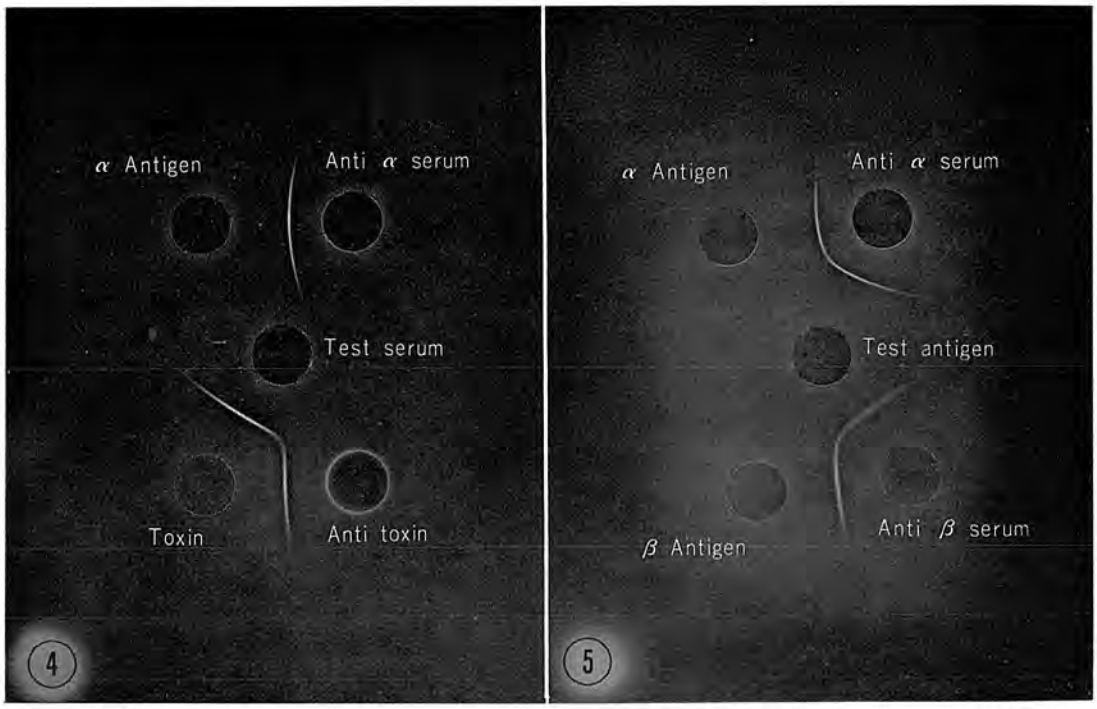

FIGURE 4 Immunodiffusion pattern demonstrating the specific absorption of α antibodies with H37Rv cells.
Test serum: A mixture of anti-α rabbit serum and diphtheria antitoxin (20 units/ml) absorbed with washed intact H37Rv cells
The protein concentrations of α antigen and of crystalline diphtheria toxin were 50 μg/ml and 30 μg/ml, respectively.

FIGURE 5 Immunodiffusion pattern demonstrating the presence of α and β antigens in the supernatant of an agitated H37Rv cell suspension.
Test antigen: Concentrated supernatant of the H37Rv cell suspension agitated in a Waring blender for 3 min
Indicator antigens: α and β antigens (each 50 μg/ml)
Antisera: Anti-α and -β rabbit sera

treated with strain H37Rv cells in the same way used for anti-α,-β rabbit sera. However, neither a decrease in antitoxin nor in antibodies against bovine-serum-albumin could be detected by Ouchterlony's immunodiffusion test (Fig. 4). Further, it was observed that bacterial cells which had been heated at 80°C for 30 minutes did not absorb antibodies against either α or β antigen. Incidentally, both α and β antigens are known to be heat labile and completely lose their ability to combine with the respective antibodies when heated at 70°C for 10 minutes (YONEDA and FUKUI, 1961 b).

3. *Liberation of α and β antigens from thoroughly washed intact cells of strain H37Rv by agitation*

From the result of the absorption experiment described above, it may be concluded that the intact bacterial cells of strain H37Rv have α and β antigens attached to their cell surface as specific binding sites for α and β antibodies. To prove this, an attempt was made to liberate

the α and β antigens in a soluble state from thoroughly washed bacterial cells.

H37Rv strain cells were grown at 37°C in synthetic medium with shaking as described in Materials and Methods. When the optical density at 590 mμ (OD) of the exponentially growing culture reached 1.6, a 30 ml aliquot of the culture was harvested and the cells were collected, washed three times with phosphate buffered saline (pH 7.0) by centrifugation and then resuspended in 30 ml of buffered saline. The whole suspension was then put into a 50 ml blending vessel and stirred at 10,000 rpm in a small Waring blender for 3 to 5 minutes. After stirring, the suspension was centrifuged and the resulting supernatant was filtered through a Millipore filter (HA. 0.45 μ), precipitated with 80 per cent saturation of ammonium sulfate and then dialyzed against buffered saline. Finally, the dialyzed material was tested in Ouchterlony's agar plates for its precipitating activity with anti-α and -β rabbit sera.

The result is shown in Fig. 5. It may be seen that a single precipitation line formed between the dialyzed filtrate and the antiserum employed. This line fused completely with the line of the indicator antigen with the specific antiserum. This clearly indicates the presence of α and β antigens in the test filtrate. Both of these were liberated in a soluble form from the bacterial cells during stirring. The DNA and RNA contents of the dialyzed filtrate were analyzed by the diphenylamine (BURTON, 1956) and orcinol (MEJBAUM, W., 1939) reactions and found to be almost negligible. The cells obtained before and after treatment in an ordinary Waring blender were stained with acid-fast stain and their morphology was studied under a light microscope. It was found that, after agitation, the cells completely retained their acid-fast stainability and no noticeable morphological changes due to this treatment could be seen. Similar results were obtained when bacterial cells were exposed to sonic oscillation even for so short a time (1 to 2 minutes) that no gross degradation of the cells occurred.

4. *Intracellular distribution of α and β antigens*

Intact washed cells from still and shaking cultures of strain H37Rv were disintegrated with quartz powder in mortars as described above. The disintegrates were first centrifuged twice at 5000 $\times g$ for 20 minutes to remove quartz powder and unbroken cells. The resulting slightly turbid supernatants were then centrifuged twice at 14,500 $\times g$ for 30 minutes, and the residues were used as test material (Pellet 1). The supernatants were again centrifuged at 105,000 $\times g$ for 120 minutes in a Hitachi Model 40P ultracentrifuge and the resulting supernatant (Sup 2) and residue (Pellet 2) were used in the test. The fractionation procedure is shown in Fig. 6.

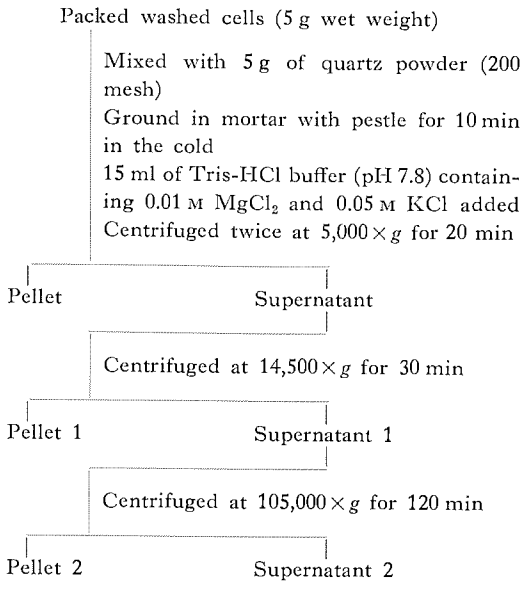

FIGURE 6 Fractionation of washed intact cells of strain H37Rv.

a) Absorption of α and β antibodies with pellet fractions

0.1 g wet weight of pellet was carefully suspended in 0.5 ml of antiserum containing 0.3 ml of anti-α and 0.2 ml of anti-β rabbit sera. The suspension was placed in a water bath and incubated at 37°C for 15 minutes. After incubation, the suspension was centrifuged at

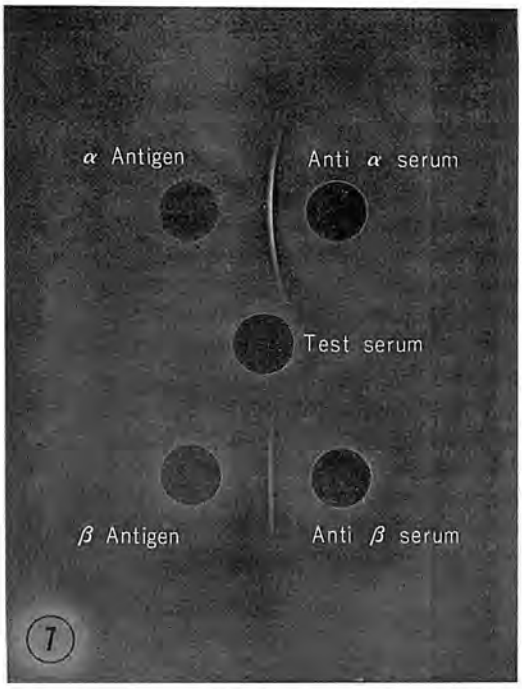

FIGURE 7 Immunodiffusion pattern demonstrating the absorption of α and β antibodies with insoluble debris (Pellet 2) of the mechanically disintegrated cellular preparation of strain H37Rv.
Test antisera: A mixture of anti-α and -β rabbit sera absorbed with Pellet 2 (cf. Figure 6)
Indicator antisera: Anti-α and -β rabbit sera
Indicator antigens: α and β antigens (each 50 μg/ml)

FIGURE 8 Immunodiffusion pattern demonstrating the presence of α and β antigens in the supernatant of the mechanically disintegrated cellular preparation of strain H37Rv.
Test antigen: Supernatant fraction of cellular disintegrated preparation obtained by centrifugation at $105{,}000 \times g$ for 120 min
Indicator antigens: α and β antigens (each 50 μg/ml)
Indicator antisera: Anti-α and -β rabbit sera

$14{,}500 \times g$ for 30 minutes and the resulting supernatant was again thoroughly mixed with 0.1 g of pellet and incubated. The absorption procedure was repeated three times with each pellet and the final supernatants obtained by centrifugation were tested for their precipitating activity with both α and β antigens in Ouchterlony's agar plates.

Fig. 7 shows the agar precipitation pattern of the antiserum treated with Pellet 2 in an Ouchterlony's plate. It can be seen that none of these antigens are precipitated with the absorbed serum while they form single clear precipitation lines with their respective antisera, indicating the absence of both α and β antibodies in the test serum. A similar result was obtained with antiserum absorbed with Pellet 1.

b) Detection of α and β antigens in supernatant fractions

Ten ml of supernatant (Sup 2) obtained by centrifugation at $105{,}000 \times g$ was concentrated about five times with Carbowax 6000 and the concentrated supernatant was employed for Ouchterlony's immunodiffusion test. The protein content was 10.8 mg per ml.

The result is shown in Fig. 8, where it can be seen that Sup 2 forms a precipitation line with anti-α or -β rabbit serum and the line fuses completely with the single precipitation line

of the indicator α or β antigen with its specific antiserum. This clearly indicates the presence of α and β antigens in the supernatant. The exact content of these antigens in the supernatant has not yet been determined by the quantitative precipitation test. However, since about ten fold dilution of the test supernatant resulted in complete loss of the precipitating activity with anti-α or -β serum, α and β antigens probably constitute a very minor part of the protein fraction in the supernatant. From rough calculations, they did not seem to constitute more than 1 per cent of this protein. Mechanical disintegration of strain H37Ra was also shown to release both α and β antigens into the medium and disintegrated preparations appeared similar to those of strain H37Rv with regard to the intracellular distribution of α and β.

DISCUSSION

It is clear from the results of the present quantitative precipitation experiments that the protein components responsible for α and β antigens comprise nearly 70 per cent of the total protein released by strain H37Rv into the medium. This value is in good agreement with that estimated from protein yields in a previous study on their fractionation (YONEDA and FUKUI, 1961 a) and also provides conclusive evidence that α and β are major protein products liberated extracellularly by the organism. YONEDA and FUKUI (1961 a, b) found that about 20 per cent of the total extracellular protein of strain H37Rv was composed of a non-antigenic fraction (mtp-2). Accordingly, it appears that α and β may constitute almost 90 per cent of the total antigenic protein fraction of the organism released into the medium under the cultural conditions employed in this study. Preliminary kinetic studies on the extracellular liberation of these antigens either in shaken or still cultures, however, suggested that the amount may vary to some extent with the phase of growth and the medium employed.

In unheated culture filtrates of strain H37Rv, serveral other antigenic protein components have been found which can be separated by a combination of ammonium sulfate fractionation, zone-electrophoresis and DEAE-cellulose column chromatography (unpublished data), but they comprise only 10 to 15 per cent of the total extracellular protein fraction and thus are considered on a quantitative basis to be minor components. Although the biologic properties of α and β still remain to be clarified, quantiative results strongly suggest the possible importance of the antigens in the serology and immunology of tuberculous infection. Indeed, as will be described in our forthcoming paper, evidence has been accumulating to suggest a very significant role of α and β as type specific antigens in mycobacteria. Thus, at least from a quantitative point of view, it seems that in antigenic studies on the extracellular protein products of tubercle bacilli these two major antigens must be taken into serious consideration.

The relationship between α and β and Seibert's A, B and C proteins (SEIBERT, 1949; SEIBERT *et al.*, 1955) was discussed previously (YONEDA and FUKUI, 1961 b). From the finding that α and β comprise nearly 70 per cent of the total protein fraction in unheated culture filtrates of strain H37Rv, it seems likely that α and β are major components of fractions A, B and C provided that these latter fractions are isolated intact. However, neither the A, B nor C protein fraction was found to contain detectable amounts of β and only small amount of α (less than 10 per cent). Since both α and β are very labile protein antigens (YONEDA and FUKUI, 1961 b; YONEDA, 1963), the result may be explained by assuming that the α and β in the A, B and C protein preparations tested lose their ability to combine with the respective antibodies during the preparation of these fractions. At least in the case of protein A, it is unlikely that the preparation was originally composed mostly of a non-antigenic fraction (mtp-2), because there are great differences in the electrophoretic and sedimentation patterns of A and mtp-2. Although the contents of

α and β in A, B and C may well vary in different preparations, largely due to the degree of denaturation occurring in the acid and alcohol precipition procedures employed, the fact that the latter fractions all contain α as a common antigen strongly indicates that A, B and C are not single distinct proteins.

Detection of α and β antigens in both intact and disintegrated cells of strain H37Rv employing absorption and immunodiffusion techniques provided evidence that both α and β are present on/in the cell of the tubercle bacilli which produce these antigens extracellularly. Evidence for this may be summarized as follows: (1) specific antibodies for both α and β can be absorbed with either thoroughly washed intact cells of the organism or with insoluble debris of mechanically disintegrated preparations; (2) α and β antigens are readily detectable in the supernatant fraction obtained from disintegrated preparations by ultracentrifugation; (3) considerable amounts of α and β antigens are released from washed intact cells by homogenizing the suspension in an ordinary Waring blender for a few minutes.

The data described in a previous section leave little doubt that the absorption of the antibodies against α or β with either intact cells or the cellular debris was not effected merely by non-specific adsorption but by a specific immunochemical reaction. It was therefore expected that the cells must have α and β antigens as specific binding sites for their respective antibodies, and this was clearly shown by demonstrating the presence of α and β antigens in the soluble fraction of mechanically disintegrated preparations of washed intact cells.

The intracellular localization of α and β is of special interest in view of the antigenic structure of tubercle bacilli. From the finding that α and β antibodies can be absorbed by intact cells and even with those prepared from cultures in the exponential phase of growth, it is suggested that at least part of these antigens exists outside the permeability barrier of the cell for antibodies. In relation with this, it is noteworthy that considerable amounts of α and β antigens were liberated in a soluble form from washed intact cells by stirring the cell suspension for so short a time that the cells were still intact morphologically. Thus, α and β antigens seem to be peeled off from the cellular surface by this agitation treatment. Whether or not the mechanical release of these protein antigens is accompanied by actual cell death is unknown. However, since no significant amount of nucleic acid was released from the cells after the agitation treatment, it may be that no serious damage of the surface structure was caused by this treatment. If damage occurred, it would subsequently cause the cell breakage and release of nucleic acid.

Although quantitative immunochemical data are necessary before a conclusion can be reached, these results strongly suggest that α and β antigens are mainly localized in a releasable form on the surface structures such as the wall and cytoplasmic membrane of the cell. The additional finding that specific anti-α or -β rabbit serum can agglutinate H37Rv strain cells supports this view. However, the exact association of these antigens with the cellular structure is unknown.

Finally it should be pointed out that all these findings are on tubercle bacilli grown in an artificial medium. It is still unknown whether α and β antigens show a similar profile in *in vivo* cultures of the organism to that obtained *in vitro*.

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