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Preparation and properties of antigen 60 from *Mycobacterium bovis* BCG

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SUMMARY

Antigen 60 (A60) is the main thermostable immunogen of both 'old tuberculin' (OT) and 'purified protein derivative' (PPD), known reagents for cutaneous tests in tuberculosis. It is recognized by bidimensional immunoelectrophoresis with anti-BCG antiserum, where it appears as the less mobile component. A60 was prepared from the cytoplasm of *Mycobacterium bovis* BCG, and purified by exclusion gel chromatography and lectin affinity chromatography. Labelled A60 was obtained by radioiodination and used for a radioimmunoassay. Composition of A60 was explored by use of organic solvents, chemicals and enzymes. It contained two fractions of free and bound lipids, as well as protein and polysaccharide moieties. After removal of both free and bound lipid fractions, the core still retained the ability to form immunoprecipitinogen lines with anti-BCG antiserum. The lipopolysaccharide and lipo-protein moieties of A60, as well as the free lipid fraction, were also complexed by antibodies. It is concluded that A60 is a lipopolysaccharide-protein complex of 10^6 to 10^7 daltons, which is a major immunogenic component of mycobacterial cytoplasm. The detailed structure of this antigen, its immunological properties, and its use for an ELISA type immunoassay for tuberculosis are described in two other publications.

Keywords mycobacteria bacterial antigens immunoassays lipopolysaccharide-proteins

INTRODUCTION

The complex immune response elicited by a mycobacterial infection is the result of an interaction of different bacterial components with the immune system of the host. Cellular immunity is known to play a key role in the development of resistance to mycobacterial infection (Collins & Mackaness, 1970b; Bloom, 1971). Delayed hypersensitivity, which occurs upon subcutaneous injection of filtrates of mycobacterial cultures (tuberculin-like preparations) and is involved in cutaneous tests for tuberculosis, is just an aspect of cellular immunity (Kanai, Youmans & Youmans, 1960; Collins & Mackaness, 1970; Neiburger, Youmans & Youmans, 1973; Daniel & Hinz, 1974; Kuwabara, 1975).

Classification of mycobacterial antigens is a complex matter, due to the large number of components and different procedures for fractionation of cell homogenates (Daniel & Janicki, 1978). Moreover, polypeptides and polysaccharides, which are able to act as immunogens, are present in both cell wall and cytoplasm, and in some cases they are found also in the extracellular fluid (Cummins, 1962; Lederer *et al.*, 1975; Barksdale & Kim, 1977; Misaki, Azuma & Yamamura, 1977; Daniel & Janicki, 1978). In certain instances, these components crossreact in immunochemical assays without being identical in their overall composition. A reference system developed by

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Daniel & Janicki (1978) has led to identifications of a dozen *M. tuberculosis* antigens, which were separated by immunoelectrophoresis. Another reference system, based on crossed immunoelectrophoresis, has been introduced by Closs *et al.* (1980): it has allowed the separation of some 30 antigens of *M. tuberculosis* and *M. bovis*.

Application of the latter reference system to analysis of *M. bovis* cytoplasm has pointed towards antigen 60 (A60) as being the less mobile polymer in the crossed immunoelectrophoresis plates. A60 has been recently reported to be the main thermostable antigen of several preparations, such as the 'old tuberculin' of R. Koch and the 'purified protein derivative' or PPD of F.B. Seibert, which are widely used as diagnostic reagents in human and veterinary medicine (cf. Harboe (1981) for review). A60 belongs to a family of major thermostable antigens, which are present not only in micro-organisms of the genus *Mycobacterium*, but also in those of the related genera *Corynebacterium* and *Nocardia* (CMN group of bacteria) (Laub, Delville & Cocito, 1978; Harboe *et al.*, 1974; Gueur *et al.*, 1983).

Moreover, it has been found that the cytoplasmic antigen M_1 of a group of corynebacteria (this antigen corresponds to A60 of BCG) crossreacted with the peripheral polysaccharide, a major cell wall component of these micro-organisms (Abou-Zeid *et al.*, 1982; 1985) (cf. also Cocito & Delville (1983; 1985) for reviews). This renders even more complicated the analysis of the subcellular distribution of this sort of antigens and related polymers.

The present work relates the purification of antigen 60 of *M. bovis* BCG (A60), and the analysis of its biological properties and immunological reactivity. This paper provides evidence of A60 being a heterogeneous polymer of lipopolysaccharide-protein nature. The structure of the lipid and sugar moieties of this antigen are described in another paper (Fabre *et al.*, 1986), whereas the analysis of the protein part is in progress. The immunological properties of A60, and its use for diagnostic immunoassays are to be described in a different work (Cocito *et al.*, unpublished).

MATERIALS AND METHODS

Micro-organisms. Most of the work has been done with the Calmette-Guérin strain of *Mycobacterium bovis* (var. BCG) from the Pasteur Institute of Paris (courtesy of Mrs Giorgiou). The NCTC 334 strain of *M. smegmatis* has been used in some experiments. These micro-organisms were grown in Dubos medium with 5% decomplemented horse serum. Cultures were carried out at 37°C in reciprocal shakers and, for preparative purposes, in 20 l fermentors (Biolafitte, France), under agitation and aeration. Radioimmunoassays were performed with the Cowan I strain of *Staphylococcus aureus* (NCTC 85308), which was propagated in CCY medium at 37°C under forced aeration, Bacteria collected by centrifugation were inactivated by incubation with 0·5% formaldehyde, washed, suspended in PBS (10% w/v) and stored at −20°C.

Preparation and fractionation of bacterial homogenates. Bacterial suspensions in either 150 mM NaCl, 10 mM Na phosphate buffer pH 7·4 (PBS), or in 40 mM NH_4Cl, 15 mM Mg acetate, 50 mM Tris-HCl Buffer, pH 7·4 (NMT), were disrupted by sudden pressure release in a French pressure cell (Aminco Instr., Silver Spring, MD) equipped with a hydraulic press (Wabash, Ind.) (8,000 psi at 4°C). Homogenates were centrifuged first at 2,000 ***g*** rev/min for 15 min at 4°C to remove unbroken cells, and then at 25,000 ***g*** for 20 min at 4°C to sediment the cell walls (angular rotor A50 from Spinco, Palo Alto). Supernatant containing whole cytoplasm was fractionated by ultracentrifugation at 160,000 ***g*** for 2 h at 4°C in the angular rotor A50 of Spinco. Supernatant containing the cytosol was analyzed as such, and in some instances was lyophilized. The pellet of ribosomes was resuspended either in PBS or in NMT, depending on the experiments, for 14 h at 4°C. Aggregates were removed by centrifugation at 8,000 ***g*** for 20 min at 4°C: the supernatant accounted for the ribosome fraction.

Column chromatography of antigen 60 and fractions. Preparation of A60 was routinely carried out by exclusion gel chromatography. For this purpose, mycobacterial cytoplasm was fractionated on columns of Sepharose 6B (Pharmacia, Uppsala, Sweden) (10–20 mg protein/1 ml sample/10 ml bed column volume), which were eluted with the indicated buffers. Some experiments were done with Sepharose 4B (Pharmacia).

Affinity chromatography was used to further purify reference samples of A60 prepared by exclusion chromatography. This step based on the use of Sepharose 4B-immobilized concanavalin A (a lectin with D-glucose and D-mannose specificity) was carried out either on a column of Con A-Sepharose (Pharmacia) or by batch adsorption as follows. A suspension of Con A-Sepharose was mixed with A60 solution (2:1, w/v mixture) and, after 1 h incubation at 20°C, centrifuged (10,000 rev/min 10 min, 20°C) to sediment particles. Sediment was resuspended in 500 mM α-methyl-D-glucoside, 50 mM EDTA, 100 mM Na acetate buffer, pH 7·4 containing 1% Na dodecyl sulphate (SDS). After 20 min incubation with agitation at 37°C, suspension was recentrifuged as above, and supernatant was desalted by passage through a G100 Sepharose (Pharmacia) column and lyophilized.

Radioiodination of A60. A60 samples (about 120 μg protein/120 μl PBS buffer) were incubated with ^{125}I preparation (300 μCi carrier-free iodide in NaOH from Amersham Radiochemical Centre, England) and chloramine T (10 μl of 6·5 mg/ml solution in PBS) for 3 min at 20°C. Reaction was stopped with Na metabisulphite (10 μl of a 8·45 mg/ml solution in PBS). Mixtures were fractionated on Sepharose 6B columns (2 ml bed volume), and exclusion peaks containing (^{125}I) A60 were isolated and diluted with 0·2% (w/v) solutions of bovine serum albumin in PBS to yield preparations ($\sim 10^3$ d/min/μl), which were used immediately.

Electrophoretic techniques. Crossed immuno-electrophoresis was performed according to the technique of Closs *et al.* (1980) on glass plates (5 × 7 cm) covered with 3 gel slabs made of 1% agarose gel in 0·02 M Tris-barbital buffer, pH 8·6. Antigen solutions (10 μl of solutions containing 1 μg A60/ml) applied to the origin, were run for 1 h at 8 V/cm at 15°C in the first dimension. Bands were cut and transferred to the final plates, on which intermediate and superior gels were placed. The intermediate gel contained either agarose only (control), or agarose plus reference antiserum (crossed immunoelectrophoresis with intermediate gel). Upper gel contained 200 μl of anti-BCG antiserum (Dako, Copenhagen, Denmark). Electrophoresis in the second dimension was run at 3 V/cm for 18 h at 15°C. Slants were washed, dried, stained with Coomassie blue, and photographed. Fractionation of labelled A60 fragments was carried out with the 'SDS-Page' electrophoresis procedure. For autoradiography of labelled A60 preparations, bidimensional electrophoresis plates (10^6 d/min (^{125}I) A60/μg A60/10 μl PBS/sample), after Coomassie blue staining, were placed over a Osray M3 film (Agfa-Gevaert, Antwerp, Belgium). The latter was exposed sequentially to a developer (G150 for 5 min at 20°C) and a fixer (G334, 10 min, 20°C).

Immunodiffusion. Double diffusion-precipitation tests were performed on plastic plates covered with 1% (w/v) agarose in 0·85% (w/v) NaCl 0·05 M Na phosphate buffer, pH 7·2. Routinely, antiserum was placed in the central well, and antigen samples in the peripheral wells. Plates were kept in humidity chambers for 24 h at 20°C and photographed by transillumination; in some instances, plates were stained with Coomassie blue before taking pictures.

Preparation of antisera. Three month-old BALB/c mice were immunized by subcutaneous injection of 200 μl aliquots of oil-in-water emulsions made of equal volumes of antigen solution (A60 and fractions) in PBS buffer, and of incomplete Freund adjuvant. Injections were repeated twice at one-month intervals, and antibody titre in blood was established before preparation of antisera. A large part of the work was carried out, however, with the reference anti-BCG rabbit antiserum (Dako, Copenhagen, Denmark).

Radioimmunoassays. Radioimmunoassays with labelled A60 were carried out in liquid phase as follows. To a fixed amount of (^{125}I) A60 (10^4 d/min/100 μl PBS containing 0·2% albumin and 0·02% NaN_3) increasing dilutions of antiserum (10 μl) were added. After 30 min incubation at 20°C, 2 ml aliquots of 1% suspension of the Cowan I strain of *Staphylococcus aureus* in PBS containing 1% (v/v) Tween 20 were added. After incubation (37°C, 15 min), samples were centrifuged, and radioactivity in the pellet was measured in a gamma spectrometer. Competition experiments were performed by adding increasing amounts of unlabelled A60 (or its fractions) to the reaction mixture described above.

Colorimetric and spectrophotometric determinations. Hexoses in polysaccharides were determined by the anthrone reagent, pentoses by the orcinol method, and hexosamines with the Morgan-Elson reagent. Quantitative determination of proteins was done with the Folin-Ciocalteu phenol reagent. The following staining reagents were used: aniline hydrogen phtalate for carbohydrates,

ammonium molybdate for phospholipids, and Coomassie blue for both proteins and lipids, and amino black for proteins. References concerning the colorimetric procedures used can be found in Janczura *et al.* (1981) and Abou-Zeid *et al.* (1982; 1985).

RESULTS

Purification of antigen A60. A60 has been prepared from the cytoplasm of *M. bovis* BCG, which was disrupted by pressure release and freed from the envelope by centrifugation. Cytoplasm was fractionated by crossed immunoelectrophoresis, whereby antigens migrated in the first dimension in a buffer-containing gel and, in the second dimension, in a gel containing anti-BCG antiserum. Coomassie blue staining disclosed the presence of some thirty components (Fig. 1A), which were identified according to a reference scheme introduced by Closs *et al.* (1980). In this scheme, A60 complex corresponds to the precipitinogen line which is closest to the origin: its R_f is, thus, the smallest among BCG-cytoplasm components reacting with the correspondent antiserum. Since A60 was defined on the basis of its immunoelectrophoretic migration, this analytical procedure has been used to follow the purification protocol.

Of numerous procedures attempted, exclusion gel chromatography of *M. bovis* cytoplasm (Fig. 1A) on 6B Sepharose proved to be the simplest and more efficient fractionation method for preparation of A60. Figure 1B shows the immunoelectrophoretic pattern of the exclusion peak, which contained virtually all cytoplasmic A60, whereas in the inclusion fraction (Fig. 1C) most cytoplasmic proteins were present.

Since a study of the composition of A60 has revealed the presence in A60 of a polysaccharide moiety containing hexoses (see below), further purification of A60 was carried out either on concanavalin A-Sepharose columns or by batch adsorption on the same support (not shown).

Quantitative determination of antigen A60. A60 being defined by immunoelectrophoretic criteria, its quantification was not a simple matter. An approach to this problem was attempted by spectrophotometric determination of the Coomassie blue-stained immunoprecipitinogen line,

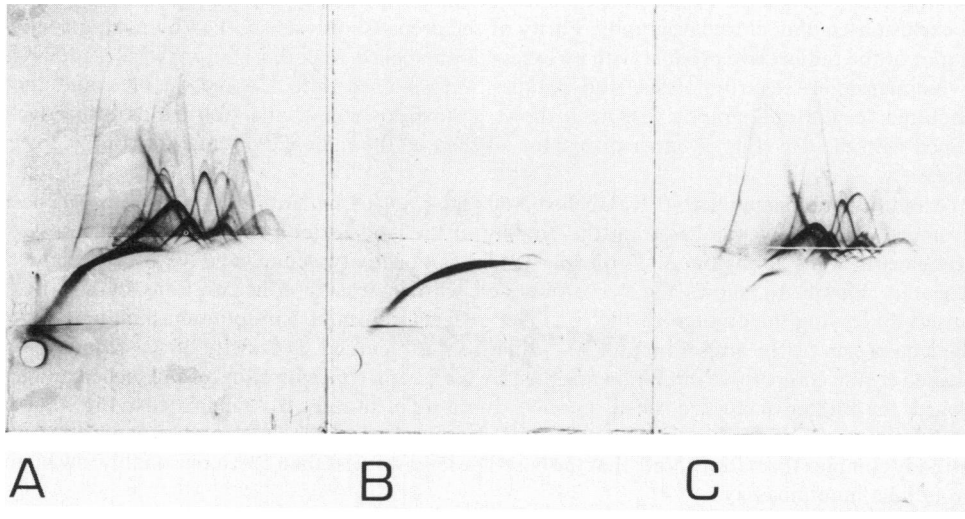

Fig. 1. Separation of A60 from the other cytoplasmic antigens. Cytoplasm of *M. bovis* BCG (A) was fractionated by exclusion gel chromatography on a Sepharose 6B column yielding an excluded fraction containing A60 (B) and an inclusion fraction with the other cytoplasmic antigens (C). The three samples were submitted to crossed immunoelectrophoresis with intermediate gel according to Closs *et al.* (1980). First dimension run = rightward; 2d dimension = upward. The three agarose layers contained (in upward direction): buffer (1st and 2nd), and anti-BCG rabbit antiserum (3d). Coomassie blue staining.

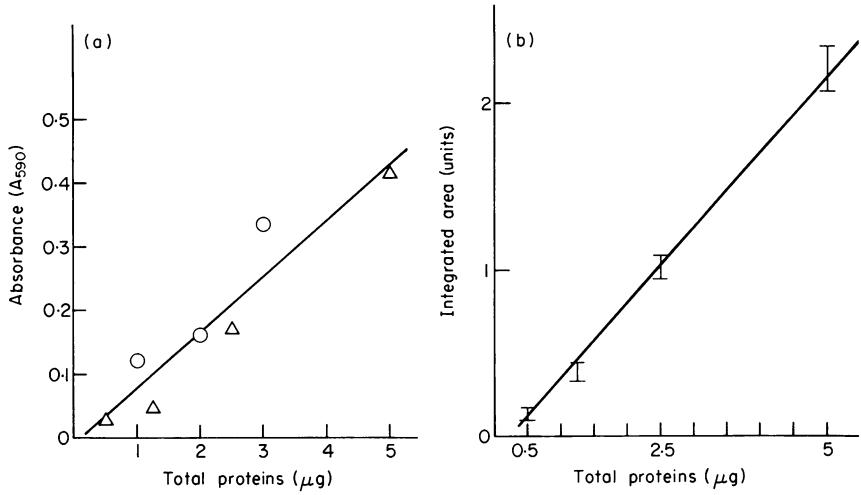

Fig. 2. Quantitative determination of A60 on electrophoresis plates. Increasing amounts of A60 (prepared as indicated in Fig. 1) were submitted to crossed immunoelectrophoresis and stained with Coomassie blue. A60 was quantified by two techniques: (a) isolation of the corresponding band, elution with methanol, and spectrophotometric measurement (A_{590nm}); and (b) direct evaluation by an integrating densitometer. Readings for different amounts of three preparations of A60 (indicated respectively by circles and triangles in (a) and by the standard deviation lines in (b)) are reported on the graphs.

upon elution of the corresponding gel band. Accordingly, an antigen unit was established as follows: 1 A60 unit = 0·1 $A_{590\,nm}$ units. This amount corresponded to about 4·5 μg of native A60. The corresponding titration curves obtained either by spectrofluorimetric determination of the eluted gel line (Fig. 2A), or by integrating densitometer measurement of the stained immunoelectrophoretic gel (Fig. 2B), are displayed in Fig. 2 for comparison.

Development of a radioimmunoassay for antigen A60. A60 was radiolabelled as follows. A preparation of the antigen was submitted to radioiodination, and the labelled product was purified by exclusion column chromatography. Purity of the preparation was checked by mixing a small aliquot of the radioactive product with an excess of unlabelled A60: this mixture was fractionated by bidimensional electrophoresis, and plaques were stained with Coomassie blue and then submitted to autoradiography. Figure 3 shows a superposition of the A60 bands respectively stained with the dye (Fig. 3, upper plates), or labelled by the radioactivity marker (Fig. 3, lower plates).

A radioimmunoassay with (^{125}I)-labelled A60 and a polyclonal anti-BCG antiserum has been developed. This assay was based on the binding of the A60-antibody complex to protein A of staphyloccal walls (Jonsson & Kronvall, 1974): radioactivity sedimented with bacteria was measured. Figure 4A shows the curve obtained with increasing concentrations of anti-BCG antiserum, leaving the amount of the (^{125}I) A60 reagent constant. An optimum binding level of labelled antigen by the antiserum at a 10^{-3} dilution was recorded. Specificity of this reaction was assessed by the competitive inhibition afforded by increasing concentrations of unlabelled antigen towards the binding of labelled A60 to a standard amount of antiserum. As indicated in Fig. 4B, at a concentration of 1 μg protein/ml, unlabelled A60 produced a near maximal inhibition of (^{125}I) A60-antibody complex formation. Note that the base-line level was less than 5%, a reasonably low blank for a direct immunoassay.

Analysis of A60 composition. Information on A60 components was gathered by submitting preparations of the antigen to the action of chemicals (organic solvents, acids and alkalis) and enzymes, according to the scheme in Fig. 5. The degraded products were submitted to crossed immunoelectrophoresis, on the one hand, and, on the other hand, to simple chromatographic separations followed by staining procedures and spectrophotometric analysis. Quantitative determinations of lipids, sugars and proteins were carried out on all fractions.

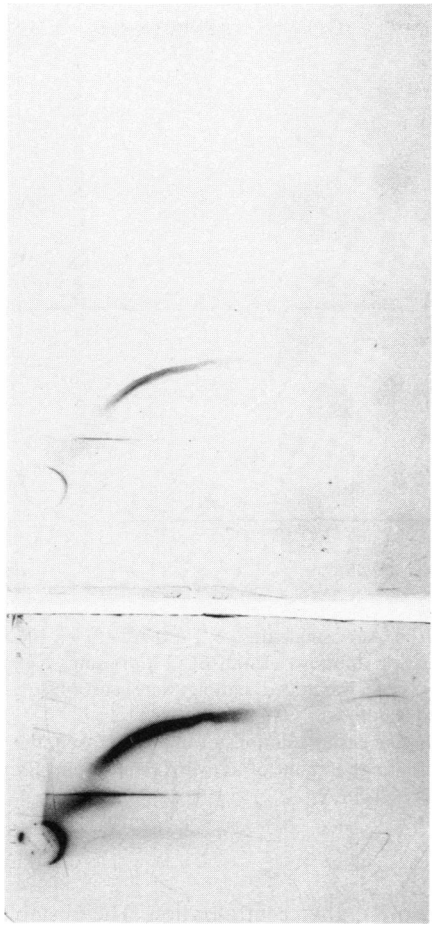

Fig. 3. Autoradiography of labelled A60. (^{125}I)-labelled A60 ($2{\cdot}5 \times 10^6$ d/min/1·5 μg A60), which was obtained as detailed in Materials and Methods, was mixed with 10 μg unlabelled A60 (carrier) and submitted to crossed immunoelectrophoresis (cf. Fig. 1), and to autoradiography (lower section) after staining with Coomassie blue (upper section).

Purified preparations of native A60 were yellowish opalescent solutions, which proved stable when stored for short times at +4°C, but yielded a sediment upon prolonged storage in cold. By dialysis and lyophilization of native A60, a dry yellowish powder was obtained, that was indefinitely stable but difficult to dissolve in buffer. Solutions of freeze-dried A60 invariably produced considerable sediments which were solubilized either by desoxycholate or by Na dodecyl sulphate. These preparations contained comparable amounts of lipid, protein and sugar components (Table 1). By extraction of lyophilized A60 with organic solvents, a free lipid extract was obtained, the residue of which yielded a yellowish opalescent solution in aqueous buffer. The white residue of defatted A60 (which was indefinitely stable in a dry form) yielded an aqueous suspension, from which a conspicuous precipitate spontaneously formed. Digestion of such a suspension with pronase produced a clear solution which, on Sepharose 6B columns, gave an exclusion fraction of high molecular weight lipo-polysaccharides (Table 1). Alkaline hydrolysis of defatted A60 (resulting in the release of bound lipids) followed by treatment with organic solvents (to extract released lipids) led to separation of a bound lipid fraction, and a lipid-free residue. The latter, which contained protein and polysaccharides in an approximate 3:2 ratio, yielded an unstable aqueous solution (Table 1). Submission of defatted A60 to acid hydrolysis gave rise to a considerable

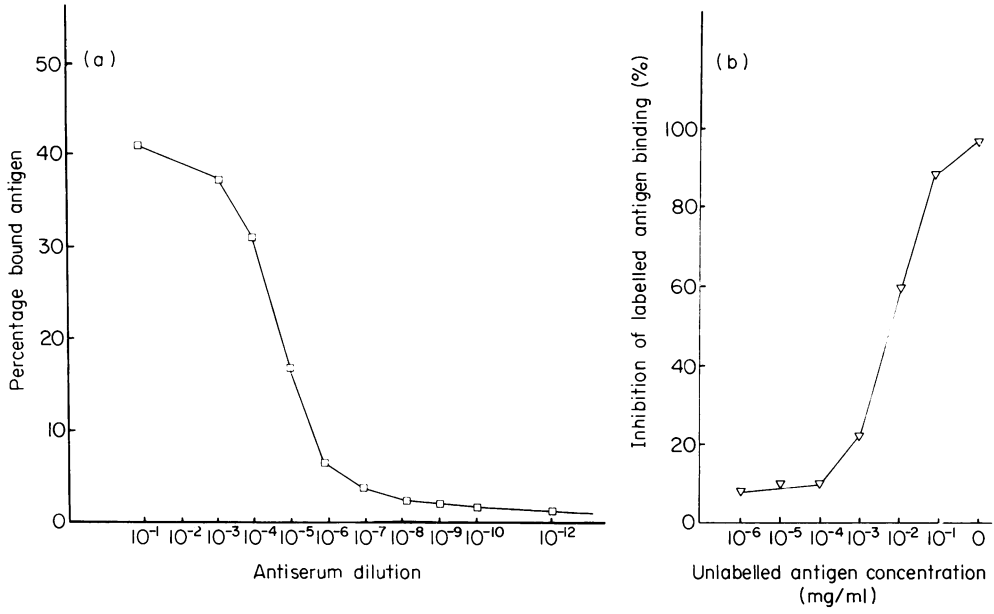

Fig. 4. Radioimmunoassay of A60. A. A single dose of (^{125}I) A60 ($0{\cdot}8 \times 10^4$ dpm/$2{\cdot}4 \times 10^{-2}$ μg protein) was added to vials containing increasing dilutions of anti-BCG antiserum. After 30 min incubation at 20°C, a preparation of protein A-staphylococci was added, samples were centrifuged, and radioactivity in the sediment was measured (cf. Materials and Methods).

B. To a series of vials containing increasing dilutions of unlabelled A60, the same quantity of (^{125}I)A60 was added, followed by the same (limiting) amount of anti-BCG antiserum. Radioactivity in antigen-antibody-protein A complexes was measured as in A.

precipitate, which partly dissolved after neutralization. The unstable solution thus formed was stabilized by desoxycholate and, upon fractionation on Sepharose 6B column, yielded an exclusion peak of lipoproteins (Table 1).

These data indicate that A60 is a lipoprotein-polysaccharide complex, made up of three moieties of free lipid, lipopolysaccharide and lipoprotein nature which can be isolated by chemical dissection of the polymer. The free lipid fraction confers stability to its partners. The other two moieties can be freed from the bound lipid components yielding lipid-free polysaccharides and proteins.

Immunological reactivity of A60 components. According to the data in the previous section, A60 is a lipo-polysaccharide-protein complex which can be cleaved into single components. It was of interest to explore the ability of these components to react and to form insoluble complexes with anti-BCG antibodies. For this purpose, fractions prepared as detailed in Fig. 5 were analyzed by both the double immunodiffusion technique using anti-BCG antiserum (Fig. 6), and crossed immunoelectrophoresis (not shown).

The starting material (native A60, or fraction 1 in Fig. 5) yielded in immunodiffusion a poorly diffusing band, which became more defined in the presence of detergent (Fig. 6–1 and 6–5).

Unstable aqueous solutions of delipidated A60 (fraction 2 in Fig. 5), in the presence of dodecyl sulphate, gave a heavy band in immunodiffusion plaques (Fig. 6–2). Surprisingly, an immunoprecipitinogen line was constantly obtained with solutions of the free lipid fraction (Fig. 6–3), particularly in the presence of detergent.

Defatted and proteolyzed A60 (fraction 4 in Fig. 5), upon fractionation on Sepharose 6B columns, yielded a high molecular weight (exclusion) fraction which produced a clear immunoprecipitinogen line in immunodiffusion (Fig. 6–4). Delipidated A60, upon alkaline hydrolysis and extraction, produced a lipid-free residue (fraction 6 in Fig. 5) yielding a clear line in immunodiffu-

Table 1. Composition of different fractions of A60 from *M. bovis*

A60 fractions			Composition‡ (mg/10 mgA60)		
Ref. No.*	Treatment	Column Fractionation†	Sugars	Peptides	Lipids
1	Lyophilization	Exclusion	3·3	3·0	3·7
2	Extraction (org. solv.): extract	—	0·2	0·1	3·4
3	Id.: residue	Exclusion	3·1	2·6	0·9
4	Proteolysis	Exclusion	2·6	0·04	ND
		Inclusion	0·5	1·0	ND
5	Alk. hydrolysis, extraction (org. solv.): extract		—	—	0·9
6	Id.:residue	Exclusion	2·9	2·1	—
7	Acid hydrolysis	Exclusion	0·4	2·2	ND
		Inclusion	2·7	0·4	ND

* A preparation of native A60 was submitted to the fractionation procedure outlined in Fig. 8: reference numbers in this Table refer to those of that Figure.

† Some samples, which were submitted to different kinds of hydrolytic procedures, were fractionated on Sepharose 6B columns to separate exclusion and inclusion fractions.

‡ Sugars, peptides and lipids were measured in each fraction as decribed in Materials and Methods, and expressed as mg/10 mg lyophilized A60.

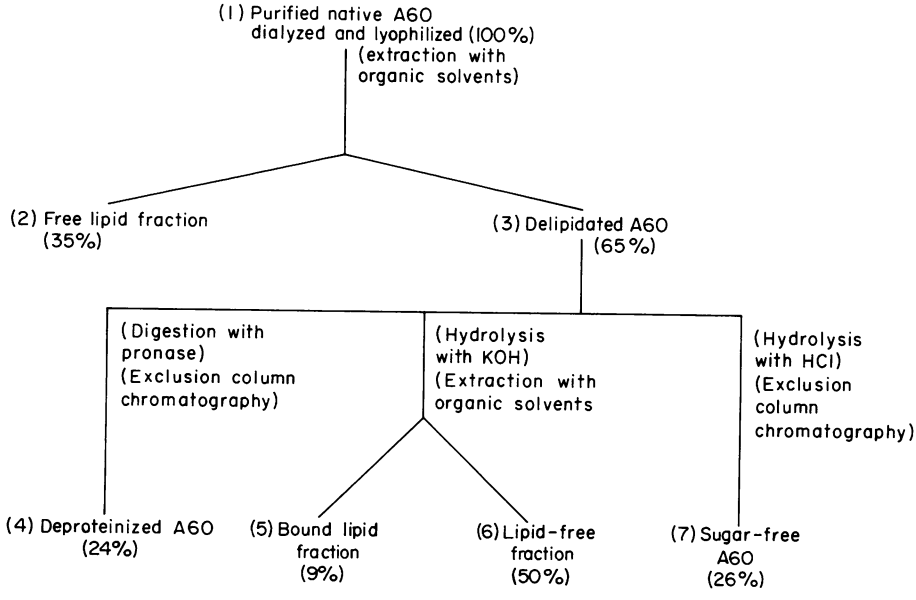

Fig. 5. Scheme of cleavage of antigen A60 by chemicals and enzymes.

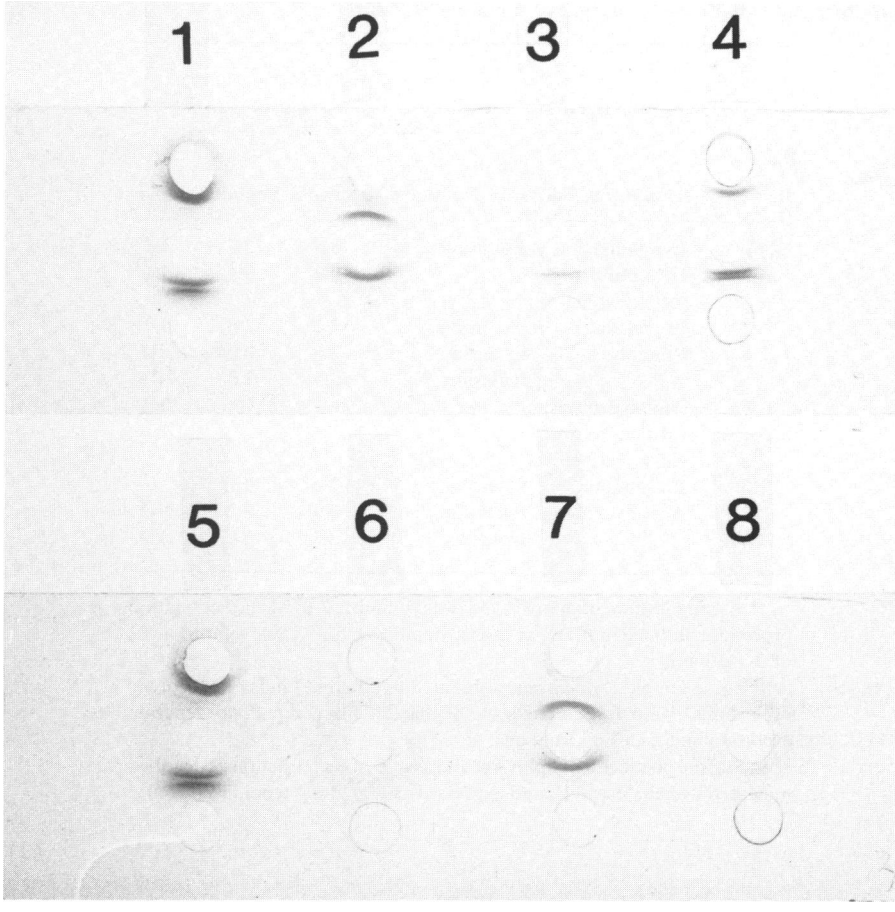

Fig. 6. Double immunodiffusion of A60 fractions and anti-BCG antiserum. A preparation of native A60 was submitted to treatments with enzymes, solvents, acids and alkalis, as outlined in Fig. 5. In each row, the central well contained anti-BCG antiserum, the upper well a sample as such, and the lower well the same sample plus 0·1% Na dodecyl sulphate. Samples: 1 and 5 = native A70; 2 = delipidated A60; 3 = free lipid fraction; 4 = deproteinized A60 (pronase); 6 = bound lipid fraction; 7 = lipid-free fraction; 8 = sugar-free A60 (1 M HCl hydrolysates).

sion plaques (Fig. 6–5), and a bound lipid fraction that was inactive in this respect (Fig. 6–6). Acid hydrolysis of defatted A60 (fraction 7 in Fig. 5). produced a poorly visible line in immunodiffusion plaques (Fig. 6–8).

It can be concluded, therefore, that the three moieties of A60 shared the ability to form insoluble immunoprecipitates with polyclonal anti-BCG antiserum.

DISCUSSION

The present work describes the preparation and purification of A60 from *M. bovis* BCG cytoplasm. The appearance of this antigen in the exclusion volume of Sepharose 6B columns (Fig. 1) agrees with data reported in another work (Fabre *et al.*, unpublished) yielding an estimate value of about 10^6 daltons for the main lipopolysaccharide component (fraction A_1) of the A60 molecule. Also, the binding of A60 to Con A-Sepharose columns (section 1 of Results) is accounted for by the demonstration that the cited fraction A_1 of 160 is a branched glucan carrying D-gluco- and D-

manno-pyranosyl residues (Fabre *et al.*, unpublished): concanavalin A is, in fact, a lectin specific for glucose and mannose.

Data in Figs. 4A and 4B are concerned with a radioimmunoassay using (^{125}I)-labelled A60 and polyclonal anti-BCG antiserum from immunized rabbits. Similar assays have been already developed in several laboratories, including ours, for A60-like antigens including antigen 7 of *Mycobacterium leprae* (Harboe *et al.*, 1979) and antigen M_1 from the LDC group of corynebacteria now renamed *Corynebacterium tuberculostearicum* (Abou-Zeid *et al.*, 1985). The immunogenic role played by this family of antigens within whole mycobacterial homogenates has been repeatedly stressed (Harboe *et al.*, 1979). Indeed, the presence of anti-A7 antibodies in leprosy, and that of anti-A60 antibodies in tuberculosis, have been reported (Harboe *et al.*, 1977; 1979).

The preparation obtained by R. Koch in 1891, and known as 'old tuberculin' (OT), was an autoclaved filtrate of *M. tuberculosis* autolysed culture. This preparation, and a fraction of it which was obtained by F. B. Seibert and coworkers in 1934 and named 'purified protein derivative' (PPD), have been extensively used for skin testing. PPD was found to contain protein components of 10^4 to 10^6 daltons and polysaccharides (Daniel & Hinz, 1974; Daniel & Janicki, 1978). A recent work (Harboe *et al.*, 1981) has shown the presence of A60 in both OT and PPD. In this publication, it was also reported that autoclaving of mycobacterial cytoplasm produced an irreversible inactivation of most antigens: among the few termostable components, A60 was prominent. Thermostability was also proved to be a peculiar feature of A60-like antigens of the CMN group of micro-organisms (Gueur *et al.*, 1983; Abou-Zeid *et al.*, 1985). Data shown in the present work are in good agreement with the cited publications and account for previous unexplained findings.

By taking into account data in Table 1 and Figs 5 and 6 it appears that all three A60 moieties retain the ability tc bind the corresponding antibodies, and to form immunoprecipitates. This is easily understandable in the case of the lipopolysaccharide and the lipoprotein components, for similar high polymers previously isolated from mycobacteria proved to be immunogenic (cf. Kanai *et al.*, 1960; Cummins, 1962; Daniel & Hinz, 1974; Kuwabara, 1975; Misaki *et al.*, 1977; for reviews see Barksdale & Kim, 1977; Daniel & Janicki, 1978). Less obvious is the immunological reactivity of the free lipid fraction which, however, can be accounted for by its peculiar composition as revealed in another work (Fabre *et al.*, 1986). It is also apparent that the polysaccharide and polypeptide components retain the ability to bind antibodies after removal of the bound lipid fraction, and that the latter is a poor hapten, as expected from current data on structure-antigenicity relationship.

Immunoelectrophoretic plaques in Fig 1B and 3 show, in addition to the A60 line, immunoprecipitates around the application well and in the proximity of it. Apparently, they are due to A60 aggregates (which are only solubilized by addition of detergent, a product interfering with antigen-antibody complex formation) and also to polysaccharides (not migrating in the electrophoresis field). Likewise, the observed 42% maximal binding of A60 by the corresponding antiserum is presumably due to the large size, complex structure, and reduced solubility of A60 (in similar assays on homogeneous protein preparations, 50% binding values are obtained).

The overall conclusion is that A60 is a major thermostable antigen of mycobacterial cytoplasm. This high polymer, which appears to be a lipo-polysaccharide-protein complex is highly immunogenic. Its chemical structure, and immunological properties are described in two other works (Fabre *et al.*, 1986; Cocito *et al.*, unpublished). The purification process of A60, which is outlined in the present paper is, in fact, the basis for an ELISA type immunoassay for tuberculosis.

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