Characterization of Mycobacterial Immunoprecipitates by Selective Staining of Enzymes

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Immune precipitation patterns of *Mycobacterium intracellulare*, *M. phlei* and *M. smegmatis* were analysed by selective enzyme staining procedures in order to characterize individual mycobacterial antigens. Enzyme activity was shown in eight precipitinogens of *M. intracellulare*, seven of *M. phlei*, and six of *M. smegmatis*. The identification of mycobacterial precipitinogens as enzymes is important since only a few mycobacterial antigens have been functionally characterized.

INTRODUCTION

Selective enzyme staining procedures following two-dimensional (crossed) immunoelectrophoresis (2D-IE) were previously employed for the analysis of mycobacterial antigen preparations (Öhman & Ridell, 1986; Ridell *et al.*, 1986). It was shown that five individual immune precipitates (precipitinogens) of *Mycobacterium phlei* each corresponded to an enzyme (glucose-6-phosphate isomerase, glutamic-oxaloacetic transaminase, isocitrate dehydrogenase, leucine aminopeptidase and malate dehydrogenase). Two of these enzymes (malate dehydrogenase, leucine aminopeptidase) also corresponded to precipitinogens of *Mycobacterium intracellulare* and two (isocitrate dehydrogenase, leucine aminopeptidase) to precipitinogens of *Mycobacterium smegmatis*.

In the present study, an extension of earlier work (Öhman & Ridell, 1986; Ridell *et al.*, 1986), precipitation patterns of *M. intracellulare*, *M. phlei* and *M. smegmatis* have been analysed by selective staining procedures for enzymes.

METHODS

Bacterial strains. *M. intracellulare* Battey-Boone TMC 1403, *M. phlei* NCTC 8151/ATCC 19249 and *M. smegmatis* NCTC 8159/ATCC 19420 were used.

Antigen preparations. *M. intracellulare* was cultivated on Watson-Reid medium, and *M. phlei* and *M. smegmatis* on Sauton medium. Cells were harvested, washed, sonicated and centrifuged as described by Öhman & Ridell (1986).

Antisera. The *M. intracellulare* antiserum was produced in a burro (donkey) and the *M. phlei* and *M. smegmatis* sera in rabbits (Öhman & Ridell, 1986).

Immunological technique. A modification of the 2D-IE technique was employed (Samuelson, 1986). This technique uses a thin agarose gel layer (0.3 mm) and the serum is applied by a special applicator. The size of the plates was 50 × 70 mm, and 170 µl immune serum was employed. The same 2D-IE modification was also used in the previous analyses (Öhman & Ridell, 1986; Ridell *et al.*, 1986).

Enzyme staining procedures. The 2D-IE plates were incubated at 37 °C in 10 ml of one of the mixtures given below. Enzyme activities are expressed as units (U): one unit converts 1.0 µmol substrate min⁻¹ (not valid for peroxidase, in which activity is not assessed exactly).

Abbreviations: 2D-IE, two-dimensional (crossed) immunoelectrophoresis; MTT, thiazolyl blue; NBT, nitroblue tetrazolium; PMS, phenazine methosulphate.

The incubation mixtures were as follows. *Aconitate hydratase* (EC 4.2.1.3): 8.2 mM-cis-aconitic acid, 153 mM-Tris (adjusted to pH 8.0 with HCl), 57.3 mM-MgCl₂, 0.12 mM-NADP, 0.12 mM-phenazine methosulphate (PMS), 0.22 mM-thiazolyl blue (MTT) and isocitric dehydrogenase (type IV; Sigma, from porcine heart), 0.24 U ml⁻¹. *Adenylate kinase* (EC 2.7.4.3): 0.96 mM-ADP (sodium salt), 86 mM-Tris (adjusted to pH 8.0 with HCl), 86 mM-glucose, 1.89 mM-MgCl₂, 0.39 mM-NADP, 0.34 mM-PMS, 0.20 mM-MTT, glucose-6-phosphate dehydrogenase (type V; Sigma, from bakers' yeast), 0.69 U ml⁻¹ and hexokinase (type III; Sigma, from bakers' yeast), 0.014 U ml⁻¹. *Aldehyde oxidase* (EC 1.2.3.1): 93.4 mM-benzaldehyde, 59.4 mM-Tris (adjusted to pH 8.0 with HCl), 0.48 mM-nitroblue tetrazolium (NBT) and 0.32 mM-PMS. *Aspartate aminotransferase* (glutamic-oxaloacetic transaminase) (EC 2.6.1.1): 7.51 mM-L-aspartic acid, 200 mM-Tris (adjusted to pH 8.0 with HCl), 0.04 mM-pyridoxal-5-phosphate, 13.7 mM-2-oxoglutaric acid and 3.6 mM-fast blue BB salt. *Glucose-6-phosphate isomerase* (EC 5.3.1.9): 0.61 mM-fructose-6-phosphate (disodium salt), 190 mM-Tris (adjusted to pH 8.0 with HCl), 0.57 mM-MgCl₂, 0.15 mM-NADP, 0.063 mM-PMS, 0.44 mM-MTT and glucose-6-phosphate dehydrogenase (type V; Sigma, from bakers' yeast) 0.057 U ml⁻¹. *Isocitrate dehydrogenase* (NADP⁺) (EC 1.1.1.42): 8.5 mM-isocitric acid (trisodium salt), 170 mM-Tris (adjusted to pH 8.0 with HCl), 1.6 mM-MnCl₂, 0.42 mM-NADP, 0.35 mM-PMS and 0.21 mM-MTT. *Leucine aminopeptidase* (EC 3.4.11.1): 2.0 mM-L-leucine-β-naphthylamide.HCl, 98 mM-KH₂PO₄ (adjusted to pH 5.5 with K₂HPO₄), 1.96 mM-MgCl₂ and 0.42 mM-fast black K salt. *Leucyl-glycyl-glycine peptidase* (EC 3.4.11.1): 1.61 mM-L-leucyl-L-glycyl-L-glycine, 198 mM-Tris (adjusted to pH 8.0 with HCl), 0.62 mM-o-dianisidine dihydrochloride, 2.48 mM-MnCl₂, snake venom (Sigma, from *Bothrops atrox*; contains L-amino-acid oxidase), 0.2 mg ml⁻¹ and peroxidase (type I; Sigma, from horseradish), 19 U (0.2 mg) ml⁻¹. *Malate dehydrogenase* (EC 1.1.1.37): 232 mM-DL-malic acid, 155 mM-Tris (adjusted to pH 8.0 with HCl), 0.66 mM-NAD, 0.38 mM-PMS and 0.59 mM-NBT. *Mannose-6-phosphate isomerase* (EC 5.3.1.8): 0.61 mM-mannose-6-phosphate (disodium salt), 75.6 mM-Tris (adjusted to pH 8.0 with HCl), 3.8 mM-MgCl₂, 0.05 mM-NADP, 0.12 mM-PMS, 0.73 mM-MTT, glucose-6-phosphate dehydrogenase (type V; Sigma, from bakers' yeast), 0.23 U ml⁻¹ and phosphoglucose isomerase (type III; Sigma, from bakers' yeast), 0.19 U ml⁻¹. *6-Phosphogluconate dehydrogenase* (EC 1.1.1.44): 2.13 mM-6-phosphogluconic acid (barium salt), 114 mM-Tris (adjusted to pH 8.0 with HCl), 39.8 mM-MgCl₂, 0.074 mM-NADP, 0.19 mM-PMS and 0.44 mM-MTT.

RESULTS AND DISCUSSION

The antigen-antibody systems representing *M. intracellulare*, *M. phlei* and *M. smegmatis* were analysed by 2D-IE followed by selective staining for various enzymes. Eleven of the enzymes sought were revealed as corresponding to precipitinogens in one or more of the three strains (Table 1). Eight precipitates corresponded to enzymes in *M. intracellulare*, seven in *M. phlei* and six in *M. smegmatis* (Table 1). Most of the enzymes revealed are involved in common metabolic pathways, such as the citric acid cycle, and have been demonstrated in mycobacteria by several investigators (for reviews see Ratledge, 1982; Wheeler, 1984; Masood *et al.*, 1985). It is probable that most enzymes are antigens and that many more would be revealed in mycobacterial precipitation patterns, if sought. Staining procedures for several enzymes, e.g. alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, glutamate dehydrogenase and phenyl-alanyl-leucine peptidase, were performed but no precipitate in any of the three patterns was stained. Some of these failures are probably due to technical difficulties such as loss of enzyme activity during the procedure, rather than to absence of the enzymes in the tested mycobacteria.

Figure 1 shows the 2D-IE pattern of *M. intracellulare* stained for 6-phosphogluconate

Table 1. *Enzymes corresponding to precipitinogens in mycobacterial 2D-IE precipitation systems*

Enzyme	<i>M. intracellulare</i>	<i>M. phlei</i>	<i>M. smegmatis</i>
Aconitate hydratase		+	+
Adenylate kinase	+	+	+
Aldehyde oxidase	+		
Aspartate aminotransferase (glutamic-oxaloacetic transaminase)		+	
Glucose-6-phosphate isomerase	+	+	+
Isocitrate dehydrogenase		+	+
Leucine aminopeptidase	+	+	+
Leucyl-glycyl-glycine peptidase	+		
Malate dehydrogenase	+	+	
Mannose-6-phosphate isomerase	+		
6-Phosphogluconate dehydrogenase	+		+

Fig. 1. Precipitation pattern of *M. intracellulare* obtained by 2D-IE combined with selective staining for 6-phosphogluconate dehydrogenase. The stained line is indicated by an arrow.

Fig. 2

Fig. 3

Fig. 2. Schematic figure of the precipitation pattern of *M. intracellulare*. The eight precipitinogens corresponding to the enzymes adenylate kinase (AK), aldehyde oxidase (AO), glucose-6-phosphate isomerase (GPI), leucine aminopeptidase (LAP), leucyl-glycyl-glycine peptidase (PE), malate dehydrogenase (MDH), mannose-6-phosphate isomerase (MPI) and 6-phosphogluconate dehydrogenase (6PG) are indicated. When the peptide was omitted from the staining mixture for PE, no activity was detected.

Fig. 3. Schematic figure of the precipitation pattern of *M. phlei*. The seven precipitates corresponding to the enzymes aconitate hydratase (ACO), adenylate kinase (AK), aspartate aminotransferase (AAT) glucose-6-phosphate isomerase (GPI), isocitrate dehydrogenase (IDH), leucine aminopeptidase (LAP), and malate dehydrogenase (MDH) are indicated.

dehydrogenase. Fig. 2 is a schematic picture of the same system summarizing the results of the enzyme staining procedures. The eight precipitation lines that corresponded to enzymes are indicated. Fig. 3 provides a schematic picture of the *M. phlei* system, with seven precipitinogens corresponding to enzymes. Certain precipitating arcs corresponding to enzymes were not visible until after the pertinent enzyme staining. The schematic figure thus contains more precipitation lines than seen in the ordinary pattern (compare Figs 1 and 2).

In the present study each single IE plate was stained by one enzyme staining mixture only. It is however possible to perform several reactions successively on the same plate, although the result becomes more difficult to interpret.

Individual enzymes are often identified by staining after electrophoresis in starch or polyacrylamide gels. However, there are problems in these techniques (see e.g. Gabriel, 1971). For example, the enzyme molecules might diffuse after the separation and/or the staining reagent might not penetrate the gel. In the present method the antibodies bind the enzyme molecules, thus overcoming the diffusion problem. The thin gel layer (0.3 mm) and the fact that the gel consists of agarose (having comparatively large pores) facilitates penetration of reagents. These advantages may explain why a variety of enzymes could be detected quite easily, and also why coupled enzyme reactions could be used without difficulty.

Little is known about the function of mycobacterial antigens, and this has hampered the development of many fields of mycobacteriology. The present technique provides possibilities for recognizing and identifying mycobacterial precipitinogens which are enzymes, and for distinguishing individual antigens in multilinear patterns.

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