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ISOLATION OF RECOMBINANT MYCOBACTERIAL ANTIGENS BY AN AUTOMATIC AND GENERALLY APPLICABLE PURIFICATION METHOD FOR β-GALACTOSIDASE FUSION PROTEINS

BERND SCHOEL and STEFAN H. E. KAUFMANN*
*Max-Planck-Institut für Immunbiologie, 7800 Freiburg (F.R.G.); and *Abteilung für Medizinische Mikrobiologie und Immunologie, Universität Ulm, Oberer Eselsberg, 7900 Ulm (F.R.G.)*
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SUMMARY

An automated two-dimensional chromatographic method has been developed for the isolation and concentration of recombinant fusion proteins with β-galactosidase. The system consists of an immunoaffinity column with anti-β-galactosidase antibodies as ligand, followed by an anion-exchange column. It was used for the purification and concentration of recombinant fusion proteins from *Mycobacterium tuberculosis* and *M. leprae*. Small amounts of crude lysates of *Escherichia coli* were loaded stepwise onto the immunoaffinity column with intermittent washing, elution and re-equilibration. After several cycles the eluate was passed through the anion-exchanger. Using an immunoaffinity gel of 5-ml volume and the anion-exchanger Mono Q HR 5/5, from 10 ml of crude *E. coli* lysate (containing up to 50 mg of protein) up to 100 μg of recombinant protein in a 2-ml volume could be isolated overnight.

INTRODUCTION

The clonal approach on the genetic level provides defined proteins of the cloned species. An expression library of the pathogens *Mycobacterium tuberculosis* and *M. leprae* has been established in the λgt11 vector system, and several recombinant protein antigens have been identified with monoclonal antibodies[1,2]. Because the host response to these pathogens is mediated by T-cells, an understanding of the antigens recognized by T-lymphocytes is of particular importance[3]. Analysis of recombinant proteins with peripheral blood T lymphocytes, however, requires separation of the antigen of interest, which makes up only a minor part of the lysate from *E. coli* components. Therefore we have developed an automated method for the purification of these recombinant proteins. This procedure is based on the fact that, in the λgt11 system, recombinant proteins are expressed as fusion proteins with β-galactosidase. This common feature of different recombinant fusion proteins makes affinity chromatography directed to β-galactosidase with substrate analogues or antibodies as ligands the method of choice[4]. Because the recombinant fusion proteins failed to

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express β-galactosidase activity, we used purified polyclonal rabbit antibodies directed against β-galactosidase as affinity ligand. With this isolation procedure we were able to purify different recombinant mycobacterial antigens for functional analysis with peripheral blood T-lymphocytes.

METHODS

Culture and lysis of recombinant E. coli clones

E. coli Y1089 containing no λgt11-phage, recombinant *E. coli* clone Y3179 expressing the 18 kDa antigen of *M. leprae*[1], and clones Y3143, Y3144, Y3147, Y3252, Y3272, Y3275 expressing the 65-kilodalton (kDa), 14-kDa. 19-kDa, 19-kDa, 71-kDa, 12-kDa antigens of *M. tuberculosis*[2], respectively, were kindly provided by R. A. Young. *E. coli* clone Y1089G is *E. coli* Y1089 infected with λgt11-phage alone (kindly provided by H. G. Simon), expressing β-galactosidase upon induction. Infection of *E. coli* Y1089 with the λgt11-phage was performed according to Huynh *et al.*[5]. To obtain the large amounts of *E. coli* required for the isolation of recombinant proteins, 500 ml of Luria–Bertani medium[6] were inoculated with a single colony of the recombinant *E. coli* lysogen and incubated on a shaker at 32°C[5]. When the culture had grown to an optical density of 0.5 measured at 600 nm, the temperature was increased to 43°C and incubation was continued for 20 min. Isopropylthiogalactopyranoside (IPTG, Sigma) was added to the cultures, which were further incubated at 37°C for 1 h. In contrast to others[5] we used 1 m*M* IPTG for induction, since with *E. coli* Y1089G we had found that this concentration of IPTG was sufficient for optimal induction of β-galactosidase expression. Cells were rapidly centrifuged for 5 min at 6000 *g* and 25–30°C, pellets were suspended in *ca.* 20 ml of Tris-buffered saline (TBS, 20 m*M* Tris, 150 m*M* sodium chloride pH 7.5) and frozen at −24°C. After thawing, phenylmethylsulphonyl fluoride (2 m*M* final concentration, Sigma) was added, and cells were disrupted by sonification. The sonicate was centrifuged for 20 min at 45 000 *g* to remove cell debris, and supernatants were frozen at −24°C until used.

Preparation of anti-β-galactosidase antibodies

A rabbit was immunized with 1 mg of β-galactosidase (grade VIII from *E. coli*, Sigma) in 0.5 ml of phosphate-buffered saline emulsified in 0.5 ml of complete Freund's adjuvant (Difco). After 4 weeks, the rabbit was boosted in a similar way, and 7–14 days later blood was collected. The immunoglobulin G (IgG) fraction was isolated from the serum with a protein-A Sepharose CL-4B column (Pharmacia), and then passed twice through a column with soluble proteins of *E. coli* Y1089 as ligand (1.2 mg of protein coupled to 1 ml of cyanogen bromide-activated Sepharose 4B (Pharmacia) as matrix) to remove cross-reacting IgG against *E. coli* proteins. Between passages the column was cleared with 0.1 *M* sodium acetate (pH 4.0) and 0.1 *M* diethanolamine (pH 11.0), and re-equilibrated with 50 m*M* Tris (pH 7.5, buffer A). The purified IgG fraction was used for immunoaffinity chromatography and immunostaining of Western blots.

Preparation of the immunoaffinity column

Purified anti-β-galactosidase IgG fraction (1 mg of protein) was coupled to 1 g of dry cyanogen bromide-activated Sepharose 4B (Pharmacia).

Western blots

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a discontinuous buffer system[7] was performed in a separation gel with a linear polyacrylamide gradient from 22% to 10%. Crude *E. coli* extracts (15 μg of protein) were loaded on each lane. After electroblotting onto nitrocellulose[8], the sheet was blocked with 2.5% bovine serum albumin (BSA) in TBS. The blot was developed by incubation with 25 ml of anti-β-galactosidase antibodies (6 μg/ml diluted in TBS containing 0.5% BSA and 0.02% sodium azide) for 1 h, followed by three washings with 0.05% Tween 20 in TBS, and incubation with an alkaline phosphatase-labelled antibody against rabbit-IgG (diluted 1:5000 in TBS–0.5% BSA–0.02% sodium azide). After 1 h the nitrocellulose was washed four times with 0.05% Tween 20 in TBS, and finally developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate, according to the immunoblot kit (Proto Blot immunoblotting system, Promega Biotec).

SDS-PAGE

SDS-PAGE of protein fractions was performed with the PhastSystem (Pharmacia) with 10–15% gradient PhastGels. Gels were stained with the silver stain[9].

Protein assay

Protein concentrations were determined with the Coomassie dye-binding assay, using BSA as standard (BioRad)[10].

Assay of β-galactosidase

β-Galactosidase was assayed with O-nitrophenyl-β-D-galactoside (Sigma) as substrate at 20°C in 0.1 *M* sodium phosphate buffer (pH 7.3) containing 0.1 *M* 2-mercaptoethanol, 1 m*M* magnesium chloride and 2.3 m*M* substrate. The increase of absorbance was recorded at 410 nm.

Two-dimensional chromatography

The immunoaffinity chromatography (IAC) column containing 5 ml of gel and the anion-exchanger Mono Q HR5/5 (Pharmacia) were connected to the fast protein liquid chromatograph (Pharmacia) under two-dimensional chromatographic conditions using the IAC column first (Fig. 1). A superloop containing 10 ml of *E. coli* lysate was used as a sample reservoir.

The affinity chromatographic cycle consisted of the following four steps: (1) re-equilibration of the IAC column with 15 ml of 50 m*M* Tris (pH 7.5, buffer A); (2) loading 1.3 ml of the sample; (3) washing with 20 ml of buffer A and collection of the protein peak; (4) antigen elution with 10 ml of 50 m*M* diethanolamine (pH 11.0). A 9-ml volume of the eluate containing the majority of eluted antigens was collected and directed into a reservoir on a magnetic stirrer with 3/40 volume of 1.5 *M* sodium phosphate buffer (pH 6.5) (for 9 ml of eluate, 0.675 ml of phosphate buffer was required to achieve pH 7.5–8.0). The flow-rate was 1 ml/min, except during the loading step (2) and for the first 4 ml of step (3), when the flow-rate was reduced to 0.34

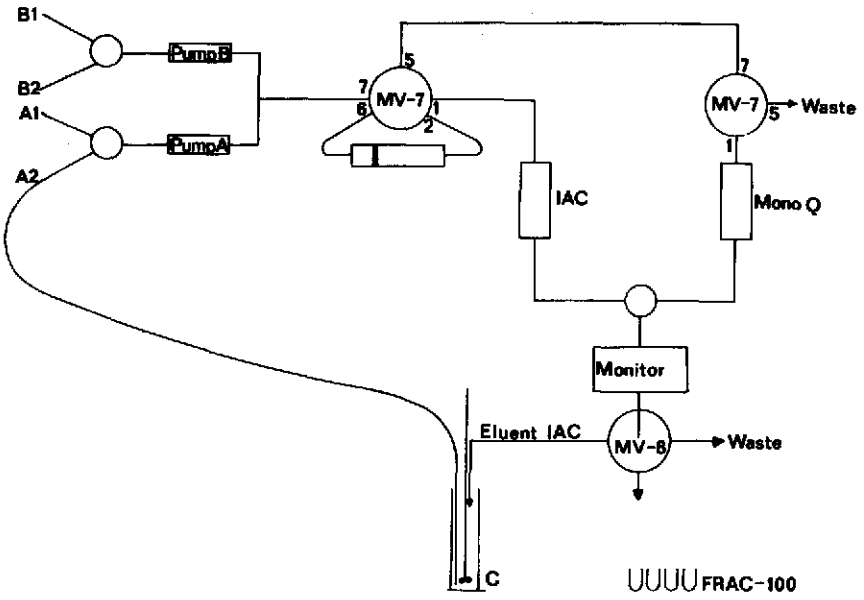

Fig. 1. Flow diagram of two-dimensional automated chromatography on the fast protein liquid chromatograph. MV-7 and MV-8 are motor valves, small circles indicate three-way valves PSV-100. Solution A1, 50 m*M* Tris (pH 7.5); solution B1, 50 m*M* diethanolamine (pH 11.0); solution B2, 50 m*M* Tris–1 *M* sodium chloride (pH 7.5); solution C, 1.5 *M* sodium phosphate (pH 6.5).

ml/min. The superloop and all fractions were stored on ice. After eight cycles the neutralized eluate was pumped onto the anion-exchanger, which had been equilibrated with buffer A. After loading and washing with buffer A the column was developed with a linear salt gradient, and within 30 ml the concentration of sodium chloride was increased to 1 *M* in buffer A. Usually the fusion proteins appeared within 2 ml at *ca.* 0.35 *M* sodium chloride. Recombinant proteins were detected in the collected fractions with a dot blot assay: 2 μl of each fraction were dotted onto a sheet of nitrocellulose, which was developed with anti-β-galactosidase antibodies as first antibody, according to the staining protocol for Western blots.

RESULTS AND DISCUSSION

Recombinant proteins expressed by the λgt11-vector are linked to β-galactosidase, and hence have higher molecular masses than β-galactosidase alone. Fig. 2 shows a Western blot of crude extracts of different *E. coli* clones expressing different proteins of *M. tuberculosis* after immunostaining with purified anti-β-galactosidase IgG. Besides the fusion proteins, in all extracts a uniform band that was recognized by these antibodies was not present in extracts of *E. coli* Y1089. This band is probably a degradation product of the fusion proteins.

In order to set up the isolation procedure for recombinant fusion proteins we used *E. coli* clone Y1089G as a model system. This clone expressed enzymatically active β-galactosidase after induction with IPTG, and hence the chromatographic conditions could be established using an enzymic assay as read-out system. In principle,

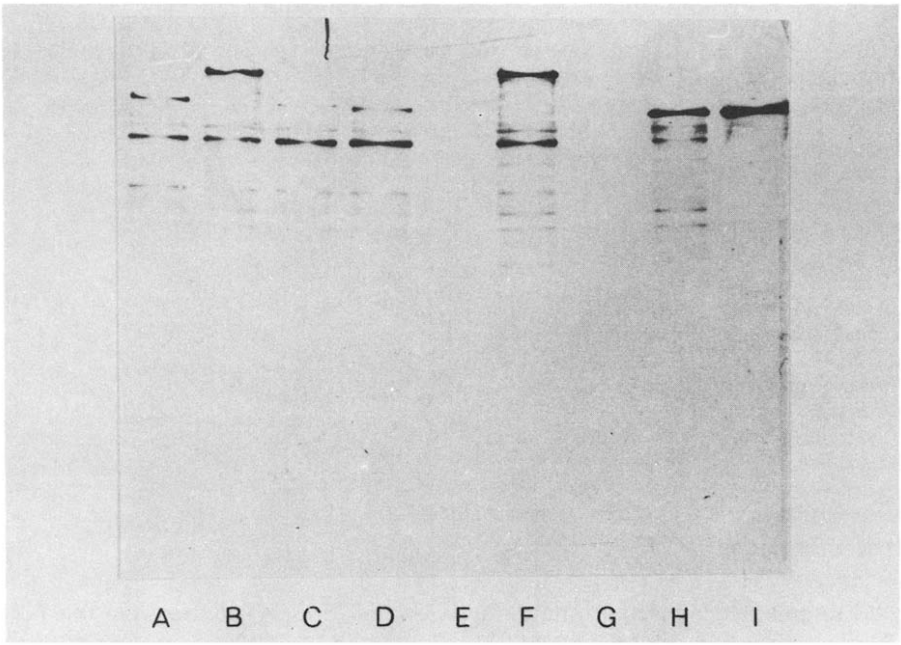

Fig. 2. Western blot of lysates of different *E. coli* clones expressing recombinant proteins of *M. tuberculosis*. Lanes A–F are lysates of clones expressing different mycobacterial proteins: A, Y3275 (12 kDa); B, Y3272 (71 kDa); C, Y3252 (19 kDa); D, Y3147 (19 kDa); E, Y3144 (14 kDa) (no expression); F, Y3143 (65 kDa); G, Y1089 (without λgt11); H, Y1089G (expressing β-galactosidase); I, 2 μg of β-galactosidase (Sigma).

different conditions can be used for the elution of bound antigens from IAC columns. Because we wanted to employ β-galactosidase as an indicator, we determined the stability of β-galactosidase under various elution conditions. The enzyme was destroyed in acidic buffers and chaotropic solvents (pH 7.0) but remained active in alkaline buffers (Table I). Hence in the subsequent experiments 50 m*M* diethanolamine (pH 11.0) was used as elution buffer.

The IAC column was loaded with crude extracts of *E. coli* Y1089G (4 mg/ml protein) and, after washing, was eluted with 50 m*M* diethanolamine (pH 11.0). Yields of up to 85% were achieved within the eluate, and an additional 10% was detected in the washing and re-equilibration fractions. During this step the antigen was diluted ten-fold. A 1-ml volume of immunoaffinity gel was capable of binding 200 μg of β-galactosidase from crude *E. coli* lysates. The purity of the eluted material after IAC is documented in Fig. 3.

Two remaining problems, namely the various binding capacities of different fusion proteins to the IAC column, and dilution during IAC, were solved by automating the chromatographic procedure on the fast protein liquid chromatograph. In a cyclic way, sufficiently small amounts of lysates were loaded onto the IAC column so that all antigen was bound. Afterwards, the IAC column was washed, antigen was eluted and the column was re-equilibrated. The material was eluted into phosphate buffer (pH 6.5) with the high molarity required for neutralization. In this system, the pH reached 7.5–8.0 at the end of all IAC steps. Subsequently the eluted antigen was

TABLE I

STABILITY OF β-GALACTOSIDASE UNDER VARIOUS CONDITIONS USED FOR ELUTION FROM AN IAC COLUMN

*Buffer**	*Remaining enzyme activity (%)***
0.1 *M* Glycine (pH 3.0)	0
0.1 *M* Glycine (pH 4.0)	20
0.1 *M* Diethanolamine (pH 10.0)	97
0.15 *M* Diethanolamine (pH 11.0)	95
0.15 *M* Diethanolamine–1 *M* sodium chloride (pH 11.0)	0
0.15 *M* Diethanolamine–2 *M* sodium chloride (pH 11.0)	27
0.15 *M* Diethanolamine–3 *M* sodium chloride (pH 11.0)	80
3.0 *M* Potassium thiocyanate	0
1.5 *M* Potassium thiocyanate	10
3.0 *M* Guanidine hydrochloride	10
1.5 *M* Guanidine hydrochloride	10

* β-Galactosidase was incubated for 30 min at 0°C.
** Percentage of remaining β-galactosidase activity.

subjected to anion-exchange chromatography on the Mono Q column. On this column β-galactosidase was bound at pH 7.5 and eluted with 100% yield at 0.35 *M* sodium chloride in a linear salt gradient. The complete two-dimensional system had an overall yield of up to 80% β-galactosidase as detected by its enzymic activity, and resulted in a five-fold concentration of the protein compared with the starting material. The process is illustrated schematically in Fig. 1.

Next, we applied this procedure to the purification and concentration of mycobacterial recombinant proteins. Using an IAC column containing 5 ml of affinity gel and the anion-exchanger Mono Q HR 5/5, we were able to isolate overnight (12

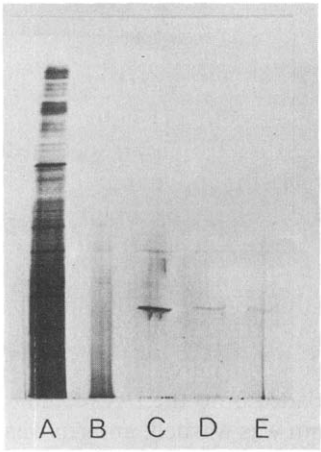

Fig. 3. SDS-PAGE on PhastSystem (Pharmacia) of *E. coli* Y1089G expressing β-galactosidase. Lanes A and B show 100 ng and 1 ng of protein of lysates, respectively; lanes C, D and E show 30 ng, 6 ng and 3 ng of immunoaffinity purified β-galactosidase, respectively.

h) up to 100 μg of recombinant protein within a 2-ml volume from 10 ml of crude *E. coli* lysate containing up to 50 mg of protein. SDS-PAGE of isolated recombinant proteins is documented in Fig. 4.

Although the recombinant proteins usually could be sufficiently purified by our method, in some cases an additional band of lower molecular mass was seen, and in case of clone Y3252 (expressing the 19 kDa protein detected by monoclonal antibody TB-C-13[11] no band was visible on the SDS gel (Fig. 4G) and on the Western blot only a faint band was detectable (Fig. 2C). Accordingly, this protein could not be isolated in sufficiently large amounts. The band of lower molecular mass was recognized by the anti-β-galactosidase antibodies, and probably represented a degradation product. We assume that these problems must be solved by modifying the culture conditions involving higher protein induction and faster lysis in the presence of potent protease inhibitors, rather than by altering the chromatographic procedure.

Recently, an IAC column with a monoclonal antibody as ligand directed against β-galactosidase became commercially available. We did not compare both IAC systems. However, because the recombinant fusion partner can have an influence on the antigenicity of the β-galactosidase molecule, we felt that an antiserum composed of multiple anti-β-galactosidase antibodies might be more appropriate to cover a broad range of fusion proteins.

Tuberculosis and leprosy are chronic infectious diseases that still cause major health problems, and effective vaccines against these pathogens are not available as yet[12]. Until recently the demand for sufficient amounts of pure antigens required for the construction of a novel vaccine could not be adequately met. The cloning of mycobacterial genes has provided a possible way of producing sufficient amounts of antigens of potential interest. Because immunity against tuberculosis and leprosy is mediated by T-lymphocytes, any rational vaccination strategy depends on the iden-

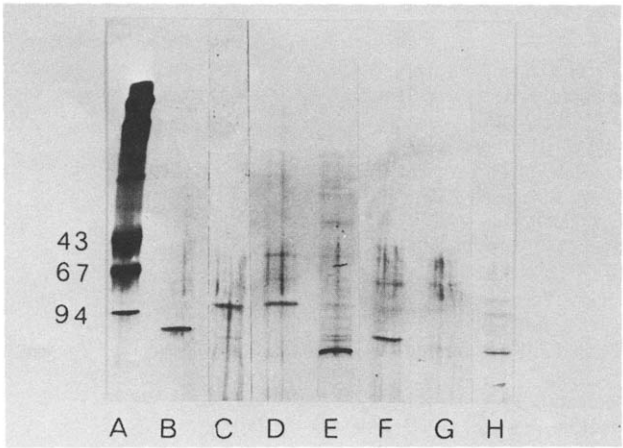

Fig. 4. SDS-PAGE on PhastSystem (Pharmacia) of isolated recombinant mycobacterial antigens. (A) Electrophoresis calibration kit (Pharmacia) indicating phosphorylase b 94 kDa, BSA 67 kDa, ovalbumin 43 kDa; (B) 30 ng of β-galactosidase isolated from *E. coli* Y1089G; (C) 17 ng of 18 kDa protein of *M. leprae*; (D–H) recombinant proteins of *M. tuberculosis*: (D) 11 ng of 19 kDa antigen; (E) 50 ng of 71 kDa antigen; (F) 20 ng of 12 kDa antigen; (G) 5 ng of 19 kDa antigen; (H) 20 ng of 65 kDa antigen.

tification of antigens on the T-cell level[3,12]. While long-term cultured T-cells can be used for testing of whole *E. coli* lysates[13–15], peripheral blood T-lymphocytes fail to respond specifically to these crude antigen preparations owing to contamination by *E. coli* components. Therefore screening of recombinant antigens with peripheral blood lymphocytes depends on prior antigen purification. As will be described elsewhere[16], the purification method introduced here makes it possible to screen recombinant antigens directly with peripheral blood T-lymphocytes.

The method described should not be restricted to recombinant proteins of mycobacterial origin, but could also be applied to other proteins of interest. Furthermore, it should be possible to apply it to fusion proteins expressed in other vector systems using β-galactosidase as fusion partner, *e.g.* the pEX-overexpression vector.

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REFERENCES

1 R. A. Young, B. R. Bloom, C. M. Grosskinsky, J. Ivanyi, D. Thomas and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.*, 82 (1985) 2583–2587.
2 R. A. Young, V. Mehra, D. Sweetser, T. Buchanan, J. Clark-Curtiss, R. W. Davis and B. R. Bloom, *Nature (London)*, 316 (1985) 440–452.
3 H. Hahn and S. H. E. Kaufmann, *Rev. Inf. Dis.*, 3 (1981) 1221–1250.
4 A. Ullmann, *Gene*, 29 (1984) 27–31.
5 T. V. Huynh, R. A. Young and R. W. Davis, in D. Glover (Editor), *DNA Cloning Techniques: A Practical Approach*, Vol. 1, IRL Press, Oxford, 1985, pp. 49–78.
6 T. Maniatis, E. F. Fritsch and J. Sambrook, *Molecular Cloning*, Cold Spring Harbor Laboratory, New York, 1982, p. 68.
7 U. K. Laemmli, *Nature (London)*, 227 (1970) 680–685.
8 H. Towbin, T. Staehelin and J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 4350–4354.
9 C. R. Merril, D. Goldman and M. L. van Keuven, *Methods Enzymol.*, 104 (1984) 441–447.
10 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248–254.
11 Letter to the Editor, *Infect. Immun.*, 51 (1986) 718–720.
12 S. H. E. Kaufmann, *Microbiol. Sci.*, 4 (1987) 324–328.
13 J. R. Lamb, J. Ivanyi, A. Rees, R. A. Young and D. B. Young, *Leprosy Rev.*, 57 (1987) (Suppl. 2) 131–137.
14 F. Oftung, A. S. Mustafa, R. N. Husson, R. A. Young and T. Godal, *J. Immunol.*, 138 (1987) 927–931.
15 F. Emmrich, J. Thole, J. v. Emden and S. H. E. Kaufmann, *J. Exp. Med.*, 163 (1986) 1024–1029.
16 M. E. Munk, B. Schoel and S. H. E. Kaufmann, *Eur. J. Immunol.*, in press.