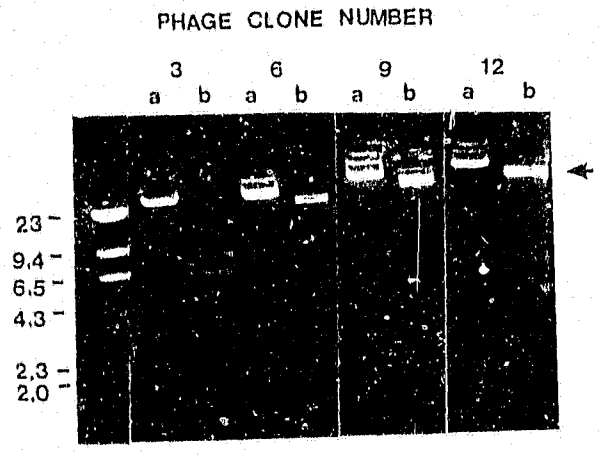

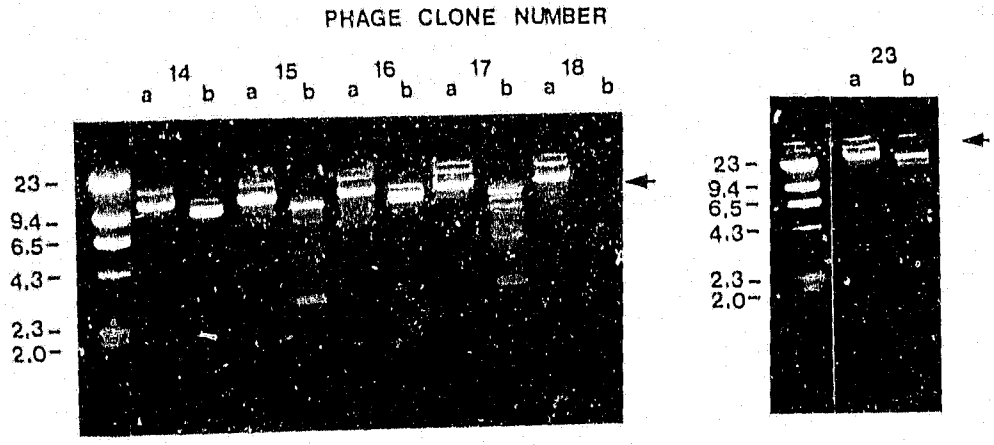

FIGURE 14 Restriction enzyme analysis of DNA purified from recombinant clones.

The DNA before (a) and after (b) digestion with Eco R1 can be seen. The positions of the molecular weight markers (kilobases) and the λ gt 11 Eco R1 arms (◄) are indicated.

3.8 GENERATION OF RECOMBINANT LYSOGENS IN E.coli Y 1089

Saturated cultures of E.coli Y 1089 were infected with each of the following phage clones: TB 3, TB 6, TB 9, TB 12, TB 14, TB 15, TB 16, TB 17, TB 18, and TB 23. The multiplicity of infection was in the order of 10. Two hundred colonies were plated per plate, and plates were incubated at 32^0C. Individual colonies were picked with a sterile toothpick and tested for growth at both 32^0C and at 42^0C. Those colonies which grew at 32^0C, but not at 42^0C, were assumed to be lysogens. Figure 15 demonstrates the results of such an experiment, whereby Y 1089 cells were infected with recombinant phage TB 6. Cells were plated in duplicate and incubated at 32^0C (Figure 15, plate A) and at 42^0C (Figure 15, plate B). Those colonies which grew at 32^0C, but not at 42^0C, were assumed to be lysogens.

Using this technique, lysogens were generated from all of the recombinant clones with the exception of clone TB 3 (Table 9). Infection of the Y 1089 cells with this clone was repeated 3 times, but no lysogens could be generated. The frequency of lysogen generation for the remainder of the recombinant clones ranged between 10% and 34%.

TABLE 9 Frequency of generation of recombinant lysogens in E.coli Y 1089

Recombinant phage *clone number*	Frequency of lysogen generation (percentage of total colony numbers)
TB 3	0
TB 6	19
TB 9	23
TB 12	22
TB 14	12
TB 15	17
TB 16	34
TB 17	15
TB 18	10
TB 23	19

FIGURE 15 Generation of recombinant lysogens in E.coli Y 1089.

Cells were infected with clone TB 6. Those colonies which grew at 32°C but not at 42°C (▲) were assumed to be lysogens.

3.9 ANALYSIS OF RECOMBINANT M.tuberculosis ANTIGENS BY SDS-PAGE AND WESTERN BLOTTING

Antigens that were expressed by the recombinant phage clones were identified and analysed by SDS-PAGE and Western blotting. The production of foreign proteins by lysogens of clones TB 6, TB 9, TB 12, TB 14, TB 15, TB 16, TB 17, TB 8, and TB 23 was induced by the addition of IPTG to the medium, and lysates of these cultures were subjected to SDS-PAGE on duplicate gels as described in Section 2.9. One set of gels was stained, while proteins from the second set of gels were transferred to nitrocellulose filters (Sections 2.9 and 2.10). Recombinant immunodominant mycobacterial antigens were identified by screening Western blots with anti-M.tuberculosis antibodies. Blots were also screened with anti-β-galactosidase antibodies in order to identify recombinant fusion proteins.

3.9.1 SDS-PAGE analysis

Total protein profiles of the recombinant lysogens can be seen in Figure 16. The sizes of the recombinant proteins expressed by the lysogens are tabulated in Table 10. Fusion proteins of molecular weights 22 kDa, 12 kDa - 13 kDa, and 6 kDa were produced by several clones. In addition, 2 non-fusion proteins of molecular weights 50 kDa and 32 kDa were also produced. The 50 kDa and 32 kDa non-fusion proteins were produced by all of

the recombinant clones, and in addition were also produced by lysogens grown in the absence of IPTG (Figure 16, lane 4). These proteins were not produced by clones containing non-recombinant λ gt 11. These results suggest that both these proteins are not normal phage proteins, and that they are not dependent on LacZ-directed expression.

Clones TB 6, TB 16, TB 15, and TB 9 produced a 22 kDa fusion protein, expressed as a recombinant protein of molecular weight 136 kDa (Figure 16, arrow "a"). Clones TB 17 and TB 23 also produced a 12 kDa fusion protein (Figure 16, arrow "b"), expressed as a recombinant protein of 126 kDa. In addition, clones TB 9, TB 15, TB 12, TB 17, and TB 23 produced a small fusion protein 6 kDa in size (Figure 16, arrow "c").

Comment

The β-galactosidase portion of the fusion proteins accounts for 114 kDa of the final protein molecular weight. The sizes of the fusion proteins were therefore calculated by subtracting 114 kDa from the size of the expressed proteins: for example, clone TB 6 produced a recombinant protein of molecular weight 135 kDa. By subtracting the molecular weight of β-galactosidase (114 kDa) from this size, the size of the recombinant fusion protein was calculated as being 22 kDa. The bands produced by these fusion proteins on gels after electrophoresis and staining were very faint (and are even

TABLE 10 Identification of recombinant protein antigens

Recombinant clone number	Recombinant protein size (kDa)	recognition by anti-*M.tuberculosis* serum	recognition by anti-β-galactosidase antibodies
TB 6	50		
	32		
	22 (fusion protein)	+	
TB 9	50		
	32		
	22 (fusion protein)		
	6 (fusion protein)	+	+
TB 16	50		
	32		
	22 (fusion protein)	+	
TB 17	50		
	32		
	12 - 13 (fusion protein)		
	6 (fusion protein)	+	
TB 23	50		
	32		
	12 - 13 (fusion protein)		
	6 (fusion protein)	+	
TB 14	32		
TB 15	50		
	32		
	22 (fusion protein)		
	6 (fusion protein)		
TB 18	50		
	32		
TB 12	50		
	32		
	6 (fusion protein)		

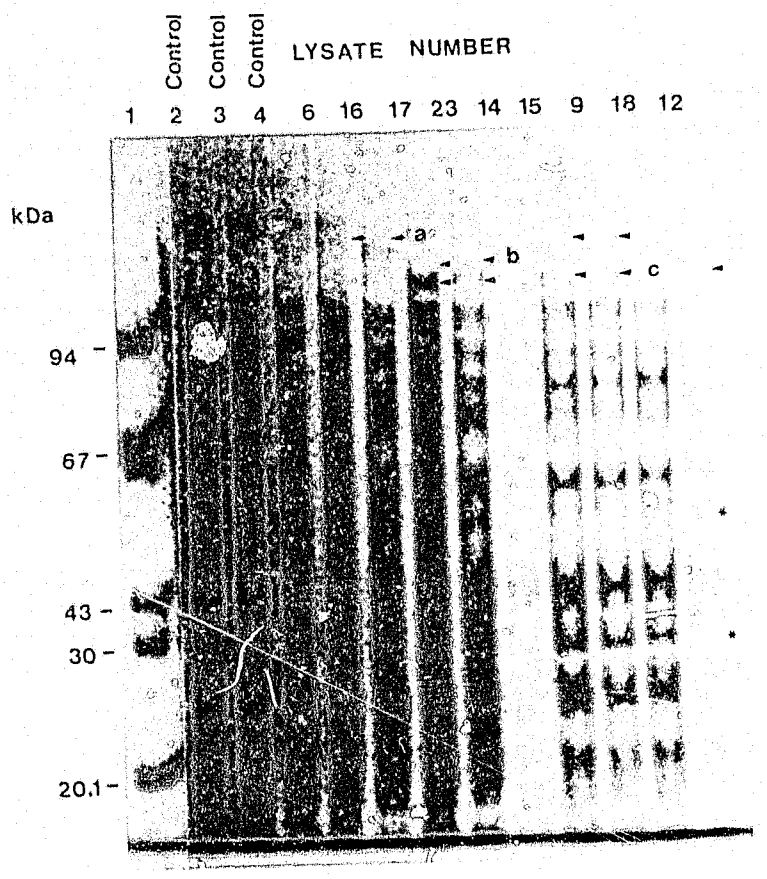

FIGURE 16 SDS-PAGE analysis of total protein profiles from recombinant clones.

Lysogens were generated as described, and lysate samples were electrophoresed on 10% polyacrylamide gels. Lane 1- molecular weight standards (kDa); lanes 2, 3 and 4- control lysogens containing no λ gt 11 (2), non-recombinant λ gt 11 (3), and clone TB 17 in the absence of IPTG (4). Fusion proteins are marked with an arrow (→); recombinant non-fusion proteins are marked with an asterisk (*).

more faint on photographs!), suggesting that very small amounts of these proteins were being synthesised by the respective clones, or that the. proteins were being rapidly degraded by the host cells.

3.9.2 Western blotting analysis

Of the recombinant proteins identified, only the 6 kDa and the 22 kDa fusion proteins were recognised by the anti-M.tuberculosis serum (Figure 17). Even though 4 clones (TB 6, TB 16, TB 15 and TB 9) produced the 22 kDa protein, the anti-M.tuberculosis serum bound only to the 22 kDa protein produced by clones TB 6 and TB 16 (Figure 17, arrow "a"). Similarly, although clones TB 9, TB 17, TB 23, TB 6, and TB 12 all produced the 6 kDa fusion protein, the anti-M.tuberculosis antibodies bound only to the 6 kDa protein produced by clones TB 17, TB 23, and TB 9 (Figure 17, arrow "b"). The reactions produced by the anti-M.tuberculosis antibodies with these 6 kDa and 22 kDa proteins were very weak.

Comment

In spite of extensive adsorption of the anti-M.tuberculosis serum with an E.coli lysate, a prominent cross-reactivity with E.coli proteins was observed (particularly with proteins of molecular weights 114 kDa, 33 kDa, 82 kDa, 59 kDa - 60 kDa,

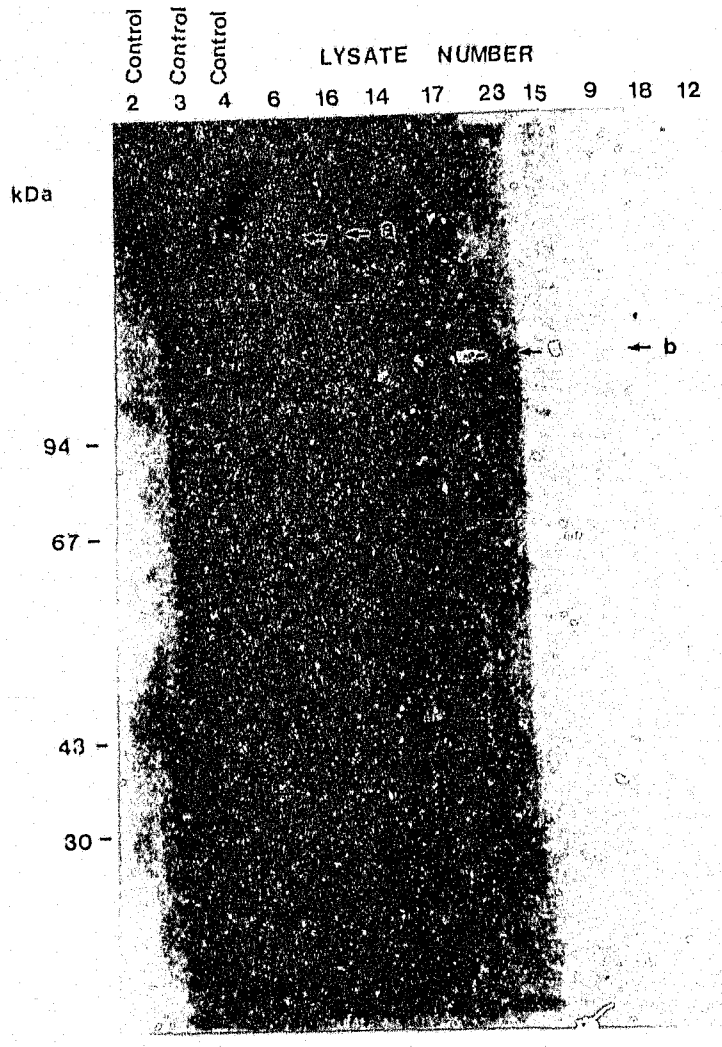

FIGURE 17 Analysis of recombinant lysates by Western blotting.

Recombinant proteins recognised by anti-M.tuberculosis antibodies are marked with an arrow (←). Some cross-reactivity of the antiserum with E.coli proteins can be seen. Molecular weight standards (kDa) are indicated on the right.

and 26 kDa) in the immunoblotting experiments. The reasons for this cross-reactivity include:

- non-specific binding of the antibodies due to the high concentration of antibodies required to detect recombinant proteins;
- possible cross-reactivity of mycobacterial proteins with some E.coli proteins;
- possible cross-reactivity of the rabbit antibodies with E.coli proteins.

When the recombinant proteins were screened with anti-β-galactosidase antibodies, only the 22 kDa fusion protein produced by clone TB 9 was recognised by the anti-β-galactosidase antibodies (Figure 18). Two additional protein bands, of molecular weights approximately 150 kDa and 200 kDa, were also recognised by these antibodies. Immunoblotting was repeated several times, altering the concentrations of the gels used for electrophoresis of the proteins (7.5%, 10%, and 15% gels were used), and increasing the dilution of the antibodies for screening (antibody dilutions of 1/1 000, 1/1 500, 1/2 000, and 1/2 500 were used). The 200 kDa protein was not visible on the gels under any of the electrophoresis conditions employed. Both the 150 kDa and the 200 kDa bands were present on immunoblots under conditions of different acrylamide concentrations, and dilution of the antibodies only resulted in very much weaker signals being produced by the

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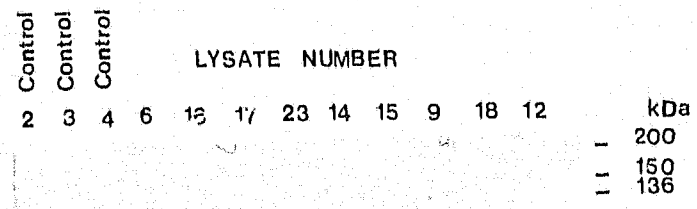

FIGURE 18 Analysis of fusion proteins by Western blotting.

Filters were incubated with antibodies to β-galactosidase in order to identify fusion proteins. The lysate from clone TB 9 demonstrated fusion proteins, the sizes (kDa) of which are indicated on the right of the blot. The 136 kDa band represents the 22 kDa fusion protein.

22 kDa fusion protein, as well as the 150 kDa and 200 kDa proteins (data not shown).

Comment

The 150 kDa protein which reacted with the anti-β-galactosidase antibodies on immunoblots was present in gels of all the lysates as a weak protein band (Figure 16). It does not appear to be a recombinant protein, since it was present in all the control lysates, including the normal E.coli Y 1089 control; the positive reaction observed with the anti-β-galactosidase antibodies could possibly be attributed to cross-reaction of the antibodies with this protein. The 200 kDa protein recognised by the anti-β-galactosidase antibodies was not present on any of the gels of the recombinant lysates, and it can only be assumed that this reaction was an artefact of the immunoblotting reaction.

At present it is not clear why positive reactions for proteins produced by more than one recombinant clone only occurred for particular clones. It is also unclear why the anti-β-galactosidase antibodies did not recognise all the fusion proteins produced by the clones (viz. the 22 kDa, 12 kDa, and 6 kDa proteins), and why the 22 kDa protein produced by clone TB 9 alone was recognised. In addition, why the anti-β-galactosidase antibodies failed to recognise the 114 kDa β-galactosidase protein of E.coli is also unclear.

CHAPTER 4 DISCUSSION AND CONCLUSION

Characterisation of individual, species-specific antigenic components has long been viewed as a potential approach to the development of vaccines, diagnostic reagents, and as a tool for the analysis of the immune response to M.tuberculosis. There are several goals of research into mycobacterial antigen structure:

i. to identify determinants with well-defined species-specificities which could be substituted for heterogenous reagents such as PPD in immunodiagnostic tests;
ii. taxonomic applications of species-specific antigens; characterisation of species-specific antigenic determinants which are involved in T cell recognition;
iii. the identification and characterisation of surface-exposed antigens and their potential role in intracellular survival;
iv. identification and characterisation of antigens which may be possible candidates for subunit or recombinant vaccines against tuberculosis.

Molecular biology and monoclonal antibody technology have provided the means whereby mycobacterial antigens encoded on linear segments of the mycobacterial genome can be systematically characterised. A systematic examination of foreign polypeptide antigens in E.coli could be possible if the factors which influence production of detectable levels of

proteins are adequately considered. There are 3 major problems associated with obtaining expression of foreign DNA as a stable antigen (Young and Davis, 1985).

The first problem is that most foreign DNA does not contain the transcription and translation signals required for expression in E.coli. Thus, the foreign gene must generally be placed under the control of an E.coli promoter that is efficiently recognised by E.coli RNA polymerase, and an E.coli ribosome binding site that can be used by the bacterial translation machinery (Young and Davis, 1985).

The second problem in expressing foreign DNA is that foreign peptides are efficiently degraded in E.coli (Young and Davis, 1983). The instability of foreign antigens can often be reduced by fusing the antigen to a stable host protein, and by using host mutants deficient in specific proteases (Young and Davis, 1983). Fusion of unstable protein antigens to the carboxy terminus of the stable E.coli protein β-galactosidase has been shown to enhance the stability of some foreign proteins (Stanley, 1983). In addition, the stability of the fusion product of β-galactosidase and a foreign antigen can be markedly increased in lon mutants of E.coli (Young and Davis, 1983). These mutants are deficient in one of the ATP-dependent proteases that are responsible for the destruction of proteins. This particular protease deficiency is especially useful since the presence of this mutation does not appear

to alter the normal growth properties of the cells (Young and Davis, 1983). The lon protease appears to have some specificity for the class of abnormal polypeptides of which carboxy-terminus β-galactosidase fusions are a member, and does not enhance the stability of other kinds of abnormal peptides (Young and Davis, 1985).

The third major problem with foreign antigen synthesis in E.coli is that constitutive high level expression of foreign polypeptides can often be lethal to the host cell (Young and Davis, 1983). A solution to this problem is to ensure that the production of the foreign antigen is transient. Thus, the expression of the DNA encoding the foreign protein is repressed during early exponential phase growth of the host. Near the end of this period, when the transcriptional and translational apparatus are still fully active, the expression of the foreign proteins is induced, and satisfactory levels of the antigen are produced before the cells become inviable.

These concepts, designed to improve the levels to which foreign antigens can accumulate in E.coli, have been incorporated into the λ gt 11 vector-host system (Young and Davis, 1983). This phage expression vector was constructed to permit insertion of foreign DNA into the β-galactosidase structural gene LacZ under the control of the lac operon (Figure 1). The recombinant DNA can be propagated lytically, and expression of the foreign DNA is repressed by the LacI

gene product (Young and Davis, 1983). Production of a recombinant fusion polypeptide can be rapidly induced by the addition of the derepressor IPTG to the culture medium. The presence of the lon mutation appears to permit accumulation of otherwise unstable novel proteins to levels which facilitate detection by immunological or physical (polyacrylamide gels) analysis (Young and Davis, 1985).

The successful isolation of genes encoding for a particular protein with the λgt 11 system depends on the quality of the recombinant DNA library and the antibody probe (Young and Davis, 1983). Both genomic and cDNA libraries have been used successfully to isolate genes with antibody probes (Huynh, Young, and Davis, 1985). The decision to employ one or the other type of library rests on several considerations: a sheared genomic library can be constructed to have insert DNA breakpoints at each base pair throughout the genome, thus increasing the probability that all coding sequences are equivalently represented (Young and Davis, 1985). Problems with this approach include concerns about intervening sequences (the degree to which intervening sequences interfere with the expression of specific epitopes is impossible to predict). In addition, screening of genomes of more than 10^8 bp is laborious and time-consuming (Young and Davis, 1985). A cDNA strategy may improve the frequency of a coding sequence, but only if information about the abundance of specific mRNA is available.

λ gt 11 recombinant DNA expression libraries constructed with cDNA (Kemp, Coppel, Cowman et al., 1983) and with DNase-digested genomic DNA (McCutchan, Hansen, Dame, and Mullins, 1984) have been used to isolate genes by using antibodies directed against Plasmodium falciparum. These libraries, however, may not contain insert DNA that is representative of all of the coding sequences of interest in a pathogen genome: cDNA is biased toward the more abundant mRNA's, and DNase can act on genomic DNA in a non-random fashion (McCutchan, Hansen, Dame, and Mullins, 1984). The sheared genomic DNA approach (Young, Bloom, Grosskinsky et al., 1985) attempts to minimise this bias: part of the impact of this approach lies in the potential to detect any epitope encoded in the genome of an organism, even if it is not always expressed.

Genomic libraries of mycobacteria have been constructed in λ gt 11 to date, largely because knowledge and characterisation of mycobacterial antigens, and more so of their mRNA's, has been limited (Young and Davis, 1985). The use of λ gt 11 libraries to analyse mycobacterial antigens was pioneered by Young, Bloom, Grosskinsky, et al., (1985). They used sheared genomic DNA from M.tuberculosis to construct the library, and used monoclonal antibodies directed against specific determinants for the detection of recombinant phage clones expressing mycobacterial antigens. This technique has since been used extensively to analyse antigens from M.tuberculosis (Young, Kent, and Young, 1987; Husson and Young, 1987; Lu,

Lien, Becker et al., 1987; Andersen, Worsaae, and Chaparas, 1988; Damiani, Biano, Beltrame, et al., 1988); M.leprae (Young, Mehra, Sweetser, et al., 1985); M.bovis (Radford, Duffield, and Plackett, 1988; Lu, Lien, Becker et al., 1987); M.africanum (Lu, Lien, Becker,et al., 1987); and M.intracellulare (Morris, Rouse, Hussong, et al., 1988).

In the present study, a λ gt 11 recombinant DNA expression library of M.tuberculosis DNA was constructed as described in Chapter Two. Genomic DNA was mechanically sheared to produce fragments of 5 kb average size. Eco R1 linkers were added to the ends of the DNA fragments to allow insertion at the unique Eco R1 site of the λ gt 11 phage. The ligated, recombinant DNA was packaged into phage heads, and this material was used to infect E.coli cells to produce a library. The aim of this approach was to generate DNA fragments with endpoints throughout the mycobacterial genome and then to produce recombinant phage in sufficient numbers such that insert endpoints would occur between most base pairs in the M.tuberculosis genome. The library constructed in this manner contained approximately 1.3×10^5 individual recombinant phage (63% of the total library) (Table 6). In order to determine whether the generation of the recombinant library had been successful, the titre for the M.tuberculosis library was compared to the titres obtained for the control reactions (Table 6). The titre for the mock packaging reaction (Table 6, reaction 1) gives a measure of any background that might be

caused by the packaging extracts. It is expected to be very low (less than 100 pfu/extract, as indicated by the manufacturers) (Promega Biotech. Madison, WI, USA). The result obtained (no plaques) indicates that the packaging extracts themselves were not contributing any background plaques to the library.

The packaging control reaction was included in these assays to provide an indication of the efficiency of the <u>in vitro</u> packaging reactions. The expected efficiency of the packaging systems, determined by the manufacturers, (Promega Biotech, Madison, WI, USA) in a standard packaging reaction using the indicated ligated λ c 1857 Sam 7 DNA, is 2.7 X 10^8 pfu/ug DNA. The results obtained in the present study, namely 2 X 10^8 pfu/ug DNA (Table 6, reaction 2), indicate that the packaging systems as a whole were performing efficiently.

The titre of the λ gt 11 arms alone (Table 6, reaction 3) gives an indication of the ligation capacity of the arms, the efficiency of the ligation reactions, and a measure of any background that may be caused by the λ gt 11 arms. Eco R1 digested, dephosphorylated, and ligated λ gt 11 arms without inserts should show fewer than 10 blue plaques, and very few clear plaques per microgram of DNA when titrated on plates containing IPTG and Xgal (Davis, Dibnner, and Battey, 1986). Clear plaques indicate either damage to the Eco R1 cloning site, or DNA contamination of the λ gt 11 arms preparation

(Davis, Dibbner, and Battey, 1986). The results obtained (Table 6, reaction 3) were within the expected range - 10^4 plaques, 100-fold less than the maximum limit of 10^6 , with very few clear plaques, indicating that the ligation capacity of the arms was good, and that minimal damage to the Eco R1 insertion site had occurred. There was an approximately 3-log difference in the titres of the packaged λ gt 11 arms alone, and the λ gt 11 arms containing control DNA, indicating that approximately 1 plaque in every 1000 plaques was a false recombinant.

The ligated and packaged positive control insert should show a significant stimulation in the number of clear plaques seen in comparison to the ligation and packaging of the vector arms without the insert. Approximately 10^6 clear plaques per microgram of inserted DNA are usually attained, these clear plaques representing "model insert" DNA clones (Davis, Dibbner, and Battey, 1986). The expected cloning efficiency for this particular control reaction, obtained by setting up a positive control reaction using a 3 kb plasmid DNA fragment with compatible Eco R1 ends, is approximately 10^7 pfu/ug DNA, with greater than 95% insertional efficiency (Promega Biotech, Madison, WI, USA). The titre obtained for the positive control reaction (Table 6, reaction 4) was 7.5 X 10^6 pfu/ug DNA, with 94% insertional efficiency, indicating a satisfactory cloning efficiency for the system.

It has been suggested (Maniatis, Fritsch, and Sambrook, 1982) that cloning efficiencies of 10^5 - 10^6 recombinants per microgram of DNA are indicative of a sufficiently concentrated library which should be large enough to represent all of the DNA sequences available for cloning. The library constructed with 200 ng M.tuberculosis DNA appeared to have the properties indicative of a successfully generated library, and was therefore chosen for amplification and screening. This library (Table 6, reaction 6) contained a reasonable percentage of recombinant phage (63.5%), and enough total recombinants (3 X 10^5 pfu/ug DNA) to be considered representative of most of the DNA sequences of M.tuberculosis.

The recombinant library was amplified in E.coli Y 1088 by small-scale liquid culture. The titre of this amplified library was 10^{11} pfu/ml and consisted of essentially greater than 90% recombinants. The recombinant mycobacterial library generated in the present study compares well with other mycobacterial libraries constructed in λ gt 11. Young, Bloom, Grosskinsky, et al., (1985) constructed an M.tuberculosis DNA library containing 4 X 10^6 recombinant phage. The titre of this library after amplification was 10^{11} pfu/ml, and consisted of approximately 40% recombinant phage. A library constructed with M.bovis DNA contained a slightly lower titre of 2.2 X 10^6 phage clones, 90% of which were recombinant (Radford, Duffield, and Plackett, 1988). A similar titre of 2.5 X 10^6 recombinant phage clones was obtained for an M.leprae library

(Young, Mehra, Sweetser, et al., 1985). This library was amplified to produce a library of 2 X 10^{11} pfu/ml, and which consisted of approximately 25% recombinants. Mehra, Sweetser, and Young, (1986) constructed an M.leprae library of 10^6 phage clones, of which 95% were recombinant. Titres of 10^6 recombinants were obtained for an M.intracellulare library (Morris, Rouse, Hussong, et al., 1988). Amplification of this library resulted in a library titre of 10^{10} phage, of which 85% were recombinant. The percentage of recombinant clones in the library constructed in the present study is high (63%), and although the titre of this library (1.3 X 10^5 pfu/ml) is slightly lower than the mycobacterial DNA library titres obtained by other investigators (Young, Grosskinsky, Bloom, et al., 1985; Radford, Duffield, and Plackett, 1988; Young, Mehra, Sweetser et al., 1985; Mehra, Sweetser, and Young, 1986; Morris, Rouse, Hussong, et al., 1988), it nevertheless compares favourably since it is only approximately 10-fold lower than that obtained for the other libraries. The amplified library titre of 10^{11} pfu/ml (>90) recombinants) obtained in the present study is in keeping with the findings of the abovementioned investigators, who obtained titres of 10^{10} - 10^{11} pfu/ml.

The amplified library was then screened for phage producing recombinant M.tuberculosis antigens using anti-M.tuberculosis antibodies, and with monoclonal antibodies to

β-galactosidase for the detection of fusion proteins. Both polyvalent (Young, Kent, and Young, 1987) and monoclonal antibodies (Young, Bloom, Grosskinsky, et al., 1985; Damiani, Biano, Beltrame, et al., 1988; Andersen, Worsaae, and Chaparas, 1988) have been used for the detection of recombinant phage clones expressing mycobacterial antigens. It has been found that the immunodominant antigens defined by monoclonal antibodies can also be detected using polyvalent serum (Young, Kent, and Young, 1987; Table 3). For successful library screening, the quality of the antibody probe has been shown to be of major importance (Young and Davis, 1985). Generally, high titre specific antibodies produced better signals than did low titre antibodies, and antibodies that produced good signals on Western blots generally produced good signals in the λ gt 11 screening procedure (Young and Davis, 1983). Different protocols exist for the detection of the primary antibodies. The ^{125}I-protein A protocol, initially used by Young and Davis (1983 b) to isolate phage clones that produced yeast RNA polymerase II, has the advantage of being sensitive and allowing controlled film exposure to maximise signal-to-noise ratios. This method is however useful only in the detection of IgG, and is prone to producing false positive signals (Young and Davis, 1983). Young, Bloom, Grosskinsky et al., (1985) modified several features of the original antibody screening protocol in order to obtain improved sensitivity and permit a more thorough and efficient screen of λ gt 11 libraries. In particular, biotinylated secondary antibody was

used to detect bound primary antibody. The secondary antibody was in turn bound by avidin-coupled horseradish peroxidase. This approach had several advantages over the use of ^{125}I-labelled protein A (Young, Bloom, Grosskinsky et al., 1985): the choice of primary antibody did not depend on its ability to bind protein A; good signal-to-noise ratios were obtained with greater primary antibody dilution; no false positives were observed, and the precise signal-producing plaque could be located by comparing the faint background pattern produced by neighbouring plaques on the agar plates.

The enzyme-labelled secondary antibody approach was used in the present study to detect phage clones producing mycobacterial antigens. Seventy five filters containing antigens from approximately 10^3 phage clones each were screened. Twenty two phage clones producing positive signals with either anti-M.tuberculosis or anti-β-galactosidase antibodies were isolated. Of these, only 10 again produced positive signals after re-isolation and amplification (Table 7). Twelve false positive signals out of 22 were obtained. These results are in contrast to the findings of Young, Bloom, and Grosskinsky, (1985) who obtained no false positive signals when screening a M.tuberculosis library. The numbers of positive clones detected in this study using antibody probes are slightly lower than the numbers obtained by other investigators who screened mycobacterial DNA libraries. Mehra, Sweetser, and Young (1986) screened 500 recombinant phage

plaques from a recombinant M.leprae library. From these, 54 clones were purified to homogeneity using monoclonal antibodies to M.leprae. Young, Mehra, Sweetser, et al., (1985) probed 10^3 recombinant M.leprae clones; 17 of these were found to produce signals, and 15 were successfully purified to homogeneity in 1 or 2 successive rescreens with a monoclonal antibody pool. Radford, Duffield, and Plackett (1988) screened 20 filters containing plaques produced by a recombinant M.bovis library; of the 88 positive clones found on the original filters, only 32 phage clones gave consistent signals when passed through primary and secondary purification steps.

Although several of the positive signals produced by recombinant phage clones in this study were distinct (Figure 12 b), in general the signals were weak, suggesting a poor level of transcription and translation of the foreign genes, or rapid degradation of the recombinant products before sufficient accumulation of such products occurred in the host cells. Alternatively, the antibodies used for screening of the recombinant library may have had low avidity for the recombinant mycobacterial antigens. Radford, Duffield, and Plackett (1988) also report variable signal strengths for an M.bovis library screened with polyclonal antiserum; the signals observed ranged from barely perceptible dots to conspicuous spots. Although no explanation as to the reasons for this observation was offered, it is possible that the abovementioned mechanisms may have occurred in their

system too.

In spite of the fact that the antiserum used in the present study was pre-adsorbed with an E.coli lysate, a strong cross-reaction with E.coli proteins still occurred (see Figure 17). It is interesting to note that libraries probed with monoclonal antibodies to M.leprae (Young, Mehra, Sweetser et al., 1985; Mehra, Sweetser, and Young, 1986) and to M.tuberculosis (Young, Grosskinsky, Bloom, et al., 1985) generally gave fewer false positive reactions than did those libraries probed with unpurified serum (Radford, Duffield, and Plackett, 1988; this study). This high incidence of false positive background plaques was probably due to the presence of E.coli-cross reactive antibodies in the unpurified serum. Radford, Duffield, and Plackett (1988) found that this high background was reduced when affinity purified antibodies were used. Adsorption with E.coli lysates either bound to nitrocellulose or added directly to the serum was found to be far less effective in reducing background signals than the use of affinity purified antiserum (Radford, Duffield, and Plackett, 1988).

DNA restriction endonuclease digestion analysis of the recombinant phage indicated that the phage contained inserts ranging in size between 1.9 kb and 7 kb (Table 8). The average length of the DNA inserts was 5 kb. This is in keeping with the findings of Young, Bloom, Grosskinsky et al (1985), who

constructed a library with M.tuberculosis inserts averaging 4 kb in length. DNA inserts with an average length of 5 kb were found in a recombinant M.intracellulare library constructed by Morris, Rouse, Hussong, et al. (1988). Smaller inserts of approximately 2 kb were found in recombinant clones constructed with M.leprae DNA (Young, Mehra, Sweetser, et al., 1985). The presence of substantially smaller inserts in the range of 200 bp - 900 bp has been reported for a M.bovis library constructed in λ gt 11 (Radford, Duffield, and Plackett, 1988). These inserts were considerably smaller than the M.tuberculosis and the M.leprae inserts discussed above, and since this size range was smaller than the size of the DNA prepared for cloning (average size 2 kb), the results suggest preferential cloning of smaller fragments. Since recombinant λ gt 11 prophage of this length are below the optimal packaging size (Young and Davis, 1983), the presence of small inserts could have affected packaging efficiency. However, it has been shown that recombinant phage with such small inserts are still able to express immunologically reactive fusion proteins (Radford, Duffield, and Plackett, 1988).

In the present study, 3 of the 10 recombinant phage clones contained inserts ranging in size from 10 000 bp to 18 000 bp (Clones TB 17, TB 18, and TB 23; Table 8). The presence of such large inserts, larger than the maximum cloning capacity of the λ gt 11 vector, could be explained by the presence of damaged λ gt 11 arms. Alternatively, one of the Eco R1 sites

flanking the insert DNA may have been lost at some stage of the cloning procedure. The absence of one or more of the Eco R1 sites in the insert DNA has also been reported for 5 out of 12 recombinant phages isolated with monoclonal antibodies to M.tuberculosis (Andersen, Worsaae, and Chaparas, 1988). Radford, Duffield, and Plackett (1988) also reported the lack of two Eco R1 sites on either side of inserted M.bovis DNA in several phage clones isolated. Similar results have been reported by Shinnick (1987) who found that only one of the Eco R1 restriction sites was present in 6 of 20 recombinants studied. These observations raise the possibility that a significant fraction of the recombinant phage in the library may have arisen from the insertion of a DNA fragment containing only one functional Eco R1 site into the λ gt 11 Eco R1 cloning site. It is also possible that some clones might have undergone some sort of recombination, rearrangement, or deletion event during propagation which removed one of the Eco R1 sites. However, further documentation is required to verify the latter suggestion.

Lysogens of the recombinant phages were generated in E.coli Y 1089 (Table 9). All except one recombinant clone, TB 3, produced stable lysogens. It is unclear why clone TB3 failed to lysogenise. A possible suggestion is that some damage occurred to the genes coding for the proteins required for intergration (cI and int), or that the att site in the bacteriophage or the bacterial genome were damaged (Maniatis,

Fritsch, and Sambrook, 1982). The frequency of lysogen generation for the remainder of the recombinant clones ranged between 10% and 34% (Table 9). These figures were within the expected frequencies of between 10% and 70%, as suggested by Huynh, Young, and Davis,(1985), and Snyder, Elledge, and Sweetser, (1987), and were thus considered satisfactory.

Cohen, Mayer, Rumschlag et al.,(1987), Young, Kent, and Young (1987), and Andersen, Worsaae, and Chaparas (1988) reported that screening of an M.tuberculosis recombinant DNA library with monoclonal or polyclonal antibodies resulted in the preferential selection of clones expressing one or more of three mycobacterial antigens which appear to be immunodominant as regards the antibody response to mycobacterial proteins. These antigen were of molecular weights 71 kDa, 65 kDa, and 14 kDa. They all appeared capable of being expressed as free proteins rather than as fusion proteins, and each of these antigens could be expressed in different molecular weight forms (Cohen, Mayer, Rumschlag, et al., 1987; Andersen, Worsaae, and Chaparas, 1988). Analysis of the protein profiles of the recombinant lysates generated in the present study has led to the identification of recombinant proteins of molecular weights 50 kDa, 32 kDa, 22 kDa, 12 kDa, and 6 kDa (Table 10; Figure 16). It is therefore tempting to speculate that the antigens isolated in this study may be related to the abovementioned immunodominant antigens of similar molecular weight.

All of the 9 recombinant phage clones produced 2 proteins of molecular weight 50 kDa and 32 kDa; 5 of these 9 clones produced a 6 kDa polypeptide; 4 clones produced a 22 kDa polypeptide; and 2 clones produced the 12 kDa polypeptide (Table 10). The predominance of these polypeptides synthesised by recombinant clones may reflect immunodominance of certain proteins. It may also indicate a selective pressure by E.coli host factors due to the fact that some recombinant proteins may be more harmful to the host cell than others. This would result in preferential accumulation of non-toxic proteins by the host cell.

The 50 kDa and 32 kDa polypeptides are of particular interest because they are non-fusion polypeptides (Table 10; Figure 16). Their synthesis was independent on the addition of IPTG to the culture medium. They are not normal phage proteins since they are not synthesised by non-recombinant lysogens, and they are not normal E.coli proteins since they are not synthesised by Y 1089 cells alone (Figure 16). Although the gt 11 system was designed for the production of antigenic proteins as fusion proteins linked to β-galactosidase (Young and Davis, 1983), there is increasing evidence that M.tuberculosis promoters are also functional in this system. The 65 kDa from M.tuberculosis has been demonstrated to be expressed as a free protein in E.coli (Thole, Dauwerse, Das, et al., 1985; Shinnick, 1987), and it has therefore been hypothesised that the mycobacterial promoter for this protein is recognised by the E.coli

transcription system. Young, Kent, and Young, (1987) have suggested that a similar hypothesis could also apply to the 71 kDa and 14 kDa M.tuberculosis antigens identified during their investigations. It is therefore also possible that a similar mechanism exists for the synthesis of the 50 kDa and 32 kDa non-fusion antigens identified in the present study.

Recognition of a mycobacterial translation initiation signal on an mRNA molecule with transcription remaining under the control of the β-galactosidase promoter would be an alternative explanation for these results (Young, Kent, and Young, 1987). If the initiation signals for these particular proteins are suitable for recognition by E.coli, then their expression in a recombinant DNA system would be independent of the orientation and reading frame of the insert. This effect could then contribute to the high frequency with which clones expressing these proteins are picked up. On the other hand, the method by which the λ gt 11 library was generated in the present study, using a large number of fragments with random endpoints, would be expected to make this effect less pronounced since the number of inserts with start points within a gene would be high compared with those containing the initiation portions of the gene.

It has been suggested (Young, Kent, and Young, 1987) that the relative importance of the abovementioned factors (i.e. the production of fusion polypeptides; the recognition of

mycobacterial promoters by the E.coli transcription machinery; or the recognition of mycobacterial initiation signals) in determining the frequency with which particular phage clones are detected depends on the nature of the epitope recognised. For an epitope consisting of a short linear sequence of amino acids, it would be expected that fusion proteins would occur most frequently. However, if a complex conformational determinant is recognised by the antibody used for screening, then the type of whole-gene transcript resulting from recognition of naturally occurring initiation sequences may provide a more satisfactory antigen (Young, Kent, and Young, 1987). However, detailed analysis of the epitopes recognised by the antibodies used for the detection of these particular antigens is necessary to provide conclusive evidence of the mechanisms operating in the mycobacterial system. Although monoclonal antibodies to the 65 kDa mycobacterial protein have been shown to recognise linear determinants of the molecule (Mehra, Sweetser, and Young, 1986), the epitopes recognised by antibodies in polyclonal serum have not yet been characterised.

The 50 kDa and 32 kDa non-fusion proteins identified in this study did not react with anti-M.tuberculosis polyclonal serum on Western blots. There are several possible reasons for this:

i. the inability of the available antibodies to recognise their target antigen in a Western blot

assay (i.e. the antigens were denatured during electrophoresis);

ii. the polypeptides are unstable in E.coli and subjected to proteolysis or other post-translational modifications;

iii. the concentration of the antibodies directed against these proteins is too low to permit detection of the recombinant antigens.

In addition, the sonicated M.tuberculosis antigens, used to immunise the rabbits for the production of antibodies, were denatured during the autoclaving step of the preparation. This raises the possibility that an antibody response to all the mycobacterial antigens in their native form was not mounted.

Cohen, Mayer, Rumschlag et al., (1987) succeeded in producing 3 recombinant E.coli strains, using pUC 13 as the vector, which produced the same 2 M.tuberculosis 35 kDa and 53 kDa non-fusion proteins. The sizes of these proteins are very similar to the 32 kDa and the 50 kDa non-fusion proteins detected in the present study. The reactivity of both proteins to rabbit antiserum produced against only the 35 kDa protein suggested that these were polymers, subunits, or degradation products of the same protein. Young, Kent, and Young,(1987) reported the isolation of a 52 kDa protein, recognised by polyclonal anti-M.tuberculosis serum, that was also recognised by a monoclonal antibody directed against a 71 kDa

mycobacterial protein. These findings led to the speculation that the 52 kDa protein was a low molecular weight form of the 71 kDa protein. Low molecular weight products of a particular protein could result from loss of the terminal region of a protein due to cloning of a partial gene in the insert DNA (Young, Kent, and Young, 1987), or they may arise from post-translational processing of original high molecular weight products (Young, Kent, and Young, 1987). Since the level of transcription and translation of non-fusion proteins cannot be controlled by external means, and since the host cells are deficient only in proteases which are active against mainly fusion proteins, the proteolysis of original high molecular weight non-fusion proteins to more stable and less toxic lower molecular weight forms is therefore a likely event.

Young, Kent, and Young (1987) also reported the isolation of a 14 kDa M. tuberculosis fusion protein from recombinant λ gt 11 clones using polyclonal antiserum. Lower molecular weight forms of this protein were also obtained. It has been suggested that it was possible that the 14 kDa protein itself was produced by partial breakdown of an original 25 kDa molecule (Young, Kent, and Young, 1987). Fusion proteins of molecular weights 22 kDa, 12 kDa, and 6 kDa were detected in the present study. It is tempting to speculate that the 22 kDa fusion protein isolated in the present study is analogous to the 25 kDa protein isolated by Young, Kent, and Young (1987).

and that the 12 kDa and 6 kDa fusion proteins are breakdown products of the 22 kDa protein.

By the same token, it is possible that the 50 kDa and the 32 kDa non-fusion proteins reported in the present study are analogous to the 53 kDa and 35 kDa non-fusion proteins described by Cohen, Mayer, Rumschlag, et al. (1987), which in turn may be related to the 71 kDa protein described by Young, Kent, and Young (1987).

However, further analysis of the antigens isolated in this study with monoclonal antibodies directed against fully characterised mycobacterial antigens is required to elucidate whether those antigens are in fact related to those reported by other laboratories. Characterisation using immunoprecipitation techniques and detailed restriction mapping and sequence analysis of the reading frames of the mycobacterial DNA inserts will also be required to further define the antigens expressed by recombinant clones, and to fully understand the origin of different molecular weight proteins produced by these clones. The 32 kDa non-fusion protein may be of particular interest for further study since a 35 kDa - 38 kDa non-fusion recombinant antigen of M.tuberculosis, containing epitopes recognised by B cells, has been reported (Cohen, Mayer, Rumschlag, et al., 1987). These epitopes were found exclusively on the M.tuberculosis

35 kDa - 38 kDa complex. Antibodies to this antigen were detected in 40% of patients with active tuberculosis (Cohen, Mayer, Rumschlag, et al., 1987). Furthermore, this antigen was able to stimulate T cells from tuberculosis patients, BCG vaccinees, and immunised guinea pigs (Cohen, Mayer, Rumshlag, et al., 1987).

Recent evidence has shown that several of the mycobacterial non-fusion proteins identified to date are unrepresentative of mycobacterial genes in general, and that they are in fact structural homologues of stress proteins (Hopwood, Kieser, Colston, et al., 1988). The amino acid sequences of these proteins are highly conserved across eukaryotes and prokaryotes, and their genes are transcribed by a specific form of RNA polymerase in E.coli (Young, 1988). Stress proteins are synthesised during periods of significant alterations in the patterns of metabolism when mycobacteria are exposed to adverse environmental conditions (Hopwood, Kieser, Colston, et al., 1988). The 71 kDa antigen of M.tuberculosis has been shown to be related to the dnaK protein of E.coli and has been demonstrated to be a member of the family of 70 kDa heat shock proteins (hsp 70) found throughout nature (Young, Lathigra, Hendrix, et al., 1988). The mycobacterial 65 kDa antigen has been shown to display strong sequence homology to GroEL, another major stress protein of E.coli (Shinnick, Vodkin, and Williams, 1988). The M.leprae 18 kDa antigen is related to a class of plant heat shock

proteins (Young, 1988). The evidence therefore indicates that recognition of stress proteins may be a significant factor in the immune response to mycobacteria. The 71 kDa antigen of M.tuberculosis, for example, has been shown to possess a significant degree of sequence homology with human hsp 70 proteins (Young, Lathigra, Hendrix, et al., 1988). It is therefore likely that immune responses to this antigen may be regulated to some degree by the self-tolerance mechanisms of the immune system (Young, 1988). A potential role for such conserved stress proteins can also be postulated in the induction of autoimmune responses in association with mycobacterial infection (Young, 1988).

Future studies based on the use of antigens which belong to the cross-reactive group of stress proteins and common antigens would have to be very carefully considered. In addition, reagents used would have to be carefully analysed to avoid complications arising from the conserved nature of these proteins. Detailed analysis of the synthesis of stress proteins within phagocytic cells, and the involvement of conserved and variable regions of such proteins in immune interactions will be important in the assessment of the role played by stress proteins during mycobacterial infection and immunity. Finally, the basis of screening recombinant mycobacterial libraries needs to be widened in order to facilitate the study of those antigens important for cell-mediated immunity against tuberculosis, and of complex

non-protein antigens. This includes the use of alternative host systems, and alternative screening procedures such as direct selection of recombinants by T cell activation tests (Lamb and Young, 1987; Oftung, Mustafa, Husson, et al., 1987). It is important that the probes which have already been developed - cloned genes (Picken, Plotch, Wang, et al., 1988), and defined antigenic determinants (Lu, Lien, Becker, et al., 1987; Andersen, Worsaae, and Chaparas, 1988; Radford, Duffield, and Plackett, 1988; Thole, Van Schooten, Keulen, et al., 1988) - should be used to assist in the investigation of the basic aspects of the immune response. This includes the analysis of the pathway of antigen processing from the level of transcription within the mycobacterium to cell surface presentation of the antigens by macrophages in conjunction with MHC molecules (Young, 1988). Questions of immunodominance of individual determinants in the context of different MHC haplotypes, and the possible association of specific T cell functions with particular antigen-MHC combinations could also be addressed (Young, 1988). The ultimate aim is to identify an immune parameter - antibody or T cell response - which is uniquely associated with either protective or defective immunity to M.tuberculosis, and to thoroughly characterise the antigenic determinant(s) associated with this response so that they can be manipulated to the advantage of better understanding, and eventually eliminating, mycobacterial infection.

Conclusion

In the present study, an *M.tuberculosis* recombinant DNA library was constructed in the expression vector λ gt 11. Nine recombinant phage clones which produced proteins recognised by anti-*M.tuberculosis* serum and anti-β-galactosidase antibodies were isolated. These phage clones were found to produce 2 non-fusion proteins of molecular weights 32 kDa and 50 kDa, and also produced β-galactosidase fusion proteins of molecular weights 22 kDa, 12 kDa, and 6 kDa. These proteins are similar, in size and reactivity patterns with anti-*M.tuberculosis* polyclonal serum, to recombinant proteins identified by other investigators using similar vector systems. It has been suggested that the non-fusion proteins identified by other investigators may be stress proteins, and may belong to the family of common antigens and heat-shock proteins: the same may be true of the non-fusion proteins identified in the present study. The recombinant proteins isolated in this study are currently being investigated as possible tools for the study of cell-mediated immunological responses to tuberculosis, and as potential reagents for the immunodiagnosis of tuberculosis.

CHAPTER 5 T CELL RESPONSES TO RECOMBINANT M.tuberculosis PROTEINS

Note:

The present experiments are preliminary studies performed to determine whether the recombinant mycobacterial antigens identified in this study (the 50 kDa and 32 kDa non-fusion proteins, and the 22 kDa, 12 kDa, and 6 kDa fusion proteins) contain epitopes recognised by T lymphocytes, and are capable of stimulating M.tuberculosis-sensitised lymphocytes from tuberculosis patients. Since these experiments are outside the scope of this dissertation, clearance to use human blood for these studies was not requested. The preparation of mononuclear cells from tuberculosis patients and healthy volunteers, and proliferation assays using these cell preparations, were therefore performed in conjunction with Dr. A.A.Wadee in the Department of Immunology, South African Institute for Medical Research, and University of the Witwatersrand, Johannesburg.

5.1 INTRODUCTION

The immunological response and resistance to mycobacterial infection is mediated primarily by the cellular immune system, and it is therefore important to identify recombinant antigens that are able to elicit a human T cell response. The recent development of an SDS-PAGE assay for screening T cell populations (Young and Lamb, 1986) provided the possibility of such an approach. The technique involves immobilising recombinant antigens onto nitrocellulose by Western blotting, cutting out the required protein band, adding the excised bands directly to lymphocyte cultures, and measuring lymphocyte proliferation. T cell activation by nitrocellulose-bound antigen has been found to be accessory cell dependent, and it is suggested that the mechanism for the T cell response involves active uptake of the solid-phase antigen by accessory cells, rather than the leaching of antigen from the nitrocellulose particles (Young and Lamb, 1986).

Abou-Zeid, Filley, Steele, et al., (1987) have modified the technique by converting the excised nitrocellulose strips into antigen-bearing particles small enough to be engulfed by macrophages. This technique enables the preparation of nanogram quantities of antigen from single protein bands. After transferring the antigens to nitrocellulose, the blots are stained to make visualisation of the required bands

possible. Several different staining protocols exist, including colloidal gold, coomassie blue, amido black, and Indian ink. The colloidal gold stain was found to be the stain of choice (Abou-Zeid, Filley, Steele, et al., 1987). Of the stains tested, it was the only one which combined lack of cell toxicity with low background proliferation. The choice of solubilising agent for the nitrocellulose is also critical (Abou-Zeid, Filley, Steele, et al., 1987): DMSO was chosen among other organic solvents (such as acetone or pyridine) as being the least toxic to the cells in proliferation assay, and unlikely to damage the antigens on the nitrocellulose. DMSO also had the added advantage of sterilising the solubilised antigens.

Direct screening of human clonal or polyclonal T cell populations with immobilised mycobacterial antigens has resulted in the demonstration that antigens previously identified with polyclonal or monoclonal antibodies, or expressed by recombinant DNA clones, are able to be recognised by sensitised T lymphocytes. Lamb and Young (1987) identified human T cell epitopes on mycobacterial proteins 16 kDa - 18 kDa, 18 kDa - 20 kDa, and 52 kDa - 55 kDa in size. Oftung, Mustafa, Husson, et al. (1987) showed that recombinant M.tuberculosis of 65 kDa and 19 kDa were able to stimulate lymphocytes from tuberculosis patients. Recombinant mycobacterial proteins 65 kDa, 35 kDa, and 28 kDa in size were shown to be able to induce a proliferative response in T cells

from the pleural effusions of tuberculosis patients (Damiani, (Biano, Beltrame, et al., 1988), indicating the presence of T cell epitopes on these antigens.

The aim of the present preliminary experiment is to determine in vitro, whether lymphocytes from tuberculosis patients and healthy donors are able to recognise any of the recombinant mycobacterial antigens identified in this study.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of solubilised antigens

Solubilised antigens were prepared essentially as described by Abou-Zeid, Filley, Steele, *et al.*, (1987). Proteins in recombinant lysates from clones TB 15, TB 16, TB 17, and TB 23 were resolved by SDS-PAGE using a 10% final acrylamide concentration as described in Section 2.9. Fifty ug of protein were loaded per lane. Fifty ug of normal, non-induced *E.coli* Y 1089 lysate were also electrophoresed. After completion of electrophoresis, the proteins were transferred to nitrocellulose sheets (Hybond C, 0.5 um; Amersham International, Buckinghamshire, England) by Western blotting as described in Section 2.10. An additional sheet of nitrocellulose was inserted between the filter paper and the gel on the cathode side of the blotting sandwich, in order to block the transfer of impurities from the filter paper onto the nitrocellulose. The presence of impurities on the blot give rise to improper staining of proteins, and high background staining.

After completion of Western blotting, the nitrocellulose sheet was incubated with PBS (Appendix 8) supplemented with 0.3% Tween 20 at 37^0C for 30 minutes. The blot was then washed 3 times, 5 minutes each wash, in 0.3% PBS-Tween, at room temperature. The blot was agitated gently during the washing

steps and during incubation with the stain. The washed blot was drained off, washed briefly with distilled water, and incubated in Aurodye forte colloidal gold solution (Janssen Life Sciences Products) for 12 hours at 37^0C. The stained blot was drained, rinsed briefly with distilled water, and dried between 2 sheets of filter paper.

The 50 kDa, 32 kDa, 22 kDa, 12 kDa, and 6 kDa recombinant protein bands were excised from the blot, divided into small pieces, and placed separately in glass vials. Normal 65 kDa and 25 kDa E.coli protein bands, as well as a band of nitrocellulose containing only background stain, were also excised and solubilised and used as controls. Two hundred and fifty ul of DMSO (Merck, Darmstadt, W.Germany) were added to each vial, and the mixtures left for 60 minutes at room temperature for complete dissolution of the nitrocellulose strips. The nitrocellulose particles were then precipitated from solution by adding an equal volume (250 ul) of filter-sterilised carbonate/bicarbonate buffer, pH 9.6 (the same buffer used for coating ELISA plates; appendix 8), dropwise very slowly while vigorously vortexing. After precipitation, the particle suspensions were transferred to sterile microcentrifuge tubes and centrifuged at 8 500 g for 5 minutes. The supernatants were carefully removed, the particles resuspended in RPMI 1640 (Highveld Biologicals, S A), and re-centrifuged at 8 500 g for 5 minutes. The particles were washed 3 times with RPMI 1640, and after the

steps and during incubation with the stain. The washed blot was drained off, washed briefly with distilled water, and incubated in Aurodye forte colloidal gold solution (Janssen Life Sciences Products) for 12 hours at 37^0C. The stained blot was drained, rinsed briefly with distilled water, and dried between 2 sheets of filter paper.

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final wash, particles were resuspended in 1 ml of the same medium. The suspensions were used immediately in lymphocyte proliferation assays, and thereafter stored at -20^{0}C.

5.2.2 Mononuclear cell preparation

Venous blood from patients suffering from clinically diagnosed tuberculosis and from healthy volunteers was collected into preservative-free heparin (Panheparin, Abbott; 10 units/ml). The cells were separated by centrifugation on a Hypaque-Ficoll gradient and the mononuclear cell fraction at the plasma-Ficoll interphase was collected. The cells were washed 3 times in RPMI 1640, and the cell count was adjusted to 1 X 10^6/ml in RPMI 1640 supplemented with 10% heat-inactivated (56^{0}C for 30 minutes) foetal calf serum (Highveld Biologicals S.A.).

5.2.3 Lymphocyte transformation

Mononuclear cells were cultured in round-bottomed 96-well microtitre plates, at a concentration of 2 X 10^5 cells per well, with whole M.tuberculosis sonicate (10 ug/ml; 20 ul/well), undiluted solubilised recombinant antigens (50 kDa, 32 kDa, 22 kDa, 12 kDa, and 6 kDa, 20 ul/well), undiluted solubilised control antigens (.5 kDa and 65 kDa; 20 ul/well) or solubilised nitrocellulose. All experiments were performed in triplicate. Plates were incubated at 37^{0}C in 5% humidified air for 5 days. Tritiated thymidine (methyl-

^{3}H-thymidine, specific activity 24 ci/uMole; Radiochemical Centre, Amersham, England) at 0.5 uCi per well was added for the final 18 hours of culture, after which the cells were harvested onto glass fibre filters. The filters were dried overnight at 37^{0}C, 5 ml of Instafluor (Chemlab) were added per filter, and the incorporated radioactivity measured in a Packard Tri-carb liquid scintillation counter. Counts were expressed as mean cpm of triplicate culture results.

5.3 RESULTS

Periphereal blood mononuclear cells from 3 patients with pulmonary tuberculosis were incubated in the presence of the 50 kDa, 32 kDa, 22 kDa, 12 kDa, and 6 kDa recombinant antigens to assess whether any of these antigens contained epitopes capable of being recognised by sensitised T lymphocytes. Several control systems were employed in this study: control cultures included solubilised nitrocellulose, two solubilised E.coli proteins of molecular weights 65 kDa and 25 kDa, and an M.tuberculosis sonicate previously demonstrated in this laboratory (Wadee, Cohen, and Rabson, 1989) to induce proliferative responses in mononuclear cells from tuberculosis patients. The results of the lyphoproliferation studies (Table 11) indicate that the patients' lymphocytes undergo blastogenesis in response to the M.tuberculosis sonicate (Table 11, line 2) and to the 32 kDa recombinant antigen (Table 11, line 9), but do not proliferate in response to E.coli antigens or nitrocellulose. Mononuclear cells from normal healthy volunteers who were not previously exposed to tuberculosis did not proliferate to any of the antigens tested (Table 11). The lymphoproliferative ability of the mononuclear cells from the patients and the controls was determined in a separate experiment, where it was shown that all of the individuals tested (patients and controls) responded well to the antigens of Candida albicans (results not shown).

Table 11 Proliferative responses of mononuclear cells from 3 patients with tuberculosis to various recombinant M.tuberculosis antigens

cell system	cpm (^{3}H-thymidine incorporated) Patient 1	Patient 2	Patient 3	mean of 3 normals
mononuclear cells alone	954	1 922	1 986	1 784
mononuclear cells + M.tuberculosis sonicate	12 108	24 191	26 307	2 762
nitrocellulose alone	1 032	1 736	2 070	2 726
E.coli 25 kDa	1 025	1 890	1 908	2 169
E.coli 65 kDa	1 532	2 080	2 118	1 577
M.tuberculosis 6 kDa	1 550	2 504	1 306	2 167
M.tuberculosis 12 kDa	1 230	1 904	1 476	2 937
M.tuberculosis 22 kDa	1 274	2 276	1 494	1 453
M.tuberculosis 32 kDa	7 078	7 720	10 763	1 569
M.tuberculosis 50 kDa	1 653	2 036	2 280	2 527

5.4 DISCUSSION

The results of the T cell proliferation assays presented in this latter section indicate that the 32 kDa recombinant mycobaterial protein is capable of activating lymphocytes from tuberculosis patients *in vitro*. The 32 kDa protein is able to significantly stimulate immune lymphocytes (it is able to induce almost half as much stimulation as that induced by the positive control containing the mycobacterial sonicate): this suggests that the recombinant 32 kDa protein is a potent T cell immunogen. The description of specific mycobacterial proteins which are immunogenic is by no means novel; several reports describing specific antigens recognised by T cells, including recombinant antigens expressed in *E.coli*, have recently appeared. Recombinant mycobacterial antigens demonstrated to be immunogenic include proteins of molecular weights 14 kDa, 16 kDa - 19 kDa, 18 kDa - 20 kDa, 32 kDa, 36 kDa - 38 kDa, 52 kDa - 55 kDa, and 65 kDa (Emmrich, Thole, Van Embden *et al.*, 1986; Lamb and Young, 1987; Mustafa, Gill, Nerland, *et al.*, 1986; Oftung, Mustafa, Husson, *et al.*, 1987; Kingston, Salgame, Mitchison, *et al.*, 1987; Lamb, Kingston, Estrada-G, *et al.*, 1988; Cohen, Mayer, Rumslach, *et al.*, 1987).

Immunogenic antigens of similar molecular weight as the antigen described in the present study (32 kDa) have previously been described. Huygen, Palfliet, Jurion, *et al.*,

(1988) demonstrated that lymphocytes from 71% of patients with active tuberculosis proliferated to a 32 kDa protein antigen present in mycobacterial culture filtrates. Young, Kent, Rees, et al., (1986) described a 38 kDa protein antigen which induced T cell proliferative responses in 60% of tuberculosis patients and healthy BCG-vaccinated subjects. Cohen, Rumslach, Mayer, et al., (1987) have described a non-fusion recombinant 35 kDa - 38 kDa protein antigen which was able stimulate T cells from tuberculosis patients, BCG-vaccinees, and immunised guinea pigs. The findings described in the present study, together with those described above, suggest that immunodominant T cell-stimulatory mycobacterial determinants exist in a M.tuberculosis antigen of molecular weight 30 kDa - 40 kDa.

The preliminary results reported herein also confirm previous reports indicating that SDS-PAGE immunoblotting could be used effectively for the analysis of the cellular immune response to a pathogenic agent during human infection (Young and Lamb, 1986; Lamb and Young, 1987).

Screening for the presence of specific gene products expressed in recombinant DNA libraries has been shown to be readily accomplished using λ gt 11 vectors in conjunction with antibody screening procedures (Young and Davis, 1983). The results reported in the present study suggest the possibility of developing analogous screening procedures using sensitised

T lymphocytes. This would facilitate the detection of cloned M.tuberculosis genes expressing antigens involved in protective or pathogenic interactions with the host. However, the present findings must be interpreted with caution, as the number of patients used are few, and the experiments need to be repeated. In addition, the recombinant 32 kDa antigens identified in this study needs to be further characterised. It is, however, tempting to speculate that this protein is similar to the previously described (Huygen, Palfliet, Jurion, et al., 1988; Cohen, Mayer, Rumschlag, et al., 1987; Young, Kent, Rees, et al., 1987) proteins, ranging in size from 32 kDa - 38 kDa, which have been shown to elicit blastogenic responses from sensitised lymphocytes. If it is determined that these antigens are in fact all the same, it would lend credibility to the hypothesis that mycobacterial antigens of molecular weights 30 kDa - 40 kDa are immunodominant proteins which may prove useful for the diagnosis and prevention of tuberculosis, and may help elucidate the mechanisms of both the humoral and cellular immune response to M.tuberculosis.

APPENDICES

APPENDIX 1 AGAROSE GEL ELECTROPHORESIS

TEA electrophoresis running buffer (10 X)

Tris (BDH, Poole, England)	42.0 g
EDTA (disodium salt) (BDH, Poole, England)	37.2 g
Glacial acetic acid	57.1 ml

Make up to 900 ml with distilled H_2O, adjust the pH to 8.2 using acetic acid, then top up to 1 litre with distilled H_2O Store at room temperature.

Bromophenol blue tracking dye for agarose gels (10 X)

50% sucrose (BDH, Poole, England)

50 mM EDTA (disodium salt)

10% Ficoll 400 (Pharmacia, Uppsala, Sweden)

0.1% bromophenol blue

(Biorad Laboratories, Richmand, CA)

Make up in distilled H_2O. Store at room temperature.

Agarose gels

The technique for casting and running agarose gels is essentially that described by Maniatis, Fritsch, and

Sambrook, (1982). Make up the required percentage agarose (Sigma Chemical Co., St. Louis, MO, USA) in 1 X TEA buffer. Dissolve the agarose completely by heating. Cool to 56°C. Add 3 ul ethidium bromide (10 mg/ml stock in distilled H_2O; Boehringer Mannheim, W.Germany) per 100 ml agarose. Mix well and pour carefully into the casting slabs. allow the gel to set completely at room temperature. Place the gel in the electrophoresis tank, and fill the tank with sufficient 1 X TEA buffer to completely cover the gel. Mix the sample to be electrophoresed with 1/10 volume bromophenol blue tracking dye, and load the sample into the wells. Carry out electrophoresis at 5 V/cm at room temperature until the dye front has migrated the required distance. Remove the gel from the electrophoresis tank, and view under ultraviolet light.

APPENDIX 2 ENZYME BUFFER STOCKS

Eco R1 methylase buffer

100 mM Tris-HCl, pH 8.0; 100 mM NaCl; 1 mM EDTA.
Make up fresh when required.

T4 DNA ligase buffer

50 mM Tris-HCl, pH 7.8; 10 mM $MgCl_2$; 20 mM dithiothreitol (Boehringer Mannheim, W.Germany); 1 mM ATP (Boehringer Mannheim, W.Germany).
Make up fresh when required.

Eco R1 buffer

100 mM Tris-HCL, pH 7.5; 50 mM NaCl; 5 mM $MgCl_2$.
Make up fresh when required.

APPENDIX 3 SPECTROPHOTOMETRIC QUANTIFICATION OF DNA

Both DNA and RNA absorb light in the ultraviolet between 250 nm and 270 nm, owing to the spectral characteristics of the 4 bases. At 260 nm, an absorbance of 1 measured in a cuvette with a 1-cm path length is indicative of double-stranded DNA at a concentration of approximately 50 ug/ml, or of RNA or single-stranded DNA at a concentration of approximately 40 ug/ml (Berger, 1987).

The ratio of the absorbance at 260 nm to 280 nm is an indication of nucleic acid purity. Values of 1.8 to 1.9 for DNA solutions, and 1.9 to 2.0 for RNA solutions, are indicative of pure solutions. The presence of protein, which absorbs at 280 nm, decreases the ratio, as does phenol, another likely contaminant of nucleic acid solutions (Berger, 1987).

To measure the absorbance of DNA solutions, dilute a small aliquot of the solution 100-fold with distilled water. Measure the absorbance of the diluted preparation in a spectrophotometer (Varian DMS 70 UV/Visible spectrophotometer) at both 260 nm and 280 nm wavelengths, using a distilled water blank.

APPENDIX 4 LURIA-BERTANI MEDIA

Luria-Bertani broth (LB broth)

Tryptone (Difco Laboratories, Detroit, USA)	10 g/l
Yeast extract (Difco Laboratories, Detroit, USA)	5 g/l
NaCl (Univar, S.A.)	10 g/l

Dissolve in distilled H_2O, adjust the pH to 7.0, and sterilise by autoclaving.

Luria-Bertani agar (LB agar)

Tryptone (Difco Laboratories, Detroit, USA)	10 g/l
Yeast extract (Difco Laboratories, Detroit, USA)	5 g/l
NaCl (Univar, S.A.)	10 g/l
Agar (Difco Laboratories, Detroit, USA)	15 g/l

Dissolve in distilled H_2O, adjust the pH to 7.0, and sterilise by autoclaving.

Luria-Bertani molten agar

Tryptone (Difco Laboratories, Detroit, USA)	10 g/l
NaCl (Univar, S.A.)	5 g/l
Yeast extract (Difco Laboratories, Detroit, USA)	5 g/l
$MgSO_4$ (Univar, S.A.)	2.5 g/l
Agar (Difco Laboratories, Detroit, USA)	10 g/l

Dissolve in distilled H_2O, adjust the pH to 7.0, and sterilise by autoclaving.

APPENDIX 5 PEROXIDASE SUBSTRATE FOR ELISA

Substrate buffer

Citric acid (BDH, Poole, England)	1.029 g/100 ml
Na_2HPO_4 (BDH, Poole, England)	1.448 g/100 ml

Dissolve in distilled H_2O, adjust the pH to 5.0, and store in the dark. Make up fresh daily.

Substrate

Immediately prior to use, add 50 ul of cold 30% H_2O_2, and 34 mg O-phenylenediamine (Sigma Chemical Co., St.Louis, MO, USA), to 100 ml substrate buffer.

APPENDIX 6 PEROXIDASE SUBSTRATE FOR IMMUNOBLOTS

Solution A

60 mg 2-chloro-1-naphthol (Biorad Laboratories, Richmond, CA) in 20 ml ice-cold methanol. Dissolve immediately prior to use, and protect from light.

Solution B

Immediately prior to use, add 60 ul of ice-cold 30% H_2O_2 to 100 ml Tris-buffered saline (see appendix 8).

Substrate solution

Mix solutions A and B at room temperature, and use immediately.

APPENDIX 7 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS REAGENTS

1. 1 M Tris-HCl buffer, pH 8.8

2. Electrophoresis running buffer (10 X stock)

Tris (BDH, Poole, England)	30.3 g
Glycine (Merck, Darmstadt, W.Germany)	144.1 g
SDS (BDH, Poole, England)	10.0 g

Make up to 900 ml with distilled H_2O, adjust the pH to 8.8, and top up to 1 litre with distilled H_2O.

3. Acrylamide stock solution (30%)

Acrylamide (Biorad Laboratories, Richmond, CA)	30 g
Bis-acrylamide (Biorad Laboratories, Richmond, CA)	0.8 g

Dissolve in 100 ml distilled H_2O. Filter, store at 4°C and protect from light.

4. 10% SDS

Make up 100 ml stock solution of SDS (BDH, Poole, England) in distilled H_2O. Store at room temperature.

5. 1.5% Ammonium persulphate

Make up 1.5% ammonium persulphate (Biorad Chemicals, Richmond, CA) in glass distilled H_2O. Store at 4^0C, and use fresh daily.

6. TEMED (N N ,N'-N'-tetramethylethelenediamine) (Biorad Laboratories, Richmond, CA).

TABLE 12 Solutions for acrylamide slab gels

volumes of solutions for percentage acrylamide gels				
solution	7.5%	8.25%	10%	15%
acrylamide stock	14.03 ml	13.40 ml	20.10 ml	22.00 ml
1 M Tris-HCl, pH 8.8	22.50 ml	18.75 ml	22.50 ml	12.00 ml
distilled H_2O	19.90 ml	14.85 ml	13.80 ml	16.50 ml
10% SDS	0.60 ml	0,60 ml	0.60 ml	0.60 ml
1.5% ammonium persulphate	3.00 ml	3.00 ml	3.00 ml	4.00 ml
TEMED	20 ul	25 ul	30 ul	35 ul

APPENDIX 8 BUFFERS

Phosphate buffered saline (0.15 M)

NaCl (Univar, S.A.)	8.0 g
KCl (Univar, S.A.)	0.2 g
$Na_2HPO_4.2H_2O$ (BDH, Poole, England)	1.15 g
KH_2PO_4 (BDH, Poole, England)	0.2 g

Dissolve in 900 ml distilled H_2O. Adjust the pH to 7.2, make up to 1 litre with distilled H_2O, and sterilise by autoclaving.

Carbonate/bicarbonate buffer pH 9.6 (0.15M)

Na CO (BDH, Poole, England)	1.59 g
NaHCO (BDH, Poole, England)	2.93 g

Make up to 900 ml with distilled H_2O. Adjust the pH to 9.6, make up to 1 litre with distilled water, and sterilise by autoclaving. Store in aliquots at -20°C.

STE buffer

10 mM Tris-HCl, pH 7.5

100 mM NaCl (Univar, S.A.)

1 mM EDTA.$2H_2O$ (BDH, Poole, England)

Make up in distilled H_2O. Sterilise by autoclaving.

TE buffer

10 mM Tris-HCl, pH 8.0

1 mM EDTA (BDH, Poole, England)

Make up in distilled H_2O. Sterilise by autoclaving.

Tris-buffered saline (TBS)

50 mM Tris-HCl, pH 8.0

150 mM NaCl (Univar, S.A.)

Make up in distilled H_2O. Sterilise by autoclaving.

SM buffer

NaCl (Univar, S.A.)	5.8 g
$MgSO_4.7H_2O$ (BDH, Poole, England)	2.0 g
1 M Tris-HCl, pH 7.5	50 ml
2% gelatin (BDH, Poole, England)	5 ml

Make up to 1 litre with distilled H_2O. Sterilise by autoclaving, and store in 50-ml aliquots.

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