Murine T-cell reactivity to cloned *Mycobacterium leprae* antigens

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The overall aim of the project is to characterise the T-cell reactive proteins of *Mycobacterium leprae* by use of murine T-cells and recombinant DNA expressed antigens. For this purpose we are screening an *M. leprae* genomic DNA library made (Young et al., *Nature* 316, 450, 1985) in λ gt11 bacteriophage and expressed in *Escherichia coli* as fusion proteins with prokaryotic β -galactosidase. Our approach is rather different to that of other groups who are examining the library in a more selective manner by taking B-cell defined antigens and testing them against sets of T-cell clones (Godal et al., *Nature* 319, 63, 1986) but we think it important to have a more general screening procedure. The feasibility of shot gun screening for T-cell reactive epitopes is important to determine as it might allow a larger selection window for T-cell reactivity. This approach is not without its drawbacks because of background proliferation with λ gt11 lysates. However we have tried to develop an assay system using nitrocellulose discs and partially purified lysates to obtain an increase in the signal to noise ratio.

Materials and methods

PHAGE AND BACTERIAL STRAINS

M. leprae library in λ gt11, *E. coli* strains Y-1088 and Y-1089 were obtained from R. Young (Boston).

FRACTIONATION AND AMPLIFICATION OF 10 000 RANDOMLY SELECTED PHAGE FROM THE *M. LEPRAE* LIBRARY

10·000 phage were randomly picked from the *M. leprae* library and divided into 10 equal pools. Each pool was further subfractionated to ten minipools. A total of 111 pools were obtained. Each pool was amplified on *E. coli* Y-1088 at 42°C, before fractionation.

GENERATION OF RECOMBINANT LYSOGENS IN EACH MINIPOOL AND PREPARATION OF PARTIALLY PURIFIED LYSATES

Overnight grown Y-1089 cells were infected with phage from each minipool at a multiplicity of approximately 50 for 20 mins at 32°C supplemented with MgCl₂. To each pool 10 ml of Luria Bertani (LB) Broth was added and incubation carried on overnight. Fresh 10 ml broth was inoculated with 100 ul of overnight grown cultures and incubated for 6 hours at 32°C. Cultures were rapidly transferred to 42°C for 20 mins, isopropyl β -D thiogalactopyranoside (IPTG) was added to 10 mM and the cultures incubated overnight at 37°C. The cells were harvested at 5000 rpm for 5 mins and lysed in Tris buffer pH 8 containing lysozyme

(1mg/ml), EDTA (0.2,M) and NaCl (50 mM). The lysed cells were transferred to eppendorf tubes and microfuged. The *E. coli* proteins in the cell debris was extracted with 0.1 % sodium deoxycholate (2X) and Triton-X (1 %). The insoluble pellet was then solubilised in Tris buffer containing 1 % SDS. Protein content in each pool was measured by the Bradford method and adjusted to 2mg/ml.

PREPARATION OF PLATES WITH ANTIGEN ABSORBED ON NITROCELLULOSE DISCS

Nitrocellulose discs were placed in each well of flat-bottom microtitre plates. Antigen was added in 10 μ l to each well. Cells were assayed in triplicate with four antigen concentrations ranging from 0.01 μ g to 10 μ g/ml. The plates were allowed to dry at 37°C and sterilised by exposure to UV for 20 mins. Each well was rinsed twice with media and the protein content in the washings estimated. Control wells contained whole irradiated *M. leprae*, Concanavalin A, lysate of λ gt11 clone containing no insert, media alone and media containing the same concentration of SDS as present in the antigen preparations.

IMMUNISATION AND PROLIFERATION ASSAY

BIOM mice were immunised subcutaneously at the base of the tail with 1×10^7 whole irradiated *M. leprae* emulsified in Freund's incomplete adjuvant. The mice were boosted after 2 weeks and a week later the inguinal and periaortic lymph nodes were harvested. Each well of a microtitre plate received 4×10^5 lymph node cells in 200 μ l of Iscove's medium containing 10 % foetal calf serum and the proliferative activity measured.

SENSITIVITY OF THE ASSAY

This was tested by a simple titration experiment whereby the number of positive clones required in a pool of negative clones to give a good signal was determined. To increasing numbers of λ gt11, constant numbers of a positive clone was added and the antigens expressed and assayed for proliferation using the above method.

Results

ANTIGEN ABSORPTION TO NITROCELLULOSE DISCS

The washings from each well was found to be negative when tested for protein suggesting that the antigens absorb strongly to nitrocellulose.

SENSITIVITY OF THE ASSAY

As observed in Table 1 at antigen concentrations of 0.1 μ g a good proliferative signal was obtained at a ratio of 1:25 of positive to negative clones. Even at a ratio of 1:200 there was still a positive signal. With increase in antigen concentration the detection limit went up; however the sensitivity of the assay decreased.

RANDOM SCREENING

The original pool with 10 000 phage was negative when tested for proliferation. Of the 10 pools in the first fractionation 4 were positive. 23 and 30 positive pools were obtained with antigen concentrations of 0.1 and 1 μ g/ml protein respectively. Data have been presented for results obtained with antigen concentration of 0.1 μ g. (Table 2).

Table 1

Ratio of +ve to -ve	Stimulation index $\mu\text{g/ml}$ of partially purified lysate					
	0.01		0.1		10	
	Expt 1	2	1	2	1	2
Only +ves	1.4	0.8	2.7	1.8*	18	8.6
1:1	1.4	0.7	0.9	1.26	14	16.7
1:10	4.9	1.4	2.8	2.5*	25	27.6
1:25	1.9	1.1	2.2	1.5*	23	30
1:50	2.6	1.0	1.6	2.6**	23	52
1:100	2.0	1.2	2.0	1.9*	10	10
1:200	1.6	0.7	1.8	1.7*	22	25.9
1:500	1.5	0.6	1.3	0.6	15	21.2
1:1000	0.7	0.6	1.5	0.5	8	16.1
Only -ves	1.4	0.7	1.1	0.8	10.5	6.6

- a) +ve clone = clone Y.3155 picked from the *M. tuberculosis* library by a monoclonal antibody TB 68.
-ve clone = λ gt11 containing no insert.
- b) The recombinant antigens expressed in *E. coli* were partially purified and tested for proliferation with lymph node cells from *M. leprae* immunised mice. The assay was carried out with nitrocellulose discs.
- c) p values obtained by Student's t-test compare test cpm responses to λ gt11 (-ve clone only) cpm responses. Levels of significant differences are: * $p < 0.05$, ** $p < 0.01$.

Discussion

We have developed a technique for partially purifying the fusion proteins expressed in bacterial lysates and absorbing them onto nitrocellulose. The fusion proteins were partially purified by extractions with the weak detergents deoxycholate and Triton-X, solubilised in 1 % SDS and absorbed onto nitrocellulose discs and then put directly into the assay. From this source signals adequate for screening purposes have been obtained with T-cells from lymph nodes of mice immunised with whole *M. leprae*.

The titration experiments establish the frequency of positive clones required in a pool of negative ones to give an adequate signal. The numbers obtained here are useful in interpreting the fractionation data.

We are aware that our random screening procedure may have picked false positives at this stage, largely because we have not used purified fusion proteins. However the first round of screening successfully eliminates a large number of negative pools. With the titration experiments we got a positive signal even at 1:200 ratio of positive to negative clones and hence a negative signal with pools of 100 phages indicates that there is no positive clone in this pool as it is within the sensitivity of our assay system. However we may be missing clones that give a low signal since the titration experiments were carried out with a clone giving good proliferative signals. Dilution analysis of *M. leprae* primed cells and their reactivity towards the positive pools would confirm the positive nature of each pool.

Table 2. Fractionation of 10,000 phage randomly picked from the *M.leprae* library.

1st 2nd	10,000 phage (Original pool)									
	A	B	C	D	E	F	G	H	I	J
	1-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90	91-100
	Original Pool – 2.0 0.1 µg/ml									
	1	2	3	4	5	6	7	8	9	10
A	0.9	1.7	1.3	1.4	0.8	0.6	1.6	11.1	1.2	2.1
B	2.0	2.5	1.6	1.2	0.5	2.4	1.7	1.8	1.6	3.0
C	1.3	1.4	1.1	0.8	3.3	6.2	6.7	2.5	6.2	4.4
D	5.0	1.8	1.3	3.6	4.6	8.6	8.2	3.0	2.8	1.8
E	5.6	7.3	2.1	1.6	2.8	2.3	1.0	0.6	0.5	0.5
F	4.6	7.0	0.8	1.2	–	–	4.0	3.2	0.6	0.4
G	2.4	2.2	3.7	1.5	–	3.5	1.6	2.0	4.4	4.1
H	3.7	4.0	4.0	7.7	3.6	4.1	6.0	5.6	2.9	10.6
I	2.1	1.9	2.1	2.1	1.5	2.4	7.8	4.4	1.3	1.8
J	1.3	1.1	2.9	3.2	9.0	0.9	1.9	4.3	1.3	2.2

The recombinant antigens expressed in *E.coli* of each pool were partially purified and tested for proliferation with lymph node cells from *M.leprae* immunised mice.

Data have been represented as stimulation index (cpm test/cpm control). The circled numbers are the positive pools. S.I. of λgt11 lysate was 2.1 and pools having an S.I. twice that of λgt11 have been considered as positive pools.

cpm of *M.leprae* (5×10^8 /ml) stimulated culture was $19,889 \pm 5,894$; ConA ($2.5 \mu\text{g/ml}$) $134,376 \pm 35,014$; λgt11 lysate $8,083 \pm 1,213$ and media alone was $3,780 \pm 1,180$.

Experiment has been duplicated with all the positive clones and one negative clone A3 with similar results.

The important question now is whether the antigens picked by our screening belong to the five antigen proteins that have been so far identified by the existing panels of monoclonal antibodies and T-cell clones. We are in the process of carrying out an immunoscreening of the positive pools with a mixture of *M. leprae* monoclonal antibodies. An important step would be achieved if we can show this and from there the technique could be adopted for screening larger pools and possibly be used in field trial studies.