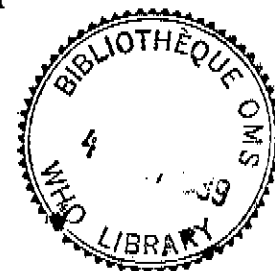




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REPORT OF THE SEVENTH MEETING OF THE SCIENTIFIC
WORKING GROUP (SWG) ON THE IMMUNOLOGY OF LEPROSY

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1.	SCIENTIFIC REPORT	

1.1 Introduction

The seventh meeting of the Scientific Working Group (SWG) on the Immunology of Leprosy (IMMLEP) was held in Geneva, 9-11 June 1987, under the auspices of the UNDP/WORLD BANK/WHO Special Programme for Research and Training in Tropical Diseases (TDR). Dr S.K. Noordeen opened the meeting with a brief review of the progress achieved by IMMILEP over the past five years. He described the setting up of field trials on the killed Mycobacterium leprae vaccine and progress in basic research on the development of second generation vaccines and immunodiagnostic tests, and announced the establishment of a Molecular Biology Subcommittee for the application of molecular biology to the immunology of leprosy. Dr Noordeen said that progress in the understanding of immunological mechanisms in leprosy was most satisfactory but that relatively little progress has been made towards an understanding of the pathology of nerve damage. He stressed the importance of relating research efforts to the clinical needs of leprosy control and management.

The Chairman of the meeting, Dr J. Ivanyi, pointed out that the main purpose of the meeting was to provide an update on recent progress in the immunology of leprosy and to outline priorities for future research.

1.2 Structural and Biochemical Aspects of M. leprae

The current state of knowledge concerning the biochemical structure of M. leprae, particularly the mycobacterial cell wall, was reviewed by Dr P.J. Brennan. The peptidoglycan content of mycobacteria is around 6% of total weight, a value closer to that of gram-negative than gram-positive bacteria. The detailed structure of M. leprae peptidoglycan is known from the work of Dr P. Draper (1). Recent research in Dr Brennan's laboratory has elucidated most of the structural features of the cell wall arabinogalactan, including the surprising finding that in contrast to all other complex polysaccharides it consists exclusively of carbohydrate residues in furanose form (2,3). The structure of lipoarabinomannan (LAM) has also been almost completely determined and shown to consist of inositol phosphate, glycerol and short- and long-chain fatty acids, in addition to arabinose and mannose (4). The presence of large amounts of arabinogalactan and lipoarabinomannan suggests that 5-linked arabinofuranosyl residues represent the major antigenic determinant of the M. leprae cell wall. A view was presented of the mycobacterial

cell wall structure in which mycolic acids combine with mycocerosic acids (from phenolic glycolipid and phthiocerol dimycocerosate) to form a structure analogous to the outer membrane of gram-negative bacteria.

Recent research has revealed the presence of a high concentration (about 26% of total cell wall weight) of firmly bound protein in the *M. leprae* cell wall. This material is not extracted by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and has an amino-acid composition quite distinct from that of peptidoglycan. By Western blot analysis it has a molecular weight of over 200K. Preparations have been prepared without the cell wall mycolates arabinogalactan and peptidoglycan. Drs B. Bloom and R.L. Modlin have recently shown that this cell wall protein is able to stimulate human T-cell proliferation and delayed-type hypersensitivity (DTH) responses in guinea pigs.

In discussion, Drs T.M. Buchanan, R.A. Young and A.S. Mustafa raised questions about the recognition of cell wall protein by monoclonal antibodies (MABs). Dr Brennan said that preliminary experiments have indicated that some MABs that recognize the 65K antigen show binding to the high molecular weight cell wall protein on Western blots.

1.3 Analysis of *M. leprae* Antigens Using Monoclonal Antibodies (MABs)

Dr A.H.J. Kolk described efforts to develop new MABs to *M. leprae* antigens. Western blots using sera from patients demonstrate antibodies to a wide range of proteins. Some of the antibodies are specific to mycobacteria; others can be removed by absorption with *Escherichia coli*. Polyclonal antisera raised in different mouse strains differ in their pattern of antigen recognition during Western blotting and such strain differences can be exploited in producing MABs to novel antigenic molecules. A new set of MABs produced in Balb/c mice was described. Most of these are directed to the same proteins as those identified by earlier MABs. Particular attention has been given to antibodies directed to the 36K antigen: some are specific for *M. leprae*, while others crossreact with *M. tuberculosis*.

Dr Buchanan reviewed the application of monoclonal antibody technology to the immunology of leprosy. He described new approaches to the preparation of anti-*M. leprae* MABs, involving immunization of mice with proteins prepared by recombinant DNA technology or with synthetic peptides. Both approaches have been used in his laboratory to generate new MABs to the 65K antigen. Synthetic peptides are used to elute immunoaffinity columns prepared with MABs and particularly good results have been obtained with the IIC8 antibody and its corresponding peptide epitope. A large number of synthetic peptides covering the whole 65K antigen have been prepared and used to carry out detailed mapping and analysis of epitopes recognized by many of the anti-65K MABs (5).

Dr J. van Embden also described the mapping of antibody epitopes on the 65K antigen with a combination of deletion mutations and beta-galactosidase fusions based on the recombinant antigen from *M. tuberculosis*. Ten different specificities have been successfully mapped, but one MAB binds only to the intact protein, not to any of the fragmented antigens.

1.4 T-cell Recognition of Mycobacterial Antigens

1.4.1 Identification of antigenic molecules

Recent research on the identification of antigens and determinants recognized by human T cells was described by Dr D.B. Young. Three approaches are used to analyse proteins capable of stimulating T-cell proliferation. The first involves separation of proteins by SDS-PAGE and determination of T-cell recognition of particular molecular weight bands. This provides an overall

survey of recognition of many different antigens by polyclonal lymphocytes, as well as making it possible to identify novel antigens recognized by individual T-cell clones. A second approach involves purification of particular antigens from mycobacterial extracts using MABs. There are technical difficulties in applying this approach to the purification of antigens from *M. leprae* extracts. The third approach is to measure T-cell proliferation in response to antigens expressed in *E. coli* from recombinant DNA. Dr Young described the effect of nonspecific inhibitory factors in crude *E. coli* extracts and outlined several techniques for presenting recombinant antigens in T-cell assays. T-cell responses to seven different mycobacterial antigens have been demonstrated with these three approaches (6).

Dr Mustafa described the generation of a large number of human T-cell clones to *M. leprae* and *M. tuberculosis* (7). A few of these clones recognize recombinant antigens identified by MABs (65K and 18K *M. leprae* proteins and a 19K *M. tuberculosis* protein). The majority of the clones appear to be directed to as yet undefined antigens. In collaboration with Dr R.A. Young, he has developed an assay system for screening the lambda gt11 recombinant library directly with T-cell clones in order to identify novel T-cell determinants. A new antigen recognized by a T-cell clone has been found through screening of 18 000 recombinant clones. Restriction analysis of the DNA insert from this clone (1.7 kb) shows that it codes for an antigen differing from those previously isolated. Analysis of the proliferative response of human T-cell lines using recombinant clones has identified six mycobacterial proteins recognized by T cells: three overlap with those identified by Dr D.B. Young.

Dr R. Kiessling described the application of the SDS-PAGE technique (8) to the screening of human T-cell responses to *M. leprae* extracts. Healthy contacts show proliferation mainly in response to low molecular weight antigens (12- 15K). The response of serum antibody and peripheral blood T cells to the same molecular weight fractions has been analysed for some individuals and little overlap found. Nitrocellulose strips cut from blots and solubilized in dimethyl sulfoxide (DMSO) are very effective in immunizing mice. This approach is being used to generate antibody probes recognizing proteins stimulatory for T-cell proliferation.

In discussion, Dr Ivanyi asked whether any limiting dilution data are available on the relative immunodominance of the 65K antigen. Dr S.H.E. Kaufmann cited data from experiments on mice immunized with mycobacterial extracts: 10-20% of T cells reactive with whole mycobacterial extracts are directed to the 65K protein, but there are no comparable data for humans infected with mycobacteria. Dr Bloom asked whether fusion proteins can perform in proliferation assays as well as native antigens. Dr van Embden reported that in his experience there is no significant difference between the two types of antigen.

1.4.2 T-cell epitope mapping

Dr D.B. Young described mapping of T-cell epitopes on the 65K antigen by a combination of sublibrary mapping, using the lambda gt11 clones previously described by Dr R.A. Young for antibody epitope mapping (9) and a synthetic peptide approach involving predictive analysis of potential T-cell epitopes from amino-acid sequences (10,11). By testing 20 amino-acid peptides two T-cell epitopes have been mapped.

Dr van Embden also described mapping of T-cell epitopes on the 65K antigen, in collaboration with workers in the laboratory of Dr R.R.P. de Vries. Epitopes recognized by 17 human T-cell clones have been mapped using the deletion library of the *M. tuberculosis* gene. A further two T-cell clones implicated in the pathology of the rat adjuvant arthritis model also recognize

the 65K antigen and have been found to map to the same region of the molecule as defined by deletion library mapping.

Dr Kolk described epitope mapping of a human T-cell line using a synthetic peptide from the 65K sequence. He also reported sequencing insert DNA from a lambda gtl1 recombinant expressing a portion of the 36K antigen. A synthetic peptide based on this sequence was found to stimulate T-cell proliferation with cells from some mouse strains (Balb/c and CBA, but not Swiss white) immunized with M. leprae. This peptide also stimulated the proliferation of human peripheral blood cells.

Dr Kaufmann presented data showing proliferative responses of human peripheral blood lymphocytes in response to several synthetic peptides based on the 65K sequence (12,13).

Dr Mustafa reported that a synthetic peptide capable of stimulating a T-cell clone specific for the M. tuberculosis 65K antigen has been identified. Partial sequencing data for the M. leprae 18K antigen have been obtained and epitope mapping of T-cell clones recognizing this protein is under way (14).

A summary of antigens recognized by T cells is given in Table 1.

Dr J.M. Ponninghaus initiated a general discussion on the significance of research on detailed epitope mapping with MABs and T cells and on the question of whether such research has any application to leprosy control. Dr Ivanyi felt that it could lead to the development of powerful immunodiagnostic tools (specific serological and skin test reagents) of considerable practical value.

Dr Bloom stated that the epitope mapping approach is important in determining the HLA restriction of responses to synthetic peptides. Understanding such effects may be crucial to the application of this type of technology to immunodiagnosis and future vaccine development.

1.5 Mechanisms of Cellular Immunity

1.5.1 Genetic restriction

Dr T. Ottenhoff summarized the results of population genetic studies that suggested an association of the DR3 haplotype with tuberculoid leprosy and DQw1 with lepromatous leprosy (15). He presented an analysis of DR3 and non-DR3 restricted responses to mycobacterial extracts. In contrast to healthy controls, the DR3 restricted response of tuberculoid leprosy patients to M. leprae extracts is lower than that of the non-DR3 response. For both patients and controls, the DR3 restricted response to purified protein derivative (PPD) is higher than the non-DR3 response (16). Dr Ottenhoff believes DR3 restricted suppressor T cells may be generated in response to tissue damage during tuberculoid disease. Recent advances in the understanding of the interaction between peptides and HLA molecules as seen by T lymphocytes were then discussed.

1.5.2 Suppressor T cells

Dr Ottenhoff described the characterization of T-cell clones to the 36K antigen of M. leprae, where both helper (CD4) and suppressor (CD8) clones are found (17,18). The suppressor T cells are irradiation sensitive and not cytotoxic. He discussed the potential role of such suppressor cells in lepromatous leprosy.

Dr Modlin described the isolation of CD8 clones from skin lesions with the ability to suppress proliferation of HLA class II-matched CD4 T-cell clones

TABLE 1. Mycobacterial antigens recognized by T cells.

<u>ANTIGEN</u>		<u>T CELLS</u>		<u>METHOD OF ASSAY</u>		
<u>M. leprae</u>	<u>M. tuberculosis</u>	Clonal	Uncloned	Purified antigen	rDNA	SDS-PAGE blot
>200 K ^a		+		+		
70 K (-)	71 K ^b		+	+		
65 K (-)	65 K	+	+	+	+	
	52-55 K	+				+
	38 K	+	+	+		
36 K		+ ^c	+	+		
28 K (-)	23 K		+	+	+	
	19 K	+	+		+	+
18 K		+	+		+	
	16-18 K	+				+
	14 K		+	+	+	
12 K		+ ^d			+	
	13B3 ^e	+			+	
PGL-I		+ ^f	+	+		

Notes:

- ^a Protein bound to cell wall of M. leprae.
- ^b From published work of Britton et al.
- ^c Includes "suppressor" clones.
- ^d Recognition by cytolytic (CD8⁺) T cells.
- ^e Antigen identified by screening rDNA library with T-cell clone.
- ^f "T-suppressor" clones.

(19,20). These cells have a conventional (alpha- and beta-chain) T-cell receptor and are also able to suppress ConA-stimulated T-cell proliferation. A high proportion of these clones recognize the synthetic disaccharide-bovine serum albumin antigen analogue of the phenolic glycolipid saccharide antigenic determinant.

1.5.3 Cytolytic T cells

Characterization of cytolytic T cells from mice immunized with M. leprae was described by Dr Kaufmann. Using antibodies directed to the T-cell recep-

tor, it is possible to demonstrate that the receptor is involved in the cytolytic effect. A model was proposed in which the combined effect of cytolytic and helper T cells working together in a coordinated response might be required for a protective immune response to mycobacterial infection (21). Preliminary experiments demonstrate an interaction between cytotoxic T cells and cultured Schwann cells infected with *M. leprae*, suggesting that these T cells can stimulate release of bacteria from the relatively "protected" environment of nerve cells and make them susceptible to killing by activated macrophages. Lysis of target cells by this type of mechanism has been shown to lead to a concomitant reduction in mycobacterial growth.

Dr Mustafa described a cytotoxic effect seen with many CD4 T-cell clones. He has investigated the HLA restriction (class II) of such killing. Both 65K and 18K antigens expressed in recombinant clones stimulate cytotoxicity. The importance of this effect was discussed in the framework of a model such as that presented by Dr Kaufmann.

1.5.4 Macrophage activation

Dr Kaufmann described the development of assays to assess killing or stasis of mycobacteria within macrophages. He described modification of the uracil uptake assay originally suggested by Dr G.A.W. Rook *et al.* (22). Dr Kaufmann found that, using bone marrow macrophages, activation of the antimycobacterial response can be demonstrated in response to gamma interferon or lymphokine preparations from mycobacteria-reactive CD4 T-cell clones. Some strains of *M. tuberculosis* are more resistant than others in this assay system.

1.5.5 Animal models

Dr Ivanyi discussed the use of animal models for the study of the immune response to mycobacteria (23). In an analysis of dormancy and reactivation of tuberculosis infection in mice, immunomodulators have little effect, although a significant effect with regard to the timing of chemotherapy and relapse of disease was found. Application of chemotherapy at later stages of infection results in a greater relapse than does early chemotherapy.

Dr Ivanyi discussed a model to assess the protective immune response to mycobacteria generated by antigens expressed in attenuated salmonella vectors using recombinant DNA techniques. Protection against live challenge with *M. tuberculosis* was obtained following immunization of C57Bl/6 mice with the attenuated salmonella. The same level of protection has been seen with control salmonella and with salmonella containing a low level expression vector with the mycobacterial 65K antigen. The possible effect of antigens which crossreact between salmonella and mycobacteria (such as the 65K antigen) was discussed.

Dr Kaufmann also presented results obtained with a salmonella strain transformed with the 65K antigen in a high level expression vector. Positive DTH responses are obtained with the recombinant but not the control strain.

1.6 Immunodiagnosis

Dr Ivanyi summarized recent applications of serum antibody competition tests (SACT) with monoclonal antibodies in tuberculosis and leprosy (24,25,26). Comparison of three serological tests has been carried out on the same set of sera - phenolic glycolipid ELISA, MLO4-SACT (*M. leprae* 35K antigen) and ML34-SACT (LAM antigen). All the tests were successful but the MLO4-SACT has the least "background" problems. An ELISA test using the peptide epitope recognized by monoclonal antibody IIIIE9 (*M. leprae* specific 65K) is negative with human sera.

The development of serological tests based on phenolic glycolipid was reviewed by Dr Brennan. Synthetic antigens containing the entire trisaccharide of phenolic glycolipid are currently accepted as optimal for such tests, since they have a slightly higher sensitivity than disaccharide-based antigens (27,28). Simple new latex agglutination assays based on phenolic glycolipid have been developed and are currently being widely tested in the Western Pacific region. With regard to antigen detection, Dr Brennan described the solid-phase immunoassays currently available (29,30) and discussed the possibility of detecting phenolic glycolipid in sera from tuberculoid leprosy patients. An analysis of the relative sensitivity of different assays suggests that complex GC-mass spectrometry with selective ion monitoring would be required in order to detect the low concentrations of the lipid expected to be present in such sera. Reverse-phase high-performance liquid chromatography (HPLC) has shown that phenolic glycolipid preparations contain a family of related structures with differences in esterified long-chain fatty acids.

Dr Kolk presented preliminary data on the application, by himself and Dr P.R. Klatser, of a competition test with the 36K monoclonal antibody (31) in a blind study on a population in Cebu. Approximately 15% of household contacts were found to be seropositive, with positivity rates of 24% (paucibacillary) and 79% (multibacillary) amongst newly diagnosed patients. Preliminary indications suggest that a synthetic antigen based on partial sequence determination of the 36K antigen can be used for serological tests.

Dr J. Convit reported the results of a serological survey of 14 000 individuals from the Venezuela vaccine trial using an ELISA test based on phenolic glycolipid antigen (Table 2). Of these 14 000, 13 were strongly positive, 25 moderately positive, 206 weakly positive and 413 "doubtful". The remainder were negative. These results have not yet been analysed with regard to sex and age.

Dr P. Fine has applied the phenolic glycolipid ELISA to sera collected in the area of the Malawi vaccine trial. Antibody levels were consistently higher in females and a distinct age-relation was observed in seropositivity with an increase up to the age of 20-30 years followed by a gradual decline. No corre-

TABLE 2. ELISA reactivity to native PGL-1 in contacts of leprosy patients in the Venezuelan immunoprophylaxis trial.

Classification	Number	Criterion	
		% activity positive control	Equivalent optical density
Strongly positive	13	≥80	≥0.8
Moderately positive	25	50-79	0.5-0.79
Weakly positive	206	30-49	0.3-0.49
Doubtful	413	20-29	0.2-0.29
Negative	13 398	<20	<0.2
Negative controls, non-endemic area	100	10 ± 7.2 ^a	0.1 ± 0.072

^a Average ± 2 S.D.

lation was found between antibody levels and contact status: household contacts did not show higher levels than non-contacts and treatment status of the index case was not related to seropositivity in contacts. A small percentage of the population had antibody levels in excess of 0.2 absorption units. Dr Fine does not feel that these results provide significant epidemiological information.

Considerable discussion was generated with regard to the role of serological tests (see 1.11, below).

1.7 Molecular Biology

Dr R.A. Young summarized progress in applying molecular biology to the immunology of leprosy, in particular in identifying protein antigens, defining T- and B-cell epitopes and assessing the diagnostic and prophylactic potential of particular antigens.

He noted that future progress in probing the molecular genetics of mycobacteria will call for (a) selection of a suitable host (initially, a rapidly growing organism such as *M. smegmatis* would be most convenient), (b) development of selectable markers: host cells carrying an auxotrophic mutation (e.g., uracil requirement) are preferable to drug resistance markers and (c) development of vectors and methods for introducing and expressing exogenous DNA in mycobacteria. He described recent progress in his own laboratory in the generation of *M. smegmatis* mutants carrying defects in the *pyrF* gene that result in pyrimidine auxotrophy. Different methods of introducing DNA into mycobacteria were described and preliminary evidence was given for successful gene conversion. Insertion of a linear section of DNA into a particular gene on the host mycobacterial chromosome results in highly stable integration. Dr R.A. Young stressed the importance of such technology for the future understanding of mycobacterial genetics and illustrated the power of this approach with an example of the functional analysis of the yeast RNA polymerase enzyme.

Dr Bloom discussed the potential development of modified BCG vaccine strains containing novel antigens expressed by recombinant DNA technology. A shuttle vector developed in collaboration with Dr W. Jacobs allows the transfer of DNA between *E. coli* and mycobacteria (32). This vector functions as a plasmid in *E. coli* and as a phage in mycobacteria and is thus termed a "phasmid". Present research is directed to the stable integration of the phasmid in mycobacteria (possibly by lysogenization). Dr Bloom described two other applications of molecular genetics in his laboratory. The first involves the use of restriction fragment length polymorphism (RFLP) analysis in the taxonomy of mycobacteria. An unusual result suggesting the presence of a repeated gene in the *M. leprae* chromosome was seen during blotting with DNA probes prepared from the region downstream of the *M. leprae* 65K gene. The second project involves the attempted establishment of a physical map of the mycobacterial genome using pulsed field gel electrophoresis. An intriguing difference between the genomes of *M. tuberculosis* H37Rv and H37Ra was noticed in early experiments.

In discussion, Dr Ivanyi asked whether molecular genetic approaches could be used to analyse the effect of deletion of particular genes from mycobacteria as well as for the addition of novel genes. It was agreed that this is a feasible approach to the definition of virulence factors.

Dr D.B. Young proposed a new model for the identification of immunodominant antigens of mycobacteria. He suggested that exposure to macrophage killing following phagocytosis could activate stress responses of mycobacteria which would involve the specific increase in synthesis of a restricted set of stress-related proteins. An analogy with the heat-shock response of *E. coli* was proposed. In support of this model several well-studied mycobacteria anti-

gens appear in fact to be closely related to *E. coli* heat-shock proteins. In particular, the 70K or 71K antigen of mycobacteria was demonstrated to have a high degree of sequence homology with the *E. coli dnaK* gene product, which is a major heat-shock protein related to the highly conserved hsp70 family of proteins found throughout nature. Partial crossreactivity of the 65K antigen of mycobacteria with an *E. coli* protein was demonstrated by a combination of monoclonal antibody tests, human T-cell proliferation and partial sequence analysis (33). It is now clear that the 65K antigen is related to the *E. coli groEL* protein, another major heat-shock protein. There are certain conserved features between heat-shock genes arrangements in *E. coli* and those in mycobacteria and there is evidence suggesting a possible heat-shock promoter sequence for the 65K protein. Dr D.B. Young discussed the potential significance of stress-related proteins as dominant antigens from the point of view of the immune response. He described a model in which presentation of highly conserved proteins as dominant antigens could lead to development of autoimmune responses.

Dr van Embden described work in his laboratory on the cloning and sequence analysis of the 65K antigen of *M. bovis* BCG. A high-level expression vector had been constructed that permitted relatively simple purification of the 65K protein. The antigen has also been expressed at a high level in attenuated salmonella strains and in vaccinia virus. These constructs are currently being used, in collaboration with Dr Kaufmann, to assess the induction of protective immune responses in animal models. Using a wide range of monoclonal antibodies, Dr van Embden and Dr J. Thole have found that several epitopes of the 65K antigen are broadly crossreactive with other bacterial genera. The 65K protein has been shown to correspond to a "common antigen" previously identified in more than 50 types of bacteria by workers at the Statens Serum Institut in Copenhagen. A combination of monoclonal antibodies and electron microscopy has shown the 65K antigen to be located mainly in the cytoplasm of mycobacterial cells. Dr D. Sweetser presented a general method for purification of fusion proteins from recombinant lambda gt11 clones. This involves ammonium sulfate fractionation followed by affinity chromatography on an anti-beta-galactosidase affinity column. SDS-PAGE analysis indicated that a high degree of purification is obtained. In discussion, Dr Kaufmann felt that further purification may be required in order to make such preparations fully active in lymphocyte proliferation assays.

In a general discussion as to whether too much attention is being focused on the 65K antigen, Dr Bloom pointed out that this protein represents an excellent model for testing many basic questions about the immune response, including the involvement of HLA restriction, and for developing experimental models to test for protective immunity.

1.8 Immunopathology

Dr Modlin reviewed his work on the analysis of T cells in leprosy lesions (19,20). The ratio of CD4 to CD8 cells within lesions is different from that in the peripheral blood and differs markedly between tuberculoid leprosy (approximately 2:1) and lepromatous leprosy (approximately 0.6:1). The number of IL-2 producing cells is ten times higher in tuberculoid than in lepromatous leprosy. Langerhans' cells within the skin have the potential to present antigen and to secrete IL-1 and therefore may be involved in T-cell activation. Such cells are present in tuberculoid but not lepromatous lesions. The possible application of *in situ* hybridization to the detection of mRNA coding for particular lymphokines may be an exciting approach to the analysis of important immunological events within leprosy lesions. Dr Modlin described work involving the cloning of CD4 and CD8 cells from skin lesions. Some of these CD4 clones proliferate in response to the cell wall protein antigen of Dr Brennan. The CD8 clones are described under the section on suppressor T cells [see above, 1.5.2].

Dr Ponninghaus asked how useful this type of immunohistology could be for the diagnosis of leprosy. Dr Modlin did not feel that this type of technique would have a practical application to routine diagnosis.

1.9 The IMMLEP M. leprae Tissue Bank

Dr R.J.W. Rees described the organization of the IMMLEP Bank with regard to the isolation of M. leprae from infected armadillo tissues and the supply of bacilli and antigen(s) to vaccine trials and to research workers (Table 3). Problems with pigments in some batches isolated from liver have been largely overcome and the introduction of a serological test for antibodies to phenolic glycolipid has been of considerable use in monitoring progress of infection in armadillos. Feedback from "consumers" of material from the bank is invited. The possibility of pooling individual batches of antigen after preliminary quality control analysis was discussed.

TABLE 3. Infected armadillo tissues (in grams) in IMMLEP Bank (1982-1987)

	Liver	Spleen	Lymph node	Skin
Stock, May, 1982	6 935	1 684	793	535
Production	36 999	7 463	3 008	8 773
Excluded	9 688	1 139	247	273
Issued	21 002	2 888	2 066	4 505
Balance, May, 1987	13 244	5 120	1 488	4 530

1.10 Vaccine Trials

Dr Mustafa summarized the results of the preliminary trials of the M. leprae vaccine in Norwegian volunteers (34,35). Both DTH and lymphocyte proliferation responses increase following vaccination and increased T-cell proliferation persists during a year of follow-up. The response to PPD does not change significantly following vaccination. The induction of M. leprae specific T cells following vaccination is clearly demonstrated by isolation of specific T-cell clones.

Dr M.D. Gupte asked whether any problems with ulcers have been found. Dr Mustafa said that ulcers have been found at high doses of vaccine and that 5×10^8 bacilli is the highest dose likely to be acceptable.

Dr Convit presented an extensive survey of progress in the vaccine trial in Venezuela, which is a controlled random double-blind trial designed to establish whether the M. leprae-BCG mixture imparts greater protective effect than does BCG alone. The intake phase has been completed, with approximately 27 000 contacts (out of 63 000 skin-tested contacts) recruited into the trial. Annual follow-up surveys of the entire trial population are now in progress. Dr Convit presented data concerning the skin-test reactivity of individuals vaccinated with BCG alone vs. M. leprae plus BCG. Sixty days after vaccination both groups show a good conversion rate, but two years after vaccination the M. leprae plus BCG group show a much higher positive (>10 mm) reaction rate.

The protective efficacy of the vaccine will be evaluated through comparison of incidence rates of the disease in the trial population only when 25 confirmed cases have occurred and been decoded by the trial monitor. Dr Corvit reiterated the generally accepted principle that the effects of a vaccine may be modified enormously by the environment in which it is employed.

Dr Fine summarized progress in the vaccine trial in Malawi. He discussed whether the reading of BCG scars gives accurate evidence of BCG vaccination. Recent evidence suggests that the BCG scar detection method may underestimate the number of individuals vaccinated and lead to an underestimate of the efficacy of BCG vaccination. Dr Fine also discussed whether HIV infection in the area of the vaccine trial could result in disseminated BCG infection and a reduction in vaccine efficiency. While it was agreed that any effects on vaccine trials are at present hypothetical, it was felt that every effort should be made to obtain data concerning levels of HIV seropositivity in trial areas.

Dr Gupta described preliminary studies in the proposed vaccine trial area at Chingleput, south India. These include the standardization of clinical diagnosis and classification and the standardization of skin-test reading.

1.11 General Discussion

Dr Ivanyi selected 10 major topics as the focus for the general discussion, during which Dr T. Godal, Director, TDR, joined the meeting.

(1) What is the role of lipoarabinomannan (LAM)? A possible role in immunomodulation is suggested by the fact that addition of LAM causes inhibitory effects in T-cell proliferation assays according to results presented by Dr D.B. Young. Dr Ottenhoff felt this could be an important result if LAM inhibits antigen processing. Results reported by Dr Modlin suggest that LAM remains in lepromatous lesions for extended periods of time, which suggests that it may play a role in mycobacterial persistence.

(2) Is there a candidate test for protective immunity? It was generally accepted that neither DTH nor LTT tests give direct information about protection. Dr Fine felt that an epidemiological approach would be most appropriate, i.e., individuals in areas where BCG vaccination has been demonstrated to be effective would be compared to those in areas where it has been ineffective. Dr Ivanyi felt that the type of approach described by Dr Kaufmann, i.e. to set up in vitro killing assays, is promising. Dr Ponninghaus felt that such a test would be of little use, while Dr P.G. Smith felt it to be vital. Dr Godal suggested that an understanding of the immune system would follow from a pragmatic approach to the study of basic immune mechanisms: this is illustrated by progress towards dissection of mycobacterial antigens and the testing of individual components in animal models.

(3) What is the difference between the vaccine potency of BCG (which needs to be viable) and that of M. leprae (which is effective in a killed form)? Dr D.B. Young suggested that if stress-related proteins are required to induce relevant immune responses, then a cultured mycobacterium would have to be able to synthesize such antigens within the macrophage; the tissue-isolated M. leprae may, he pointed out, already have a high content in these proteins.

(4) What is the role of HLA restriction? Dr Ivanyi felt that Dr Ottenhoff's finding regarding DR3 restricted responses is most interesting. Dr Godal stressed the usefulness of the synthetic peptide approach to analysis of this question.

(5) How many more clones (hybridoma? rDNA? T-cell?) are needed? Dr Ivanyi stressed that there is a need to ensure that past efforts are not duplicated. Dr R.A. Young felt that it is important to purify the already identified antigens in bulk and to complete their thorough immunological analysis.

(6) Is a subunit vaccine feasible? Major questions remain to be answered, e.g., how to identify a "protective" antigen and how to deliver that antigen in a form suitable for induction of a protective immune response?

(7) Will the 65K "topography" justify the concept of the existence of functionally different epitopes? Will different epitopes, for example, be involved in induction of helper and suppressor effects?

(8) What is the strategy for serology? Dr Ivanyi suggested possible roles of serology in case finding of lepromatous patients and in prognostic epidemiology. Dr Gupte could see no role for serology in diagnosis and Dr J. Grosset felt that active case-finding is not realistic. Dr Noordeen felt that a supportive role in diagnosis is possible but that the most important goal is to develop tests suitable for monitoring therapy and predicting relapse. Concern was expressed that the phenolic glycolipid-based ELISA tests are not sensitive enough since only a minority of tuberculoid patients are seropositive. Dr Ivanyi and Dr Kolk felt that their competition-based assays may have greater sensitivity. Dr Bloom stressed that a considerable amount of information about glycolipid antibodies is now available, suggesting that any new tests should be carefully compared to the glycolipid assay as a "benchmark" for serology. Dr Smith suggested that closer collaboration between research scientists and epidemiologists would be needed to develop and assess new serological tests.

(9) Should we take a "shuttle flight" by phasmid or plasmid?

(10) Are heat-shock proteins immunodominant or immunosuppressive? Dr Bloom suggested that construction of mutants could be useful in assessing the physiological role of heat-shock proteins.

2. FUTURE PRIORITIES AND RECOMMENDATIONS FOR RESEARCH

2.1 Biochemical Aspects and Immunodiagnosis

The SWG identified five major areas for further research:

2.1.1 Proteins and peptides

The 65K polypeptide is regarded as a model antigen from a relevant parasite for detailed immunochemical analysis. Already five or six T-cell-reactive sites and some 16 antibody-binding regions have been identified. With such definition, it is ideally suited for examination of HLA-restriction patterns.

A similar type of epitope analysis could be extended to the 28K, 36K, 71K and 18K proteins. Already crossreacting and species-specific monoclonal antibodies have been prepared and some T-cell epitopes have been identified on these antigens.

The origin of low molecular weight proteins (15K, 12K) should be analysed in detail using MABs to investigate whether they have any structural relationship to higher molecular weight proteins.

The fascinating topic of heat shock and the protein profile of M. leprae should help to focus on immunologically relevant proteins.

2.1.2 Cell wall

Further chemical and antigenic analysis of the M. leprae cell wall remains a priority, e.g., what are the T-cell stimulating components within that complex? Existing monoclonal antibodies and cloned T cells with known specificities should be used to define the antigens present in the cell wall.

2.1.3 LAM

The structure of lipoarabinomannan should be fully elucidated. Are there antigenic determinants other than 5-linked arabinofuranosyl residues? What are the differences between M. leprae and M. tuberculosis products? Is LAM present in immune complexes? What is the immunopathological significance of the variable titre of anti-LAM antibodies in leprosy sera and the high titres in treated lepromatous leprosy and in tuberculoid leprosy? The possibility of synthesizing the LAM antigenic determinant for testing in serodiagnosis was raised.

2.1.4 Immunodiagnosis

Increased emphasis should be placed on the use of protein and peptide antigens for immunodiagnosis. Competition assays based on epitopes of the 36K and 35K proteins have demonstrated high titre antibodies to these proteins in lepromatous leprosy sera. Further work is needed to develop direct binding assays using synthetic epitopes of these proteins, either as free peptides or as conjugates. Eventually there will be a need for a workshop to test the predictive value of these tests and their potential to identify subclinical infection in comparison to assays based on other protein and nonprotein antigens.

2.1.5 New antigens

There is a need for a continuing search for additional antigens. These investigations should emphasize proteins for which a T-cell response has been demonstrated. The ability to react in immunoblots with antibodies from human leprosy sera is also an important consideration. MABs and T-cell clones should be prepared along these lines.

2.2 Cellular Immune Responsiveness and Immunopathology

The following items were discussed and recommendations for future research made with regard to cellular immune responsiveness against M. leprae antigens.

2.2.1 Antigen characterization

Antigens of M. leprae and related mycobacteria have been analysed for their ability to activate human T cells. There is an obvious need to extend these studies in order to better define the potential T-cell antigen/epitope repertoire. Approaches to be followed include:

- screening of recombinant M. leprae gene expression banks;
- screening of immunoblotted M. leprae antigens;
- screening of purified, native M. leprae cell wall antigens.

Other techniques are needed to identify important antigens. One approach would be the development of a gene bank that yields high-level expression of preferably nonfusion proteins that would be nontoxic for human T cells. Precise epitope mapping, e.g., by overlapping peptides, should follow antigen identification.

Since HLA class II genes appear greatly to influence T cell specificity, studies should be conducted on T-cell responsiveness in the context of all known DR, DQ or DP specificities.

In order to investigate T-cell responsiveness, large amounts (100 mg to 1 gm) of purified antigens are needed.

2.2.2 Immunodominance

The immunodominance of antigens/epitopes should be assessed in humans as well as in immunized experimental animals, e.g., by limiting dilution assays. Qualitatively such antigens/epitopes should be characterized for their ability to activate certain immune functions. In addition to proliferation assays, tests should be carried out for lymphokine production (identification of cells and their frequency both at the periphery and in localized lesions; correlation of lymphokine production with other functions; characterization of macrophage activation factors other than gamma-interferon; the roles of tumour necrosis factor, B-cell stimulating factor, etc); cytotoxicity (types, phenotypes and frequency of such cells; antigen specificity; differences in induction vs. the effector phase); antigen presentation (character of antigen/epitope; interactions; effect(s) of lymphokines or M. leprae components on antigen presenting cells).

2.2.3 Immunoregulation and immunopathology

(a) Nerve damage in leprosy

A well-characterized model for the study of nerve damage should be developed in order to further characterize the function of Schwann cells for, e.g., MHC class I and II expression; antigen presentation; M. leprae antigens or autoantigens; type of cells involved (T helper and/or T cytotoxic, etc.) and mechanism of destruction (bystander or direct effect; role of lymphokines, etc.). Culturing of human Schwann cells and T cells will also be crucial for the identification of cells and antigens involved in nerve damage. The direct study of the epidermis of patients in reaction as well as of individuals immunized for prophylactic or therapeutic purposes is important. In addition to phenotype studies, more functional studies, such as on lymphokine production, should also be carried out.

(b) Unresponsiveness in lepromatous leprosy

Research should focus on mechanisms of unresponsiveness; the relative importance of suppression by T cells (e.g., by limiting dilution assays) and macrophages; mechanisms of suppression by suppressor cells (antigen recognition, etc.); strategies for the induction of responsiveness to M. leprae in anergic patients (e.g., by using lymphokines, immunotherapy and/or selected antigenic determinants).

(c) Idiotypes

Very little is known about possible anti-idiotypic regulation in lepromatous leprosy, either at the T-cell or the B-cell level.

2.2.4 Protective immunity

To date, the best in vivo models consist of experimentally immunized animals (preferably of several species and MHC phenotypes). There is a clear need for a good in vitro model for the elimination of mycobacteria from host cells. Systems developed so far have proved to be complicated and not always reproducible. Immunological monitoring of vaccine trials is also an important

area of research for the identification of parameters which correlate with protective immunity.

2.3 Molecular biology

2.3.1 Antigens

The interface between recombinant DNA expression of *M. leprae* genes and immunology should continue to be supported by: (a) further development of tools to assess the humoral and cell-mediated immune response to infection and/or vaccination, especially T-cell probes to screen rDNA libraries; (b) production of recombinant protein antigens through the use of over-expression vectors and hosts and purification of proteins for seroepidemiological or T-cell-based studies. This will require sequence analysis of the genes and facilities for large-scale production and subsequent purification of the proteins/peptides.

2.3.2 Function

- (a) The biological functions of protein antigens should be studied by sequence homology with known proteins to study their role in disease.
- (b) Genes controlling the synthesis of complex biological molecules, such as cell-wall components and the transcription apparatus, should be isolated and studied.

2.3.3 Genome

Genes and genome organization should be studied by: (a) identifying and isolating gene expression signals for use in genetic manipulation; (b) mapping genes of interest on the *M. leprae* genome.

2.3.4 Gene manipulation

Methods of manipulating genes in mycobacteria should be developed by: (a) isolating selectable markers and host mutants with deficiencies in selected genes, and producing vectors to introduce DNA into laboratory strains; (b) introducing genes from foreign sources and investigating molecular mechanisms necessary for the stability of gene introduction and expression.

2.3.5 Protective immunity

- (a) Recombinant vaccine vehicles should be used to carry expressed *M. leprae* genes for tests of immunization and protective efficacy.
- (b) Simplified schemes using genetically manipulated bacteria should be developed to assess mechanisms of pathogenesis and virulence.

2.3.6 Population genetics

A possible human genetic propensity to develop leprosy or a particular form of leprosy disease should be investigated once the human genome is fully mapped (in about two years).

2.4 Vaccine Trials and Related Activities

2.4.1 Vaccine trials

- (a) Support for the ongoing trials in Venezuela and Malawi, and the proposed trial in south India, should be maintained.

- (b) A single review group should be set up to examine all three trials in a uniform manner.
- (c) Revaccination strategies should be assessed and their possible implementation decided upon.
- (d) The implications of a successful vaccine should be considered.
- (e) Potential areas for trials of future "nth" generation vaccines should be explored.

2.4.2 IMMLEP M. leprae Tissue Bank

- (a) Supplies of M. leprae should be ensured for ongoing, planned and possible future trials.
- (b) Sources of M. leprae provided to the Bank should be assessed and, if necessary, regulated.

2.4.3 Immunoassays

- (a) The development of immunological methods for early diagnosis of leprosy in endemic regions should be encouraged.
- (b) The epidemiological value of immunological tests should be evaluated in the field, e.g., in a workshop to assess the current epidemiological status of tests.
- (c) Easily monitored immunological correlates of protective immunity against clinical leprosy should be identified and studied.
- (d) Better skin-test antigens, e.g., synthetic peptides, and better methods for their standardization should be developed.
- (e) Areas, including trial areas, should be identified and developed for immunoepidemiological studies (Brazil is one possibility).

2.4.4 Additional topics

- (a) Studies (e.g., case control studies) of protective efficacy of BCG, including dose-response effect(s), should be encouraged.
- (b) Research on the mechanisms of nerve damage and the means of controlling it should be encouraged, in collaboration with THELEP.
- (c) The impact of human immunodeficiency virus (HIV) infection on the immunological manifestations of leprosy should be evaluated.

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