Memoranda
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Memoranda are statements concerning the conclusions or recommendations of certain WHO scientific meetings; they are signed by the participants in the meeting.

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Plan of action for research in the immunology of tuberculosis: Memorandum from a WHO Meeting*

A meeting was held to outline a comprehensive research plan, based on recent advances in biomedical sciences, for the development of improved methods for the control of tuberculosis. Such a plan requires a stepwise approach. The meeting focused on the initial activities to be carried out and on setting priorities.

In the setting of priorities, the meeting considered not only feasibility and potential benefit of various studies but also activities already being undertaken and their funding. Thus, emphasis was put on the exploitation of new technological approaches, such as recombinant DNA technology, T-cell cloning and hybrid-cell methodology, and the production of monoclonal antibodies and immunoregulatory substances. The following subjects were proposed for targeted research: molecular biology, monoclonal antibodies, immunoregulation in human tuberculosis, experimental immunology of tuberculosis, and cloning of mycobacteria.

A strategic plan has been drawn up to indicate the link between the initial activities and the ultimate goals of the programme. It was recommended that a comprehensive research programme on the immunology and molecular biology of tuberculosis should be established and, as funds become available, the programme should be advertised to the scientific community on a worldwide basis.

GENERAL CONSIDERATIONS

During the past few years, since the failure of BCG in the trial in South India, a considerable amount of imaginative thinking has gone into the explanation of the results obtained, and proposals for research have been made. Reports on these activities are available (*1*, *2*) and, to a large extent, they form the basis for what is reported here. While studies are well under way in some fields (e.g., retrospective studies to determine the efficacy of BCG in infants and young children in tropical and subtropical areas, and experimental investigations to test some of the hypotheses to explain the failure of BCG in South India), no concerted efforts have yet been initiated with regard to other activities. Recent advances in the basic biomedical sciences have provided new methods (such as recombinant DNA technology, T-cell cloning, and monoclonal antibodies), which offer new opportunities for improved immunoprophylaxis and other control methods.

Although the original intention was to apply modern immunological techniques to find an explanation for the variations in BCG protection, the scope of the programme has expanded into the search for more efficient tools for tuberculosis control. The control methods at present being used in developing countries are based on passive case-finding by microscopy, ambulatory chemotherapy, and BCG immunization of children. How much impact these methods can be expected to have on the tuberculosis problem in developing countries is not established, but it seems that, even with optimal implementation, the effects in terms of problem reduction will be limited.

* This Memorandum was drafted by the signatories listed on page 785 on the occasion of an informal discussion held in Boston, MA, USA, on 7–9 February, 1983. Requests for reprints should be addressed to: Chief, Tuberculosis and Respiratory Infections, World Health Organization, 1211 Geneva 27, Switzerland. A French translation of this article will appear in a later issue of the *Bulletin*.

The need to develop better control methods is fully justified by the public health importance of tuberculosis in the developing world. A steady decline in the available resources and staff for tuberculosis research during the past twenty years has resulted in a lag in the knowledge of the epidemiology and pathogenicity of tuberculosis, compared with the progress made in other communicable diseases.

The present report outlines an integrated international research plan to permit the proper analysis of objectives, the setting of priorities, and the consideration of resource requirements.

ESTABLISHING A PLAN OF ACTION

Many basic features of *Mycobacterium tuberculosis* have first to be considered. The simplest situation is where no important antigenic and biological differences exist between different strains of the species. A situation of intermediate complexity would exist if there were important antigenic and biological differences between substrains of *M. tuberculosis*. Further complexity would arise if phenotypic variation of a single genotype should turn out to be important in relation to pathogenesis.

The plan has been based on the assumption that strain heterogeneity may be a factor of importance in mycobacterial pathogenesis. This will allow both the development of simpler tools, e.g., species-specific tools, and the understanding of more complex situations, e.g., phenotypic variations of a single genotype ("adaptive antigenic variation"). Any of these situations will require modifications in the plan.

There is considerable evidence favouring heterogeneity in the species of *M. tuberculosis*, especially from phage-typing studies (*3*). However, evidence favouring the importance of variations in the antigenic and biological properties of *M. tuberculosis* in pathogenesis is at present limited, but studies both in simpler organisms (e.g., viruses) and more complex organisms (e.g., parasites such as trypanosomes) have shown that antigenic variation due to mutational events or DNA rearrangement mechanisms may play a fundamental role in the survival of pathogenic organisms. With regard to tuberculosis, the possibility of heterogeneity of *M. tuberculosis*, both between different geographical regions and intraregionally, has to be entertained. New methods, such as monoclonal antibodies, T-cell cloning, and recombinant DNA technology, permit, for the first time, detailed investigation into this subject.

As envisaged in the plan, the approach includes a comparative study of different *M. tuberculosis* strains since the subtle differences in biological behaviour in non-immune cells (e.g., monocytes) and immune cells (e.g., activated monocytes) of different strains are likely to provide important information. Studies in this area will start with cloned samples of specimens from existing *M. tuberculosis* strain collections. As technology develops, these studies will be expanded to include more samples from the same areas and from different regions.

Studies on immunoregulatory dysfunction should include different categories of patients with tuberculosis, because such investigations are likely, even in a limited sample, to indicate the immunoregulatory dysfunctions and the most useful methods for detecting them. Such studies should also be extended to healthy subjects in order to identify those phenomena that may be related to pathogenesis and to identify deficiencies in the responses to immunization with the aim of improving the immunization procedures (see below, Proposed Areas of Research, section C).

The probability of correcting immunoregulatory dysfunction is likely to increase in the future as new drugs and highly-purified immunoregulatory molecules (e.g., lymphokines and monokines) from T-cell hybridomas or recombinant DNA technology become available. These substances will make it possible to investigate immunological restoration in patients during or after short-term therapy to reduce the risk of relapse.

In order to develop an efficient vaccine for tuberculosis, it is important to evaluate: (*a*) the role of atypical mycobacteria on the host's protective response to *M. tuberculosis*, (*b*) the possibility of strain heterogeneity or of antigenic variation, (*c*) the immunoregulation changes accompanying infection, and (*d*) the genetic basis of host susceptibility.

For the purposes of the programme, we consider that a detailed analysis of a few areas to uncover the basic rules governing the action of *M. tuberculosis* will be more cost-effective than the attempt to do too much from the very beginning.

Priorities

The methodology of molecular biology has progressed in recent years to the extent that it can now be applied directly to a number of health problems, including tuberculosis (see below, Proposed Areas of Research, section A). Since few activities are going on at present in this area, this deserves to be given top priority in the initial phase of the programme.

Extensive attempts to develop more specific skin tests (for diagnosis of infection) have been made over the last ten years by the biochemical fractionation of

M. tuberculosis. However, no component with satisfactory superiority to PPD (purified protein derivative of tuberculin), with a few suggestive exceptions that require consideration, has been identified. In fact, the picture that has emerged of the antigenic mosaic of mycobacteria is that most, if not all, macromolecules of these organisms contain antigenic determinants (hereafter called epitopes), some of which are unique to each strain while others are shared. Thus, for this programme, the biochemical fractionation approach to improved diagnostic tests has not been given high priority.

The chemical characterization of mycobacterial cell walls and other lipid-containing components has made important progress in recent years. While this remains a key area of research, it appears likely to attract funds on its own merits (e.g., immunological adjuvants) and has, therefore, not been given high priority for funding from this programme. However, knowledge from this area will be essential for the structural analysis of specific epitopes and their synthesis.

Another area of rapid development in tuberculosis should be the preparation of monoclonal antibodies (see below, Proposed Areas of Research, section B). Claims for species-specific and strain-specific antibodies have already been made (*4*). On the basis of experience from other fields, it may be anticipated that a workshop to minimize repetitive and overlapping work should be held in the future when sufficient information has accumulated. The purpose of such a workshop should be to clarify the antigen and mycobacterial strain specificity of antibodies available and to evaluate their usefulness for antigen characterization. The workshop should also include the comparison of tests used for detecting such antibodies and the design of screens for developing "second" generation antibodies.

In relation to immunization, monoclonal antibodies may not necessarily detect antigens involved in protective (T-cell) immunity. It is therefore important that the methodologically more complex field of T-cell cloning for tuberculosis should get started as early as possible (see below, Proposed Areas of Research, section D, (1)). This should also be done in humans to identify variant-specific epitopes recognized by T cells and for studying in detail the immune regulation in patients and control subjects.

T-cell cloning now appears to be the best approach to acquiring information on the nature of protective antigens. Such studies have to be carried out in animals. While the model to be chosen may not be valid for humans in detail, the emphasis on this approach is based on the judgement that general extrapolations will hold, e.g., the role of surface antigens as compared to cytoplasmic antigens in protective immunity. Is protection limited to a few essential antigens, or do they form a broad type of hierarchy? It will be important to know this for the selection of epitopes to be included in new vaccines.

Our insight into the regulation of the immune response remains fragmentary, especially in humans where key membrane molecules like the HLA-DR group have not yet been fully characterized. While it will be important to determine if the immune response to BCG is altered in areas where BCG is not effective, these studies will have to be repeated with modifications following new developments in basic immunology. Nevertheless, this area deserves high priority from the very beginning, especially at the methodological level (e.g., development of T-cell clones for analysis of immune regulation), as it is likely to have an important impact on the probability of developing better vaccines (see below, Proposed Areas of Research, sections C and D).

The immune system has, of course, not evolved as a result of artificial immunizations (using vaccine given by a syringe and needle) and it will take some time before such approaches will have had a selective impact on the immune system. As more and more details of the immune system become known, it appears that the most effective protective responses are elicited in the target organ of the natural infection. This may apply not only to the type of response but also to specificity, e.g., influenza-specific lymphocytes turn out to have a selective affinity for the respiratory tract. Thus, tuberculosis immunization using the respiratory route should be investigated again.

Earlier studies have clearly suggested that aerogenic immunization may be more effective than intradermal immunizations (*5*). This area, therefore, requires to be considered again. Since the delivery of a precise dose to the lung will be difficult, one of the first problems to be studied should be the relationship between the minimal effective dose and the dose causing complications. If this difference turns out to be large, then the prospect of developing this approach for practical use should be good. Regarding techniques for the delivery of aerogenic vaccines, it is possible to make use of developments from other fields, e.g., viral vaccines. Some fundamental studies related to this approach have also been outlined, e.g., behaviour of *M. tuberculosis* in alveolar macrophages and the nature of immune responses elicited from the lung as compared to other routes of infection or immunization, including the homing properties and antigenic specificity of pulmonary-induced T-cell responses (see below, Proposed Areas of Research, sections C and D).

Resource requirements

The exact amount of funds needed for such a programme is difficult to predict, mainly for two reasons:

(1) In a number of areas of research initiated by WHO, a substantial amount of work has been carried out with little or no financial support from the Organization, but this varies considerably from one disease to another and also depends on the availability of support for biomedical research in general.

(2) It is essential that "organic growth" should be allowed to take place from the initial phases of such a programme, i.e., there should be an increase of funding in the course of time, since initial over-funding may lead to the support of low-priority projects.

With these reservations in mind, one can, in this particular case, consider a programme of considerable potential and efficiency, given an annual budget of about US$1 million.

PROPOSED AREAS OF RESEARCH

The programme for research in the immunology of tuberculosis (IMMTUB), initiated by WHO, will start by concentrating on the following five areas:

— Molecular biology
— Monoclonal antibodies
— Immunoregulation in human tuberculosis
— Experimental immunology of tuberculosis
— Cloning of mycobacteria.

A. Molecular biology

The goals include establishing the genetic and biochemical bases for *M. tuberculosis* pathogenicity and developing methods to identify strain heterogeneity. These goals should be accomplished by initiating basic research in the following areas:

(1) Establish DNA clone banks using available bacterial host-vector systems. Clone banks should only be established from a virulent *M. tuberculosis* strain derived from a single cell (see below, section E).

(2) Develop and/or identify host-vector systems to obtain expression of *M. tuberculosis* gene encoding proteins.

(3) Identify DNA probes to differentiate *M. tuberculosis* from all other species and to differentiate between virulent and avirulent *M. tuberculosis* strains. This would include:

(*a*) the determination of the presence of plasmids, prophages, and movable genetic elements;
(*b*) the development of DNA probes for use in epidemiological studies and for diagnosis of *M. tuberculosis* in biological specimens;
(*c*) the correlation of the above data with virulent and avirulent phenotypes as revealed by biochemical and immunological techniques.

(4) Determine the genetic and biochemical bases for drug resistance.

B. Monoclonal antibodies to *M. tuberculosis*

The further development of monoclonal antibodies to various components of *M. tuberculosis* is very important to this programme. They can serve several purposes, such as:

(1) Detection of mycobacterial proteins expressed by organisms containing recombinant DNA.

(2) Purification and characterization of antigens that bind with monoclonal antibodies. These would be of use in a variety of immunological analyses, including those for epidemiological use.

(3) The use of monoclonal antibodies in antibody competition tests. This makes it possible to detect antibody responses to given antigens without isolation of the antigens concerned.

(4) Detection of mycobacterial antigens in clinical specimens.

(5) Use of monoclonal antibodies to subdivide serotypes of *M. tuberculosis* and *M. bovis* for epidemiological use. They could also be helpful for the rapid identification of other mycobacterial species or their subdivision into serotypes.

(6) Use of appropriate antigens, purified by monoclonal antibodies, in specific skin tests or *in vitro* to establish which species of mycobacteria had infected human or animal subjects. These antigens might be of great value in differentiating exposure to *M. tuberculosis* from exposure to BCG in humans, and exposure to *M. bovis* from exposure to other mycobacteria in cattle, etc. They could be useful in exploring the relationship between immunization and previous contact with environmental mycobacteria, a matter that may determine the efficacy of vaccines in different parts of the world.

C. Immunoregulation in human tuberculosis

The immunological reactions of the human host to *M. tuberculosis* should be further investigated. With knowledge of these reactions, it should be possible in the future to devise rational modes of enhancing protective and decreasing suppressive immune reactions, of eliminating latent infections, and of improving chemotherapy.

The groups of people to be studied should include:

(1) Patients with different stages of tuberculosis, including assessment before, during and after chemo-

therapy. The last should include patients who have relapsed.

(2) Prospective studies on subjects who will receive BCG, including comparative studies between populations that have shown protection following BCG and those that have not. The feasibility of determining the presence or absence of hypersensitivity to atypical mycobacteria prior to immunization with BCG should be explored in each of the above.

(3) Prospective studies on high-risk populations.

(4) Studies on the influence of factors known to depress cell-mediated immunity, such as viruses (measles) and parasites (malaria), and their effect on the immune response to *M. tuberculosis*.

Studies should be carried out on the blood of human subjects and, when possible, on cells or tissues from other organs. This work should be aimed at the determination of phenotype and function of T cells (T-suppression, lymphokine-production, T-helper function, etc.), assessment of macrophage function (monocyte/macrophage activation, "killing" of *M. tuberculosis*, chemotaxis, etc.), and modification of MHC (major histocompatibility complex) and surface markers. The antibody response could also be assessed.

Of special interest is the comparison of the above with cells from lymph nodes (taken at biopsy), from tuberculosis effusions, from lung specimens and, when appropriate, from bronchial lavage. Such comparison would allow the assessment of the possible compartmentalization of the immune response to *M. tuberculosis* in humans (see below, section D, (4)).

It is important that, in longitudinal studies on patients with tuberculosis, cells should be cryopreserved so that samples obtained at different times can also be compared at the same time. Samples can also be examined in the future with new reagents as they become available.

D. Experimental immunology of tuberculosis

(1) *Development and characterization of T-cell clones*

T-cell clones specific for *M. tuberculosis* should be developed in human and rodent models. Primary emphasis should be placed on T-cell clones associated with delayed-type hypersensitivity, protection, and/or immunoregulation for use in the characterization of particular antigenic epitopes involved in the development of protective immunity. Quantitative assessment of epitope recognition in mixed cell populations and the biological functions of these epitopes should also be carried out by the method of limiting dilution analysis. Where *in vitro* assays for T-cell activity are used, attempts should be made to associate this activity with *in vivo* functions. Such cloned T cells should also be useful for the study of immunomodulatory agents (lymphokines, monokines, or synthetic immunopharmacological drugs). T-cell clones should also be useful in the study of antiidiotypic antibodies and immunoregulation.

(2) *Macrophages*

Mechanisms involved in the bacteriostatic and bacteriocidal activity of macrophages against *M. tuberculosis* should be examined. This should involve the development of reproducible assays of intracellular killing and/or bacteriostasis of tubercle bacilli. Comparative studies involving a variety of strains of tubercle bacilli, especially virulent versus avirulent, should be carried out. The importance of killing mechanisms, dependent on and not dependent on oxygen, should be defined for these pathogens. Mechanisms by which the mycobacterium resists intracellular killing should be investigated. This will include the effects of mycobacterial cells and their components on macrophage function. The possibility of the existence of extracellular killing mechanisms should be investigated. The modulatory effects of *M. tuberculosis*-infected macrophages and their products on the immune response to this pathogen should be examined. This would include changes in antigen presentation by the macrophage and alteration of MHC-encoded products and other surface markers. T cells and their products obtained from tuberculous lesions should be assessed for their regulatory influence on macrophage function.

(3) *Immunomodulation*

Monoclonal antibodies directed against macrophages presenting *M. tuberculosis*-derived antigens (processed antigens) on their membranes or against *M. tuberculosis*-reactive T cells should be developed for the purpose of dissecting the response of cell-mediated immunity. The different cell types (T_S, T_H, etc.) infiltrating the tuberculous lesion at various times in the course of the infection should be analysed. Assessment of immunomodulatory substances shown to be effective *in vivo* should be tested in a variety of *in vitro* systems, using selected or cloned T-cell populations. Studies on the suppressive effects of mycobacterial products on T cells and on macrophages should be pursued.

(4) *Local versus systemic immunity*

The antituberculous activity of defined cell populations should be compared in cells collected from a variety of anatomical sites in both normal and immune subjects. These studies should be developed to compare the local immune response (especially in

the lung) with its expression in other host organs. Quantitative comparisons of aerogenic and systemic routes of immunization and challenge should be made. Since the adoptive transfer system is important to define the cellular components of the antituberculous response, the organ distribution of mononuclear cells from different sites in adoptively-transferred animals should be correlated with the expression of an effective protective response. The biochemical changes associated with immunological activation of macrophages in different organs should be compared with the cells' ability to limit the intracellular growth of the pathogen.

E. Cloning of mycobacteria

To investigate the genetics and biochemistry of tubercle bacilli, and for other purposes as well, it is important that clones of mycobacteria derived from a single cell of the various strains should be obtained. Such clones should be compared with their parent strains to see whether they have retained the important biological characteristics (listed in Table 1), especially virulence, and as many as possible of the additional characteristics of the strains. All strains should be maintained by storage of aliquots at −70 °C or lower to preserve their phenotypes. These aliquots should be distributed and used in research projects with a minimum of subcultivation.

Strains of tubercle bacilli are currently classified as *M. tuberculosis*, *M. bovis*, and *M. africanum*. *M. tuberculosis* is the predominant causative organism of tuberculosis in humans throughout most of the world, with *M. bovis* only occasionally encountered. *M. africanum* is found in West and Central Africa, the West African strain being more closely related to *M. bovis* and those in Central Africa more nearly related to *M. tuberculosis*. These strains, when freshly isolated from patients, are almost always of high virulence in the guinea-pig. A variant of *M. tuberculosis*, termed South Indian, has been found to be attenuated in the guinea-pig when given by the intramuscular, intravenous or aerogenic routes. Low virulence is found in about 70% of strains isolated in Madras, South India, and in a lower proportion of strains in northern India, Burma, Thailand and East Africa. The South Indian strains are characterized by increased susceptibility to hydrogen peroxide (without loss of catalase activity) and the presence of the attenuation indicator lipid; these strains frequently have moderate resistance to thiophen-2-carboxylic acid hydrazide, and are of intermediate (I) phage type. Catalase-negative, isoniazid-resistant strains of *M. tuberculosis* are also attenuated in the guinea-pig and have increased susceptibility to hydrogen peroxide. Catalase-negative, isoniazid-resistant varieties of South Indian strains are even more attenuated than their parent strains. When strains of *M. tuberculosis* or *M. bovis* have been passaged in the laboratory, a loss of virulence often occurs, examples being the H37Ra and BCG strains.

An example of eight strains representative of these

Table 1. Biological characteristics of some strains of mycobacteria

Virulence in the guinea-pig	Catalase activity	Susceptibility to H_2O_2	A I lipid	Origin	Whether attenuated by *in vitro* passage	Possible strains[a]
High	Normal	Resistant	Absent	USA, Europe	No	Erdman
High	Normal	?	?	W. Africa	No	*M. africanum*
Intermediate	Normal	Resistant	Absent	USA	Partial	H37Rv
Low	Normal	Resistant	Absent	France	Yes	BCG Paris 1789
Very low	Normal	Resistant	Absent	USA, France	Yes	H37Ra
Low	Normal	Susceptible	Present	S. India	No	79112[b] 79157[c]
Low	Absent	Susceptible	Absent	USA, Europe	No	TM303
Very low	Absent	Very susceptible	Present	S. India	No	INH-resistant Indian
High	Normal	Resistant	Absent[d]	France	No	Ravenel

[a] All except *M. africanum* are available from the Trudeau Culture Collection. See also LEFFORD, M. J., ed. *Mycobacterial culture collection*, 2nd ed. Bethesda, Department of Health and Human Services (DHHS publication No. (NIH) 80–289).

[b] This strain has been studied more than 79157, but has abnormally high sulpholipids.

[c] Normal sulpholipids, but less thoroughly studied.

[d] Possibly not actually tested.

different types is shown in Table 1. These strains include fully virulent *M. tuberculosis* and *M. africanum* together with examples exhibiting all known varieties of animal attenuation; these are likely to be of particular value in studies on mechanisms of virulence and of macrophage activity.

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F. Assaad, Division of Communicable Diseases, World Health Organization, Geneva, Switzerland
I. Azuma, Institute of Immunological Science, Hokkaido University, Sapporo, Japan
T. M. Buchanan, Seattle Public Health Hospital, Seattle, WA, USA
F. M. Collins, Trudeau Institute Inc., Saranac Lake, NY, USA
R. Curtiss, Department of Microbiology, University of Alabama, Birmingham, AL, USA
J. R. David, Department of Tropical Public Health, Harvard School of Public Health, Boston, MA, USA
P. Draper, National Institute of Medical Research, Mill Hill, London, England
T. Godal, The Norwegian Radium Hospital, Oslo, Norway
M. Goren, Division of Research, National Jewish Hospital and Research Center, Denver, CO, USA
B. W. Janicki, Immunobiology and Immunochemistry Branch, National Institute of Allergy & Infectious Disease, Bethesda, MD, USA
S. H. E. Kaufman, Max-Planck Institute for Immunobiology, Freiburg-Zahringen, Federal Republic of Germany
D. A. Mitchison, Royal Postgraduate Medical School, London, England
A. Pio, Tuberculosis and Respiratory Infections, World Health Organization, Geneva, Switzerland
G. Torrigiani, Immunology, World Health Organization, Geneva, Switzerland

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