Monoclonal Antibodies and Recombinant DNA Technology: Present and Future Uses in Leprosy and Tuberculosis*

Leprosy (Hansen's disease) and tuberculosis (TB) are both chronic granulomatous infections of man caused by related mycobacteria. They are found worldwide, although the most seriously affected areas are the developing countries of Asia and Africa. Historically, leprosy is possibly a recent disease, whereas tuberculosis has been known since 23,000 B.C.[1]

According to the World Health Organization (WHO), over 10.5 million people are estimated to have leprosy and between 1.2–1.4 billion are exposed to the risk of contracting the disease.[2] There are some 30 million new cases of tuberculosis per year.[3] However, only 50% of patients with mycobacterial disease are registered, and only 50% again receive regular treatment.[3] This failure to register and treat patients is largely due to an inadequate public health infrastructure and poor patient compliance. As a result of these factors, both antileprosy and antituberculosis drugs have failed to control and eradicate these diseases. In addition, both primary[4, 5] and secondary[6, 7] drug-resistant bacteria have now been documented.

Primary prevention, in the form of vaccination, continues to be a major area of research. The BCG program for TB, although effective in some areas, has been a total failure in others.[8] Rapid diagnosis is hampered by the slow *in vitro* culture of *Mycobacterium tuberculosis* and the inability to grow *M. leprae* except *in vivo* in mice,[9] armadillos,[10] and monkeys,[11] thus further delaying therapy.

The use of monoclonal antibodies (MABs) and recombinant DNA technology will, hopefully, herald a new era in which the generation in bulk of mycobacterial products may reveal much about the biochemistry and surface structure of these organisms. Knowledge thus acquired should speed diagnosis and has obvious roles in therapy and prevention.

This review attempts to collate work done in these areas and to indicate the potential benefits to be accrued.

MONOCLONAL ANTIBODIES

In 1959, Burnet[12] proposed the theory of clonal selection which predicted that one plasma cell would produce only one immunoglobulin specificity. Confirmation came from studies of human myeloma proteins and from animals with B-cell lymphoproliferate disorders. The discovery of cell hybrids formed by spontaneous fusion of two different cells in culture was made in

* This review was written in 1984 by Michael J. Seckl, B.Sc., University College, London, while a third-year medical student at University College Hospital. It won First Prize in the annual competition set up by the British Leprosy Relief Association (LEPRA) for essays on various aspects of leprosy. We take pleasure in publishing this review. Mr. Seckl's address is 30 Fairmead Road, London N19 4DF, England.

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[11] Meyers, W. M., Walsh, G. P., Brown, H. L., Fukunishi, Y., Binford, C. H., Gerone, P. J. and Wolf, R. H. Naturally acquired leprosy in a mangabey monkey (*Cercocebus* sp.). Int. J. Lepr. **48** (1980) 495–496.

[12] Burnet, F. M. *The Clonal Selection Theory of Acquired Innumity.* Cambridge: University Press, 1959.

1960,[13] but it was not until 1975 that Milstein and his colleagues[14] published a successful technique for the production of immortalized mouse B-cell hybridomas secreting MABs raised to specific antigens.

Synthesis

Hybridomas are formed by fusing together two different cells so that the resulting hybrid cell survives, whereas the nonfused parent cell populations do not. Lines of myeloma cells, preferably not secreting immunoglobulins, have been established in long-term culture for several species,[15–17] including man. By fusing such cells (using inactivated Sendai virus or polyethylene glycol)[18] with another population, for example, spleen cells from an immunized mouse, it has proved possible to produce immunoglobulin-secreting hybrids of which as many as 10% may secrete a specific antibody. The non-myeloma parent cells that do not fuse die naturally in culture, but the same is not true of nonfused parent myeloma cells. These have to be eliminated by growth of the mixed culture in a selective medium containing hypoxanthine, aminopterin, and thymidine (HAT).[19] A selective enzyme defect can be induced in the myeloma line (for example, deficient hypoxanthine guanine ribosyl transferase or thymidine kinase) so that when the cells are grown in HAT, they cannot use hypoxanthine and, since the only other route to nucleotide synthesis is blocked by aminopterin, they die. The hybridoma survives because of the ability conferred by the immune cell genome to use hypoxanthine in nucleotide synthesis.

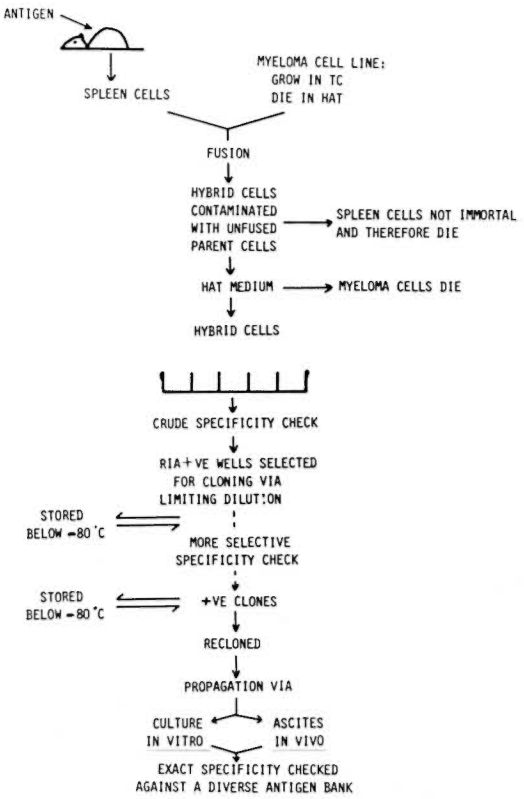

FIG. 1. Technique for mouse monoclonal antibody synthesis (much simplified). TC = tissue culture; HAT = hypoxanthine, aminopterin and thymidine; RIA = radioimmunoassay.

Hybridization takes less than one hour. The next stages in the production of MABs may take several months. Briefly, the hybrid cells are distributed into wells in a plastic tray and by 7–14 days the cell growth is sufficient to test for the presence of antibodies by immunoassay. The cells from the antibody-positive wells are then cloned by limiting cell dilution[20] as shown in Figure 1. Final mass production of the MAB may be achieved via an ascitic tumor in mice[21] or in culture media. MABs do not suffer the

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[21] Wright, P. Production of monoclonal antibody ascitic fluid and sera in mice with hybrid myeloma tumors. J. Inst. Animal Technicians **31** (1980) 113–118.

disadvantages of polyclonal antisera, such as batch variation, low titers, and extensive tissue absorportions required to obtain specificity.[22-24]

Mouse vs human MABs

The use of mouse MABs (mMABs) to study and to intervene in human disease has several shortcomings: a) mMABs used in man are immunologically foreign and may provoke immune responses to them by the host. b) The antigenic determinants selected may vary considerably between different species, and less so between different members of the same species. Thus, immunizing a mouse against antigens A, B, and C may produce a response against A and B but not against C. The same procedure carried out in man may produce antibodies to B and C. Clearly, this has far-reaching implications, including the hampering of attempts to develop serodiagnostic tests and new vaccines (see Serodiagnosis). c) There is recent evidence to suggest that anti-idiotypes may be important in the regulation of immune responses to *M. tuberculosis* and *M. leprae*.[25, 99] Immunomodulation using anti-idiotypes has therapeutic potential, but mMABs are not easily raised against idiotypic determinants of human antibodies. d) The raising of mMABs cannot provide the means of defining or comparing the range of specificities and idiotypes present in the antibody responses of patients and immune individuals. Thus, human MABs appear of greater scientific and therapeutic interest.

Human MABs

Human (hMABs) can be raised in two different ways: fusion of human B cells with a human myeloma line (human/human hybrid),[26] and fusion of human B cells with a mouse myeloma line (human/mouse hybrid).[27] Both methods have drawbacks. The human/human hybrids have three disadvantages: a) The current human myeloma lines are secretors of immunoglobulin (Ig), so there is contamination with irrelevant antibodies. b) The rate of Ig secretion is low. c) Most authors report a low percentage of secreting hybrids.

The main disadvantage of human/mouse hybridization is contamination with mouse components. The genetic instability problem has been overcome by re-cloning every three weeks for the first two months. Work is now under way to synthesize these human/mouse hybrids against mycobacterial antigens which will hopefully replace many of the mMABs used today. Such hybrids have already been generated against human idiotypes.[28] The dangers of injecting foreign protein into man to generate B cells for hMAB production must not be forgotten.

Epidemiology

Various methods have been employed to classify mycobacteria into species and strains (extensively reviewed elsewhere).[29] The most successful immunological methods have involved precipitation of soluble an-

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tigen in gel matrices by double diffusion, immunoelectrophoresis, and crossed immunoelectrophoresis. Essentially, there are two problems with these techniques: a) They require hyperimmune antisera of very high quality. As already mentioned, MABs have several advantages over polyclonal antisera. b) Even with the best antisera, it has been difficult to distinguish between different members of the tuberculosis complex (*M. tuberculosis, M. africanum, M. bovis,* and *M. microti*) and impossible to detect other strains.[30] Nevertheless, several groups have now produced mMABs to mycobacteria (Table 1).

Tuberculosis taxonomy. *M. tuberculosis, M. africanum,* and *M. bovis* are currently distinguished by morphological and biochemical criteria and by phage typing (A, B, and I).[41] The report by Coates, *et al.* in 1981[31] of mMABs against tuberculosis gave hope for both a better mycobacterial taxonomy and the possibility of a serodiagnostic test. A variety of whole tuberculosis and BCG strain bacteria were inoculated to generate MABs against intact cell surface antigens. The resulting MABs were then tested against a bank of mycobacteria for their species and strain specificity. Unfortunately, this bank was not extensive enough and notably did not include *M. africanum, M. vaccae,* and *M. leprae,* nocardia, or corynebacteria. Furthermore, although the MABs were generated against whole bacteria, they were tested against pressates (broken-down bacteria). Thus, there is a possibility of crossreaction between external and internal components of different organisms. Some biochemical analysis of these antigens has been made (personal communication, Dr. T. Coates). MAB TB68 binds a four-chain glycoprotein of 120KD and MAB TB72, a two-chain protein of 35KD. The tentative conclusions were that these MABs were able to detect differences between particular strains of *M. tuberculosis,* virulent *M. bovis,* and *M. bovis* BCG.

Leprosy taxonomy. The development of mMABS against *M. leprae* has recently been reviewed.[42] Gillis and Buchanan[35] were the first to produce mMABs of which only two (IVD8-IgG$_1$ and 111E9-IgG$_{2a}$) were specific against *M. leprae,* binding to cytosolic antigens. The methods of antigen production for immunization and for MAB-specificity testing were the same, the antigens being a mixture of extracellular and intracellular components. For epidemiological purposes this is a far more sensitive method of identifying differences between strains, since these differences may not be confined to the

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[41] Grange, J. M. and Redmond, W. B. Host-phage relationship in the genus *Mycobacterium* and their clinical significance. Tubercle **59** (1978) 203–225.

[42] Ivanyi, J. Application of monoclonal antibodies towards immunological studies in leprosy. Lepr. Rev. **55** (1984) 1–9.

TABLE 1. *Summarizing mMABs against* Mycobacterium tuberculosis, *BCG, and* M. leprae, *demonstrating the confusing nomenclature.*

Footnote reference	Example of mMABS produced or used	Chemical structure and specificity	Molecular weight (KD) and no. of chains	Localization	Crossreactivity
31, 32	TB68-IgG_1	Glycoprotein	120 4 chains	Cell wall	Narrow[a]
31–33	TB72-IgG_1	Protein or glycoprotein	35 2 chains	Cell wall	Narrow[b]
34	BCG SA12-IgG	Protein	10	Cell wall	Narrow[c]
35	IVD8-IgG_1	—	—	Cytoplasm	None
35	11H9-IgG_1	Protein	68	Cytoplasm	Broad
36	MLO6/MY1a-IgG_1	Protein	12	Cytoplasm	None
36, 37	MLO4/MY2a-IgG_1	Protein	—	Cytoplasm?	Marginal
36	MLO2/MY4a-IgG_3	Polysaccharide	40–50	Cell wall	Broad
36	ML35/MY4b-IgM				
38	PG_1 B5B-IgM	PGL-I-*M. leprae*-specific disaccharide	?	Cell wall	Marginal[d]
	PG_1 B2G-IgM	PGL-I-*M. leprae*-specific disaccharide	?	Cell wall	Moderate
39	PG_1 46.7-IgG_1	PGL-I-*M. leprae*-specific disaccharide	?	Cell wall	Not shown
39	Y_1-IgG_1	Protein	68	?	Not shown
	SA1 D2D-IgG_1[e]	Protein	28	Cytoplasm	Broad
	SA1 A1B-IgG_1[e]	Protein	28	Cytoplasm	Narrow
40	A494-IgM	?	?	Cell wall	All mycobacteria

[a] *M. tuberculosis* strains H37Ra, H37Rv, 6067, 729, and S1.
[b] *M. tuberculosis* strains and *M. bovis* BCG and Vallée.
[c] *M. bovis* BCG, *M. tuberculosis* H37Rv (other strains not tested).
[d] *M. terrae* and *M. nonchromogenicum.*
[e] Personal communication, Dr. D. B. Young.

bacterial cell wall. In this respect, genome analysis will provide the ultimate sensitivity.

In a later report,[38] Buchanan, *et al.* described a set of IgM mMABs against the *M. leprae*-specific trisaccharide (Fig. 2). Confirmation that this trisaccharide was only found in *M. leprae* has come from structural[43] and serological[44] studies. However, some the MABs which apparently only bound to the intact trisaccharide (i.e., *M. leprae* specific) also crossreacted with *M. bovis, M. nonchromogenicum,* and *M. terrae.*[38] At least two possibilities therefore exist: a) The trisaccharide, although unique structurally, antigenically is not, and the previous serological studies were not sufficiently sensitive. Indeed, the latest investigations have shown crossreactivity with *M. bovis* and *M. kansasii* (although this crossreactivity may be phenol-ring related).[45] b) Nonspecific binding occurred to the other organisms. Although this occurs with IgM-class antibodies, it seems unlikely since only some organisms were stained. However, these MABs have already been of great use in investigating the T-suppressor-cell activity generated by the trisaccharide.[39]

One of the disadvantages of trying to use MABs to isolate species-specific antigens is, indeed, their extreme specificity. While such MABs recognize an epitope on a molecule which may be species specific, the other epitopes may be crossreactive, and vice versa. An example of this has just been reported (personal communication, Dr. D. B. Young). Five mMABs have been synthesized which

[43] Payne, S. N., Draper, P. and Rees, R. J. W. Serological activity of purified glycolipid from *Mycobacterium leprae.* Int. J. Lepr. **50** (1982) 220–221.

[44] Yanagihara, D. L., Knisley, C. V., Barr, V. L., Hunter, S. W. and Brennan, P. J. ELISA and the specific glycolipid antigens of mycobacteria. Fed. Proc. **42** (1983) 2022.

[45] Brett, S. J., Payne, S. N., Draper, P. and Gigg, R. Analysis of the major antigenic determinants of the characteristic phenolic glycolipid from *Mycobacterium leprae.* Clin. Exp. Immunol. **56** (1984) 89–96.

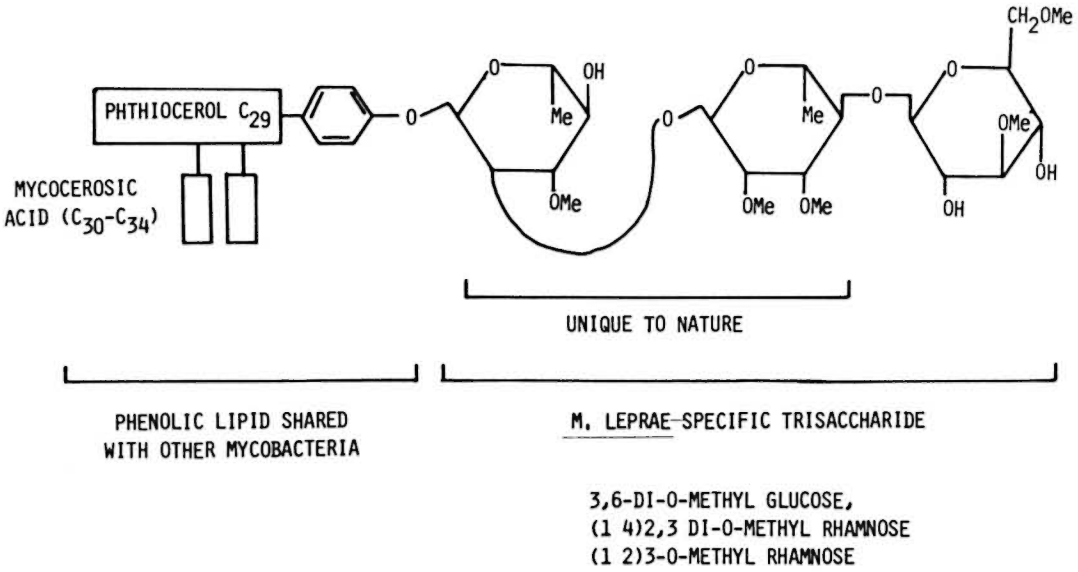

FIG. 2. Chemical structure of the *M. leprae* trisaccharide.

bind to an intracellular antigen of 28KD found in all mycobacteria. Two of these MABs are *M. leprae* specific, one is cross-reactive with all mycobacteria, and two are partially specific (*M. leprae, M. triviale,* and *M. phlei*). Interestingly, competition studies have suggested that the species-specific epitopes are closely clustered while the cross-reactive epitopes are on another distinct part of the molecule.

MAB taxonomy. A rapidly enlarging array of MABs exist against mycobacteria (Table 1). A classification system, perhaps that of Ivanyi,[33] should be formed. Workshop meetings would produce much useful interchange. (A good example of this has been the development of a human leukocyte MAB classification scheme.[46, 47])

Other epidemiological uses. The use of MABs in taxonomy is not promising since most are crossreactive. Nevertheless, the MABs which are *M. leprae* or *M. tuberculosis* specific could be used to explore modes of transmission of infection, organism virulence, host susceptibility, the epidemiology of small epidemics, and such interesting questions as whether or not tuberculosis originates by an infection with a single or several bacilli.

MABs and immunopathology

Tuberculosis, and particularly leprosy, exist as disease spectra. In leprosy, at one extreme are patients who mount a vigorous cell-mediated immune (CMI) response, tuberculoid leprosy (TT); at the other extreme are patients who have a profound absence of CMI, lepromatous leprosy (LL). The failure of CMI, particularly in tuberculosis, may be fatal for the patient. The exact mechanism(s) involved and why they occur only in some patients are still unclear. In order to understand the possible roles of MABs, the CMI pathway and its regulation are outlined hereafter and pertinent recent advances in immunology are highlighted.

CMI pathway (Fig. 3). Mycobacteria penetrate the body through a variety of sites. For *M. tuberculosis* the usual portal of entry is the lungs, "wild-type" mycobacteria probably enter during their passage along the gastrointestinal tract, while *M. leprae*'s portal is unknown. The bacteria are then phagocytosed by macrophages and partially digested ("antigen processing"), allowing some bacterial components to be extruded

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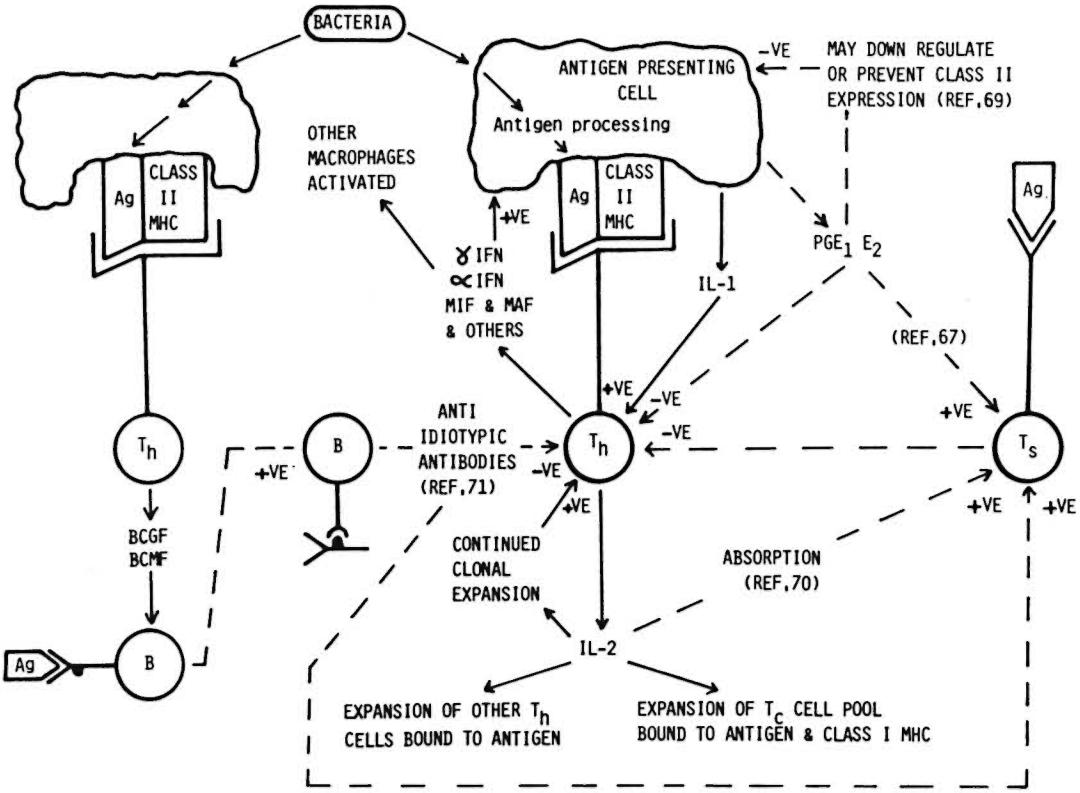

FIG. 3. A much-simplified view of the immune pathways in leprosy and tuberculosis. Suppressor mechanisms (-----) may therefore include: a) an antigen-presenting cell defect, b) a hole in the Th repertoire, c) IL-1/IL-2 and/or PGE_1/PGE_2 defect, d) Ts cells, e) anti-idiotypes, f) a lymphon defect. Ag = antigen; MHC = major histocompatibility complex; IL-1 = interleukin-1; PGE_1 = prostaglandin E_1; IFN = interferon; MIF and MAF = migration inhibition and macrophage activation factor, respectively; Th = helper T lymphocyte; Ts = suppressor T lymphocyte; B = B lymphocyte; BCGF and BCMF = B cell growth and maturation factors, respectively; +ve = positive effect; −ve = negative effect.

onto the cell surface for antigen presentation.[48] Effective presentation can only occur when the foreign antigen is combined with class I (HLA A B C) or class II (HLA D DP, DQ, DR)[48b] major histocompatibility (MHC) molecules. Class I MHC molecules are found on all nucleated cells; whereas class II MHC molecules only occur on antigen-presenting cells (APCs) (usually of monocyte origin), some T and B cells, thymus epithelium, and a few other cell types.[49]

The use of highly purified allo- and hetero-antisera and MABs have permitted differentiation and analysis of "functional" T-cell subsets (Table 2). In addition, antigen- or mitogen-activated T cells may be distinguished from resting T cells by their increased density of class II antigens[50, 51] and Fc receptors for IgG.[52]

[48] Unanue, E. R., Beller, D. I., Lu, C. Y. and Allen, P. M. Antigen presentation: comments on its regulation and mechanism. J. Immunol. **132** (1984) 1–5.

[48b] Bodmer, J. and Bodmer, W. Histocompatibility 1984. Immunol. Today **5** (1984) 251–254.

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[52] Kaszubowski, P. A., Goodwin, J. S. and Williams, R. C., Jr. Ia antigen on the surface of a subfraction of T cells that bear Fc receptors for IgG. J. Immunol. **124** (1980) 1075–1078.

TABLE 2. *Functional T-cell subset identification on mouse and man.*

T-cell function	Mouse phenotype	Human phenotype
T helper/inducer (Th/i)	Lyt 1+/23−[53]	TH_2−[54, 55] OKT4+[56]
T suppressor/cytotoxic (Ts/c)	Lyt 1−/23+[53]	TH_2+[52, 53] OKT5+, OKT8+[57]

The functional purity of these T-cell subsets is questionable. For example, mouse suppressor T cells (Ts) have been found which are Lyt-1+/2,3−[58, 59] or Lyt-1+/2,3+.[60] Helper T cells (Th) may also express different Lyt phenotypes.[61] Similar functional heterogeneity is known to exist in man.[62]

It has been shown that Th cells will only respond to foreign antigen when it has been processed and is properly presented (usually in conjunction with self-class II molecules).[49] Cytotoxic T cells (Tc) are similarly restricted but usually to self-class I antigens.[63] Clearly, any defect in macrophage processing and antigen presentation may alter Th or Tc cell responsiveness to foreign antigen. (Indeed, HLA DR3-negative individuals mount a poor immune response to *M. leprae*.[64])

The binding of Th cells to an antigen plus class II MHC complex triggers interleukin-1 (IL-1) release from the antigen-presenting cell (APC).[48] This, in turn, causes Th cell release of various humoral, non-immunoglobulin molecules (lymphokines) which mediate a variety of biological effects. At this point the two arms of the immune response, CMI and humoral immunity, diverge. The types of lymphokines secreted are dependent upon the type of antigen presented and, thus, upon the responding Th cell. If the antigen creates a predominantly T-cell-dependent, B-cell response, then most of the lymphokines released will activate antigen-bound B cells (for example, B-cell growth factor).[65] Conversely, antigens stimulating CMI are associated with the secretion of macrophage and/or Tc-activating lymphokines. These include IL-2, alpha-interferon (α-IFN), macrophage inhibition factor (MIF), and macrophage activating factor (MAF).[48, 66] In leprosy and tubercu-

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losis, the most important part of the immune response is the effective activation of macrophages to enable mycobacterial destruction.

The immune response, like most biological systems, may be "down" regulated. This can be achieved through a variety of mechanisms involving suppressor T cells (Ts), excess circulating antibodies, or idiotypic antibodies. Thus, the suppression can be both cell mediated and humoral. Recently, there has been increasing evidence that prostaglandins, PGE_1 and PGE_2, produced on the surface of APCs can "down" regulate the response by inhibiting Th cells[67] (probably by prolonged activation of adenylate cyclase; cAMP inhibits lymphocyte transformation), activating Ts cells,[68] or "down" regulating the expression of class II MHC antigens on APCs.[69]

Finally, it is important to remember the role of the lymphon, the collective name for the primary and secondary lymphoid organs and their constituent cells. All too often the cellular interactions and functions underlying immune responses are considered out of the context of their physiological environment. The crucial importance of the cyto-architecture (e.g., the role of dendritic reticular cells in germinal center formation) and the concept of lymphocyte traffic, together with its disturbance by antigen,[72] needs to be considered when studying deficient or abnormal immunology.

Histology. In addition to their use in subdividing T cells, MABs have been developed against various class II molecules,[73] IL-2,[74] the IL-2 receptor,[75] and subpopulations of APCs.[76] Some of these MABs are now being used in the study of histological sections of leprosy granulomas. Initial results have shown that the well-organized epithelioid granulomas of tuberculoid leprosy (TT) lesions contain large numbers of OKT4+ (helper/inducer T cells, Th/i) lymphocytes surrounded by a heavy "mantle" of predominantly suppressor/cytotoxic T cells (Ts/c). The overall Th : Ts ratio is normal (2:1). The diffuse lepromatous lesion, by contrast, has few lymphocytes, and there is controversy over the existence of a decreased Th : Ts/c ratio.[2, 77, 78] As already mentioned, subdividing T cells by their surface phenotypes is functionally very misleading. In their most recent paper, Modlin, *et al.*[79] have attempted to overcome this problem by MAB analysis of IL-2-producing and IL-2-accepting cells in TT and LL biopsy specimens. Using double-staining techniques, they found that in TT lesions there were large numbers of IL-2-producing (Th phenotype) cells in intimate contact with

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macrophages surrounded by a mantle of mainly IL-2-accepting Th phenotype cells. The IL-2-producing cells did not possess IL-2 receptors. By contrast, the disorganized LL lesions had few IL-2-producing cells and decreased numbers of IL-2-accepting cells of both helper and suppressor phenotypes. From these data it is tempting to suggest that defective CMI in LL is associated with decreased IL-2 production, but this seems unlikely to be the primary defect. Biopsy work with borderline lepromatous, borderline tuberculoid, and indeterminate leprosy patients should now be done in an attempt to clarify the situation.

Unfortunately, similar investigations have not yet been performed in tuberculosis lesions. This presumably reflects both the difficulty in obtaining lung biopsies and the comparative paucity of workers in the field.

Clearly, MABs can play a vital role in the analysis of cell types and cell products in histological sections, and this is likely to yield far more relevant data than the investigation of peripheral blood cells.

MABs and erythema nodosum leprosum (ENL). Classically, type II lepra reactions (ENL) have been thought to result from an Arthus type of hypersensitivity—circulating immune complexes (CIC) being deposited in capillary beds and then activating inflammatory processes via the complement cascade.[80] However, there is considerable evidence to suggest that the CIC and alterations in complement levels found are secondary phenomena for the following reasons: a) Similar levels of CIC occur in LL patients with or without ENL.[81] b) In areas with endemic leprosy, CIC may be found throughout the entire leprosy spectrum, although ENL only develops in LL patients.[82] c) Thalidomide, used in the treatment of ENL, has no suppressive effect on experimental Arthus reactions. d) Local levels of C3d in lepromatous lesions do not correlate well with the circulating levels of C3d.[83]

The latter point may suggest that local complement production by tissue macrophages may be more important in ENL. Complement fragments such as C3a are extremely chemotactic for neutrophils.[72] An alternative pathogenesis for ENL reactions could involve local tissue dissemination of bacteria, triggering the release of local complement and, thus, neutrophil chemotaxis toward multiple sites in the body, where degranulation would cause tissue injury. Indeed, neutrophil chemotaxis is depressed in untreated LL patients but enhanced by dapsone chemotherapy.[84] Thus, the increased occurrence of ENL in LL patients during their first year of dapsone therapy may be related to increased chemotaxis. However, not all LL patients on chemotherapy develop ENL. Although such a hypothesis has obvious weaknesses, it would be interesting to investigate further the role of macrophages and neutrophils in ENL reactions, perhaps using MABs to define specific cell types and products.

Serodiagnosis

The possibility of accurate serodiagnosis from a drop of blood, urine, or cerebrospinal fluid in leprosy or tuberculosis has been the dream of many investigators. It would be of particular use in the following: a) cases with uncertain diagnosis; b) paucibacillary cases with negative immediate staining or long-term culture; and c) in a case-finding control program together with, or as an alternative to, biological examination.

Such a test would have the following requirements: a) species-specific antigens; b) evoke an antibody response in all infected individuals, including severely ill patients; and c) evoke a response which wanes rap-

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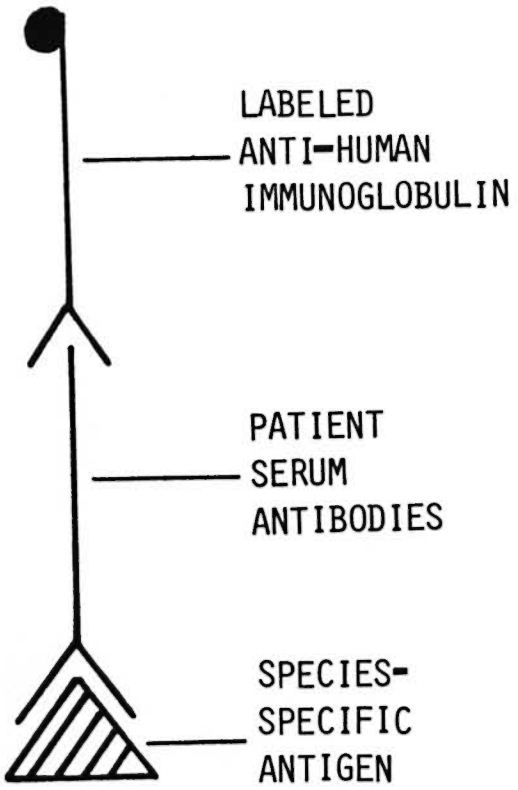

FIG. 4. Principles of an indirect antibody assay to a species-specific antigen.

idly when infection is no longer active or after effective vaccination.

In principle, the presence of infection would be determined either by detection of circulating species-specific antigens or by detection of circulating antibodies generated by an immune response to species-specific antigens. Clearly, the presence of antigen is better proof of infection than an antibody assay, but it is not yet clear whether *M. tuberculosis* releases sufficient antigen to make this a realistic possibility. Even in LL, where the bacterial load is enormous, it has proved remarkably difficult to demonstrate mycobacterial antigen in the serum, partly because much of it is circulating as immune complexes.[82] It is conceivable that MABs will allow the development of assays of sufficient sensitivity.

To date, all attempts at serodiagnosis have relied on detecting circulating antibodies to species-specific antigens. This has been performed by solid- or liquid-phase binding assays using enzyme or radiolabeled second-layer antibodies (Fig. 4). Such assays are not dependent upon secondary antibody characteristics, such as complement fixation or agglutination, and thus, in theory, can detect all antibody classes which bind to the antigen in question. Unfortunately, no antigen has yet been found with epitopes specific to a single species. This was previously attributed to the physical characteristics of mycobacteria, which do not break down into sub-units of predictable molecular weights and properties.[85] However, the recent MAB work has overturned this view and has delineated reproducible antigens for both *M. leprae* and *M. tuberculosis* (Table 1). Yet even these have crossreacting epitopes (Fig. 5).

Two possible solutions exist. Firstly, species-specific epitopes (e.g., the *M. leprae* disaccharide) could be synthesized as shown in Figure 4. An alternative and simpler approach would be the use of whole molecules in an inhibition assay (Fig. 6).[32, 33, 37]

The so-called *M. leprae*-specific, phenolic glycolipid-I (PGL-I), as aforementioned, has been investigated by MABs[38] and serology.[44, 45] Both methods have revealed that the terminal saccharide is the most important epitope. One group has recently synthesized the terminal disaccharide and conjugated it to bovine serum albumin (BSA).[86] Preliminary results in screening sera from healthy controls, tuberculosis patients, and leprosy patients have, however, shown some cross-reactivity between mycobacterial species. This has been attributed to nonspecific BSA binding, but this seems unlikely since the MABs also show species crossreactivity. In addition to this, there are two other problems with such assays. Firstly, when the immune response of an outbred species such as man is presented with an immensely complex antigenic structure such as mycobacteria, only some of the thousands of epi-

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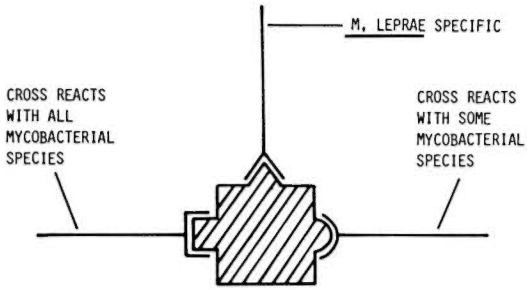

FIG. 5. A single molecule (antigen) with varying epitope specificities.

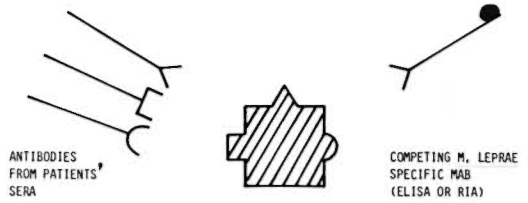

FIG. 6. Principles of an inhibition assay.

topes evoke an antibody response. Which ones are selected will depend partly on genetic factors and partly on the previous priming of antibody responses by, for example, "wild type" mycobacteria. Several reports illustrate this point.[87, 88] When sera from tuberculosis patients were tested against a range of glycolipids isolated from *M. tuberculosis,* different individuals were found to have responded to different selections of these molecules.[88] Thus, a diagnostic assay may require a battery of species-specific, MAB-defined epitopes. Interestingly, a bank of TB-specific MABs[32] was no more successful than a single TB-specific MAB[33] in serodiagnostic testing. Neither approach was particularly encouraging, achieving 74% positive diagnoses in a highly selected population of patients. The recent developments of hybrid hybridomas producing bispecific MABs may reduce the total bank of MABs necessary for serodiagnosis, provided the species-specific epitopes occur close together on the same molecule. Some tentative evidence may exist for this (see Leprosy taxonomy).

The second problem with any assay of antibody titer against infecting mycobacteria has been with the serum antibody concentration. Severely ill tuberculosis patients who are not on treatment have low antibody levels.[89] This has been attributed to depressed immunity. Conversely, LL patients with severely depressed CMI have high levels of circulating antibodies, some of which are *M. leprae* specific.[90, 91] Furthermore, the response to *M. tuberculosis* is often delayed, with the highest titers and the greatest variety of antibodies occurring several weeks after the disease is initially diagnosed.[88] Thus, subclinical diagnosis in epidemiological studies may not be possible for tuberculosis. Abe's fluorescent antibody absorption test has successfully detected *M. leprae*-specific antibodies in subclinical infections.[92, 93] Clearly, leprosy has better prospects for serodiagnosis than tuberculosis.

A recent paper has attempted to hasten the search for new *M. leprae*-specific MABs by testing LL sera against *M. leprae* components of known molecular weights derived from *M. leprae* sonicates run on SDS-PAGE.[94] Six of these were only identified

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by sera from leprosy patients and not by sera from normal controls. Obviously, this study needs to be extended to include sera from the whole spectra of leprosy and tuberculosis. Such *M. leprae*-specific antigens may then be useful for generating MABs for immunodiagnosis.

Hopefully, the creation of human MABs will reveal a species-specific epitope which will react with more than 95% of patient sera. Then we may have a truly useful tool.

Treatment

Immunomodulation. Several theories have been proposed to explain the deficient CMI response to mycobacteria observed in some patients with TB or leprosy. The evidence for and against these hypotheses is outlined below.

Ts cells. Bloom, *et al.*, in a series of well-controlled experiments, have shown that exposure of peripheral blood mononuclear cells from lepromatous (LL) or borderline (BL) patients to Dharmendra lepromin (D. lepromin) *in vitro* consistently resulted in the suppression of allogenic lymphocyte transformation in response to concanavalin A (a polyclonal T-cell activator).[95] The suppressor activity was shown to originate from monocytes and OKT8 phenotype T cells (Ts/c).[96, 97] These Ts/c cells also expressed Fc and Ia receptors, indicating an active cell population,[97] although this activity may be nonspecific. Removal of the OKT8 phenotype T cells from blood samples of BL and LT patients allowed proliferation of OKT4 phenotype T cells in response to D. lepromin. In LL patients, removal of the Ts cells did not permit proliferation, suggesting extremely low levels of D. lepromin-responsive Th cells. This group's most recent results have indicated that some of the suppression may be mediated through Ts cell recognition of the PGL-I terminal disaccharide.[39] MABs specific for this *M. leprae* disaccharide completely inhibited the suppression induced by PGL-I. In patients who have improved following vaccination with the new Convit mixture of killed *M. leprae* and live BCG, there is an almost total reduction in both D. lepromin-induced and PGL-I-induced suppression.[39] This suggests that the Ts cells reactive with PGL-I are relevant to the immunological anergy of LL patients. Suppression has also been found against BCG and tuberculin, in the cases mediated by MHC-restricted OKT4 phenotype cells.[98]

In animal models, BCG-activated Ts cells have been shown to elaborate an as yet uncharacterized nonspecific suppressor factor which inhibits *in vitro* DNA synthesis.[99] This is associated with a lack of IL-2 production.[100] A lack of IL-2 has been reported in LL[101] and in *M. lepraemurium* infections.[102] Thus, it is conceivable that Ts cells, in response to certain mycobacterial epitopes, secrete a suppressor factor (SF) which may act directly on Th cells or indirectly on APCs (by reducing IL-2 production and secretion; Fig. 7). There is also skin-test evidence for such circulating SFs.[110] The fact that IL-1 production appears to be normal in LL patients[103] suggests that SF may not act on the APC (but, of course, this is not ruled out). Normal IL-1 levels may also imply

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normal antigen presentation even in HLA DR3-negative individuals.

If this suppressive mechanism exists, then the following roles for MABs can be predicted: a) Bloom, *et al.* have suggested the use of OKT8 MABs[96] either as complement activators or conjugated to the potent plant toxin ricin.[104] This would involve the destruction of the entire circulating Ts/c cell pool. Such a drastic measure might lead to unwanted reactions (e.g., autoimmune disease and viral infections). This is clearly undesirable and, until we can develop human MABs to *M. leprae*-specific Ts cells, such methods are unlikely to be used. Perhaps Ts cells which bind the *M. leprae*-specific disaccharide could be isolated and then used to generate specific MABs. An alternative approach may involve the use of recombinant DNA technology to isolate the DNA coding for Ts cell receptor diversity. b) hMABs against mycobacterial suppressor epitopes; c) hMABs to purify IL-2. In at least some cases, IL-2 extracts have been shown to reverse *in vitro* suppression seen in LL.[98] However, IL-2 has been used in AIDS patients with mixed success.[105] Another disadvantage is its short half-life.[100] Perhaps IL-2 will be useful in treating downgrading reactions (leprosy patients who rapidly progress toward the LL pole).

Some discrepancies exist in this Ts cell model. Nath, *et al.* have been unable to show Ts cell activity in LL.[106] Indeed, suppression was seen at the tuberculoid end of the spectrum. This difference could be attributed to the different *M. leprae* preparations used. In addition to these antigenic differences, there was considerable variation between the patient populations and their treatment status investigated by these research groups. Probably of most relevance is the antigen overload, particularly of PGL-I, which occurs in LL.[2] This is likely to give secondary immune suppression,[107] and raises the important question of whether or not the T suppression observed by Bloom, *et al.* is a primary or secondary phenomenon. Rook, *et al.* have indicated that leprosy patients only respond to the species-specific mycobacterial antigens (group iv). This is suggested from delayed-type hypersensitivity (DTH) responses to dermal challenge with various mycobacteria.[108, 109] LL patients do not respond to *M. leprae* antigens but following treatment, many patients respond to antigen preparations from other crossreactive species. If DTH corresponds to CMI, then the Ts cells specific to *M. leprae* determinants may not be relevant, and we will need to look for the mechanism of suppression of common mycobacterial antigens. It seems unlikely that both types of suppression exist and may be important in differing circumstances.[110–113] Again, MABs could be of great use in defining and analyzing the roles of group i and group iv an-

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[106] Nath, I., van Rood, J. J., Mehra, N. K. and Vaidya, M. C. Natural suppressor cells in human leprosy: the role of HLA-D-identical peripheral lymphocytes and macrophages in the *in vivo* modulation of lymphoproliferative responses. Clin. Exp. Immunology **42** (1980) 203–210.

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[113] Raff, M. C. Do antigen presenting cells distinguish self from non-self? Nature **298** (1982) 791–792.

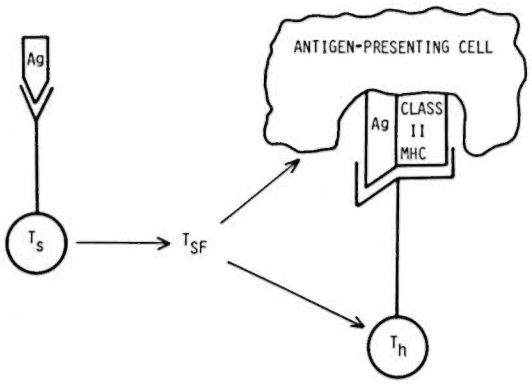

FIG. 7. Possible targets of a T-suppressor factor. T_{SF} = T-suppressor factor (see Figure 3 for other abbreviations).

tigens on slow- and fast-growing mycobacterial species.

Macrophage-Th cell axis. A macrophage defect may reside in the failure to process, present, destroy, or elaborate factors in leprosy or tuberculosis. Little is known about the mechanisms of antigen processing.[113] MABs can be used in pulse-chase experiments to detect what happens to individual epitopes as they are processed. This may reveal which epitopes are selected for presentation (and how they are modified) and which are discarded. Perhaps antigen processing is not required at all.[114] It is possible that only certain macrophage subpopulations are capable of correct presentation. These subpopulations may be analyzed by MABs.[76] MABs generated against sulfatides[115] may reveal the details by which *M. tuberculosis* and *M. leprae* prevent phagosome-lysosome fusion and *M. leprae* can escape from phagosomes and exist within the cytosol.[116] Armed with such knowledge, we may hope to develop new therapies, perhaps by encouraging lysosome-phagosome fusion.

At present there is no concrete evidence of a specific macrophage defect.[117] An association between HLA DR3-negative individuals and unresponsiveness to *M. leprae* has been claimed.[64] However, this group's latest results confuse the matter—HLA DR3-negative macrophages present better than HLA DR3-positive macrophages.[118] Nath, *et al.*[106] and other workers[95] have described a macrophage-derived suppressor factor. If this exists, then MABs directed against it would block unwanted suppression.

A hole in the Th cell repertoire is unlikely for several reasons, and it seems improbable that Th cells are unresponsive to all *M. leprae* antigens. The development of MABs, T-cell clones, and recombinant DNA technology may all help to solve the unanswered problem of T-cell restriction and, hence, the ongoing immunological argument of determinant selection versus hole in repertoire.

Idiotypes. Collizzi, *et al.* have recently published exciting work demonstrating the possible role of idiotypes in regulating the immune response in tuberculosis and leprosy.[119] Mice heavily infected with BCG produced antibodies which specifically inhibited DTH to TB purified protein derivative (PPD) in transfer experiments. There was some evidence to suggest that these antibodies bind to T cells.[99] MABs could be used to define which T-cell subpopulation was involved and might indicate whether or not the T-cell receptor or its associated molecules are the target of anti-idiotypes in mycobacterial suppression. Daus, *et al.*[120] have shown that antibody subclass is important in determining suppression or stimulation of T and B cells in immune responses to BCG in guinea pigs. The

[114] Kaye, P. M., Chain, B. M. and Feldman, M. Accessory cell heterogeneity in anti-mycobacterial responses. Immunobiol. **167** (1984) 155–156.

[115] Goren, M. B., D'Arcy Hart, P., Young, M. R. and Armstrong, J. A.Prevention of phagosome lyosome fusion in cultured macrophages by sulfatides of *Mycobacterium tuberculosis.* Proc. Natl. Acad. Sci. U.S.A. **73** (1976) 2510–2514.

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[117] Rook, G. A. W. The immunology of leprosy. Tubercle **64** (1983) 297–312.

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[119] Ferluga, J., Colizzi, V., Ferrante, A., Colston, M. J. and Holborow, E. J. Hypothesis: possible idiotype suppression of cell-mediated immunity in lepromatous leprosy. Lepr. Rev. **55** (1984) 221–227.

[120] Daus, H., Hammer, H. J., Rajki, K. and Mauch, H. Influence of subclass-specific anti-idiotypic antibodies on the kinetics of the immune response to BCG. Immunology **52** (1984) 697–702.

relationship between Gm allotypes and idiotypes[121] may provide another genetic link with the susceptibility of individuals. MABs against Gm allotypes may help to define this. Another potential role of idiotypes would be to mimic antigen, and thus stimulate a host immune response.[141] Similarly, idiotypes resembling the Ts-cell receptor might allow the host to overcome deleterious suppressor mechanisms.

MABs could, of course, be developed to idiotypes or idiotopes in order to modulate the immune response in a beneficial way. It seems likely that several mechanisms of suppression are at work in mycobacterial disease, and MABs will play a major role in analyzing these. However, for the present, the use of MABs in the treatment of patients cannot be foreseen, not only because of our lack of understanding but also because of the difficulty in transport (refrigeration) and administration. A vaccine is the best hope for the Third World.

Prophylaxis. Traditionally, antibodies have been used to provide passive immunity. Clearly, in leprosy and tuberculosis, where the organisms are concealed from circulating antibodies, such prophylaxis is useless. The ideal vaccine for developing countries should have the following properties: a) inexpensive to produce, b) provides full immunization after one dose, c) easily distributed and administered (e.g., no refrigeration required), and d) has no side effects.

MABs in conjunction with recombinant DNA technology are being used to make a new tuberculosis vaccine. The details and prospects for this are outlined in the section, Prophylaxis—new vaccines.

Prognosis

Many different factors may relate to the prognosis of mycobacterial disease. For example: a) There may be changes in antibody subpopulations (e.g., circulating idiotypes against Th cells) which may affect the prognosis.[99] b) Many patients with LL have CIC but do not suffer ENL. MABs may be used to analyze which CICs correspond with ENL. Recent work has indicated that the number of mycobacterial constituents in these complexes is rather restricted and, to date, only one protein antigen (67KD) has been identified,[122] possibly that recognized by MAB 11H9.[35] c) Serum markers may be discovered (e.g., SF). d) It may become possible to measure the proportion of *M. leprae*- or *M. tuberculosis*-specific Ts cells.

Thus, MABs will provide both the definition of disease markers and the precise methods of monitoring changes in their levels.

RECOMBINANT DNA TECHNOLOGY

Recombinant DNA technology or genetic engineering is legally defined as "the formation of new combinations of heritable material by the insertion of nucleic acid molecules, produced by whatever means outside the cell, into any virus, bacterial plasmid, or other vector system so as to allow their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation."

The historical background for genetic engineering is difficult to trace because of its multiple origins within science. For example, the discovery of useful restriction endonucleases came from virologists in 1970,[123, 124] transformation from bacteriologists in 1970,[125] and effective DNA separation by agarose gel electrophoresis from biochemists in 1977.[126] Some of the relevant principles of this technology are briefly

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[121] Binz, H., Wigzell, H. and Bazin, H. T cell idiotypes are linked to immunoglobulin heavy chain genes. Nature **264** (1976) 639–642.

[122] Chakrabarty, A., Maire, M., Saha, K. and Lambert, P.-H. Identification of components of IC purified from human sera. II. Demonstration of mycobacterial antigens in immune complexes isolated from sera of lepromatous patients. Clin. Exp. Immunology **51** (1983) 225–231.

[123] Smith, H. O. and Wilcox, K. W. A restriction enzyme from *Hemophilus influenzae*. I. Purification and general properties. J. Mol. Biol. **51** (1970) 379–391.

[124] Kelly, T. J. and Smith, H. O. A restriction enzyme from *Hemophilus influenzae*. II. Base sequence of the recognition site. J. Mol. Biol. **51** (1970) 393–409.

[125] Mandel, M. and Higa, A. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53** (1970) 159–162.

[126] Johnson, P. H. and Grossman, L. I. Electrophoresis of DNA in agarose gels: optimizing separations of conformational isomers of double-stranded and single-stranded DNAs. Biochem. **16** (1977) 4217–4225.

TABLE 3. *Properties of classes of vectors for recombinant DNA technology.*

Vector class	Maximum size of foreign DNA insert in kilobases (kb)	Size of vector DNA (kb)
Plasmid	15	3–15
Phage	25	50
Cosmid	50	25

discussed below and more comprehensive reviews may be found elsewhere.[127]

The principles

DNA isolated from bacteria must first be broken into manageable lengths using restriction endonuclease enzymes. These enzymes form the basis of our present-day DNA technology. Over 200 have been described, and there is now a complicated classification system. The restriction endonuclease is able to precisely cut DNA at a specific site and does this reproducibly. The fragments are then cloned to form a genomic library. Cloning involves the bulk production of each DNA fragment isolated. This is achieved by inserting the DNA into vectors which are taken up by dividing cells (e.g., *Escherichia coli*). The DNA fragment is then copied whenever the cell divides. Three vector classes have been described, and their properties are summarized in Table 3.

The advantages and disadvantages of these vectors have been reviewed.[127] In order to distinguish between replicating cells (for example, between *E. coli* containing the vector with the DNA fragment and *E. coli* containing the vector alone, or no vector), certain characteristics such as penicillin resistance may be pre-incorporated into the vector (Fig. 8). This principle is similar to the selection of MAB-producing hybridomas with HAT medium. Because of the very rapid growth of *E. coli* (even if only one organism in 10,000 has taken up a recombinant plasmid), mass cultures consisting exclusively of a specific small subpopulation can be produced in a matter of days.

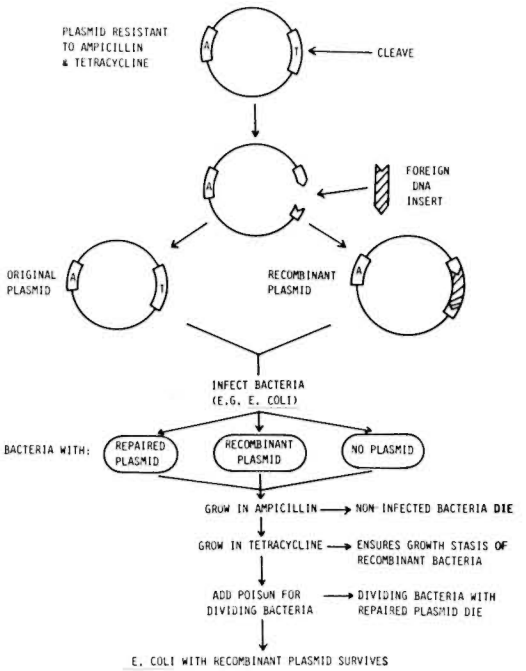

FIG. 8. Principles involved in the selection of recombinant plasmids. A = ampicillin resistance; T = tetracycline resistance.

It is now possible to mix the collection of random fragments of total genomic DNA with vector DNA (e.g., plasmid) in linear form. Physical conditions are adjusted to give the maximum likelihood of closure of each plasmid circle by insertion of just one genomic fragment. Thus, a heterogeneous population of *E. coli* will be produced containing plasmids with different inserted fragments. Conventional bacteriological procedures, however, allow colonies of *E. coli* to be grown from single organisms, and if all of the original DNA has been incorporated, virtually every DNA fragment from the original mixture is represented in the set of isolates forming a genomic library. Such libraries now exist for some strains of the tuberculosis complex and *M. leprae* (personal communication, Dr. T. Coates).

The next, rather tedious, step involves reading the libraries. The vector DNA is separated from *E. coli* DNA by centrifugation. Recombinant plasmid DNA is then cleaved by the same restriction enzyme used for the insertion step and separated from the foreign DNA by agarose gel electrophoresis. The phenomenon of hybridization is

[127] Old, R. W. and Primrose, S. B. *Principles of Gene Manipulation.* 2nd ed. Oxford: Blackwell Scientific Publications, 1981.

then exploited, in which single-stranded DNA (ssDNA) always pairs with its complementary DNA sequence (guanine opposite cytosine, adenine opposite thymidine). In this way, biotin or radiolabeled complementary DNA (cDNA) probes of 15 base pair length constructed based upon, e.g., amino acid sequences of a particular protein, can be used to locate the protein-producing DNA. The techniques involved in locating a desired gene from a clone bank include Southern blotting (after Dr. E. M. Southern), where cDNA hybridizes with DNA;[128] northern blotting, where RNA probes hybridize with DNA;[129] and western blotting, where antibody binds to protein produced from the clone bank.[130]

Epidemiology

Theoretically, the most sensitive methods of detecting differences between species and strains of mycobacteria would operate at the genomic level. This problem can be approached in several ways. Imaeda, *et al.* have used whole genome hybridization to analyze species homology.[131] A more sensitive method involves DNA fragmentation by restriction endonucleases and separation into electrophoretic DNA patterns. Each mycobacterial species would then have its own DNA "fingerprint." However, differences may occur in non-coding parts of the genome. Thus, these tests may suffer from poor specificity.

One group working in New Zealand has recently published a preliminary report using the latter method.[132] While they could successfully distinguish *M. tuberculosis* from *M. bovis,* they were unable to demonstrate any difference between *M. tuberculosis* strains H37Ra and H37Rv (17 types of restriction enzymes were tried). If strain differences reside in single amino acid substitutions, then only full DNA sequencing will allow distinctions to be made. Such substitutions may determine infectivity and pathogenicity of mycobacteria.

In addition to studying mycobacterial virulence, recombinant DNA techniques can be used to determine the genetics and mechanisms of host susceptibility. Indeed, T-cell receptor research has been greatly advanced by genetic engineering, and the genes coding for part of this elusive receptor have been revealed.[133–135] While we are close to understanding the generation of Th-cell receptor diversity, we still do not possess the molecular explanation for T-cell restriction or the nature of the Ts-cell receptor. Such answers have far-reaching implications, not only in terms of host susceptibility but also for the development of effective vaccines.[136]

Recombinant DNA technology could be used to analyze interesting phenomena, for example, a) the relationship between corynebacteria and *M. leprae.* Preliminary work has shown great DNA homology between corynebacteria isolated in LL patients and *M. leprae.*[131] Certainly, corynebacteria and *M. leprae* ribosomes are closely related.[29, 85] b) Why is *M. leprae* more pathogenic than *M. tuberculosis*? Despite numerous claims of *in vitro* growth of *M. leprae,* none has yet been substantiated.[137] Research into the biochemical pathways of *M. leprae* has been hampered by the small numbers of bacteria available from *in vivo* growth systems (250 mg dry weight bacteria from 50 g infected tissue) and the impure samples obtained even with NaOH extraction.[137] Genetic en-

[128] Southern, E. M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98** (1975) 503–517.

[129] Alwine, J. C., Kemp, D. J., Parker, B. A., Reiser, J., Renart, J., Stark, G. R. and Wahl, G. M. Detection of specific RNAs or specific fragments of DNA by fractionation in gels and transfer to diazobenzyloxy-methyl paper. Methods Enzymol. **68** (1979) 220–242.

[130] Thomas, P. S. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. **77** (1980) 5201–5205.

[131] Imaeda, T., Kirchheimer, W. F. and Barksdale, L. DNA isolated from *Mycobacterium leprae*: genome size, base ratio and homology with other related bacteria as determined by optical DNA-RNA reassociation. J. Bacteriol. **150** (1982) 414–417.

[132] Collins, D. M. and De Lisle, G. W. DNA restriction endonuclease analysis of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG. J. Gen. Microbiol. **130** (1984) 1019–1021.

[133] Williams, A. F. The T-lymphocyte antigen receptor—elusive no more. Nature **308** (1984) 108–109.

[134] Robertson, M. T-cell antigen receptor—the capture of the snark. Nature **312** (1984) 16–17.

[135] Owen, M. J. T-cell receptor companions. Nature **312** (1984) 406.

[136] Mitchison, N. A. Immunology—rational design of vaccines. Nature **308** (1984) 112–113.

[137] Wheeler, P. R. Metabolism in *Mycobacterium leprae*: its relation to other research on *M. leprae* and to aspects of metabolism in other mycobacteria and intracellular parasites. Int. J. Lepr. **52** (1984) 208–230.

gineering could be used to generate large quantities of pure enzymes of analysis. Thus, differences between *M. leprae* and host metabolic pathways may be discovered, paving the way for the development of new therapeutic approaches and convenient culture media.

Diagnosis

In diagnosis, as in many other fields of mycobacterial research, recombinant DNA techniques have yet to be fully exploited. Perhaps they could be used in conjunction with MABs as the basis of serodiagnostic tests (an example of this already exists for hepatitis B).[138] Alternatively, cDNA probes might be synthesized to detect circulating bacterial DNA. At present, Stanford, *et al.* are attempting to provide evidence for a mycobacterial etiology of Crohn's disease by using cDNA probes in tissue biopsy specimens. However, these methods are not straightforward and are unlikely to be appropriate for the development of a clinically applicable blood test. If mycobacterial DNA does circulate in the blood, it will be difficult to determine the relative importance of the various fragments and their specificity in different individuals. This is a problem not dissimilar to that encountered with serodiagnosis.

Treatment

Recombinant DNA technology is a relatively inexpensive method of generating protein products in bulk. There are a number of obvious applications for this (for example, IL-2 or IFNs). The role of IL-2 has already been discussed. Kiderlen, *et al.* have provided some tentative evidence that recombinant γ-IFN induces mouse macrophage killing of *Listeria monocytogenes in vivo.*[139] The immune response to *L. monocytogenes* and its cellular localization are very similar to those of *M. leprae* and *M. tuberculosis.* It would be interesting to examine the role of γ-IFN in mycobacterial disease.

Foreign DNA transfer into human cells to promote resistance. The possibility of inserting useful foreign DNA into the human genome has excited scientists for many years. The best example of such a technique is provided by Palmiter's "super mouse."[140] A DNA fragment containing the promotor of the mouse metallothionein I gene fused to the structural gene of the rat growth hormone (GH) was micro-injected into the pronuclei of fertilized mouse eggs. Several of these transgenic mice had extraordinarily high levels of serum GH and grew into giant mice. Second-generation mice had variable gene expression. Thus, the stability of these genes in vertical transmission is questionable. It seems very unlikely that there will ever be a requirement for embryo therapy, but the possibility of using control sequences, such as the metallothionein promotor, to correct gene disorders or to alter immune imbalances in isomatic cells of affected patients is very exciting. Such corrective procedures might be achieved using viral vectors. For example, the nucleotide sequence of a protein antibiotic capable of protection against mycobacterial disease could be inserted into viral DNA and used as a vaccine. Depending on the virus used, the antibiotic coding DNA could be inserted into the host genome (retro viruses) or replicated within the host cytosol (vaccinia). A single dose of recombinant virus might then be able to protect the host for life.

Clearly, there are many problems to be surmounted both technologically and ethically. Until we can precisely insert and remove DNA from animals[142] without otherwise causing harm (e.g., neoplasia[143]), such procedures cannot be performed in man.

Prophylaxis—new vaccines. The development of an effective, cheap vaccine against

[138] Murray, K. New routes to drugs, diagnostic agents and vaccines. Lancet **2** (1984) 1194–1198.

[139] Kiderlen, A. F., Kaufmann, S. H. E. and Lohmann-Matthes, M. L. Protection of mice against the intracellular bacterium *Listeria monocytogenes* by recombinant immune interferon. Eur. J. Immunol. **14** (1984) 964–967.

[140] Palmiter, R. D., Brinster, R. L., Hammer, R. E., Trumbauer, M. E., Rosenfeld, M. G., Birnberg, N. C. and Evans, R. M. Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature **300** (1982) 611–615.

[141] Cooke, A., Lydyard, P. M. and Roitt, I. M. Autoimmunity and idiotypes. Lancet **2** (1984) 723–724.

[142] Lerner, R. A. Synthetic vaccines. Sci. Am. **248** (1983) 48–56.

[143] Palmiter, R. D., Wilkie, T. M., Chen, H. Y. and Brinster, R. L. Transmission, distortion and mosaicism in an unusual transgenic mouse pedigree. Cell **36** (1984) 869–877.

mycobacterial disease would have a tremendous impact, particularly in South India where BCG has failed to provide adequate levels of protection against either tuberculosis or leprosy.[8] The reasons for this failure are not clearly understood. However, there is increasing evidence that pre-sensitization to certain "wild-type" mycobacteria may induce suppression which is enhanced or unchanged with BCG, *M. tuberculosis*, or *M. leprae*.[108-112] This suppression is probably against the common mycobacterial antigens (group i).

Developments in molecular immunology, immunochemistry, and recombinant DNA technology have provided the foundations for the production of a new generation of vaccines. Many of the antigens associated with immune disorders in infectious diseases are attributed to protein molecules. Clearly, knowledge of the molecular features responsible for the antigenicity of protein molecules has at the basis of understanding, in molecular terms, the cellular events of the immune response. Localization and synthesis of appropriate epitopes of mycobacteria should afford valuable synthetic vaccines which will, in principle, be expected to have few or no side effects.

Recombinant DNA technology could therefore be used to make new vaccines by: 1) synthesis of epitopes for direct injection;[144] 2) synthesis of epitopes to be combined with a) an adjuvant. Unfortunately, none of the adjuvants used in animals may be used in man, largely because of local skin reactions. Recently, a new adjuvant (Iscom) derived from tree bark has been discovered. Initial experiments have shown no side effects in animals.[145] b) class II molecules in liposomes; 3) insertion of epitope-producing DNA into a vector, e.g., vaccinia virus (see below).

It is difficult for the "vaccinologists" to isolate a mycobacterial antigen capable of generating a beneficial immune response. The use of MABs has shown that each antigen has many epitopes. Just which epitope will generate the required immune response in unknown. It may be important to distinguish between B-cell and T-cell epitopes. Although B-cell responses can be important in idiotypic regulation, it is the primary T-cell response which generates CMI against mycobacteria. Matthews, *et al.* have created a set of T-cell clones to the MAB-purified TB68 antigen.[146] At least three epitopes have been defined which are capable of inducing MAF release from the T-cell clones. One is species specific; one, partially specific; and one, broadly cross-relative (corroborating the antibody work mentioned above). Unfortunately, all of the clones analyzed were selected for helper activity. It would have been interesting to investigate the suppressor/cytotoxic subset for epitopes triggering suppression on the TB68 antigen. If no such suppressor epitopes exist, then we may have an antigen which could promote a beneficial immune response.

Coates, *et al.* (personal communication) are now developing a new TB vaccine using recombinant DNA technology. At present, they are trying to amino acid sequence the TB68 and TB72 antigens. Thus, an N-terminal, 15-base pair, cDNA probe could be synthesized in an attempt to isolate the genes coding for these antigens. Unfortunately, such oligo-nucleotide probes are affected by degeneracy of the code (e.g., serine has four codons). Thus, all the possible probes may have to be synthesized or, alternatively, an amino acid sequence with least degeneracy selected. Furthermore, short probes are likely to give rise to more false-positives (i.e., binding to irrelevant genes with similar homology). Despite these failings, this method is probably the most rapid to detect the gene (providing it is present in the clone bank) producing the antigen required. Having isolated this gene, Coates, *et al.* intend to insert it into modified vaccinia virus for use as a live vaccine (Figs. 9 and 10).

Bloom and colleagues have approached the problem in a slightly different way (personal communication, Dr. T. Coates) (Fig.

[144] Murray, K., Bruce, S. A., Hinnen, A., Wingfield, P., van Erd, P. M., de Reus, A. and Schellekens, H. Hepatitis B virus antigens made in microbial cells immunize against viral infection. EMBO J. **3** (1984) 645–650.

[145] Morein, B., Sundquist, B., Hoglund, S., Dalsgaard, K. and Osterhaus, S. Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. Nature **308** (1984) 457–460.

[146] Matthews, R., Scoging, A. and Rees, A. D. M. Mycobacterial antigen-specific human T-cell clones secreting macrophage activating factors. Immunology **54** (1985) 17–23.

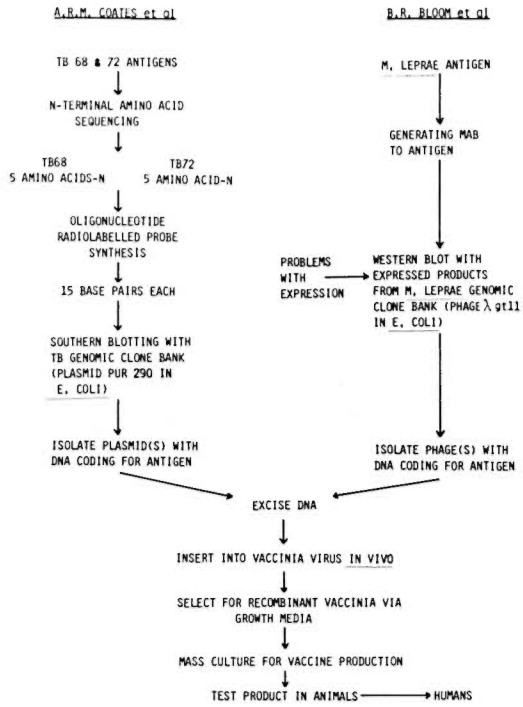

FIG. 9. Stages involved in vaccine production using recombinant vaccinia virus.

8). Working with a *M. leprae* clone bank (λ gt11 vector), they have used MABs in western blots to detect expression of antigen. Unfortunately, they and others (e.g., R. Curtiss, III) have suffered expression problems. Failure of expression can occur as a result of some of the following: a) an incomplete genomic library leading to the absence of a required product, b) nonfunctioning promotors, c) incomplete genes, d) suppressed genes, e) toxicity of a gene product to the host cell, and/or f) instability of a novel peptide in the host cell environment. Hopefully, these difficulties with gene expression will be surmounted, allowing analysis of the *M. leprae* products. Bloom, like Coates, then intends to construct a recombinant vaccinia vaccine.

Vaccinia for vaccines. Following the eradication of smallpox, medical interest in the vaccinia virus has dwindled, but a flood of recent papers has revealed a major new role for this virus in vaccination. Recombinant DNA technology has been used to reconstruct vaccinia into a "DNA-carrier" of larger than 25,000[147] base pairs which is infectious but not pathogenic. The advantages of such a vaccine include: a) economy of manufacture (only a few cents per dose during the smallpox campaign); b) stability in dried form without refrigeration, enabling easy transportation in tropical countries without loss of potency; c) simple administration, e.g., jet gun for use by relatively medically unskilled field workers; d) large DNA capacity, allowing multiple antigen expression and thus polyvalent vaccines; and e) generation of both CMI and humoral responses.

To date, vaccinia virus recombinants expressing the hepatitis B virus surface antigen,[148, 149] influenza virus hemagglutinin,[150, 151] herpes simplex virus (HSV) glycoprotein D,[149] *Plasmodium knowlesi* sporozoite antigen[152] and, most recently, rabies virus glycoprotein[153] have been constructed. Moreover, single intradermal vaccinations with these recombinants have either protected or prevented clinical signs of infection with hepatitis B,[154] influenza,[150] and rabies[153] in animals. Intraperitoneal in-

[147] Smith, G. L. and Moss, B. Infectious poxvirus vectors have capacity for at least 25,000 base pairs of foreign DNA. Gene **25** (1983) 21–28.

[148] Smith, G. L., Mackett, M. and Moss, B. Infectious vaccinia virus recombinants that express hepatitis B virus surface antigen. Nature **302** (1983) 490–495.

[149] Paoletti, E., Lipinskos, B. R., Samsonoff, C., Merger, S. and Panicalli, D. Construction of live vaccines using genetically engineered poxviruses: biological activity of vaccinia virus recombinants expressing the hepatitis B virus surface antigen and the herpes simplex virus glycoprotein D. Proc. Natl. Acad. Sci. U.S.A. **81** (1984) 193–197.

[150] Smith, G. L., Murphy, B. R. and Moss, B. Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza virus hemagglutinin gene and that induces resistance to influenza virus infection in hamsters. Proc. Natl. Acad. Sci. U.S.A. **80** (1983) 7155–7159.

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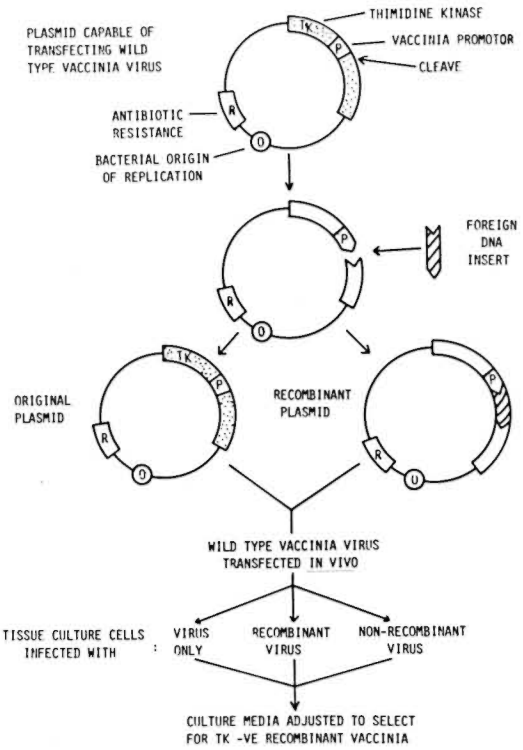

FIG. 10. Construction of vaccinia virus recombinants. R = antibiotic resistance; O = origin of bacterial replication; TK = thymidine kinase; P = vaccinia promotor.

jection of the herpes recombinant vaccine[148] prevented HSV infection, but inoculation of rabbits with the malarial recombinant vaccine produced variable results.[152] Some rabbits responded with a high antibody titer; others, with a low titer.

The difficulties encountered with recombinant vaccinia vaccines include: a) variable expression of foreign genes. This may be overcome by using stronger promotors or by inserting multiple gene copies. b) risk of complications including mutation into a more virulent virus. In the U.S.A., smallpox vaccination had a serious complication rate of $1:10^5$ (and nine deaths from 14.5 million vaccinations). Clearly, the risks of the primary disease outweigh the risks of vaccinations in smallpox. Nevertheless, attempts to attenuate the virus and to improve vaccine safety would be welcome.

Vaccinia for tuberculosis and leprosy? The recombinant vaccinia vaccine clearly fills three of the four requirements of a vaccine outlined above. Exactly how successful such a vaccine would be in improving the existing BCG is not clear. It may well be that Coates, *et al.*'s TB68 and TB72 antigens contain epitopes generating clinically beneficial immune responses (as discussed above). If, however, the epitopes generate suppression or a deleterious type of T help (or, indeed, Tc-cell activity), then we may expect such a vaccine to fail. A perfect illustration of our lack of understanding of mycobacterial vaccines is provided by Convit, *et al.* who had empirical success in reversing LL anergy with a mixture of *M. leprae* and BCG.[155] Exactly why this particular mixture of antigens should be effective is obscure. Hopefully, the use of MABs, T-cell clones, and recombinant DNA technology will pave the way to a clearer understanding of these processes.

CONCLUSION

The development of MABs and recombinant DNA technology has permitted the exploration of vast new areas of research. In 1984, Köhler and Milstein were awarded the Nobel Prize for their work with MABs. Do these new techniques offer any hope of a solution to the mycobacterial misery of the Third World?

Present uses

The exquisite specificity of MABs clearly allows a remarkable opportunity to define, purify, and analyze bacteria and immune cells and their structures and products. An array of mouse MABs has now been synthesized against *M. leprae* and *M. tuberculosis*. The initial results show that these are of limited use in taxonomy and serodiagnosis. By contrast, MAB studies of T-cell function in histological sections have provided structural evidence of the immunopathology present.

Recombinant DNA technology, while having enormous potential uses, has not as yet produced results. Only a limited number of mycobacterial clone banks have been

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made; thus only a few strains may be analyzed. The *M. leprae* clone banks have not, by and large, expressed proteins successfully, and attempts at mycobacterial taxonomy using restriction enzyme patterns have failed. Nevertheless, these techniques have been of great use in analyzing T-cell receptor diversity, although the final solutions to T-cell restriction and the Ts-cell receptor are still lacking. Such work is of great relevance in trying to comprehend mycobacterial-induced anergy.

The Future

Clearly, much of the existing MAB work needs to be expanded, and a classification scheme for the mycobacterial MABs should be introduced. In immunopathology, the importance of common and species-specific antigens (from fast- and slow-growing mycobacteria) in triggering suppressor circuits may be examined with MABs. Furthermore, selective inhibition of one suppressor mechanism with MABs may allow analysis of the relative effects of other suppressor mechanisms. Temporal studies comparing healthy populations, exposed groups, and those with disease may help to resolve whether or not the CMI deficit in mycobacterial disorders is the primary cause of the pathology or a secondary result of other processes. The possibility of immunomodulation with MABs is also speculative and will depend on both a better understanding of the immunopathology and the development of hMABs (MABs raised from other species may induce graft versus host responses). Of course, any MAB destined for human use would undergo extensive testing for undesirable crossreactions with other tissues.

Recombinant DNA technology will provide for "in bulk" generation of uncontaminated *M. leprae* and *M. tuberculosis* products. In this way, we may be able to derive further knowledge of both the external and internal biology (e.g., metabolic pathways) of these organisms and, hence, develop new therapeutic approaches and, perhaps, the correct conditions for *in vitro* growth of *M. leprae*.

Genetic engineering techniques are currently providing a new generation of cheap vaccines ideal for use in developing countries. The attempts to construct a recombinant vaccinia virus expressing *M. tuberculosis* and *M. leprae* antigens are very encouraging. However, since we do not understand the nature of the CMI suppression seen in mycobacterial disease (and against which epitopes this is directed), the success of these vaccines cannot be predicted.

Hopefully, the combined use of MABs, T-cell clones, and recombinant DNA technology will help to solve the puzzles of mycobacterial disease, and both leprosy and tuberculosis will dwindle into the historical annals of medical literature.

—Michael J. Seckl

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Note added in proof. Since typing, Krambovitis, *et al.* (Lancet 2:1984, 1229–1231 and editorial, p. 1254) have published an encouraging preliminary report on the rapid diagnosis of tuberculous meningitis, based on an antigen agglutination test. An absorbed rabbit antiserum was generated against one of the *M. tuberculosis* plasma membrane antigens. This effectively diagnosed 17 of 18 patients (the remaining patient was only proved positive at autopsy). The reason for this crossreactivity was unclear.

MABs would have obvious advantages over the heteroantisera used in this study. If these results are substantiated, then we may look forward to earlier treatment of a disease which is all too often misdiagnosed.