Tuberculosis: Pathogenesis, Protection, and Control
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Chapter 28

Mechanisms of Pathogenesis in Tuberculosis

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KOCH PHENOMENON, TISSUE DAMAGE, AND PROTECTIVE IMMUNITY IN THE PATHOGENESIS OF TUBERCULOSIS

In the 1890s, Robert Koch observed that a primary infection of guinea pigs with *Mycobacterium tuberculosis* in the skin produced a nonhealing lesion and that reinoculation of the animals after several weeks produced only a firm, red nodule that necrosed and finally healed. These observations first suggested the existence of immunity to tuberculosis infection. When tuberculous guinea pigs were challenged intradermally with a culture supernatant of *M. tuberculosis* (old tuberculin) or with live organisms, there was necrosis both locally in the challenge site and at a distance in the preexisting tuberculous lesion (Koch, 1891). This reaction, now known as "the Koch phenomenon," protected against virulent organisms, perhaps among other reasons because the local necrosis caused sloughing of the tissue containing the organisms, since similar necrosis in deep sites or in the lungs failed to eliminate the bacteria. Thus, if guinea pigs were preimmunized by protocols that gave rise to necrotizing skin test reactivity equivalent to the local necrosis elicited by Koch, they were rendered more rather than less susceptible to infection by intramuscular injection of a small number of virulent organisms. In contrast, immunization protocols priming small tuberculin reactions were protective (Wilson et al., 1940). These observations led to endless confusion. For instance, is the Koch phenomenon an exaggerated version of the tissue-damaging process seen routinely in tuberculosis lesions? If so, what is the relationship between this tissue-damaging response and protection? The problem is that we do not know the mechanisms involved at the cellular or molecular level. Therefore, we do not know whether the tissue damage, the Koch phenomenon, and the protection are "excessive" and "regulated" manifestations of similar pathways or whether they are the results of qualitatively different immunological mechanisms (Dannenberg, 1968). The balance between cell-mediated immunity and tissue damage throughout the course of this disease determines what form the disease takes and may not be dissimilar to the spectrum in leprosy that has been correlated to the balance of Th1 and Th2 lymphocyte subsets (Salgame et al., 1992; Bloom et al., 1992). In the ideal

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case, which is the situation for the large proportion of tuberculin-positive individuals who have been infected but show no evidence of disease, an early and appropriate cell-mediated immune response develops and controls the infection. While the tissue-damaging component is excessive in only a small number of individuals infected with *M. tuberculosis*, it is largely responsible for the clinical manifestations of the disease, and the control of this excess would reduce much of the pulmonary destruction that occurs. The little that we do know about these mechanisms is discussed in this chapter and in chapter 27.

Activated Macrophages: Discrepancy between Mouse and Human

The topic of activated macrophages is discussed in detail in chapter 27, but in brief, the microbicidal mechanism is uncertain, particularly in relation to humans. Since the work of Mackaness (1968), mostly with *Listeria monocytogenes*, demonstrated the enhanced nonspecific bactericidal activity of macrophages activated by mediators released from lymphocytes primed and stimulated to specific antigens, activated macrophages have usually been assumed to be important effectors in mycobacterial diseases as well. This view is compatible with histological evidence, as outlined above. However, most of the published work involves mouse macrophages. Murine macrophages can be activated to inhibit or destroy virulent *M. tuberculosis* in vitro. This can be achieved with class II major histocompatibility complex (MHC)-restricted T-cell lines (Rook et al., 1985) or with lymphokines (Rook et al., 1986a; Flesch and Kaufmann, 1990). More recently, it has become apparent that by activation with gamma interferon (IFN-γ) and either lipopolysaccharide or tumor necrosis factor alpha (TNF-α), murine macrophages can be triggered to release nitric oxide (NO), which is required for the mycobactericidal activity of macrophages (Denis, 1991a; Chan et al., 1992).

The situation is still less clear when we consider human macrophages. To our knowledge, only two authors have claimed to be able to induce killing of virulent *M. tuberculosis* by human monocytes or monocyte-derived macrophages in vitro (Denis, 1991b; Crowle, 1990). The authors of this chapter have been unable to demonstrate such killing. At best, a slowing of the rate of intracellular replication by about one generation in four was achieved following addition of recombinant IFN-γ and calcitriol (Rook et al., 1986b). IFN-γ alone frequently causes increased growth of *M. tuberculosis* in human cells (Rook et al., 1986a, b). Moreover, it seems that human monocytes are unable to generate tetrahydrobiopterin, which is an essential cofactor for arginine-dependent NO synthesis (Stuehr et al., 1991). We are careful to note that these studies have been carried out with blood monocytes, not tissue macrophages. Since other human cell types, e.g., endothelial cells and liver cells, can produce this NO in large quantities, it is not clear whether human macrophages are unable to do so or whether the correct combination of cytokines and culture conditions or tissue sources has simply not yet been found.

Cytotoxic T Cells

While T-cell-derived lymphokines and the activated macrophage represent a necessary condition for protection, it is certainly not the whole story even in the mouse. In vitro evidence exists that cytotoxic T cells recognizing mycobacterial antigens do develop in both humans and mice (Kaufmann, 1988; Ottenhoff et al., 1988; Orme et al., 1992). Transgenic animals whose gene for β2-microglobulin is disrupted and who are unable to express class I MHC on the cell membrane have greatly increased susceptibility to tuberculosis

(Flynn et al., 1992). This implies a role for $CD8^+$ T cells, possibly cytotoxic cells. Interestingly, β2-microglobulin "knockout" mice were unaffected by infection with BCG or avirulent *M. tuberculosis* H37Ra. One way that an antigen known initially to be taken up into an endosomal compartment could be presented to MHC class I-restricted cytotoxic lymphocytes (CTL) would be by its ability to escape from phagolysosomes into the cytoplasm, as has been found for *Listeria* and *Shigella* spp. In mycobacterial infeciton, the issue remains controversial. There is electron microscopic evidence that virulent *M. tuberculosis* can escape from the phagosome (Myrvik et al., 1984; McDonough et al., 1993). In the latter work, it is noteworthy that in the 7-day macrophage cultures studied, only the virulent H37Rv and not the avirulent *M. tuberculosis* H37Ra or BCG strain was observed in the cytoplasmic compartment. On the other hand, when rapid freezing techniques are used to preserve membranes, there is evidence that *M. tuberculosis* retains host lysosomal membrane antigens and may still be within a vesicle (Xu et al., in press). It is possible that processing of antigen via the class I pathway is important, allowing the host to kill parasitized cells that are failing to exert bactericidal effects. This failure could result from an unusual intracellular location of the organisms or, according to a recent report, from a failure to present the antigens of such organisms (Pancholi et al., 1993) (further discussed below).

γδ T Cells

A large proportion of human peripheral blood γδ T cells, even from PPD-negative donors, will proliferate in response to mycobacteria (Kabelitz et al., 1990; Uyemura et al., 1991). In vitro, these cells secrete a pattern of cytokines similar to those of Th1 cells (see below) and are cytotoxic. It is reasonable to speculate that they may be involved early in immunity to tuberculosis, but there is only circumstantial evidence for this (Barnes et al., 1992). Knockout mice unable to generate these T cells may reveal their relevance to the control of mycobacterial infection.

Lymphokines and Cytokines

Recent experiments involving *M. tuberculosis* infection of transgenic mice are providing new and interesting information on the role of some lymphokines and cytokines. As would be expected, knockout mice with a disruption in the gene for IFN-γ succumb rapidly (within 3 weeks) to *M. tuberculosis* infection. In contrast, mice will eventually die, although after many weeks, from BCG (Dalton et al., 1993; Cooper et al., 1993; Flynn et al., 1993). While knockout mice for TNF-α are not yet available, it has been possible to treat mice with neutralizing anti-TNF antibodies. This has led to dissemination of BCG infection (Kindler et al., 1989) and rapid death of *M. tuberculosis* (Flynn et al., submitted). Thus, TNF is a necessary condition for protection, again suggesting the importance of NO and reactive nitrogen intermediates in protection. Together, these data suggest to us that multiple immune mechanisms are required for protection. On the basis of available data, we believe that both MHC class II-restricted T-cell-derived cytokines, at least IFN-γ, macrophage-derived TNF-α, and MHC class I-restricted T-cell responses, probably CTL, are necessary conditions for protection; none is sufficient, and the roles of γδ T cells and other cytokines remain to be elucidated.

TISSUE-DAMAGING MECHANISMS IN TUBERCULOSIS

Direct Toxicity and Enhanced Susceptibility of Individual Cells to TNF-α

M. tuberculosis has some toxicity for cells in vitro. This is particularly noticeable

with monocytes, which often die if they take up more than five bacilli. Conversely, monocytes or macrophages can take up very large numbers of *M. avium* without obvious toxic effects. It had previously been suggested that *M. tuberculosis* has the ability to inhibit lysosomal fusion, which could contribute to the survival of the pathogen in macrophages (Armstrong and Hart, 1975). In those studies, fusion could be blocked by agents that prevented lysosomal acidification, and *M. tuberculosis* produced ammonia in abundance (Gordon et al., 1980). In this context, recent studies by Sturgill-Koszycki et al. (in press) indicate that vacuoles formed around *M. avium* fail to acidify below pH 6.3 to 6.5. Immunoelectron microscopy indicated that the vacuoles containing the mycobacteria contain lysosomal proteins but not the proton-ATPase responsible for acidification. Because other lysosomal membrane markers were present, this result indicates remarkably selective fusion of vesicular membrane proteins. Because of the difficulty in securing *M. tuberculosis* cultures that are 100% viable, it is always difficult to ascertain with certainty whether fused vesicles contain primarily nonviable organisms and whether the bacilli found in nonfused vesicles are responsible for most of the damage. Recent studies by McDonough et al. (1993) indicate that lysosomal fusion occurred very rapidly in murine or human macrophages infected in vitro with live or dead *M. tuberculosis* or BCG. However, the intracellular fates differed. BCG essentially remained in fused phagolysosomes for the entire 7-day observation period. Both H37Rv and H37Ra *M. tuberculosis* strains rapidly appeared to bud or extrude from the fused phagolysosome to form a unique vesicle, with the organisms enclosed by a very tightly apposed membrane. Over time, fusion of secondary lysosomes failed to occur in these tightly membrane-apposed containing vesicles, and much of the multiplication of *M. tuberculosis* occurred in these vesicles. Between 4 and 7 days, only the virulent strain of *M. tuberculosis*, H37Rv, escaped from these tightly apposed membrane vesicles and entered the cytoplasm. These results, with electron-dense markers used for lysosomes, thus confirmed and extended the earlier observations by Myrvik et al. (1984) indicating that *M. tuberculosis* could escape from phagolysosomes into the cytoplasm. However, using immunoelectron micrographic techniques designed to preserve membranes, Xu et al. (in press) found that all bacilli appear to be surrounded with host cell membrane and membrane antigen and may not be free within the cytoplasm. Escape from phagolysosomes would represent one of the few biological differences between avirulent and virulent strains of *M. tuberculosis* that could be directly related to virulence, and so the issue is an important one to resolve. Not only is it relevant for antigen presentation to MHC class I-restricted CTL, but *M. tuberculosis* that enters the cytoplasm of macrophages could exert direct toxic effects on the cells or may increase the susceptibility of infected cells to TNF-α that is discussed below.

M. tuberculosis is readily taken up by a wide variety of nonmacrophage cell types in vitro (Shepard, 1958; Filley and Rook, 1991; Filley et al., 1992), and such cells are much less susceptible to the toxicity of the organism. This has been shown for several cell lines and also for human endothelial cells and fibroblasts. This is paradoxical, because unlike *M. leprae*, *M. tuberculosis* is not seen inside such cells in vivo. One possibility is that in vivo these cells are killed quickly, so that parenchymal cells infected with bacilli are rarely seen in histological sections of tissues. An alternative answer may lie in the observation that cells containing *M. tuberculosis* are rendered exquisitely sensitive to killing by TNF-α (Filley and Rook, 1991; Filley et al., 1992). Therefore, macrophages infected in vitro may be killed by their own production of TNF-α, while nonmacrophage cells survive

in vitro in the absence of TNF-α but are rapidly killed in vivo, since TNF-α is probably abundant in lesions, as discussed below. The ability to increase sensitivity to TNF-α was prominent in virulent strains of *M. tuberculosis* but weak in H37Ra and virtually absent from *M. avium* and BCG strains (Filley and Rook, 1991). Finally, if CTL are engaged, they would have the capability of lysing parenchymal cells expressing mycobacterial antigens in association with MHC class I.

The Koch Phenomenon

As outlined above, Koch noted that 4 to 6 weeks after establishment of infection in guinea pigs, intradermal challenge with whole organisms or culture filtrate resulted in necrosis locally and in the original tuberculous lesion (Koch, 1891). Similar phenomena occur in humans. The tuberculin test is frequently necrotic in subjects who are or have been tuberculous. This is not an inevitable consequence of the delayed hypersensitivity response to tuberculin, because necrosis does not occur when the same test is performed in healthy BCG recipients or in tuberculoid leprosy patients. Moreover, Koch sought to exploit this phenomenon for the treatment of tuberculosis and found that injection of larger quantities of culture filtrate (old tuberculin) subcutaneously into tuberculosis patients would evoke necrosis in established tuberculous lesions at distant sites (Anderson, 1891). This resulted in necrosis and sloughing of the lesions of skin tuberculosis (lupus vulgaris, usually caused by bovine strains), but when similar necrosis was evoked in deep lesions in the spine or lungs, the results were disastrous and merely provided further necrotic tissue in which the bacteria could proliferate. This treatment was therefore abandoned.

This phenomenon shows parallels with the Shwartzman reaction. Shwartzman observed that a site primed by an injection of gram-negative bacteria (though endotoxin will substitute) undergoes necrosis if a second dose of gram-negative organisms (or endotoxin) is injected intravenously 24 h later (Shwartzman, 1937). Several early workers demonstrated that mycobacterial lesions will undergo necrosis if the animal is subsequently challenged intravenously or subcutaneously with endotoxin-rich bacteria (Bordet, 1931), endotoxin (Shands and Senterfitt, 1972), or muramyl dipeptide (Nagao and Tanaka, 1985), and this necrosis is accompanied by massive systemic release of TNF-α (Carswell et al., 1975). It is thought that the "prepared" inflammatory site is abnormally susceptible to circulating cytokines and activated cells resulting from the second challenge injection. Direct injection of cytokines, particularly TNF-α, into such sites will cause similar necrosis (Rothstein and Schreiber, 1988). Recent studies suggest, however, that the susceptibility of mycobacterial lesions to such necrosis differs in one fundamental way from that studied by Shwartzman. The injection of mycobacterial components (if genuinely endotoxin free) will not prepare a site for TNF-α-mediated necrosis unless $CD4^+$ T-cell reactivity has previously been primed (Al Attiyah et al., 1992). Moreover, recent studies have revealed that such sites undergo necrosis only if the $CD4^+$ T-cell-mediated response involved is mixed Th1-Th2 or Th0, while in contrast, mycobacterial immunization schedules leading exclusively to Th1 cytokine release yield T-cell-mediated inflammatory sites that are not sensitive to TNF-α-mediated damage (Hernandez-Pando and Rook, unpublished data). Perhaps, therefore, the role of TNF-α depends on what the T cells are doing. Is the Koch phenomenon a "T-cell-dependent" Shwartzman reaction in a susceptible, mixed Th1-Th2 inflammatory site?

A second set of mediators that must be considered is reactive oxygen intermediates and reactive nitrogen intermediates. Much evidence has shown that macro-

phages can be activated by IFN-γ to release reactive oxygen intermediates, O_2, H_2O_2, and hydroxyl radicals. Chan et al. (1992) showed that these compounds were not microbicidal for *M. tuberculosis*. They further showed that IFN-γ and TNF-α activated mouse macrophages to release reactive nitrogen intermediates that were mycobactericidal. One mechanism by which NO is cytotoxic for mammalian cells is by binding to the Fe-S centers present in some critical enzymes, including ribonucleotide reductase (Nathan and Hibbs, 1991). The triggering of oxygen radicals and nitric oxide release by infected macrophages could in fact damage those and adjacent cells and contribute to tissue pathology.

Even if these speculations are correct, they do not provide a complete account of the mechanism of tissue damage in tuberculosis. More important, they leave us uncertain as to the link between the acute necrotic phenomenon evoked by Koch and the slowly evolving necrosis leading to caseation, liquefaction, calcification, and cavity formation described above.

We cannot rule out several other possible mechanisms. CTL were considered above as possible components of the protective pathway, but increased activity of such cells could also contribute to the increased killing of infected macrophages. Indeed, we know too little to rule out any effector cell type.

Excessive Cytokine Release

In view of the ability of *M. tuberculosis* to increase the sensitivity to TNF-α of individual cells, the ability of the $CD4^+$ T-cell-mediated response to render a whole tissue sensitive to the same cytokine, and the Shwartzman-like nature of Koch's "cure" for tuberculosis, we must consider the possibility that TNF-α in synergy with other cytokines is a component of tissue destruction in humans. The bacteria produce potent triggers of cytokine release (Rook et al., 1987; Valone et al., 1988; Moreno et al., 1989; Silva and Faccioli, 1988). Blood monocytes (Takashima et al., 1990) and alveolar macrophages (Rook and Al Attiyah, 1991) from tuberculosis patients release TNF-α "spontaneously" in large quantities, and the cytokine is present in the lung (Barnes et al., 1990). In view of the weight loss seen in humans, it is interesting that cytokine-induced wasting can be evoked by injecting very small quantities of trehalose dimycolate (cord factor) dissolved in oil into the peritoneal cavities of mice (Silva and Faccioli, 1988). Circulating levels of TNF-α inhibitors are also high in the serum of tuberculosis patients (Foley et al., 1990). These are extracellular domains of receptors shed in response to TNF-α release. Thus, TNF-α is certainly released in the human disease. Its role is uncertain, but recent studies show that administration of thalidomide, which reduces mRNA levels for TNF-α, to tuberculosis patients causes rapid symptomatic improvement and weight gain (Kaplan, 1993), as it did for reducing erythema nodosum in leprosy (Sampaio et al., 1991). In the mouse, on the other hand, TNF-α is clearly necessary for protection, though as discussed in the previous section, this is probably not the whole story, and protection may depend on the type of T-cell reactivity at the site into which TNF is released. Studies by Kindler et al. (1989) showed that treatment of mice with polyclonal anti-TNF-α antibodies inhibited granuloma formation, leading to disseminated infection with BCG. However, in *M. tuberculosis*-infected mice treated with neutralizing monoclonal antibodies to TNF-α, Flynn et al. (submitted) have evidence that granuloma-like structures formed, but the mice were unable to control the infection. Interestingly, the granulomas in the anti-TNF-α-treated mice were necrotizing, while the control mice, which were able to restrict the growth of *M. tuberculosis*, had nonnecrotizing granulomas. Thus, although TNF-α probably plays a role,

these data indicate that high levels of TNF-α are not necessary for induction of tissue necrosis in mice. It will be crucial to resolve the discrepancies between the results using the polyclonal and monoclonal anti-TNF antibodies through transgenic knockout mice unable either to express the appropriate TNF receptors or to produce TNF-α itself.

Tuberculin Shock

As will be discussed below, tuberculosis patients have defective adrenal function, and some have reduced ability to increase cortisol levels in response to adrenocorticotropin, so they have little "adrenal reserve." This adrenal deficit may explain a tendency to go into "tuberculin shock" (systemic Koch phenomenon?) if chemotherapy causes rapid release of bacterial antigens and cytokine triggers (Scott et al., 1990). This complication is most often seen in patients with severe disseminated disease and in patients with protein malnutrition and liver damage. It may be relevant that in experimental models, liver damage greatly enhances susceptibility to the toxicity of TNF-α released in response to bacterial components (Freudenberg and Galanos, 1991).

REGULATION OF PROTECTIVE AND TISSUE-DAMAGING RESPONSES IN TUBERCULOSIS

In the absence of certainty about the effector mechanisms involved, any discussion of the regulation of the protective and tissue-damaging responses must be somewhat speculative.

Th1 and Th2 Cells: Selection of Functional T-Cell Subsets and Disease Progression

Functional T-cell subsets in mice (Mosmann and Moore, 1991) and humans (Romagnani, 1991; Salgame et al., 1991) have been defined by the patterns of lymphokines they produce (see chapter 25 for more detailed discussion). Type 1 $CD4^+$ and $CD8^+$ CTL produce IFN-γ, interleukin-2 (IL-2), and lymphotoxin. Type 2 $CD4^+$ (Th2) cells and $CD8^+$ T cells produce predominantly IL-4. Each subset exerts negative regulation upon the other (Romagnani, 1991; Maggi et al., 1992), and together with the major regulatory cytokines IL-12 and IL-10 produced primarily by macrophages, they largely determine the type of T-cell response that ensues. Thus, the cytokine profile observed in mycobacterium-responsive human cells from healthy donors may be influenced by the conditions used (Haanen et al., 1991; Barnes et al., 1993) and can be altered by the addition of cytokines or cytokine-neutralizing antibodies (Maggi et al., 1992). In many experimental intracellular infections, e.g., with *Leishmania* spp., the type 1 response is protective and the type 2 response leads to disease progression. In helminth infections, the reverse may be true. In human leprosy, the type 1 response is associated with resistance and the type 2 response is associated with the lepromatous or unresponsive form (Bloom et al., 1992). One wonders whether the anergy seen in a quarter of tuberculosis patients may be related to Th2 function, and evidence for effects of type 2 cytokines in tuberculosis has been discussed by Rook (1991; Rook et al., 1993b).

In many chronic infections or inflammatory diseases, there is a permanent or transient switch from a Th1 pattern of response to Th2. Such a switch appears to occur, for example, in schistosomiasis (Grzych et al., 1991) and syphilis (Fitzgerald, 1992) patients. Does the evidence for a Th2 component in the response of tuberculosis patients mentioned above suggest a similar trend in this disease? It is certainly interesting that the necrosis that occurs around the ova in murine schistosomiasis occurs at precisely the time when a Th2 response becomes superimposed on a preexisting Th1 pattern (Grzych et al., 1991). It is not

impossible that a similar mixed pattern of response lies behind the necrosis seen in tuberculosis (Barnes et al., 1993), and as pointed out above, it is these "mixed" responses that are TNF-α sensitive in one murine model.

Current dogma states that the cytokines released by Th1 cells enhance Th1 activity and inhibit Th2. Why, then, does the Th1 → Th2 shift occur? We should remember that the Th1/Th2 ratio is not determined only by cytokines. The release of prostaglandins from activated macrophages downregulates Th1 cells (Phipps et al., 1991), and there are two striking endocrine changes in tuberculosis patients that would indeed be expected to have this effect.

(i) Formation of calcitriol in mycobacterial lesions. The macrophages of tuberculosis patients, following activation by IFN-γ, express an active 1a-hydroxylase and rapidly convert 25(OH)-vitamin D3 to calcitriol (Rook et al., 1986b; Rook, 1988). Their T cells may also produce this enzyme (Cadranel et al., 1990). This is a potent phenomenon, leading occasionally to leakage of calcitriol into the periphery and to hypercalcemia, though it has in the past been difficult to understand its role in the disease (Rook, 1988). It now seems possible that this is a feedback mechanism that tends to downregulate Th1 and enhance Th2 responses, because the active vitamin D3 metabolite, $1,25(OH)_2$ cholecalciferol (calcitriol), inhibits production of IFN-γ and IL-2 and increases production of IL-4 and IL-5 (Daynes et al., 1991).

(ii) Adrenal dysfunction in tuberculosis. The pituitary-adrenal axis is disturbed in patients with tuberculosis (Sarma et al., 1990; Ellis and Tayoub, 1986; Barnes et al., 1989a). There is a striking reduction in adrenal function, reflected by low levels of essentially all steroid metabolites in 24-h urine collections (Rook et al., unpublished data). Most significantly, patients have very low or absent levels of dehydroepiandrosterone sulfate (DHEA-S) (Ellis and Tayoub, 1986; Rook et al., unpublished data). This may have serious consequences, because the desulfated form, DHEA, is a genuine antiglucocorticoid, the specific receptors for which are found in T cells (Meikle et al., 1992). DHEA enhances Th1 activity (Suzuki et al., 1991; Daynes et al., 1990) and inhibits the effects of glucocorticoids, including their tendency to suppress Th1 lymphocytes and enhance Th2 (Blauer et al., 1991; Daynes et al., 1990; Fischer and Konig, 1991). For instance, a single dose of DHEA given before dexamethasone can block the ability of the latter to cause depletion of thymocytes and temporary unresponsiveness of peripheral T cells to mitogens (Blauer et al., 1991). Therefore, the T cells of tuberculosis patients may be chronically exposed to glucocorticoid effects unopposed by the antiglucocorticoid influence of DHEA. This may not only encourage a Th1-to-Th2 switch but even contribute to the fall in $CD4^+$ T-cell count and in CD4/CD8 ratio that is well documented in tuberculosis patients (Singhal et al., 1989; Ainslie et al., 1992) and of course in human immunodeficiency virus-infected patients, in whom DHEA is also low and correlates directly with CD4 counts (Wisniewski et al., 1993). This has been developed further as a hypothesis elsewhere (Rook et al., 1993b).

At present, no information exists on the patterns of lymphokines and T-cell subsets in healing and progressing lesions in tuberculosis. It is clear that there is anergy associated with a significant proportion of tuberculosis patients and that the prognosis for these patients prior to chemotherapy was generally poorer than for those with tuberculin hypersensitivity. That picture is suggestive of the type 2 (Th2) predominance in many patients with lepromatous leprosy. We suggest the hypothesis that in the proportion of individuals infected with *M. tuberculosis* who progress to primary progressive or recrudescent forms of disease, the relative ratios of type 1 (Th1) to

type 2 (Th2) are altered. We propose further, on the basis of the requirement for MHC class I-restricted T-cell function for resistance to tuberculosis in mice and the finding that protection can be engendered by the 65-kDa antigen presented in transfected mammalian cells (Silva and Lowrie, in press), that the number of $CD8^+$ CTL may similarly be critical.

Roles of Different Antigenic Components of *M. tuberculosis*

Is there any evidence that protective cell-mediated immunity and the Koch phenomenon or tissue-damaging pathways represent responses to different components of the organism? It has long been the dream of many workers to create a novel vaccine, perhaps a modified BCG, that primes bactericidal cell-mediated immunity but not necrosis. The practical advantages would be even greater if nonnecrotizing skin test responsiveness could also be dissociated from induction of protective immunity. Such a vaccine, if achievable, would have many potential benefits (adapted from Dannenberg [1990]). (i) Vaccinated individuals would not be appreciably tuberculin positive, so that tuberculin testing of such persons would still be a useful procedure for diagnosing infection with virulent tubercle bacilli. (ii) A vaccine that evoked little or no delayed hypersensitivity could be given more than once to create high levels of immunity (especially in high-risk groups). (iii) If available, such a vaccine might replace isoniazid in preventive therapy for persons who recently became tuberculin positive, obviating hepatotoxicity and possibly bacillary resistance to the drug. (iv) Such a vaccine could be given to patients with active disease in order to boost the bactericidal pathways (immunotherapy). Tuberculin and BCG cannot be used for immunotherapy, because they evoke the Koch phenomenon, as Robert Koch discovered to his cost (Anderson, 1891).

How realistic a proposal is this? First, as discussed in detail in chapter 28, there is unambiguous evidence that tuberculin skin test reactivity can be dissociated from protection in humans. For example, while greater than 85% skin test conversion was observed in most of the controlled trials of BCG against tuberculosis, protection varied from 0 to 77%. Similarly, in the British Medical Research Council trials, one lot of vole bacillus vaccine produced very few skin test conversions yet protected well against tuberculosis. This and other studies with different strains of BCG (Fine, 1989) suggest that tuberculin positivity may not correlate with protective immunity in humans. At present, there is no evidence that different antigens are involved. If, as suggested by Kaufmann (1988) and Flynn et al. (1992, 1993), both type 1 CD4 T-cell function and lymphokines and $CD8^+$ MHC class I-restricted T cells are required for protection, it might be expected that different antigens and certainly different T-cell epitopes would be involved. On the other hand, there is good evidence that the necrosis-inducing components can be separated from at least some potentially protective antigens, as explained below.

The tissue-damaging responses are evoked by tuberculin, which is a very crude culture supernatant from old autolysing bacterial cultures precipitated by trichloroacetic acid or ammonium sulfate. Such supernatants contain fragments of essentially all the antigens of *M. tuberculosis*. Therefore, it is not meaningful to speak of removing the "tuberculin" antigens from BCG. However, the Koch phenomenon (manifested as a necrotic skin test reaction) appears to be targeted preferentially toward the species-specific epitopes of *M. tuberculosis*. When tuberculosis patients are skin tested with antigens derived from other mycobacteria, they do not exhibit necrotic responses. In fact, their responses to the common, cross-reactive mycobacterial antigens or epitopes (which must include the

heat shock proteins [HSPs]) are diminished (Kardjito et al., 1986). Moreover, there is strong evidence that cell-mediated immunity to mycobacteria can be initiated, perhaps mediated, by responses to the common, cross-reactive epitopes. The more obvious reasons for this assertion are as follows. (i) BCG protects against leprosy as well as or better than it does against tuberculosis (Fine et al., 1986). (ii) Small positive tuberculin reactions in non-BCG recipients correlate with protection (Palmer and Long, 1966; Fine, 1994). These reactions are caused by contact with environmental mycobacteria. (iii) A common cross-reactive protein (the 65-kDa HSP) from *M. leprae* that is expressed in murine macrophages can engender protection against tuberculosis in a murine model (Silva and Lowrie, in press). (iv) Tuberculosis patients may lose their skin test responses to common cross-reactive antigens (Kardjito et al., 1986).

At present, the simplest way to achieve a preparation with suitable adjuvant properties that contains a broad spectrum of common but not species-specific antigens of *M. tuberculosis* appears to be use of related mycobacterial species. Preliminary results of immunotherapy with an autoclaved member of the fast-growing subgenus *M. vaccae* are encouraging (Onyebujoh and Rook, 1991; Rook et al., 1994). For immunotherapy as an adjunct to chemotherapy, such preparations are at present the only possibility. It is dangerous to administer species-specific components of *M. tuberculosis* to patients, since they may trigger the Koch phenomenon and tuberculin shock (Anderson, 1891).

Tuberculin Skin Test: Significance in Apparently Healthy People

One must consider at the outset, from the presently available evidence, that the tuberculin test, like the lepromin skin test, represents the consequences primarily of type 1 (Th1) CD4 T cells in response to mycobacterial antigens. If CD8 CTL are necessary for protection, their presence is unlikely to be readily detected by skin testing with tuberculoproteins that do not have access to the cytoplasmic compartment of antigen-presenting cells and are not presented in the context of MHC class I antigens.

In countries with a high standard of living, a positive test can be a diagnostic clue, but in developing countries, much tuberculin skin test positivity is due to frequent contact with ubiquitous environmental species. In such populations, a positive test has little validity as a diagnostic tool. On the other hand, a negative test (unless the patient has evidence of advanced disease or AIDS and is anergic) renders tuberculosis unlikely. Nevertheless, epidemiologically, even in developing countries, the test has some predictive power in healthy people. As already mentioned, small nonnecrotizing responses in people who have not received BCG correlate with a significantly decreased risk of developing tuberculosis, while large reactions (Koch phenomena?) correlate with an increased risk of disease, perhaps because many such individuals are in fact already infected (Palmer and Long, 1966; Fine, 1994).

A large or necrotic tuberculin reaction, remaining years after the primary disease has healed, probably signifies that a few dormant bacilli are still present in inapparent caseous foci. Such bacilli may be released from time to time and then rapidly destroyed, which gives a booster effect to the whole immune system, including to the level of tuberculin sensitivity.

Individuals who have been infected with the tubercle bacillus can in time become tuberculin negative with or without antimicrobial treatment. In some of these individuals, the tubercle bacillus may have been eradicated. In many, a recall of tuberculin sensitivity is produced by the antigens in tuberculin (purified protein derivative) in-

jected for skin testing. When retested with intermediate-strength purified protein derivative, a person who was negative 3 weeks earlier may now be tuberculin positive as a result of the booster effect of tuberculin itself (Thompson et al., 1979).

Significance in Patients with Active Tuberculosis

The size of the dermal tuberculin reaction in patients is of limited prognostic significance (Lurie, 1964), though individuals who are very ill with tuberculosis may show a negative tuberculin skin test. However, when they are recovering, they again show a positive skin test. Several mechanisms could be involved, including compartmentalization of T cells, Th2 cell dominance, or suppressive cytokines from infected macrophages. Regarding compartmentalization, tuberculous lesions may collect most of the relevant circulating T cells, so that few are available to participate in the tuberculin reaction. This concept receives support from the fact that lymphocytes in bronchoalveolar lavage and pleural fluids (and presumably in other diseased tissues) contain a greater proportion of antigen-specific T cells, secrete greater quantities of lymphokines, and show a greater tendency to proliferate (in the presence of specific antigen) than T lymphocytes in the peripheral blood (Barnes et al., 1989b, 1990). Tuberculin-negative patients with active tuberculosis also have a greater number of monocytes and lymphocytes in their peripheral blood that exert apparently suppressive effects in vitro (Ellner and Wallis, 1989). Production of prostaglandin E_2 (Ellner and Wallis, 1989), or possibly IL-10 or transforming growth factor β, by these monocytes may contribute to their suppressive effects. Finally, as mentioned above, the balance of type 1 (Th1) to type 2 (Th2) T-cell subsets may be critical, and a switch from type 1 to type 2 responses (or to mixed responses) could result in anergy or suppressive effects on type 1 T-cell function and macrophage activation.

Persisting Viable Tubercle Bacilli

In human beings, after even an inapparent tuberculous infection heals, the lungs may contain one or more small encapsulated caseous foci. In such foci, tubercle bacilli may persist in a dormant and nonmetabolizing state, insusceptible to sterilization by antimicrobial agents. The bacilli may remain viable in the host for life and cause active disease when resistance is lowered by old age, corticosteroids, immunosuppressants, AIDS, or other factors. It is the presence of these bacilli that necessitates prolonged (6-month) courses of chemotherapy, with the resulting problems of cost, compliance, and drug resistance. It is not certain that drugs able to kill dormant or stationary-phase mycobacteria can be devised, since most microbicidal agents depend on actively metabolizing or dividing cells. Persistence of viable tubercle bacilli may also be the reason the positive tuberculin reaction is usually maintained for life. Each time the bacillus multiplies, the immune system may be stimulated.

In addition, tubercle bacilli may possibly persist within macrophages as forms with unusual cell walls (Stanford, 1987), and there are reports of mycobacterial genomic material in the tissues of some patients with sarcoidosis (Bocart et al., 1992; Fidler et al., 1993), in spite of the absence of bacteria detectable by conventional means. It is conceivable that similar forms exist in tuberculosis patients, and new studies with in situ polymerase chain reactions should help us explore this point.

Mycobacteria and Idiopathic or "Autoimmune" Diseases

Tuberculosis patients have a spectrum of autoantibodies that is remarkably similar to that seen in rheumatoid arthritis patients (Shoenfeld and Isenberg, 1988). They also

have a striking change in the glycosylation of the immunoglobulin G heavy chain, and this is also characteristic of patients with rheumatoid arthritis or Crohn's disease and a subset of patients with sarcoidosis (Rook et al., 1993a). The immunotherapy discussed above leads to a rapid fall in percent agalactosyl immunoglobulin G in tuberculosis patients (Rook et al., 1994), though the significance of this remains to be elucidated.

The known capacity of mycobacterium-containing adjuvants (Freund's complete adjuvant) to facilitate experimental induction of autoimmunity and evidence that mycobacterial disease can be accompanied by a sterile arthropathy (reviewed by Rook et al. [1993a]) have reawakened speculation that some autoimmune syndromes may be cryptic infections or may be triggered by past encounters with mycobacterium-like organisms (Rook et al., 1993a). Interest was further increased by the discovery that T cells that will passively transfer the arthritis evoked in susceptible rat strains by Freund's complete adjuvant are able to recognize the mycobacterial 65-kDa HSP (van Eden et al., 1988).

HSPs are involved in assembly, folding, and transport of other cellular proteins and are expressed under various conditions of stress. These functions are fundamental to the survival of all life-forms, particularly under stressful conditions, when synthesis of HSPs may increase while synthesis of other proteins is reduced. HSPs are highly conserved throughout evolution, and there is striking sequence homology between the HSPs of microorganisms and those of higher animals. These facts, together with the demonstration that T cells mediating an experimental autoimmune disorder recognize the mycobacterial HSP65, have led to the following hypotheses, for each of which there is some evidence (compare reviews by Young and Elliott [1989], Polla [1991], and Cohen and Young [1991]). (i) The immune response to bacterial HSPs may cross-react with host HSPs, leading to autoimmune disease. (ii) The immune response may focus its attention on HSPs because they are so conserved and may be induced by the stress of infection or inflammation. This may enable rapid recognition of any pathogen. (Inadvertent autoimmunity is a consequent risk.) (iii) Self-HSP-derived peptides presented by MHC molecules may be targets for cytotoxic cells. This would enable the immune response to detect and eliminate stressed autologous cells, which might facilitate recognition of transformed or infected cells.

At present, the evidence for recognition of HSPs by T cells from synovia of joints of patients with inflammatory arthritides or from thyroid tissue of patients with thyroiditis (reviewed by Young [1992]), despite the best efforts of several laboratories, have mostly been unsupportive of the HSP cross-reactivity hypothesis as the basis for autoimmunity, and sporadic reports of reactivity to the human homolog in juvenile chronic arthritis (De Graeff-Meeder et al., 1991) or in *Yersinia*-associated reactive arthritis (Hermann et al., 1991) are unconvincing, rely on few cell lines, and fail to exclude the possibility of contamination of the recombinant protein with the *Escherichia coli* products.

In tuberculosis, such autoimmune reactions could contribute to both caseous necrosis and liquefaction, but there is at present no direct evidence for this. In fact, desensitization to mycobacterial antigens prevented cavity formation in animals (Yamamura et al., 1974), indicating that if there is any autoimmune component, it is dependent on initiation by a mycobacterium-specific cellular immune response. We suggest that although the evidence for recognition of HSPs by CD4 Th cells is negligible, possibility must be considered that common cross-reactive HSP antigens could be recognized by MHC class I-restricted CTL. The logic is that HSPs are expressed as cytoplasmic antigens and would be ex-

pected to be presented in association with MHC class I antigens. Since any pathogen—viral, bacterial, or protozoal—that invaded the cytoplasm of infected cells would induce the same conserved antigens, there would be existing memory for CTL activity that could provide an early response. Cytotoxic CD4 T cells in humans have been reported to kill macrophages pulsed with the mycobacterial 65-kDa HSP (Ottenhoff et al., 1988) and stressed autologous cells in the absence of mycobacterial antigen, presumably through cross-reactive recognition of the autologous HSP (Koga et al., 1989), although this has not yet been confirmed. Finally, the results of Silva and Lowrie (in press) indicate that expression of the 65-kDa heat shock cognate protein of BCG in mouse macrophages provided effective immunization against challenge with *M. tuberculosis* and suggest that there must be some immune responses against HSP65 that can provide protection. While definitive evidence for a role of HSPs in either protection or autoimmunity is lacking, the available data are consistent with the hypothesis that if HSPs are involved, rather than being important at the level of MHC class II-restricted CD4 cells, they would preferentially engage $CD8^+$ MHC class I-restricted CTL. They could then exert cytotoxic activity on infected macrophages containing more bacilli than they are able to kill and presumably could liberate the bacilli to enable them to be phagocytosed by infiltrating macrophages that may be activated by the IFN-γ released locally by the $CD8^+$ CTL or $CD4^+$ Th1 cells in granulomas and be more effective in killing at lower multiplicities of infection. CTL might also kill somatic cells in the vicinity either infected with *M. tuberculosis* or stressed to express endogenous HSPs in association with MHC class I. Events that can be interpreted in this manner are seen in histopathological studies (Dannenberg, 1991). Clearly, it will be important to undertake experiments to establish the existence of human CTL, the nature of the antigens they recognize, and their relation to protection, tissue damage, and autoimmunity.

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