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Special Article

THE IMMUNOGENICITY OF KILLED MYCOBACTERIA

Introduction

The question of the immunogenicity of killed mycobacteria has become of enormous importance in relation to the future design of vaccines for leprosy. Much attention has been focused on the fact that a single subcutaneous injection of relatively large numbers of leprosy bacilli killed by heat or by irradiation induces in mice a state of delayed skin-test positivity, which can be elicited with soluble antigen a few days later.[1,2] Moreover, the killed organisms evoke resistance to live leprosy bacilli,[3] resistance to an intravenous challenge with *Mycobacterium tuberculosis* and local resistance to *Listeria monocytogenes.*[1] There is an increasing tendency to regard this immunogenicity of killed organisms as exceptional and as an indication of some bizarre property unique to *Mycobacterium leprae.* This tendency is a consequence of a widespread misconception that other species of mycobacteria lose their immunogenicity when killed, whereas this is in fact true only of a minority of species, in certain strains of mice.

The immunogenicity of killed mycobacteria in guinea-pigs and rabbits

The first report of the induction of positive tuberculin reactivity in guinea-pigs with killed *Mycobacterium tuberculosis* was published by Baldwin in 1911 and subsequently discussed by Petroff[4] in 1923. This author extended the studies of Baldwin and observed that from 1–3 intraperitoneal injections of 12.5 mg (dry weight) of dead tubercle bacilli, even if autoclaved at 121°C at 15 lb in^2, was as effective a way of inducing cutaneous tuberculin-test positivity as was a virulent infection. Using this protocol, Petroff and Stewart[5] subsequently demonstrated that guinea-pigs immunized with killed bacilli were also indistinguishable from infected ones on the basis of susceptibility to delayed fatal haemorrhagic tuberculin shock and showed a similar degree of protection from an intracutaneous challenge with virulent organisms.

These results were obtained in spite of the fact that the intraperitoneal route is not an efficient one, but very large doses of killed organisms were

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required. Freund and Opie (1938),[6] working with rabbits, tried various routes and were able to show that a far smaller dose of killed organisms (0.2 mg at weekly intervals) injected subcutaneously or intracutaneously was as effective as BCG, although repeated injections were required for most animals. Thus, in their own words (p. 296): 'Rabbits (and human beings) differ widely in the rapidity with which they undergo sensitization with heat-killed tubercle bacilli, but after repeated injections all animals become sensitized.'

Similarly Wilson *et al.* (1940)[7] injected guinea-pigs intramuscularly with various doses of heat-killed *M. tuberculosis* (at 5–7-day intervals for 12–20 weeks) and reported that after a variable number of injections all the animals became skin-test positive, some of them exquisitely so. Highly significant protection from subsequent intramuscular challenge with from 12–300 live *M. tuberculosis* organisms was also seen in those animals with weak or moderate responses.

Numerous other examples could be quoted (see Weiss, 1959, for 263 refs)[8] but these suffice to indicate that in rabbits and guinea-pigs killed *M. tuberculosis* is immunogenic. Moreover, the method used to kill the organisms seems to be of secondary importance since workers using heat, ultra-violet light, prolonged storage, formalin, urea or phenol, reported essentially similar findings.[8] However, large doses, or repeated injections, or both, are needed in order to evoke responses comparable to those evoked by li e organisms. (In the early years this quantitative difference was less obvious because even live organisms were used in quite unnecessary doses, often of several milligrams.) At first sight the explanation for this difference between the immunogenicity of live and dead organisms seems likely to be a trivial one. Thus live organisms proliferate and synthesize more antigen and so constitute a far greater and more prolonged immunogenic stimulus than an equivalent number of killed organisms. Large or repeated injections of the latter clearly compensate for this difference. However, the situation is in reality rather more complex. Although killed organisms injected into a large number of outbred guinea-pigs will evoke in at least some of the animals all of the phenomena associated with the response to live ones (necrotic or non-necrotic skin-test responses, tuberculin shock and protection), it is clear from studies by Raffel[9] that the percentage of the animals showing the *necrotic* type of skin-test reactivity is lower when killed organisms are used, even when skin-test reactions of a similar size are obtained. Moreover, the relationship between skin-test antigen dose and reaction size is different. Killing *M. tuberculosis*, therefore, has qualitative as well as quantitative effects on its immunogenicity.

The immunogenicity of killed mycobacteria in mice

The significance of these observations becomes clearer when one considers the immunogenicity of killed mycobacteria in mice.

Our present concepts of the cellular basis for immunity to facultative or obligate intracellular parasites originate in the studies of immunity to *Listeria monocytogenes*, *Brucella abortus* and *Salmonella typhimurium*, in mice reviewed by Mackaness.[10]

These organisms do not evoke protective immunity when injected dead and Mackaness suggested that this is because they lack 'built-in' adjuvanticity. This view is supported by the more recent observation that certain adjuvants, such as polyanions, or the cationic surface-active agent dimethyl, dioctadecyl ammonium bromide, when mixed with killed *Listeria monocytogenes*, can render it protective.[11] However, Mackaness also made the point that mycobacteria are different from these other genera, in that they *do have* 'built-in adjuvanticity' and many are immunogenic in mice when killed, though as in rabbits and guinea-pigs larger doses are required than when live organisms are used.[10, 12] Numerous examples in mice were also reviewed by Weiss.[8] In this respect many mycobacterial species resemble *Corynebacterium parvum*, which has a similar adjuvant-active cell wall and is routinely used killed.

More recently workers who use mice have lost sight of this well-established immunogenicity of most killed mycobacteria and have begun to make misleading generalizations such as that 'immune reactivity to mycobacteria or other intracellular parasites develops only when bacilli multiply in the phagocyte'.[13] Such statements are obviously in direct conflict with a huge amount of published evidence. How has this apparent disagreement come about? One possibility, for which there is good evidence, is that killing does not have the same effect on the immunogenicity for mice of all mycobacterial species. It is often possible to predict whether or not killing an organism will reduce its immunogenicity for a particular mouse strain, by studying the type of response evoked in that mouse strain by the same organisms injected live. Thus delayed skin-test (foot-pad test) responses in mice have distinct 24-hour and 48-hour components.[2, 13, 14] Those organisms which evoke little skin-test positivity during the first 2–3 weeks, but then cause a response with a powerful 48-hour component, are the ones which are not immunogenic when killed. The best examples of this are *Mycobacterium kansasii*,[2] *M. lepraemurium*,[13] some *M. avium* strains and some, but not all, strains of BCG (own published observations) or *M. tuberculosis*. These, although a minority of mycobacterial species, are the most commonly used organisms and therefore the view that killed mycobacteria are not immunogenic has dominated. Indeed these organisms, when injected killed, sometimes not only fail to evoke skin-test positivity or protective immunity, but actually trigger mechanisms which allow increased proliferation of a simultaneously administered live inoculum.[13]

Other organisms, however, evoke mainly the 24-hour component. This type of response more closely resembles that induced by *Listeria monocytogenes*,[10] or *Corynebacterium parvum*. It appears within a few days of infection, reaches a maximum or plateau by about 10 days and peaks 18–24 hours after skin-test

challenge with soluble antigen. (The 2–3-week delay is not seen and the 48-hour component is weak or absent.) Those mycobacteria which evoke this '*Listeria*-like' response, when injected live, will do so equally well when injected dead if a dose of at least 2×10^7 organisms is used. This property, which is characteristic of *Mycobacterium vaccae*[2] and *M. nonchromogenicum*, is shared by Glaxo BCG, *Corynebacterium parvum*, *Mycobacterium leprae*,[2] and probably other, non-pathogenic species.

These types of response also differ by several other criteria but it is not yet clear whether the basis for the differen
level.[2] Both types of response can correlate with protection, though under different circumstances (Rook, Bahr and Stanford, submitted for publication). Thus the 48-hour type of response correlates very strongly with protection against *dissemination* of *M. avium* (McIntyre and Rook, unpublished observations) or *M. lepraemurium*[14] from superficial sites. On the other hand, it is the '*Listeria*-like' response which correlates with the best protection against systemic challenge with these organisms. Moreover, the '*Listeria*-like' response gives full protection against foot-pad challenge with *M. Kansasii* (Rook and Stanford, submitted for publication) or *M. leprae*. The importance of these findings lies in the fact that, as discussed above, killing mycobacteria preferentially reduces the 48-hour component in mice, and the necrotic or 'Koch' component in guinea-pigs (perhaps these are analogous). We clearly need to understand better the protective relevance to man of the human equivalents of these types of response.

The immunogenicity of killed mycobacteria in man

During the first 25 years of this century approximately 23,000 individuals in Italy were vaccinated with heat-killed *Mycobacterium tuberculosis* by scarification.[15] Subsequently more than 50,000 children were vaccinated with intradermal injections of formalinized organisms (1.5 mg, wet weight.[15] Unfortunately these trials were almost entirely uncontrolled, and evidence for their success is anecdotal. Nevertheless, this evidence is suggestive and the disappearance of the killed vaccine appears to have been due not so much to its own failure, as to the proven success of BCG in well-organized trials. However, two 'defects' of the killed vaccine were noted. First, 30–50 times as many organisms were required, and these, even when given in divided doses, often resulted in distressing lesions. Secondly, there was a theoretical objection to the fact that unlike BCG, the killed vaccine caused little local lymph-node involvement. It was felt that the ideal vaccine should mimic the 'primary complex' of a tuberculous infection. This led to the interesting suggestion that killed organisms failed to reach the draining node, and that greater lymph-node involvement could be achieved by adding hyaluronidase to the vaccine (reviewed[15]).

Surprisingly, our knowledge of the immunogenicity for man of killed *M. leprae* is even more fragmentary. It is still not clear whether Lepromin acts as an unusual skin-test reagent, or as an immunizing stimulus, or both. There are of course numerous reports that repeated Lepromin tests can cause positive Mitsuda responses in previously negative individuals,[16] and that Mitsuda positivity correlates with a decreased risk of disease, particularly of the lepromatous type.[17] However, these studies have involved small selected groups, already exposed to living leprosy bacilli, and do not constitute proof that one or more doses of killed *M. leprae* will evoke either Mitsuda positivity or protection in normal, previously unexposed populations.

Is killed *Mycobacterium leprae* unique?

It is obvious from the work reviewed above that the immunogenicity of killed *M. leprae* in mice is not in itself unique. On the other hand, the studies of Shepard and his colleagues point to two ways in which *M. leprae* does appear to differ from all the other species which have been studied, though we cannot at present be certain that unusual properties of the available preparations of *M. leprae* bacilli are not due to unusual methods used in this preparation. First, only *M. leprae* causes *chronic* lymph-node enlargement when injected killed.[3, 16] This contrasts with the relative failure of killed *M. tuberculosis* to cause a lymph-node reaction, which was discussed earlier. It will be interesting to know whether this property of killed *M. leprae* should be attributed to a tendency to localize in the draining node, or to very slow degradation. The latter could be explained by the unexpectedly high proportion of glycine in the wall of *M. leprae*[19] which may be related to an unusual peptidoglycan structure of extreme stability.

The second possibly unique feature of the immunogenicity of *M. leprae*, is that killing may a

any property of an organism which affects the way in which its antigens are presented to the host's antigen-recognizing cells and which is dependent on the viability of the organism, will be relevant to the consequences of killing it. Live *M. leprae* can escape from phagosomes, and it gets into the cytoplasm of many cell types, including macrophages, muscle cells and fibroblasts. Moreover, it can get into nerves, which may constitute an 'immunologically privileged site'. Antigens leaking from such sites into the blood-stream have been shown to evoke suppression rather than immunization.[20] (This mechanism has been discussed recently in relation to leprosy.)[21] When killed, the leprosy bacillus presumably does not get into these 'hiding places', but rather into phagosomes of phagocytic cells, capable of 'processing' antigen and presenting it to the antigen-recognizing system. Thus, killing would be expected to increase immunogenicity.

Conclusion

The variable effect of killing on the immunogenicity of different mycobacterial species is clearly trying to tell us something fundamental about the biology of the organisms. It is therefore essential to build up an accurate picture of how *Mycobacterium leprae* compares with other species.

(1) The immunogenicity of killed *M. leprae* is not in itself unique (although it has unusual features). There is a huge neglected literature on the ability of killed mycobacteria to evoke *both* skin-test positivity *and* protection.

(2) It is possible that killing *M. leprae* causes *qualitative* changes in the type of response evoked. This is true of pathogenic members of the slow-growing subgenus, which, when killed, evoke less of the necrotizing component. We therefore need to know more about the relevance to protection of these different components. However, the response to killed *M. leprae* in mice resembles that evoked by several non-pathogenic members of the fast-growing subgenus, rather than the response to pathogenic slow-growers.

(3) We know that BCG, a living vaccine, can protect man against leprosy. There is at present no evidence that killed *M. leprae* is immunogenic (skin-test positivity or protection) in people not previously exposed to living leprosy bacilli, but a review of the literature involving killed mycobacteria suggests that it will be. It remains, nevertheless, an act of faith.

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