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Influence of BCG-induced immunity on the bactericidal activity of isoniazid and rifampicin in experimental tuberculosis of the mouse and guinea-pig

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Summary. The influence of previous BCG vaccination on the bactericidal activity of isoniazid and rifampicin has been studied using serial counts of viable tubercle bacilli in the spleens and lungs of mice and guinea-pigs infected intravenously with *M. tuberculosis*, strain H37Rv. In mice, BCG vaccination decreased the bactericidal activity of isoniazid in the spleen, but did not affect its activity in the lungs, where immunity is less strongly expressed. BCG did not influence the bactericidal activity of rifampicin in either organ. In contrast, previous BCG vaccination in the guinea-pig increased the bactericidal activity of isoniazid and rifampicin in the spleen and lungs. The differences between the animal species might result from the immune response being mainly bacteriostatic in the mouse but bactericidal in the guinea-pig.

Keywords: BCG vaccination, immunity, isoniazid, rifampicin

A review by the International Union against Tuberculosis and the World Health Organisation (Joint IUAT/WHO Study Group 1982) concluded that the most powerful method for controlling tuberculosis is the combination of case-finding and chemotherapy, while BCG vaccination, although it can prevent tuberculosis (usually in childhood) in those vaccinated, has only a small epidemiological effect in reducing the risk of infection in the community. Thus, one of the ways in which current interest in the immunology of tuberculosis might usefully develop is towards the introduction of immunotherapy to increase the efficacy of chemotherapeutic drugs in control programmes. Although it is often assumed that an increase in immunity would favour chemotherapeutic activity, there is little experimental evidence to support the assumption. We have shown previously that murine recombinant interferon-γ reduced the bacterial counts of *Mycobacterium tuberculosis* in the spleens and lungs of mice, but had no effect on the bactericidal activity of isoniazid or rifampicin (Khor *et al.* 1986). However, the reduction in organ counts in animals treated with interferon was small, in contrast to the much larger effect expected with previous BCG vaccination. Some 30 years ago, previous BCG vaccination was found to increase the bactericidal activity of isoniazid in experimental tuberculosis of the guinea-pig (Mitchison & Selkon 1956). We decided to continue this work by exploring the interaction between vaccination and chemotherapy in the mouse as well as in the guinea-pig and by using rifampicin as well as isoniazid, since these are

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the two most potent drugs used in the chemotherapy of tuberculosis.

Materials and methods

Chemotherapeutic drugs. Rifampicin (Ciba Laboratories, Horsham, West Sussex) was suspended in 0.5–0.7% methyl cellulose and isoniazid (Sigma Chemical Co., Poole, Dorset) was dissolved in distilled water. Both drugs were administered in 0.2 ml volumes to mice and in 0.4–0.8 ml volumes to guinea-pigs by oral gavage. The dose sizes were 20 mg/kg body weight isoniazid and 25 mg/kg body weight rifampicin in both animal species.

BCG vaccination. BCG was freeze-dried Glaxo vaccine (Evans Medical Ltd, Dunstable, Bedfordshire). Mice were inoculated with a suspension of the isoniazid-resistant BCG strain containing 2×10^5–4×10^6 colony-forming units (cfu) divided among five subcutaneous sites. Guinea-pigs were inoculated with 1 mg (moist weight) of the isoniazid-sensitive BCG into the right thigh muscle. BCG was given 7–8 weeks before challenge to half of the animals.

Challenge. A mouse-passaged, virulent strain of *M. tuberculosis* H37Rv was obtained and stored at −70°C, as described by Khor *et al.* (1986) before inoculation into mice and guinea-pigs.

Procedures in mice. After BCG vaccination the BALB/c mice (18–20 g weight initially) were challenged intravenously and chemotherapy was started in half of the animals 3 days after challenge. At intervals of a few days, four mice were killed in each of the four treatment groups and quantitative cultures of spleen and lungs were set up on plates of selective 7H11 medium as described by Khor *et al.* (1986), except that 2 mg/l thiophenecarboxylic acid hydrazide (Aldrich Chemical Co. Ltd., Gillingham, Dorset) was added to some 7H11 medium plates to suppress growth of BCG and 1 mg/l isoniazid to other plates to allow counting of BCG in the presence of *M. tuberculosis* H37Rv.

Procedures in guinea-pigs. Dunkin Hartley guinea-pigs (410–520 g weight) were BCG vaccinated and then infected intravenously through the lateral ear vein (Markham & Kent 1951) with 1×10^7 cfu *M. tuberculosis* suspended in 0·1% gelatin in normal saline. Chemotherapy was started in half of the animals 1 day after challenge. At intervals, spleens and lungs were taken with aseptic precautions at autopsy from two animals in each treatment group and viable counts set up as for mice.

Statistical treatment. Results were examined by analysis of variance, using the GLIM statistical package, when there was any doubt as to their interpretation. The statistical significance of the effect of BCG vaccination on the bactericidal activity of isoniazid and rifampicin was assessed on organ viable counts taken during drug treatment, from the interaction between the effects of time and BCG vaccination in the mice given BCG or no BCG and then treated with the drug.

Results

Mice treated with isoniazid

The viable counts obtained in the first experiment with mice treated with isoniazid are shown for the spleens and lungs in Fig. 1. The spleen counts at day 0 were apparently much lower in BCG-vaccinated animals (4.0 $\log_{10}$ cfu/spleen) than in unvaccinated control animals (4·7 $\log_{10}$ cfu/spleen). Since the counts for corresponding animals in all other experiments were much closer, the point for the vaccinated animals was redrawn arbitrarily at the same count as for the unvaccinated animals. From 3 days onwards until the end of the experiment at 7 days, the counts on the vaccinated mice were lower than those for the control mice by 0.5–0.7 $\log_{10}$ cfu/spleen. When treatment with isoniazid was started at day 3, the spleen counts decreased slightly during the first day and then fell rapidly in the unvaccinated mice but only decreased slightly in those previously vaccinated, the difference between

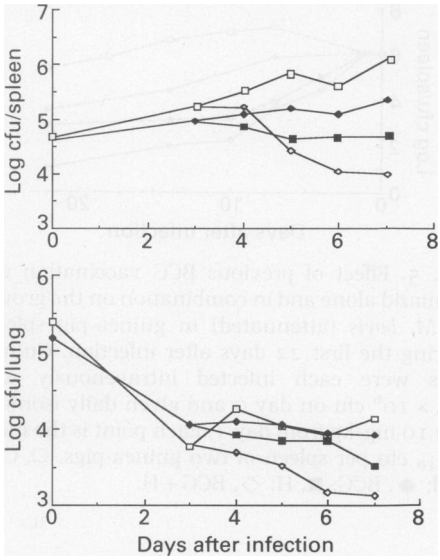

Fig. 1. Effect of previous BCG vaccination and isoniazid alone and in combination on the growth of *M. tuberculosis* in the spleens and lungs of mice during the first 7 days after infection. Mice were each infected intravenously with 1.0×10^6 cfu on day 0 and given daily isoniazid (H) 25 mg/kg from day 3. Each point is the mean $\log_{10}$ cfu per organ of four mice. □, Control; ◆, BCG; ■, BCG+H; ◇, H.

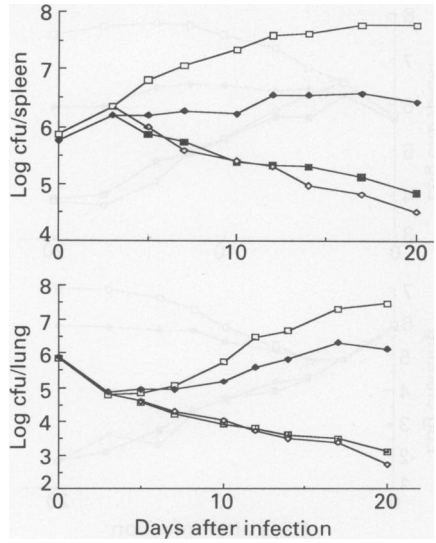

Fig. 2. Effect of previous BCG vaccination and isoniazid alone and in combination on the growth of *M. tuberculosis* in the spleens and lungs of mice during the first 20 days after infection. Mice were each infected intravenously with 2.5×10^6 cfu on day 0 and given daily isoniazid (H) 25 mg/kg from day 3. Each point is the mean $\log_{10}$ cfu per organ of four mice. □, Control; ◆, BCG; ■, BCG+H; ◇, H.

the rates of fall being statistically significant ($P<0.001$). Thus BCG vaccination slowed the bactericidal activity of isoniazid. The counts on the lungs showed little difference between the curves for the vaccinated and control mice that were not treated with isoniazid, and BCG vaccination did not alter the bactericidal activity of isoniazid ($P>0.05$). The residual mean square, derived from the replicate mice at each time point, in the analyses of variance for this experiment and for others, was about 0.03 for the spleen and the lung counts. The results of a second similar experiment, lasting 20 days, are shown in Fig. 2. BCG vaccination reduced the counts on the spleens and lungs of mice receiving no chemotherapy. Previous BCG vaccination slowed the bactericidal activity of isoniazid slightly in the spleens ($P<0.001$) but had almost no effect in the lungs ($P=0.03$).

Mice treated with rifampicin

The results of a 20-day experiment in which mice were treated with rifampicin in place of isoniazid are shown in Fig. 3. Among animals which received no chemotherapy, the curves were appreciably lower in vaccinated than in control mice. Previous BCG vaccination did not alter the bactericidal activity of rifampicin in either organ (spleens, $P=0.04$; lungs, $P>0.05$). A further experiment was done in which rifampicin was given for 10 days. The viable counts in the spleens (Fig. 4) and lungs (data not presented here) again showed no effect of BCG vaccination on the activity of rifampicin ($P>0.05$ for both organs).

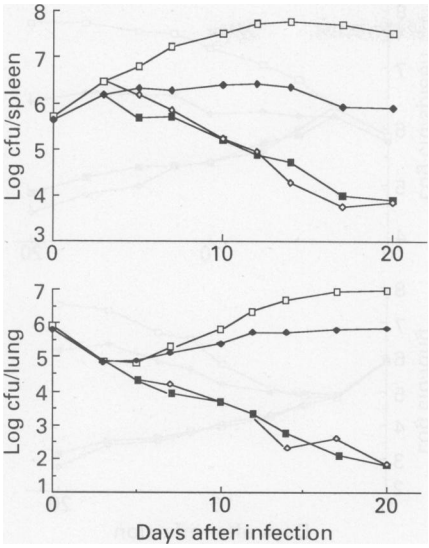

Fig. 3. Effect of previous BCG vaccination and rifampicin alone and in combination on the growth of *M. tuberculosis* in the spleens and lungs of mice during the first 20 days after infection. Mice were each infected intravenously with 2.2×10^6 cfu on day 0 and given daily rifampicin (R) 20 mg/kg from day 3. Each point is the mean $\log_{10}$ cfu per organ of four mice. □, Control; ◆, BCG; ■, BCG+R; ◇, R.

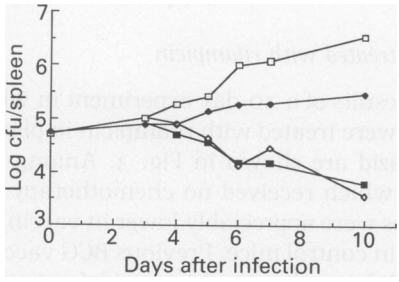

Fig. 4. Effect of previous BCG vaccination and rifampicin alone and in combination on the growth of *M. tuberculosis* in the spleens of mice during the first 10 days after infection. Mice were each infected intravenously with 3.6×10^5 cfu on day 0 and given daily rifampicin (R) 20 mg/kg from day 3. Each point is the mean $\log_{10}$ cfu per organ of four mice. □, Control; ◆, BCG; ■, BCG+R; ◇, R.

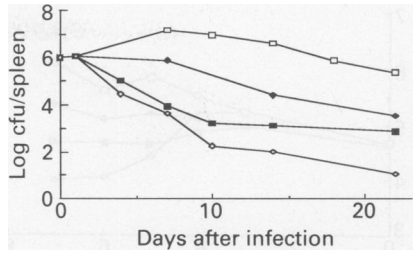

Fig. 5. Effect of previous BCG vaccination and isoniazid alone and in combination on the growth of *M. bovis* (attenuated) in guinea-pig spleens during the first 22 days after infection. Guinea-pigs were each infected intravenously with 3.5×10^6 cfu on day 0 and given daily isoniazid (H) 10 mg/kg from day 1. Each point is the mean $\log_{10}$ cfu per spleen of two guinea-pigs. □, Control; ◆, BCG; ■, H; ◇, BCG+H.

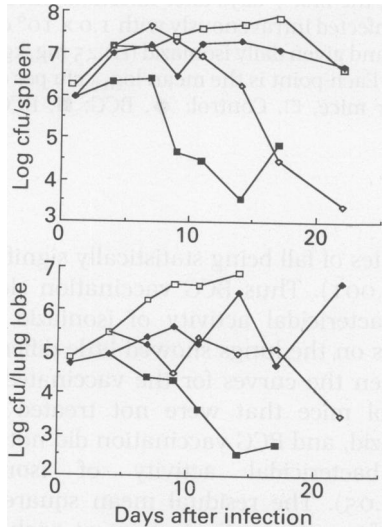

Fig. 6. Effect of previous BCG vaccination and rifampicin alone and in combination on the growth of *M. tuberculosis* in the spleens and lungs of guinea-pigs during the first 22 days after infection. Guinea-pigs were each infected intravenously with 1.0×10^7 cfu on day 0 and given daily rifampicin (R) 20 mg/kg from day 1. Each point is the mean $\log_{10}$ cfu per organ of two guinea-pigs. □, Control; ◆, BCG; ■, BCG+R; ◇, R.

Guinea-pigs treated with isoniazid

The viable counts on the spleens of guinea-pigs are shown in Fig. 5 redrawn from a previous experiment described by Selkon & Mitchison (1956). This experiment differed from those described here in several minor respects. The BCG used was the Copenhagen strain. The challenge organism was an attenuated variant of *M. bovis*, strain Branch. Treatment with isoniazid was started 1 day after challenge. A similar experiment was also done with the virulent parent Branch strain but this could only be continued for 11 days because control animals then began to die from tuberculosis. The spleen counts (Fig. 5) fell more rapidly in vaccinated than in control animals, and previous BCG vaccination accelerated the bactericidal activity of isoniazid throughout the 22 days of the experiment. Similar conclusions could be drawn from the data (not presented here) for the experiment with the virulent Branch strain.

Guinea-pigs treated with rifampicin

Spleen and lung viable counts are shown in Fig. 6. The counts in both organs of the BCG-vaccinated guinea-pigs were lower than those in the unvaccinated controls. Previous BCG vaccination accelerated the bactericidal activity of rifampicin over the first 9–14 days of the experiment.

Discussion

Previous work has shown that recombinant murine interferon-γ, a potent lymphokine, reduced the counts of viable bacilli in the spleens and lungs of BALB/c mice infected intravenously with *M. tuberculosis* H37Rv (Khor *et al.* 1986). The reduction in the counts was usually about 0·2–0·4 $\log_{10}$ cfu, apparent about 1 day after dosage with interferon and not increased by subsequent doses. In the present study, using the same strain of mice and similar techniques, the effect of previous BCG vaccination on the organ counts was considerably greater. The difference between vaccinated and unvaccinated animals increased throughout each experiment, usually reaching more than 1.0 $\log_{10}$ cfu in the spleens and rather less in the lungs. Thus the effects of BCG vaccination cannot be attributed solely to increased release of interferon-γ.

The first of our findings relating immunity to chemotherapy was that BCG-induced immunity in the mouse slowed the bactericidal activity of isoniazid in the spleen though not in the lungs. An inhibitory effect of BCG vaccination on isoniazid activity was also found by Kanai & Kondo (1971) in the spleens of mice challenged with a streptomycin-resistant variant of *M. tuberculosis* H37Rv. Inhibition was attributed to immunity slowing the rate of growth of the challenge organism. This explanation was supported by experiments in which the rate of growth of a challenge with a streptomycin-dependent variant of the H37Rv strain could be maintained by treating the mice with streptomycin or inhibited by giving them no streptomycin (Kanai 1966). Isoniazid was bactericidal only when the mice were given streptomycin to allow mutiplication of the challenge organisms. Rees & Hart (1960) also showed that the stability of viable counts of *M. tuberculosis* in the lungs of mice with chronic tuberculosis was due to a slowing of the rate of growth of the organisms and not to a balance between growth and killing. The bactericidal activity of isoniazid can be reduced or abolished *in vitro* by a variety of conditions that inhibit the growth of *M. tuberculosis* including suspension of the bacilli in non-nutrient medium (Hobby & Lenert 1957; Peizer *et al.* 1954), the use of stationary phase rather than log phase organisms, anaerobic culture (Mitchison & Selkon 1956), low pH and low temperature (Dickinson & Mitchison 1981). It is therefore reasonable to suppose that immunity in the spleens of mice, expressed mainly as bacteriostatic rather than bactericidal activity, slows the bactericidal activity of isoniazid in a similar manner though the effect might be

less marked because the influence of immunity on growth rates is less extreme than the in-vitro conditions just considered. The failure of BCG-induced immunity to slow the isoniazid kill in the lungs might reflect the lesser expression of immunity in the lungs than in the spleen; in each experiment, the difference between the spleen counts in the vaccinated and unvaccinated groups not given isoniazid was appreciably greater than the difference between the lung counts.

In contrast to its effects on isoniazid activity, BCG vaccination did not reduce the bactericidal activity of rifampicin in mouse spleens. In-vitro studies have shown that rifampicin was more bactericidal than isoniazid when bacterial stasis was interrupted by short periods of active metabolism (Dickinson & Mitchison 1981) as might well occur in stationary phase cultures and also in the organs of mice with BCG-induced immunity. Thus the failure of BCG vaccination to inhibit the bactericidal activity of rifampicin in the spleens of mice might be due to its superior activity against semi-dormant organisms.

An increase in the bactericidal activity of isoniazid and rifampicin was found in the spleens and lungs of guinea-pigs. An explanation for the difference between mice and guinea-pigs in this effect might arise from consideration of the immune responses in the two species. Cultures of *M. tuberculosis* which are highly resistant to isoniazid or are derived from South Indian patients are attenuated in the guinea-pig (Cohn *et al.* 1954; Mitchison *et al.* 1963; Mitchison 1964). When injected intramuscularly they produce persisting lesions at the site of injection but the small lesions in the spleen, liver and lungs heal rapidly and the animals survive. When injected intravenously, growth for first 2–3 weeks occurs at much the same rate as with virulent cultures, but the organ counts then decline fairly rapidly (Cohn *et al.* 1954; Wallace *et al.* 1961). Isoniazid-resistant and South Indian attenuated cultures are more susceptible to the bactericidal activity of H_2O_2 than usual suggesting that they might be attenuated because of their susceptibility to H_2O_2 generated in host macrophages (Coleman & Middlebrook 1956). More recent evidence on the role of H_2O_2 in host defence is reviewed by Lowrie & Andrew (1988). If intracellular H_2O_2 is indeed a major host defence mechanism in the guinea-pig, it would seem to be essentially bactericidal in its action. A predominantly bactericidal immune mechanism would be expected to be additive or synergistic with the bactericidal action of isoniazid and rifampicin. In contrast, the virulence of isoniazid-resistant or South Indian cultures that are attenuated in the guinea-pig have been found to be of similar or only slightly lower virulence in the mouse as compared to isoniazid-sensitive cultures from Western countries (see Wallace *et al.* 1961) suggesting that the bactericidal H_2O_2 mechanism is less important in the mouse macrophage. Thus, the differences between the two animal species in the effects of BCG-induced immunity on the activities of isoniazid and rifampicin might be due to a predominantly bacteriostatic immune response in the mouse and a more bactericidal response in the guinea-pig.

The implications of these findings for immunotherapy in man can only be speculative. Guinea-pig attenuation probably implies only a small change in virulence for man. Pulmonary disease due to either isoniazid-resistant strains or to South Indian attenuated strains progresses in its usual, often fatal, manner in man unrelated to guinea-pig virulence (Ramakrishnan *et al.* 1961, 1962), whereas disease due to these strains is often self-limiting in the guinea-pig. There are however two lines of evidence suggesting that guinea-pig attenuation implies some loss of virulence in man. First, a study of tuberculosis arising in close family contacts of patients under treatment with isoniazid alone and therefore excreting isoniazid-resistant organisms, suggests that these resistant strains are unlikely to infect the contacts possibly because of their low virulence (Devadatta *et al.* 1970). Secondly, there is a strong tendency for the most attenuated

strains (those highly resistant and catalase-negative) to be selected out during passage from patients with acquired isoniazid-resistance to their contacts (Canetti *et al.* 1964). Thus, the response to peroxide-susceptible attenuation in man appears to be closer to the limited response found in the mouse than to the much greater response of the guinea-pig. It therefore seems likely that the immune processes in man are more similar to those in the mouse than to those in the guinea-pig and that an increase in immunity will hinder or fail to influence the action of chemotherapeutic drugs rather than increase their action. So far, two clinical studies suggest that altering the immune status during the early stages of chemotherapy has little effect on the activity of the antibacterial drugs. In the first of these, high-dosage steroids, which might be expected to reduce immunity, were given during the first 8 weeks of short-course regimens and failed to influence the rate of conversion of sputum to culture negativity or the relapse rate after chemotherapy had been finished (Tuberculosis Research Centre, Madras 1983). In the second study, levamisole, which might improve the immune response, again failed to influence sputum conversion or the relapse rate when given for the first 8 weeks of chemotherapy (Kenyan/Zambian/British Medical Research Council Collaborative Study 1988).

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