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Comparison of the Immunogenicity of Vaccines Prepared from Viable *Mycobacterium bovis* BCG, Heat-Killed *Mycobacterium leprae*, and a Mixture of the Two for Normal and *M. leprae*-Tolerant Mice

CHARLES C. SHEPARD,* ROSALIND M. VAN LANDINGHAM, LAURA L. WALKER, AND SHUN-ZHANG YE†

Leprosy Section, Division of Bacterial Diseases, Centers for Disease Control, Atlanta, Georgia 30333

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Intradermal vaccines consisting of viable *Mycobacterium bovis* BCG, heat-killed *Mycobacterium leprae*, or mixtures of the two were titrated in mice in doses of $10^{5.2}$, $10^{5.8}$, $10^{6.4}$, $10^{7.0}$, and $10^{7.6}$ acid-fast bacilli. The immune response was measured by sensitization (48 to 72 h foot pad enlargement on challenge with $10^{7.0}$ heat-killed *M. leprae*) and by protection against infection with a viable *M. leprae* challenge. There was increasing response with increasing dose of vaccine, and overall the responses to the three vaccines were similar. At the lowest dose, however, the combination of BCG and *M. leprae* gave superior protection. The local reaction to the vaccines in the lower dose range was less severe with the *M. leprae* vaccine. In another experiment, the three vaccines were compared in normal mice and in mice that had been rendered tolerant by intravenous injection of *M. leprae*. The tolerant mice developed no measurable sensitization on vaccination with *M. leprae*, but they developed partial but distinct sensitization on vaccination with BCG, alone or in combination with *M. leprae*. The tolerant mice developed little or no protection with any of the vaccines, however.

In studies of cultivable mycobacteria as possible vaccines against *Mycobacterium leprae*, the only culture we found that provides consistent and solid protection against *M. leprae* infection in mice is the BCG strain of *Mycobacterium bovis* (10). BCG is also unique among mycobacterial cultures in its ability to stimulate sensitization, as measured by 48- to 72-h foot pad enlargement (FPE) after challenge with *M. leprae* antigen. In the dose and route used, 10^7 acid-fast bacteria (AFB) in intradermal injections, viable BCG was approximately equivalent to heat-killed *M. leprae* in stimulating sensitization and protection against infection.

The usual dose of antileprosy vaccine for this work in mice, about 10^7 AFB, was chosen for its optimal effect. The dose in humans, however, will need to be adjusted according to the side effects, probably chiefly local induration and ulceration if the dose is too great. Consequently, we wanted to determine the effects of smaller doses of the vaccines, and the first experiment reported is a titration of heat-killed *M. leprae*, living BCG, and equal mixtures of the two to determine their efficacy in inducing sensitization (FPE) and protection against infection.

Recently a method was described for the production of immune tolerance in mice by the intravenous injection of *M. leprae* (13). After the intravenous injection of 10^7 *M. leprae*, the tolerance, as measured by reduction in FPE, ranged from 80 to 100% and lasted for at least 168 days. Because the immunological status of these mice may be the same as that of Mitsuda-negative persons in endemic areas, we compared the three vaccines in *M. leprae*-tolerant and normal mice in a second experiment. In a third experiment, we followed up on possible explanations for some of the results in the second experiment.

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MATERIALS AND METHODS

The methods have been described in detail elsewhere. In brief, 13- to 16-week-old CFW female mice were distributed five per cage, and the cages were randomized. There were 10 mice per group for measurements of FPE and 30 mice per group for measurements of protection except as noted. The intravenous

† Permanent address: Institute of Dermatology, Chinese Academy of Medical Sciences, Taizhou, Jiangsu Province, People's Republic of China.

injections were given into a tail vein in a volume of 0.2 ml, the intradermal injections (9) into the flank in a volume of 0.01 ml, and the foot pad injections in a volume of 0.03 ml. For FPE measurement of sensitization (8), the thickness of the foot was measured just before and at 24, 48, and 72 h after the foot pad injection of 10^7 heat-killed *M. leprae*. Corrected FPE signifies that the average FPE in unimmunized controls has been subtracted. The maximum corrected FPE is seen at 48 or 72 h, and the values reported are those observed at the time the maximum is reached in the positive control group. To measure protection against infection (12), 5,000 mouse-passaged *M. leprae* were inoculated into a hind foot pad. Counts of *M. leprae* in pools of four unvaccinated mice were then carried out at appropriate 28-day intervals, and after the *M. leprae* growth curve had reached about 10^6 per mouse, harvests of eight individual mice were carried out from each vaccinated group and from the two control groups. The harvests were repeated in 90 days. The figure given for protection is $\log_{10}$ of the average AFB per mouse in control groups $-$ $\log_{10}$ of the average AFB per mouse in the vaccinated group. The reaction at the site of vaccination and the size of the regional lymph nodes (9) were measured 28 days after intradermal vaccination. *M. leprae* for intravenous injection, intradermal immunization, and foot pad challenge for measurement of sensitization was purified by the Percoll method of Draper (4) from experimentally infected armadillo livers that had been gamma irradiated with 2.5 megarads. For intradermal immunization and foot pad challenge, these *M. leprae* suspensions were heated to 100°C for 30 min. The *M. leprae* for infectious challenge were freshly harvested from mice infected with a "fast" strain (7) of *M. leprae* in mouse passage.

Differences between groups were analyzed statistically by the two-sample rank test. The probability values (P) calculated were for the two-tailed test.

RESULTS

Comparative titration of the three vaccines. In the first experiment (Fig. 1 through 3), mice were vaccinated in doses of 1.56×10^5, 6.25×10^5, 2.5×10^6, 1×10^7, and 4×10^7 *M. leprae*, BCG, or a combination of the two mycobacteria (in the combination, the dose of each organism was that shown). Groups were tested 28 days later for FPE on *M. leprae* challenge. Other groups were tested for protection against infection by living challenge, also given 28 days after infection.

The FPE (Fig. 1) was significantly increased ($P < 0.002$ versus the unvaccinated controls) in all vaccinated groups except the one receiving $10^{5.2}$ *M. leprae* ($P < 0.10$); there was a general increase in FPE with increasing dose of vaccine. Overall, the FPE was similar with all three vaccines given in the same dose. However, with the lowest two doses of vaccine ($10^{5.2}$ and $10^{5.8}$), the FPE in the mice vaccinated with *M. leprae* was less than that in mice vaccinated with BCG or BCG plus *M. leprae* (*P* values ranged from $<$ 0.01 to $<$ 0.05). With the highest vaccine dose ($10^{7.6}$), the FPE in the *M. leprae*-vaccinated mice was greater than that in the BCG-vaccinated mice ($P < 0.02$) but not significantly greater than that in the mice infected with BCG plus *M. leprae*. Protection against infection followed the same trends. Protection that was very significantly increased over that of unvaccinated controls ($P < 0.002$) was seen with all groups except those receiving the lowest two doses of BCG ($P < 0.05$ and $P < 0.01$ at 7 months, and $P < 0.01$ and $P < 0.02$ at 10 months) and those receiving

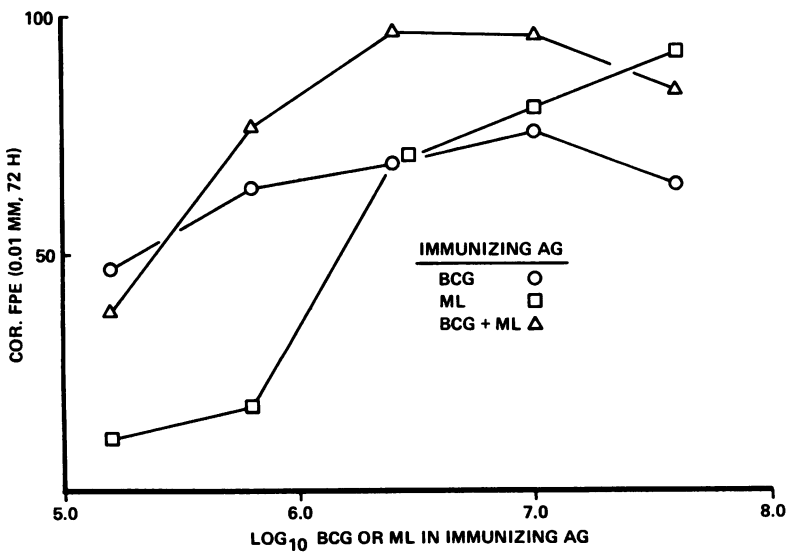

FIG. 1. Immunogenicity of various intradermal doses of viable *M. bovis* BCG, heat-killed *M. leprae* (ML), and mixtures of the two, as measured by corrected FPE (Cor. FPE) on challenge with *M. leprae* suspensions at +28 days. The uncorrected FPE in unimmunized mice was 15 U.

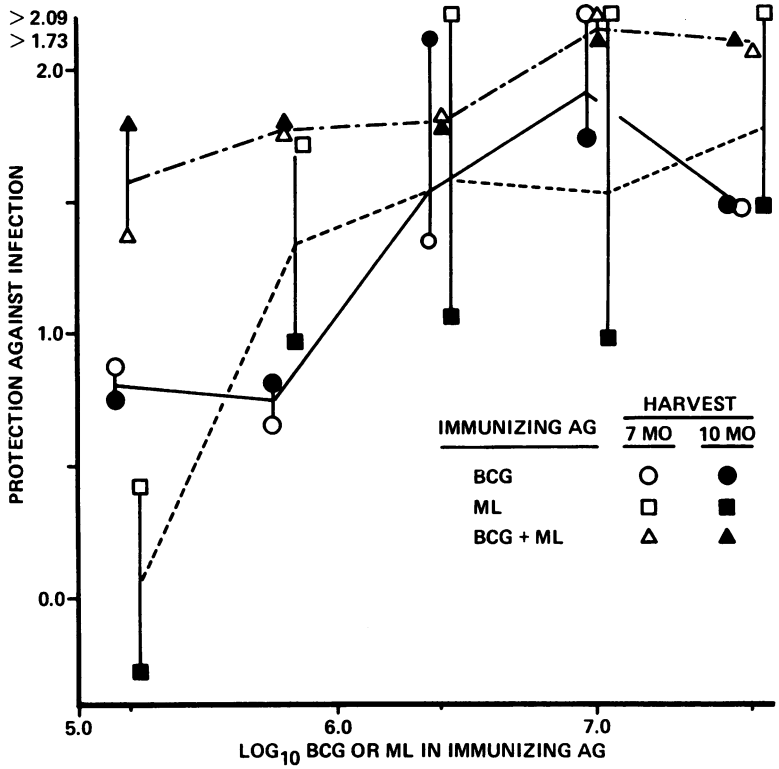

FIG. 2. Immunogenicity of various intradermal doses of viable *M. bovis* BCG, heat-killed *M. leprae* (ML), and mixtures of the two, as measured by protection against infection. After challenge at +28 days with 5,000 *M. leprae* in mouse passage, the AFB counts in unvaccinated controls were $<1.4 \times 10^4$ at 76, 3.43×10^4 at 84, 3.46×10^5 at 113, 5.83×10^5 at 141, 1.39×10^5 at 168, 7.00×10^5 at 196, and 8.40×10^5 at 224 days. Harvests were then carried out at 227 days (7 months) and 318 days (10 months) from 8 individual mice from each vaccinated group and 16 from the control groups. Protection against infection was then estimated according to the formula in the text. The average harvests in the control groups were $10^{5.99}$ and $10^{5.68}$ at 7 and 10 months, respectively.

the lowest dose of *M. leprae* ($P < 0.10$ at both intervals). There were no significant differences between the responses to the different vaccines at the same dose level, except in the case of *M. leprae* versus BCG plus *M. leprae* in doses of $10^{5.2}$ ($P < 0.01$ at 7 months, $P < 0.10$ at 10 months).

The local reaction at 28 days at the site of vaccination is presented in Fig. 3. Again there was an increase in reaction with increasing dose of vaccine. The reaction to the *M. leprae* vaccine, however, was less than that to BCG alone or BCG plus *M. leprae* in the same dosage (the *P* values for the difference ranged from < 0.01 to not significant, but the difference was consistent at all dosages except the greatest, $10^{7.6}$). The enlargement of the regional (inguinal) lymph node followed similar trends, with the lymph nodes of the *M. leprae*-vaccinated mice averaging a smaller size at each dosage level, including the top dosage, $10^{7.6}$ AFB.

Thus, the overall result of the first experiment was that the three vaccines gave similar results when given at the same dose. At the lowest dose ($10^{5.2}$ AFB), however, the combination of BCG and *M. leprae* gave superior protection. Also, in the lower dose range the *M. leprae* vaccines produced less local reactions than the vaccines containing BCG. As a result, with vaccines giving local reactions averaging 0.5 to 1.5 mm, the *M. leprae* and BCG plus *M. leprae* vaccines gave somewhat more protection than the BCG-only vaccine.

In earlier studies (8), a dissociation was observed between sensitization and protection against infection in subcutaneously vaccinated mice, and it was pointed out that the dissociation might be explained on a quantitative basis, with protection being demonstrable at a level of sensitization that was not elicitable with the FPE conditions used. In the present experiment, a similar result was obtained with intradermal vaccination with $10^{5.8}$ *M. leprae*; FPE was at a very low level, but protection was distinct.

Comparison between *M. leprae*-tolerant and normal mice. In the second experiment, we

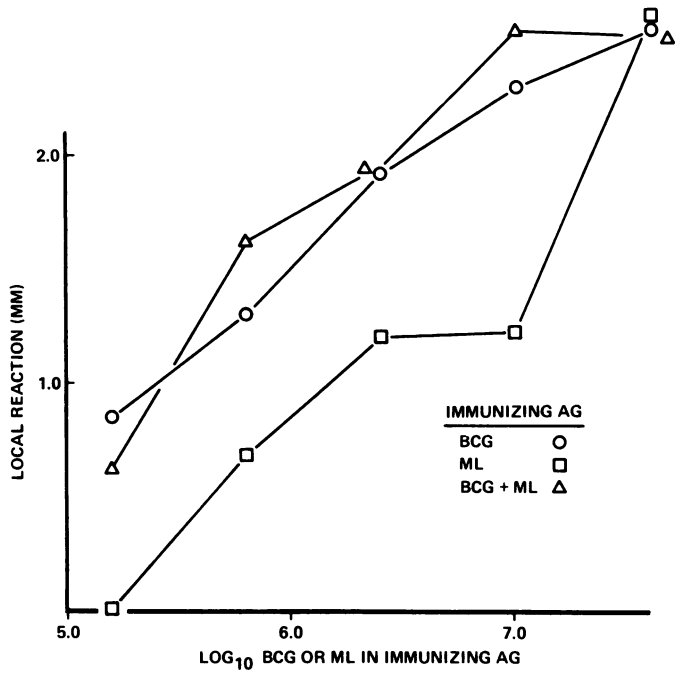

FIG. 3. Local reaction to various intradermal doses of viable *M. bovis* BCG, heat-killed *M. leprae* (ML), and mixtures of the two.

compared the effects of the three vaccines in *M. leprae*-tolerant mice (13) versus normal mice (Fig. 4 through 6). Thus, at 0 days certain groups were injected intravenously with 10^7 *M. leprae*. At +14 days certain groups were injected intradermally in the right flank with 10^7 BCG, *M. leprae*, or a mixture containing 10^7 each of BCG and *M. leprae*. Groups D and I, which received BCG in the right flank, also received *M. leprae* in the left flank; this was done to look for an adjuvant effect of one antigen on the other in the mixture (groups C and H). At +42 days some of the groups received 10^7 heat-killed *M. leprae* in the right hind foot pad for measurements of FPE, and other groups received living *M. leprae* challenge for measurements of protection against infection. Since protection against infection can be effected late, even after challenge (11), at a time when sensitization as measured by FPE might have changed from that observed at +42 days, the sensitivity measurements were repeated at +126 days.

FPE measurements (Fig. 4) showed that the intravenous injections of *M. leprae* produced good tolerance (>100%) to intradermal challenge with *M. leprae* (group B) and did not sensitize (group E). The normal mice (groups F through I) were well sensitized by the intradermal injections. The tolerant mice that received BCG intradermally were sensitized. (The *P* values for the differences of groups A, C, and D from the negative control group J were < 0.002 to < 0.02. The *P* values for the differences of A, C, and D from their respective positive control groups F, H, and I were in the range < 0.001 to < 0.05.) The addition of *M. leprae* to the BCG had little effect. The results at +112 days were very similar to those at +42 days, thus indicating considerable stability of the immunological status in this interval. (The FPE results at +42 days have been presented previously [13]).

Protection against infection (Fig. 5) gave somewhat different results, however. Although the normal mice were well protected by the vaccines (groups F through I), the tolerant mice were not. There were some protection in groups A, C, and D in the 6-month harvest, but this had largely disappeared in the 9-month harvests. The protection in group E (no intradermal vaccine) at 6 months had also disappeared at 9 months.

The local reactions at 28 days after vaccination are given in Fig. 6 because of their analogy to the Mitsuda reaction (13). Tolerant mice receiving intradermal *M. leprae* (groups B and D) showed little local reaction compared with their normal controls (groups G and I). Thus, these tolerant mice gave negative Mitsuda reactions, whereas normal mice (and intradermally immunized mice [13]) give positive Mitsuda reactions. Mitsuda reactivity receives considerable weight in the development of antileprosy vaccine strategies because it is widely accepted

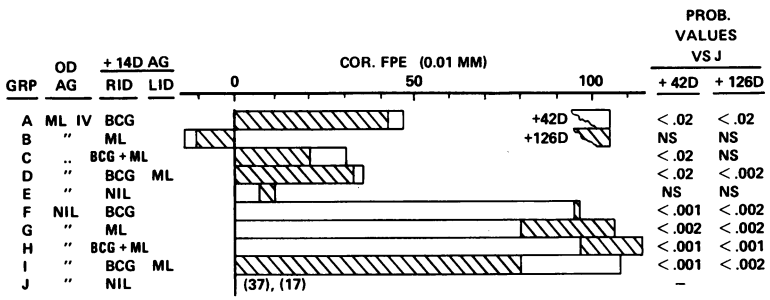

FIG. 4. Immunogenicity intradermal of 10^7 viable *M. bovis* BCG, heat-killed *M. leprae* (ML), and a mixture of the two in *M. leprae*-tolerant and normal mice, as measured by corrected FPE (Cor. FPE) 48 h after challenge with *M. leprae* suspensions 28 and 112 days after intradermal vaccination. The challenge antigen was *M. leprae* given at +42 or +126 days. The uncorrected FPE in the unimmunized control group (J) is shown in parentheses for the two respective intervals. Abbreviations: RID, right intradermal; LID, left intradermal; NS, not significant.

in the leprosy literature as an index of immunity to infection by *M. leprae*.

Thus, in experiment 2, mice were rendered tolerant by intravenous injection of *M. leprae*, and such mice were no longer capable of responding to intradermal vaccination with *M. leprae* by the development of sensitization, as demonstrated by FPE on foot pad challenge with *M. leprae* antigen. They were capable of responding to intradermal vaccination with BCG by the development of partial sensitization to *M. leprae*, however. On the other hand, the tolerant mice developed little or no protection against infection with *M. leprae* when vaccinated intradermally with *M. leprae*, BCG, or a mixture of the two.

The measurement of sensitization in mice (foot pad injections) differs from that in humans (intradermal injections) in that the foot pad route is not immunogenic with the *M. leprae* dose used (13), whereas the intradermal route in humans very probably is. Thus, the conversion to Mitsuda positivity of Mitsuda-negative patients and family contacts after vaccination with a combination of BCG and *M. leprae* (but not by vaccination with either component alone) (3) might conceivably be a consequence of sensitization by the Mitsuda testing itself. Consequently, we explored the possibility of such an explanation for the difference between the results in mice and those reported in humans by the third experiment. This time, intradermal injections were repeated at +28 days, and the final foot pad testing took place at +56 days (Fig. 7).

Another *M. leprae* antigen was used. The amount of tolerance induced was not as great

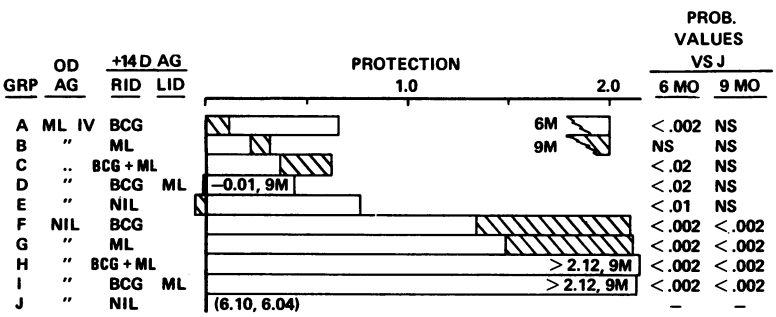

FIG. 5. Immunogenicity of 10^7 viable *M. bovis* BCG, heat-killed *M. leprae* (ML), and a mixture of the two in *M. leprae*-tolerant and normal mice, as measured by protection against infection. After challenge, 28 days after intradermal vaccination with 5,000 *M. leprae* in mouse passage, the AFB counts in unvaccinated controls were $<2 \times 10^4$ at 70, 8.1×10^4 at 98, 6.35×10^5 at 133, and 9.67×10^5 at 154 days. Harvests were then carried out on individual mice from all vaccinated and control groups at 185 days (6 months) and 279 days (9 months) to measure the protection against infection. Protection was estimated according to the formula given in the text. The average harvest in the unvaccinated groups (J) is shown in parentheses in $\log_{10}$. Abbreviations: 0D and +14D, 0 and +14 days, respectively; AG, antigen; IV, intravenous; RID, right intradermal; LID, left intradermal; M, months; NS, not significant.

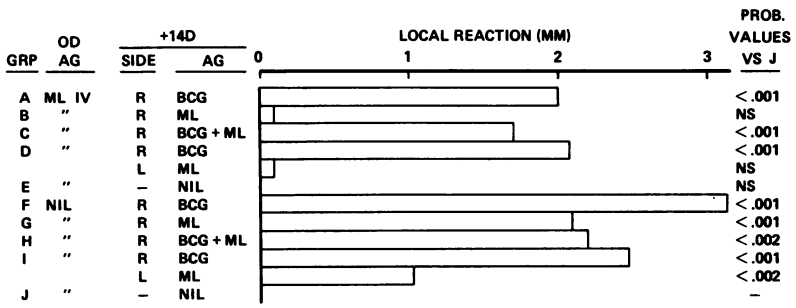

FIG. 6. Local reaction of *M. leprae*-tolerant and normal mice to intradermal vaccinations with viable *M. bovis* BCG, heat-killed *M. leprae* (ML), and a mixture of the two. Abbreviations: 0D and +14D, 0 and +14 days, respectively; AG, antigen; IV, intravenous; R, right; L, left; NS, not significant.

(63% in group A), although the amount of sensitization was satisfactory. With only one injection, the combination of BCG and *M. leprae* was marginally more immunogenic than either component alone at +28 days (group A versus C, not significant; group B versus C, not significant) and at +56 days (group D versus F, $P < 0.01$; group E versus F, not significant) in the tolerant mice but not in the normal mice. In the tolerant mice receiving an intradermal injection at both +28 and +56 days, the *M. leprae* sensitization was better when the two antigens were different than when they were the same (group G versus H, $P < 0.01$; group G versus I, not significant; group J versus H, $P < 0.01$; group J versus I, not significant). In the normal mice, all the injections were strongly immunogenic (K through T).

There was some suggestion that the tolerant mice could be better sensitized to *M. leprae* by intradermal immunization with both BCG and *M. leprae* rather than with either antigen alone, but the responses when the two antigens were separated temporally were at least as good as when they were administered simultaneously. This result would tend to speak against the explanation that has been advanced for the

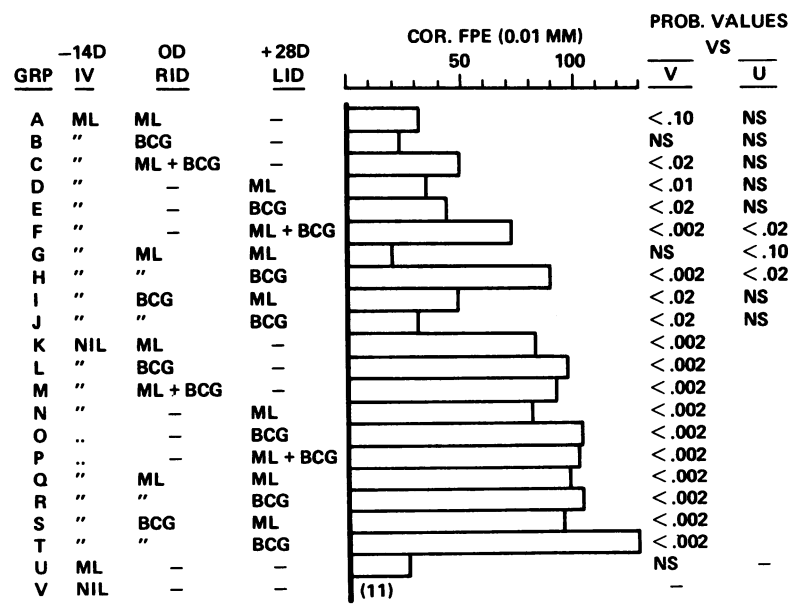

FIG 7. Sensitization of *M. leprae*-tolerant and normal mice to repeat intradermal vaccination with viable *M. bovis* BCG and heat-killed *M. leprae* (ML). The uncorrected FPE in the unvaccinated control group (V) is given in parentheses. Groups K, L, N, O, U, and V contained 10 mice per group, and all others contained 5 mice per group. The challenge antigen was *M. leprae* given at +56 days. Abbreviations: −14D, 0D, and +28D, −14, 0, and +28 days, respectively; IV, intravenous; RID, right intradermal; LID, left intradermal; NS, not significant.

greater efficacy of the combination vaccine (3), namely that the macrophage activation in the site of BCG vaccination in a Mitsuda-negative individual results in more efficient presentation of admixed *M. leprae* antigen.

DISCUSSION

Three vaccines were compared. (i) The *M. leprae* vaccine is a new product that has not yet been tried in humans. It is made possible now by the greater amounts of *M. leprae* that can be grown in experimentally infected armadillos (11) and by the heat stability of *M. leprae* immunogenicity (6). Its first testing as an immunogen in humans is expected soon. (ii) BCG has been tested against leprosy in four large vaccine trials. The first three trials were initiated some years ago, and their status was recently reviewed. (Sixth IMMLEP Scientific Working Group meeting, UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases, Geneva, Switzerland, 7–9 June 1982.) The trial in Uganda (family contacts) (2) has continued to show about 80% protection (I. Sutherland, personal communication) (14). The trial in New Guinea (entire community) (5) has continued to show about 50% protection (G. Scott, personal communication). The trial in Burma (children) (1) has shown about 30% protection for one lot of BCG. (T. Sundaresan, personal communication). A more recent trial in India (entire community) has shown about 35% protection in the first assessment (S. P. Tripathy, personal communication). In these leprosy trials, examination for leprosy has been relatively frequent and the disease seen in both vaccine and control groups has been mild. Thus, it was not clear whether BCG vaccination prevented multibacillary leprosy, which is responsible for transmission of the disease. (iii) The combination of viable BCG and heat-killed *M. leprae* has been suggested by Convit et al. (3) on the basis of the observation that Mitsuda-negative patients and family contacts could be converted to Mitsuda positivity by vaccinations with mixtures of the two materials but not by either one alone.

The theoretical basis for choosing among these three products for field trial is somewhat incomplete. Probably the best that can be done in humans at present, short of the field trial itself, is first a determination of the acceptable dosage of the vaccine based on side effects, and second a determination of the conversion rate with a delayed-type skin test carried out with soluble (nonimmunogenic) *M. leprae* antigen after vaccination with each product at its acceptable dosage level. The precise relationship between such delayed-type hypersensitivity and resistance to infection is probably not known for leprosy any better than it is for tuberculosis, where the polemic is about as old as the experimental field itself. The results we report here in the second experiment illustrate the possible separation between sensitization and protection. Mice that had been injected intravenously with *M. leprae* were rendered tolerant, that is, they developed little or no sensitization to *M. leprae* antigens (measured by FPE), when they were vaccinated intradermally with *M. leprae* suspensions. However, such mice could be partially but distinctly sensitized to *M. leprae* antigens by intradermal vaccinations with BCG. Nevertheless, the tolerant mice, when vaccinated intradermally with BCG, *M. leprae*, or the combination, developed little or no protection against infection. The discrepancy between the two results could not be explained on the basis of relative sensitivity of the two tests, since the results of the first experiment indicate that protection against infection is inducible with smaller doses of vaccine than FPE.

In the consideration of vaccination in leprosy endemic regions, one of the unknown factors is the *M. leprae* immune status of the various members of the population. Some individuals, especially those of young ages, will not have responded immunologically to exposure to *M. leprae* antigens. The rest will have, and they will consist of several categories: (i) those who have already been fully immunized to *M. leprae* antigens, (ii) those who may benefit from vaccination because their exposure has not resulted in maximum immunity or because they have lost immunity after their exposure has ceased, and (iii) those whose exposure to *M. leprae* has resulted in the development of specific immunological tolerance to *M. leprae*. It is the last category whose numbers and response to vaccination is least known. Mitsuda negativity may well be an indicator of *M. leprae* tolerance (13), but the prevalence of negativity to properly injected, fully potent integral lepromin is disputed. Such a group might include those incubating multibacillary leprosy and those whose future exposure to *M. leprae* may result in the development of multibacillary disease, if genetic or acquired deficiency in the immune response to *M. leprae* plays a role. Thus, this group, though least defined, may be the most important to immunize if the future transmission of leprosy in the community is to be prevented. Unfortunately, the present results do not indicate a method of immunizing such a group.

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