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Lack of a Sustained Protection Against Murine Leprosy in C3H Mice Vaccinated with Extracts of *Mycobacterium lepraemurium* in Admixture with *Mycobacterium bovis* (BCG)[1]

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Mycobacterium leprae, isolated and purified from the tissues of experimentally infected armadillos, can theoretically be obtained in sufficient quantities for the preparation of a vaccine against human leprosy ([17]). For obvious reasons, live nonattenuated armadillo-isolated *M. leprae* cannot be utilized as such. It has been suggested ([3]) that killed *M. leprae* in association with an appropriate adjuvant (e.g., Bacillus Calmette-Guérin) might constitute an efficient vaccine for human use. The efficacy of such a mixture can be tested in the animal model of murine leprosy. While immunologically intact mice exhibit only a restricted infection with *M. leprae* ([13]), many inbred strains of mice are highly susceptible to *M. lepraemurium* (MLM) infection which produces a fatal disease ([2,6,10]).

During the last few years, several groups of investigators ([7,8,11]) have found that preimmunization of mice with live BCG alone inhibited the proliferation of MLM at the inoculation site and also inhibited the dissemination of the bacilli to lymphoid organs. Very little is known about the effects of BCG in admixture with the antigens of MLM on the evolution of murine leprosy. Hanks and Fernandez ([5]), however, have reported that such a preparation significantly reduced the incidence of MLM-induced lepromas in the skin of rats as compared to those in animals immunized with BCG or MLM alone.

In this paper, we compare the ability of a mixture of live BCG and extracts of MLM with that of either BCG or MLM alone to inhibit the multiplication of MLM in the foot pad and their dissemination to the draining lymph node and spleen during the evolution of murine leprosy. The C3H mouse, a strain highly susceptible to murine leprosy when infected subcutaneously in the foot pad ([1,16]), was used. A single vaccinating dose was administered intradermally one month prior to the experimental infection. The results indicate that all the vaccinating preparations have beneficial effects either at the foot pad level alone or, in addition, at the lymph node and spleen levels. MLM extracts in admixture with BCG did not induce a letter protection than BCG alone. On the other hand, no prolongation of the survival time was observed following vaccination with any preparation.

MATERIALS AND METHODS

Mice. Inbred female C3H/HeNCr1 mice (hereafter referred to as C3H) were obtained from Canadian Breeding Farm and Laboratories, Ltd, Laprairie, Quebec, Canada, and were used at six to eight weeks of age. They were maintained under standard laboratory conditions and fed Purina Chow and water *ad libitum*.

BCG. A lyophilized preparation of the Montreal substrain of BCG (Institut Armand-Frappier, Laval-des-Rapides, Quebec, Canada) was used. The content of each vial was reconstituted with sterile saline immediately before vaccination of mice. One mg of the reconstituted vaccine contained about 1×10^7 viable units when plated on Dubos solid medium.

M. lepraemurium. The Hawaii strain of MLM was propagated in C3H mice as previously described ([16]). When needed, the bacilli were isolated (see below) either from fresh spleen or liver of heavily infected mice or from these organs kept at −70°C.

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Disruption of *M. lepraemurium*. A suspension of MLM containing 5×10^8 bacilli per ml of phosphate buffered saline was submitted four times to a French pressure cell operating at 4°C and at 1200 pounds per square inch. Under similar conditions, the growth of BCG on specific mycobacterial culture media was completely abolished. In some experiments, disrupted MLM were separated by means of ultracentrifugation at $40,000 \times g$ for 3 hr into soluble (cytoplasmic) and insoluble (particulate) fractions.

Vaccination of mice. Four groups of 25 C3H mice were injected into the right flank with either a) a single dose of 1×10^6 viable units of BCG, b) disrupted MLM derived from 1×10^6 bacilli, c) a mixture of these two preparations (2×10^6 total bacilli) in equal proportion, or d) with sterile saline as control. Twenty-four hr before vaccination, a hair remover cream was applied to an area on the right flank. The vaccinating dose in a volume of 20 μl was administered intradermally in this area through a 30-gauge needle taking care to produce a small but distinct bleb in the skin. BCG and MLM preparations were mixed immediately prior to inoculation.

Challenge with *M. lepraemurium*. Twenty-eight days after vaccination, the vaccinated and control mice were challenged in the plantar surface of the right hind foot with 1×10^5 freshly harvested MLM suspended in 20 μl of sterile phosphate buffer.

At two different times (15 and 30 weeks) after challenge, eight randomly chosen mice in each group were sacrificed and the acid-fast bacilli (AFB) were counted in the right hind foot pad, in the right popliteal lymph node, and in the spleen. The mice remaining in each group were kept to evaluate survival times.

Counting of the bacilli. The right hind foot (amputated at the ankle and freed of bone), the draining popliteal lymph node, and the spleen were suspended, respectively, in 15, 15, and 25 ml of phosphate buffer (0.15 M, pH 7.4) containing 0.1% w/v bovine serum albumin (BSA), and homogenized separately for 10 sec in a Lourdes disintegrator (Model MM1D) running at maximum speed. Homogenates were first centrifuged at $500 \times g$ for 5 min to eliminate tissue debris. Supernatants were further centrifuged at $10,000 \times g$ for 10 min and the sediments were suspended in 1 ml of phosphate buffer-BSA. The number of AFB was counted using the spot-slide method of Shepard and McRae ([14]). The lower limit of sensitivity (when one AFB was seen in 50 fields examined at magnification $\times 1000$) was about 2×10^4 AFB per organ. When counts were made early after the challenge, a pool of bacillary sediments obtained from three to four mice was suspended in 1 ml of phosphate buffer prior to enumeration in order to increase the sensitivity of the method.

Statistical analysis. Results of bacillary counts and the survival times of each group of mice were expressed as the mean $\pm$ standard error of the mean (SEM). The data being normally distributed, the Student's t test was used to determine the significance of differences between groups. A probability value (p) of 0.05 was considered as the limit of statistical significance.

RESULTS

Multiplication of *M. lepraemurium* in non-vaccinated mice

To determine the most appropriate infecting dose to be used, non-vaccinated C3H mice were infected subcutaneously into the right hind foot pad with three (10^3, 10^5, and 10^7) doses of freshly harvested MLM and AFB were counted at the infection site, in the draining popliteal lymph node and in the spleen over a 36-week period after infection. As seen in The Figure, the time at which AFB became first detectable depended on the anatomical site and on the size of the inoculum. For instance, with 10^3 bacilli, AFB were detectable at 12, 16, and 24 weeks after infection, respectively, in the foot pad, popliteal lymph node, and the spleen. This variation was partly due to the fact that 24 hours after infection 9.5% $\pm$ 2.3 of the inoculated bacilli remain in the foot pad, and less than 1% had disseminated into the popliteal lymph node and into the spleen (R. Turcotte, unpublished observations); and also to the fact that more than 2×10^4 AFB per organ must be present in order to be detectable by the present method of enumeration.

In the foot pads of mice inoculated with 10^3 and 10^5 bacilli, the rates of multiplication were high for up to 16 weeks after in-

TABLE 1. *Counts of AFB in the foot pads and popliteal lymph nodes of vaccinated C3H mice 15 weeks after challenge with MLM.*

Mice vaccinated with	Mean no. of AFB ($\times 10^5$) ± S.E.M. (N = 8)	
	Foot pad	Lymph node
BCG plus disrupted MLM	1.8 ± 0.5[a]	0.9 ± 0.1[b]
BCG	2.5 ± 0.4[a]	1.2 ± 0.3[c]
Disrupted MLM	2.9 ± 0.8[a]	1.8 ± 0.6[d]
Saline	7.6 ± 0.7	3.2 ± 0.6

[a] $p < 0.001$, Student's *t* test, compared to saline controls.
[b] $p < 0.01$, Student's *t* test, compared to saline controls.
[c] $p < 0.02$, Student's *t* test, compared to saline controls.
[d] Not statistically significant.

fection. The amount of bacilli remained more or less stable for the following 8 to 12 weeks and increased again but at a lower rate by the end of the observation period. In the foot pads of mice infected with 10^7 bacilli, the rate of growth was very low throughout the whole observation period since, as mentioned above, about 10% of the infecting dose (i.e., 10^6 bacilli) was already present in the foot pad 24 hours after infection. In contrast, in the lymph node and the spleen, the rate of multiplication was constant during the whole observation period and was not influenced by the size of the inoculum. According to the slope of the growth curves, multiplication of MLM occurred at a faster rate in the spleen than in the foot pad (except early after infection), while the rate of multiplication in the lymph node was in between. For instance, during the 24- through 36-week period, the doubling time was found to be 11.4 ± 1.1, 19.5 ± 1.8 and 65.6 ± 7.8 days (mean values obtained with the three infecting doses), respectively, in the spleen, lymph node and the foot pad. In mice infected in the foot pad with 10^3 and 10^5 MLM, the doubling time for the first 16-week period after the challenge was 16.8 and 17.6 days, respectively. Because of the relatively early detection and the rapid growth of bacilli in the foot pad over 16 weeks, 10^5 MLM was the challenge dose retained for the following experiments and bacillary enumeration was performed only after the 15th week.

Vaccination of mice with disrupted *M. lepraemurium* alone and in admixture with BCG

Table 1 shows the number of AFB detected in the foot pads and the popliteal lymph nodes of mice vaccinated with live BCG, mechanically disrupted MLM, or with a mixture representing the sum of these two preparations. At 15 weeks after challenge, significantly fewer bacilli were detected in the foot pads of all three vaccinated groups and in the draining lymph nodes of those vaccinated with the BCG-containing preparations as compared to the controls. No significant differences in the numbers of bacilli at these two anatomical sites were observed between the three vaccinated groups. At 30 weeks after the challenge (Table 2), although fewer AFB were detected in the foot pads of mice vaccinated with the three preparations and in the spleens of those vaccinated with BCG alone, these differences were not statistically significant. Moreover, the data on the mean survival times which did not differ among all groups

TABLE 2. *Counts of AFB in the foot pads, popliteal lymph nodes, and spleens of vaccinated C3H mice 30 weeks after challenge with MLM.*[a]

Mice vaccinated with	Mean no. of AFB ($\times 10^5$) ± S.E.M. (N = 8)			Mean survival time in days ± SEM
	Foot pad	Lymph node	Spleen	
BCG plus disrupted MLM	280.7 ± 108	31.3 ± 10.2	1242 ± 467	327 ± 6.9
BCG	284.2 ± 95.7	20.3 ± 6.5	486 ± 135	329 ± 6.7
Disrupted MLM	141.5 ± 73.3	33.7 ± 11.8	1406 ± 227	322 ± 7.3
Saline	447.1 ± 208	28.5 ± 9.2	1152 ± 375	326 ± 6.5

[a] None of the differences between the experimental and control groups were statistically significant.

TABLE 3. *Counts of AFB in the spleens of vaccinated C3H mice 22 weeks after challenge with MLM.*

Mice vaccinated with	Mean no. of AFB ($\times 10^5$) ± S.E.M. (N = 8)	Mean survival time in days ± SEM
BCG plus disrupted MLM	1.72 ± 0.3[a]	311.8 ± 6.9
BCG	1.67 ± 0.4[a]	321.4 ± 7.4
Disrupted MLM	3.04 ± 0.8[b]	311.2 ± 6.7
Saline	4.27 ± 0.7	308.5 ± 7.5

[a] $p < 0.01$, Student's *t* test, compared to saline controls.
[b] Not statistically significant.

TABLE 4. *Counts of AFB in the foot pads of vaccinated C3H mice 15 weeks after challenge with MLM.*

Mice vaccinated with	Mean no. of AFB ($\times 10^5$) ± S.E.M. (N = 8)	Mean survival time in days ± S.E.M.
Whole disrupted *M. lepraemurium*	0.79 ± 0.15[a]	353.4 ± 5.5
Cytoplasmic fraction	1.12 ± 0.13[b]	358.5 ± 8.6
Particulate fraction	0.65 ± 0.12[a]	361.9 ± 10.8
BCG alone	0.81 ± 0.23[a]	384.2 ± 11.2
BCG plus cytoplasmic fraction	0.75 ± 0.22[a]	388.0 ± 11.6
BCG plus particulate fraction	0.64 ± 0.10[a]	387.4 ± 10.0
Saline	2.86 ± 0.20	376.5 ± 12.3

[a] $p < 0.01$, Student's *t* test, compared to saline controls.
[b] $p < 0.02$, Student's *t* test, compared to saline controls.

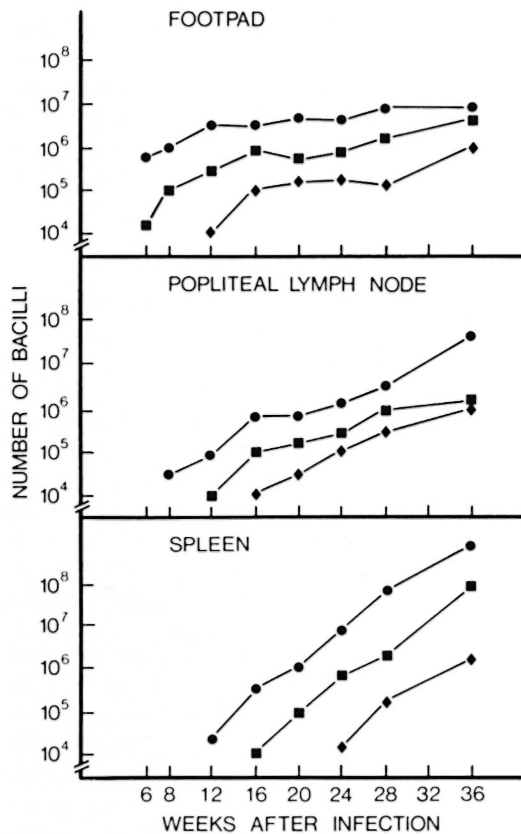
of mice (see right hand part of Table 2) agreed well with the lack of significant differences in bacterial counts at 30 weeks.

The counts of AFB in the spleen when determined at 15 weeks after challenge were too low to see if vaccination prevented the dissemination of MLM into this lymphoid organ. Therefore, an additional experiment was performed in which splenic counts were determined at 22 weeks (Table 3). As already observed at 15 weeks for the draining lymph node, BCG, alone and in admixture with MLM, protected mice against bacillary dissemination to the spleen; whereas MLM alone lead to a low but nonsignificant protection. Here again, the survival times of vaccinated mice were not significantly improved over those of control mice.

Some of the bacillary sediments obtained from the foot pad, popliteal lymph node, and the spleen of BCG-vaccinated mice were cultured on Dubos solid medium at 15 weeks after infection (19 weeks after vaccination) to evaluate the contribution of BCG bacilli to the number of detectable AFB. No viable BCG were found in the foot pad. Viable AFB were found occasionally in the popliteal node and regularly in the spleen. However, the amount never exceeded 8×10^3 bacilli per organ. Therefore, the contribution of viable BCG bacilli to the total number of AFB was negligible.

Vaccination of mice with fractions of disrupted MLM

The cytoplasmic and particulate fractions were used alone and in association with BCG to vaccinate mice in order to compare their protective activity. Since, as shown above, the resistance induced by the whole disrupted MLM preparation was mainly localized at the challenge site and occurred relatively early after the infection, bacillary counts were determined only in the foot pad and at the 15th week after challenge. As seen in Table 4, significantly fewer AFB were detected in the foot pads of all vaccinated groups as compared to the control group. Although not statistically significant, the particulate fraction of MLM appeared more active in limiting the rate of multiplication than the corresponding cytoplasmic fraction. The addition of BCG to the cytoplasmic fraction seemed to improve its activity while it did not affect that of the particulate fraction. When considering the mean survival times, it is noted that all the BCG-containing preparations slightly prolonged the lives of mice, while the MLM preparations slightly reduced them in comparison with the control group. However, these differences were not statistically significant. The amount of AFB detected in the foot pads of control mice was lower and their survival times were longer in these experiments when compared to the results



THE FIGURE. Counts of AFB in the foot pad, draining popliteal lymph node, and spleen of C3H mice infected with different doses of MLM. Mice were infected in the right hind foot pad with 10^3 (◆), 10^5 (■), and 10^7 (●) freshly harvested bacilli. Each point represents the mean value obtained from four mice. (The standard errors of the mean were omitted to retain clarity.)

presented in the previous tables, but the reductions in bacillary counts in mice vaccinated with BCG or whole disrupted MLM were proportionally the same.

DISCUSSION

Intradermal vaccination of C3H mice with either live BCG, MLM extracts, or a mixture of both, significantly reduced the number of AFB detected at the infection site, that is, in the hind foot pad, at the beginning of the disease. A decrease in the rate of multiplication of MLM or a lower bacillary retention at the inoculation site could explain this local bacillary reduction. In addition, vaccinations with BCG alone and with the mixture of BCG and MLM extracts caused a delay in the dissemination of infectious MLM to the draining popliteal lymph node and the spleen. These findings agree well with the recent results of Lefford, *et al.* ([8]) and Lagrange and Hurtrel ([7]) who evaluated the influence of BCG vaccination against murine leprosy, even though the experimental conditions differed markedly from one laboratory to another. Surprisingly, no significant delay in bacillary dissemination was observed in mice vaccinated with MLM antigens alone. This difference could be due to the fact that live BCG continued to divide to some extent in the mouse, and thus lead to a more adequate immunizing dose. Vaccination of mice with mechanically disrupted BCG instead of live BCG should be of aid in answering this point. Another explanation could be that the disintegration process, as used in this study, partially inactivated the antigens responsible for protection against murine leprosy. In a recent preliminary experiment from our laboratory, no reduction of MLM multiplication was observed at the foot pad level in C3H mice vaccinated with MLM killed by 2.5 Mrads of gamma irradiation.

The addition of BCG to MLM antigens seemed to increase their activity since, by comparison to MLM alone, such preparations were able to limit the dissemination of MLM to lymphoid organs. However, it must be emphasized that the augmented activity never exceeded that of BCG alone, thus suggesting that this additional effect was due solely to the BCG present in the mixed preparation. Lagrange and Hurtrel ([7]) have reported that the inoculation of one or two doses of heat-killed MLM to BCG-presensitized C3H mice prior to the challenge with live MLM did not significantly reduce bacillary dissemination to the draining lymph nodes. Therefore, it would appear that under our conditions, the addition of antigens of MLM to the BCG preparation did not improve immunization.

Although vaccination seemed to reduce the local proliferation of MLM and markedly reduced their dissemination to lymphoid organs at the beginning (15 weeks) of the infectious process, no significant differences in the bacillary counts and in the mean survival times were observed between the vaccinated and non-vaccinated mice at 30 weeks after infection. It is still unknown

whether our vaccination conditions have induced and maintained an optimal level of protection or not. On the other hand, the degree of induced protection could be adequate initially, but it might be neutralized during the evolution of the disease. Experiments are in progress to study the influence of suppressor cells, as detected recently in the spleen of leprous C3H mice ([15, 16]), upon the early protective effects of BCG as shown in this study. The lack of a sustained protection in vaccinated mice could also be explained by the possibility that vaccination reduced the multiplication of MLM only in a limited number of organs and not in all the anatomical sites that MLM can invade. Furthermore, the fact that no significant differences in bacillary counts were observed at 30 weeks after infection between vaccinated and non-vaccinated mice suggests that, after a temporary reduction of MLM multiplication, the bacilli could grow at a faster rate.

It is important to note that, according to the parameters used by various investigators who have evaluated the effects of BCG vaccination in murine leprosy, opposite conclusions have been reached. For instance, when the mean survival times of animals or the histological structures of leprous lesions were considered, BCG was found to have no beneficial effect ([4, 9]). On the other hand, it exerted some protective activity when other criteria such as the local proliferation of MLM, its dissemination to lymphoid organs, the incidence of skin lepromata, etc., were evaluated at an early stage of the infection ([5, 7, 8, 11]). This raises the question of which are the more appropriate parameters for evaluating protection in murine leprosy. Obviously in any vaccination experiment prolongation of the survival time must not be neglected.

Differences in susceptibility to murine leprosy seem to depend more upon the route of infection and the size of the inoculum than upon a particular strain of inbred mice ([12, 16]). The absence of a sustained protection as shown in this study could be due to the fact that a mouse strain was used which is highly susceptible to murine leprosy when infected in the foot pad. This experimental model was chosen because in the human situation, the individuals who need an adequate protection are those who are susceptible to develop the lepromatous form of leprosy. On the other hand, it is unlikely that very different results would have been obtained with another strain, e.g., the C57BL/6 mouse, since this strain is even more susceptible than the C3H when infected intravenously.

SUMMARY

Inbred C3H mice were vaccinated intradermally with a single dose of live BCG, whole extracts of mechanically disrupted *Mycobacterium lepraemurium* (MLM), or a mixture of both these agents. Four weeks later, they were infected in one hind foot pad with freshly harvested MLM. Vaccination with BCG-containing preparations significantly reduced the multiplication of MLM in the infected foot pad and the bacillary dissemination to the draining popliteal lymph node and the spleen, while vaccination with MLM extracts solely limited the growth of MLM in the foot pad. MLM antigens in admixture with BCG did not offer a better protection than BCG alone. The protective effect was observed near the 15th week after the infection. At 30 weeks post-infection, no significant difference in bacillary counts was noted between the vaccinated and unvaccinated mice. In addition, the mean survival time of vaccinated mice did not significantly differ from that of control mice. Thus, in the C3H mouse, vaccination was able to limit temporarily the growth and dissemination of MLM, but these effects were unable to stop the fatal progression of murine leprosy.

RESUMEN

Se vacunaron ratones C3H, intradérmicamente, con una sóla dosis de BCG viable, con extractos de *Mycobacterium lepraemurium* (MLM) obtenidos por rompimiento mecánico, o con una mezcla de ambos materiales. Cuatro semanas después se infectaron con MLM recién cosechado en un cojinete plantar trasero. La vacunación con las preparaciones que contenían BCG redujo considerablemente la multiplicación del MLM en el cojinete plantar y la diseminación bacilar a los gánglios linfáticos popliteos regionales y al bazo, mientras que la vacunación con los extractos de MLM solamente limitó el crecimiento del MLM en el cojinete plantar. La mezcla del extracto de MLM con BCG, no confirió mejor protección que el BCG sólo. El efecto protector se observó hacia la semana 15 después de la infección. Treinta semanas después de la infección, no se observaron diferencias significativas en las cuen-

tas de bacilos entre los grupos vacunado y no vacunado. Además, el tiempo de sobrevida media de los ratones vacunados no difirió significativamente del de los controles. La vacunación de los ratones C3H fue capaz de limitar temporalmente el crecimiento y la diseminación del MLM pero no logró detener el progreso fatal de la enfermedad.

RÉSUMÉ

On a vacciné par voie intradermique des souris endogames C3H avec l'une des trois préparations suivantes: une dose unique de BCG vivant, des extraits entiers de *M. lepraemurium* (MLM) détruit mécaniquement, ou un mélange de ces deux préparations. Quatre semaines plus tard, ces souris ont été infectées dans le coussinet plantaire de l'une des pattes arrières, avec du MLM fraîchement recueilli. La vaccination avec des préparations contenant du BCG ont réduit de manière significative la multiplication de MLM dans le coussinet plantaire infecté, et ont diminué la dissémination bacillaire dans les ganglions lymphatiques poplités drainant la région, de même que dans la rate. Par contre, la vaccination avec des extraits ne contenant que MLM n'a réussi qu'à circonscrire la croissance dans le coussinet plantaire des bacilles inoculés. Les antigènes de MLM, ajoutés au BCG, ne confèrent pas une meilleure protection que le BCG seul. L'effet protecteur a été observé aux environs de la quinzième semaine après l'infection. Trente semaines après l'infection, aucune différence significative n'a été observée dans les énumérations bacillaires, chez les souris vaccinées et chez les souris non vaccinées. De plus, le temps moyen de survie des souris vaccinées n'était pas significativement différent de celui des souris témoins. Dès lors, on peut conclure que chez la souris C3H, la vaccination permet de limiter temporairement la croissance et la dissémination de MLM, mais est incapable d'arrêter la progression inéluctable de la lèpre murine.

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