INTERNATIONAL JOURNAL OF LEPROSY

Volume 55, Number 2
Printed in the U.S.A.

Immunization of Mice with *Mycobacterium leprae/Mycobacterium bovis* BCG Admixtures: Modulation of the Acquired Response to BCG[1]

Ian M. Orme[2]

There is now good evidence to suggest that vaccination of individuals with a mixture of killed *Mycobacterium leprae* plus living BCG can result in an upgrading of the immune status of both lepromatous and borderline leprosy patients, measured primarily as an improved capacity to express cutaneous sensitivity to an injection of lepromin ([1, 7]). The mechanism underlying this upgrading remains unproven, but may involve the triggering by BCG of acquired mechanisms of immunity that are cross-reactive with *M. leprae*-specific epitopes. In view of this, it is reasonable to speculate that the *M. leprae*/BCG admixture vaccine may prove to be of important prophylactic value.

One question that is raised by the use of such admixtures consists of the nature of the qualities, in terms of the efficacy of generation of various parameters of acquired immunity, conferred by the use of the admixture over that of the use of the BCG vaccine alone. However, because of the well-known difficulties in using viable *M. leprae* in animal models, a direct answer to such a question will have to rely on the accumulation of data from clinical trials now currently in progress ([2]). A secondary question that can be answered using animal models, however, consists of the manner in which the presence of killed *M. leprae* may modulate the host response to the viable BCG component in an admixture vaccine. Indeed, because killed *M. leprae* is known to possess both adjuvantive and inflammatory properties, it is reasonable to assume that some degree of modulation will take place.

The present study has investigated this possibility and has found, probably as a direct result of slower drainage of the BCG infection to the popliteal lymph node in mice inoculated with the *M. leprae*/BCG admixture, that the generation of T cells mediating delayed-type sensitivity, and of protective T cells capable of passively transferring antituberculous resistance, is delayed in mice receiving the admixture vaccine. It will show, in addition, that neither of these parameters, nor the generation of immunological memory, has substantially enhanced by the presence of the killed *M. leprae*. Finally, data will be presented to show that while the prophylactic effect of BCG against a number of nontuberculous mycobacterial infections was not affected by the administration of the admixture, the acquired resistance to one strain, *M. avium* 706, was considerably enhanced by the admixture vaccine over that conferred by BCG alone.

MATERIALS AND METHODS

Animals. All experiments were performed using specific-pathogen-free C57BL/6 female mice between 8 and 12 weeks of age.

Bacteria. *M. bovis* BCG Pasteur, *M. tuberculosis* Erdman, *M. intracellulare* D673, *M. simiae* 1226, *M. avium* 706 (serotype 1), and *M. avium* 724 (serotype 2) were obtained from the Trudeau Institute, Saranac Lake, New York, U.S.A. *M. intracellulare* 571-8 was a kind gift of Dr. P. Gangadharam, National Jewish Hospital, Denver, Colorado, U.S.A. Seedlots were grown to mid-log phase in filter-sterilized Proskauer Beck nutrient medium in roller bottles ro-

[1] Received for publication on 27 October 1986; accepted for publication in revised form on 30 January 1987.

[2] I. M. Orme, Ph.D., Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523, U.S.A.

tated at 10 rev/min. Armadillo-liver-derived *M. leprae* was kindly provided by Dr. P. J. Brennan of this Department. Briefly, liver extracts were exposed to 2.5×10^6 rad of ionizing radiation delivered by a ^{137}Ce source at a dose rate of 2.6×10^3 rad/min. Killed bacteria were then isolated by differential centrifugation and by velocity sedimentation centrifugation using Percoll (Pharmacia, Uppsala, Sweden).

Experimental infections. In order to attempt to mimic field vaccination conditions ([1, 8, 9]), mice were inoculated in a hind foot pad with an admixture of 10^6 viable BCG plus 10^7 killed *M. leprae*. A second test group received BCG alone, and a control group received an injection of saline.

In further experiments, mice were inoculated via a lateral tail vein with 0.2 ml phosphate-buffered saline containing suspensions of mycobacteria at desired concentrations.

Generation of delayed-type hypersensitivity (DTH). Mice were tested at various intervals for their capacity to mount a delayed cutaneous response to 5 μg PPD tuberculin suspended in 40 μl pyrogen-free saline. The swelling response was followed against time using dial-gauge calipers.

Generation of acquired specific resistance. The emergence of protective T cells capable of expressing antituberculous immunity was measured by passive cell transfer. Spleen cells were harvested and then enriched for T cells by negative selection, panning on plastic petri dishes coated with antiserum to mouse immunoglobulin as previously described ([3]); this method results in >95% Thy-1.2 positive staining cells. These cells were then infused in one-spleen equivalents into T-cell-deficient, sublethally irradiated, age- and sex-matched, syngeneic recipients, which were then challenged intravenously with 10^5 viable *M. tuberculosis*. Protection transferred was measured as the difference between the $\log_{10}$ value of the mean numbers of bacteria recovered 10 days later from the spleens of mice receiving T-cell-enriched normal cells, and the $\log_{10}$ of the mean numbers of bacteria recovered from mice infused with T-cell-enriched immune cells. This assay is described in detail elsewhere ([3]).

Generation of acquired immunological memory. The generation of memory immunity to vaccination was determined by exposing mice, 1 month after immunization, to a protracted (90 day) course of isoniazid chemotherapy (Pfizer, New York, New York, U.S.A.; 100 mg/l drinking water). Ten days after cessation of chemotherapy, spleen equivalents of T cells were passively transferred into *M. tuberculosis*-challenged recipients as above.

Growth of BCG in vaccinated mice. The course of the BCG infection in vaccinated mice was followed by harvesting the draining popliteal lymph node and plating serial dilutions of the homogenized tissue on nutrient Middlebrook 7H11 agar (Difco, Detroit, Michigan, U.S.A.). Bacterial colony formation was counted after 10–14 days incubation at 37°C in a humidified cabinet.

Vaccination against nontuberculous mycobacteria. We have demonstrated previously that BCG vaccination, if given prior to infection with certain nontuberculous mycobacteria, can have some prophylactic activity ([4]). To determine if this activity was modulated by the presence of the killed *M. leprae*, groups of mice (N = 5/group) vaccinated at prior intervals were infected intravenously with 10^4 of a number of mycobacterial strains. After appropriate intervals, the growth of the nontuberculous infections was determined in the spleens of test and control mice by plating homogenates on 7H11 agar as above.

RESULTS

Emergence of DTH. Consistent with previous observations ([6]), DTH to tuberculin peaked at around day 10 in mice vaccinated with BCG (Fig. 1). In contrast, this peak was delayed until about day 20 in mice vaccinated with the killed *M. leprae*/BCG admixture. In addition, no evidence for enhancement of the response was obtained.

Emergence of protective T cells. In similarity to the DTH response, the emergence of protective T cells capable of passively transferring antituberculous resistance was delayed in mice receiving the admixture vaccine (Fig. 2). Thus, in mice receiving the BCG vaccine alone, peak immunity was expressed on day 20 and remained at elevated levels throughout the experiment. Comparative levels were reached on day 30 in mice

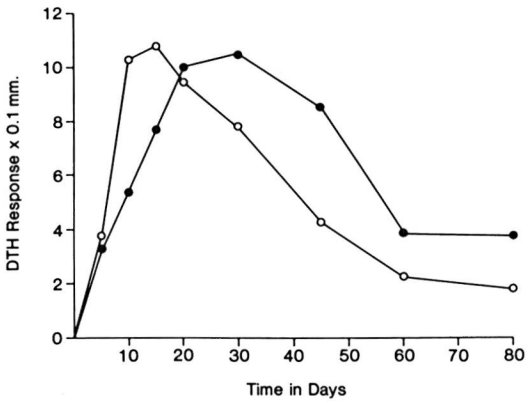

FIG. 1. Emergence of delayed sensitivity to 5 μg tuberculin in mice vaccinated subcutaneously with BCG (○) or with admixture (●). Data expressed as mean response (N = 4–5; S.E.M. omitted, it never exceeded 0.9).

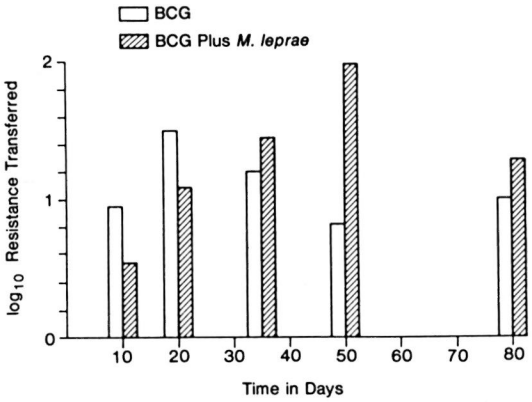

FIG. 2. Emergence of protective T-cell population capable of passively transferring acquired resistance to challenge infection with *M. tuberculosis* in vaccinated mice.

receiving the admixture; interestingly, however, these levels continued to rise to day 50, when substantial levels of acquired immunity could be transferred.

Course of BCG infection. In order to try to determine the basis for the delay in emergence of acquired immunity in mice receiving the admixture, the course of the vaccine infection in the draining lymph node was followed against time. It was found (Fig. 3) that significantly lower numbers of viable BCG could be recovered from the lymphoid tissue of mice given the admixture during the early stages of the experiment, thus indicating that drainage of bacteria from the foot pad to the popliteal node was initially much slower in these animals. This finding could not be attributed to an increased leakage to the bloodstream (no viable BCG were found in the spleen) or to bacterial load in the foot pad (drainage was normal in mice inoculated with 10^6 viable plus 10^7 killed BCG; data not shown).

Generation of immunological memory. To determine the effect of the admixture vaccine on memory generation, T cells from isoniazid-treated mice were passively transferred into *M. tuberculosis*-challenged recipients. No evidence was found (Table 1) for an adverse effect on the generation of this parameter in mice receiving the admixture.

Resistance to nontuberculous mycobacterial infections. Administration of BCG alone to mice conferred excellent protection against a subsequent intravenous inoculum of *M. simiae* (Table 2), but only weak protection (containment) against two strains of *M. avium* and two strains of *M. intracellulare*. In 4 out of the 5 challenge infections, no enhancement or reduction of protection was observed in mice receiving the admixture vaccine. The exception to this was the *M. avium* 706 infection, in which protection was substantially enhanced in mice receiving this preparation. Finally, in all cases, varying the time between vaccination and challenge had no effect on the subsequent resistance of the host to the challenge infection.

TABLE 1. *Immunological memory following vaccination.*

500 Rad-irradiated recipients infused with[a]	No. bacteria in spleen on day 10 (log_{10} mean ± S.E.M.)[b]	Log_{10} resistance transferred
Normal T cells	6.91 ± 0.09	
Memory T cells from BCG-vaccinated mice	5.80 ± 0.11	1.11
Memory T cells from admixture-vaccinated mice	5.69 ± 0.13	1.22

[a] Mice received one spleen equivalents of cells.
[b] Mice challenged i.v. with 1 × 10^5 *M. tuberculosis* Erdman.

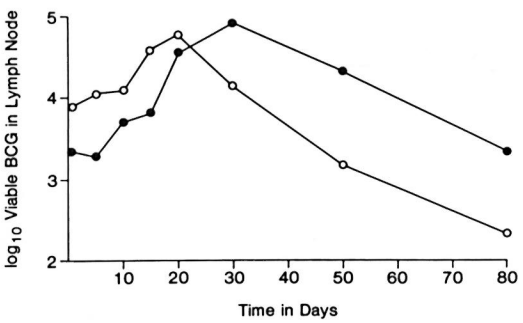

FIG. 3. Growth of BCG in the popliteal lymph node in mice vaccinated with BCG (O) or admixture (●). Data expressed as mean number of bacteria recovered (N = 4–5; S.E.M. omitted, it never exceeded 0.23).

DISCUSSION

This paper shows that the presence of killed *M. leprae* in an inoculation admixture with living BCG does not quantitatively change the capacity of the vaccine to generate adequate levels of acquired immunity, measured as the expression of delayed sensitivity, the presence of circulating protective T cells, and the retention of a sustained state of immunological memory. On the other hand, however, no conclusive evidence was obtained to suggest that such parameters could be enhanced by the presence of the killed *M. leprae*. A 30%–35% increase in peak levels of resistance transferred in mice receiving the admixture was statistically significant ($p < 0.01$), but whether this represented true enhancement is open to interpretation. Moreover, an initial delay in the emergence of these parameters probably simply reflects a slower drainage of viable BCG from the foot pad to the popliteal node, possibly as a result of a more intense inflammation occurring in mice at the sites of inoculation of the admixture vaccine.

These data, coupled with clinical field evidence ([1, 2, 7]), would support the hypothesis that a broadening of the specificity of the acquired response, rather than enhancement of reactivity, is probably the primary consequence of the administration of a combined *M. leprae*/BCG vaccine. Thus, one can envisage a scenario whereby specifically reactive T-cell clones in the nonresponsive or, alternatively, immunosuppressed host could be triggered to responsiveness by crossreactive epitopes expressed by the actively metabolizing BCG organism. In addition, a further possibility could be that macrophages, activated by the T-cell response to BCG, could then acquire the ability to catabolize and express *M. leprae*-specific relevant antigens present in the

TABLE 2. *Resistance of vaccinated mice to nontuberculous mycobacterial infection.*

Challenge infection[a]	Interval (days)	Log_{10} bacteria in spleens[b]			Log_{10} resistance	
		Controls	BCG	Admixture	BCG	Admixture
M. intracellulare 571-8	30	5.60	5.08	5.06	0.52	0.54
	60	5.31	4.59	4.47	0.72[c]	0.84[c]
	90	5.22	4.59	4.51	0.63[c]	0.71[c]
M. intracellulare D673	30	6.91	6.59	6.48	0.32	0.43
	60	6.47	5.96	6.16	0.51	0.41
	90	6.75	6.46	6.27	0.29	0.48
M. simiae 1226	30	7.29	6.22	6.00	1.07[c]	1.29[c]
	60	7.10	5.49	5.38	1.61[c]	1.72[c]
	90	7.01	5.22	5.33	1.79[c]	1.68[c]
M. avium 706	30	6.99	6.70	6.32	0.29	0.67[c,d]
	60	7.20	6.66	6.22	0.54[c]	0.98[c,d]
	90	7.35	6.87	6.33	0.48[c]	1.02[c,d]
M. avium 724	30	7.15	6.97	6.90	0.18	0.25
	60	7.10	6.69	6.80	0.41	0.30
	90	7.41	6.99	7.14	0.42	0.27

[a] Growth of challenge infection (1×10^4 i.v.) assessed in spleen 60 days later, except for *M. simiae* (40 days later).

[b] Mean values (N = 5), S.E.M. omitted; they never exceeded 0.26.

[c] Significant resistance to challenge ($p < 0.05$).

[d] Significantly higher than BCG alone ($p < 0.01$).

admixture. Such an hypothesis would thus explain the observed upgrading of lepromin positivity.

Some indirect evidence for the above hypothesis was provided by the interesting observation that acquired cross-protective resistance to the 706 strain of *M. avium* was substantially enhanced by administration of the admixture over that of the BCG vaccine alone, and that this enhancement could be observed regardless of the vaccination-challenge interval, and of levels of specific resistance observed in these animals. This presumably indicates that epitopes possessed by BCG are identical or crossreactive with those of *M. avium* 706, and that clonal expansion of T cells reactive to these epitopes is further stimulated by sets of crossreactive epitopes expressed by *M. leprae.* Such an hypothesis would also be consistent with the observation of immune upgrading in patients inoculated with the ICRC vaccine ([2]). In this case, the ICRC organism is almost certainly a member of the *M. avium* group (based upon its glycolipid profile; P. J. Brennan, personal communication), and the clinical data is suggestive that it bears epitopes identical or crossreactive to those possessed by lepromin. Thus, it can be speculated that epitopes expressed by the ICRC bacillus trigger T-cell clones, in this case mediators of delayed-type hypersensitivity, that crossreact with epitopes expressed by *M. leprae.* Indeed, the importance of such common or crossreactive epitopes to protective immunity between mycobacterial strains is already established by direct laboratory evidence ([5]).

SUMMARY

The generation of acquired immunity to BCG in mice was compared to that in animals receiving an admixture of BCG and killed *Mycobacterium leprae.* No significant qualitative differences were observed between the two groups in terms of their generation of delayed sensitivity, of protective T cells, and of immunological memory. In addition, the admixture was as effective as BCG in conferring protective immunity against certain nontuberculous mycobacteria, and in one case, that of *M. avium* 706, significantly augmented protection.

RESUMEN

Se comparó la generación de inmunidad adquirida contra el BCG entre animales inoculados con BCG y animales inoculados con una mezcla de BCG y *Mycobacterium leprae* muerto. No se observaron diferencias cualitativas significantes entre ambos grupos en términos de la generación de sensibilidad retardada, de células T protectoras, y de memoria inmunológica. Además, la mezcla fue tan efectiva como el BCG en conferir inmunidad protectora contra ciertas micobacterias no relacionadas con tuberculosis y en un caso, el del *M. avium* 706, ocurrió un aumento significativo en la protección.

RÉSUMÉ

On a comparé chez la souris l'apparition d'une immunité acquise au BCG avec celle présentée par des animaux qui avaient reçu un mélange de BCG et de *Mycobacterium leprae* tués. Aucune différence qualitative significative n'a été observée entre les deux groupes en ce qui concerne l'apparition de la sensibilité retardée, de cellules T protectrices, ou d'une mémoire immunologique. De plus, le mélange était aussi efficace que le BCG pour conférer une immunité protectrice contre certaines mycobactéries non tuberculeuses, et même pour l'une d'entre elles, *M. avium* 706, le mélange augmentait la protection.

Acknowledgment. I thank Alan Roberts for excellent technical assistance.

REFERENCES

1. Convit, J. A., Aranzazu, N., Ulrich, M., Zuniga, M., De Aragon, M. E., Alvarado, J. and Reyes, O. Investigations related to the development of a leprosy vaccine. Int. J. Lepr. **51** (1983) 531–539.
2. Noordeen, S. K. Vaccination against leprosy; recent advances and practical implications. Lepr. Rev. **56** (1985) 1–3.
3. Orme, I. M. Kinetics of emergence and loss of mediator T lymphocytes acquired in response to infection with *Mycobacterium tuberculosis.* J. Immunol. (1987) (in press)
4. Orme, I. M. and Collins, F. M. Prophylactic effect in mice of BCG vaccination against nontuberculous mycobacterial infections. Tubercle **66** (1985) 117–120.
5. Orme, I. M. and Collins, F. M. Crossprotection against nontuberculous mycobacterial infections by *Mycobacterium tuberculosis* memory immune T lymphocytes. J. Exp. Med. **163** (1986) 203–208.
6. Orme, I. M. and Collins, F. M. Aerogenic vaccination of mice with *Mycobacterium bovis* BCG. Tubercle **67** (1986) 133–140.
7. Ponnighaus, J. M. and Fine, P. E. M. Sensitization studies with potential leprosy vaccine preparations in Northern Malawi. Int. J. Lepr. **54** (1986) 25–37.
8. Samuel, N. M., Stanford, J. L., Rees, R. J. W.,

FAIRBAIRN, T. and ADIGA, R. B. Human vaccination studies in normal and contacts of leprosy patients. Indian J. Lepr. **56** (1984) 36–47.

9. SMELT, A. H. M., REES, R. J. W. and LIEW, F. Y. Induction of delayed-type hypersensitivity to *Mycobacterium leprae* in healthy individuals. Clin. Exp. Immunol. **44** (1981) 501–506.