ENHANCEMENT OF RESISTANCE TO MURINE LEPROSY BY BCG PLUS SPECIFIC ANTIGEN[1]

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Although specific immunization is theoretically a correct principle for the prophylaxis of leprosy, this procedure has appeared impractical both because of the inadequate supply of leprosy bacilli and because of their apparently poor antigenicity in susceptible individuals. BCG vaccination incites a positive Mitsuda reaction in the majority of persons, and is widely proposed as a basis for preventive programs.

Nevertheless, the importance of specific response to leprosy bacilli is illustrated in lepromatous patients by a discordance between the response to intradermal injection of tubercle bacilli and of leprosy bacilli (5), and by the failure of BCG to modify readily or permanently the Mitsuda reaction (2, 14). It seems of interest, therefore, to search for principles whereby the more available types of mycobacteria (or other agents) might be employed to enhance specific response to leprosy bacilli.

The present experiments with murine leprosy compare protection afforded by BCG vaccination with that induced by the specific antigens in heat-killed *Mycobacterium leprae murium*. They were designed in particular to ascertain whether the principle of the Dienes phenomenon (3, 7) and of the adjuvant effects of tubercle bacilli (6) might produce a more effective response to the specific antigens of the murine leprosy bacillus. The experimental proposition may be stated as follows: Will a modest amount of specific antigen, if included with the BCG suspension, result in greater protection than that afforded by BCG or by larger amounts of specific antigen given separately?

Pilot studies in this model seemed of interest for two reasons: 1. The susceptibility of the rat to a lepromatous type of mycobacterial infection is so great that significant enhancement of resistance by one or two

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injections of antigens seemed improbable without application of effective principle.[3]

2. There is a continual effort to measure or to explain, by means of the tuberculin reaction and the Koch and Mitsuda phenomena, induced increases of resistance to mycobacterial infection. The failure of the immunized rat to develop these accessory manifestations of immune response serves to remind students of mycobacterial diseases that the resistance incited by the administration of antigens probably depends on more fundamental physiologic modifications.

METHODS

Experiment 1.—In the first experiment, rats of the relatively resistant Wistar family (9) were subdivided with respect to age and potential resistance by assigning litter mates to each of the following four experimental groups:

A. Thirteen nonimmunized, normal controls;

B. Thirteen animals which received 3 mgm. (moist weight) of *M. leprae murium* killed by heat (100°C for 5 minutes), in four intracutaneous sites on the first day of the experiment and 11 mgm. in six sites on the 30th day (total 14 mgm.);[4]

C. Thirteen which received in two sites 0.15 mgm. and 0.015 mgm., respectively, of freshly-harvested BCG culture;[5]

D. Thirteen which received in each of two skin sites 0.5 mgm. of heat-killed *M. leprae murium* mixed with the dosages of BCG culture indicated above.

All immunizing doses were administered intracutaneously (ventral aspect) in order that the evolution of any BCG papules and Koch- or Mitsuda-type skin reactions could be observed. To test whether the *M. leprae murium* antigen or BCG incites any cutaneous response, special subgroups of rats were included for skin-testing after 30 days. These animals thereafter were regarded as possibly not comparable to those in Group C above, because they had not received BCG alone. They were not comparable to those in Group D because the *M. leprae murium* antigen had not been injected concomitantly with BCG, or in the BCG skin sites.

Since no information seems available on the multiplication or persistence of BCG in rats,[6] recovery of this organism from the local sites was attempted four months

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[3] Early work on the immunization of rats against murine leprosy, reviewed by Lowe (13), provides no satisfactory evidence of protection. Nakamura *et al.* (18) have stated that human and avian tubercle bacilli deprived of acid-fastness inhibit the development of rat leprosy. Although Muir and Henderson (cited by Lowe) noted no protection following BCG, Azulay (1) has recently described retardation of cutaneous lesions and apparently a more effective protection against intraperitoneal infection by BCG vaccination. The incidence of positive lesions in subcutaneously challenged sites were, 43 per cent in BCG animals and 83 per cent in controls at four months; and 97.3 per cent in BCG animals and 100 per cent in controls at six months. After intraperitoneal challenge: 0 per cent in BCG animals and 100 per cent in controls after 6 months.

[4] The use of heat-killed bacilli, although conventional, is possibly not an ideal procedure (4).

[5] Phipps strain; 8-day growth on Dubos-type medium containing 0.5 per cent purified bovine albumin, and 0.02 per cent Tween 80; kindness of Dr. Emanuel Suter, Department of Bacteriology, Harvard Medical School.

after inoculation by means of direct cultivation from scraped skin incisions. Viable BCG was not recovered.

Each rat was challenged on the 63rd day of the experiment (dorsal body surface) as follows: 2 sites subcutaneously, 2 sites intracutaneously, and 2 sites by scratching the skin with the inoculating needle to make a definite abrasion, but with an attempt not to cause bleeding.[7] The injected sites received 0.1 cc. of *M. leprae murium* in fresh 0.5 per cent testicular homogenate. In respect to bacterial numbers, this challenge dose corresponded to that in the usual 0.1 cc. of 5 per cent clarified homogenate from subcutaneous lepromas. The bacilli, however, were probably considerably more infectious than any used hitherto, since the homogenate had been prepared at a low temperature in purified bovine albumin 5 per cent and yeast supplement 5 per cent to enhance the metabolism and infectiousness of the bacilli (10), and as partial protection against inhibitors which interfere with experimental transmission (11). The scratches introduced smaller, more variable numbers of bacilli, but reproduced one of the natural modes of transmission (16).

Data on the development of infection were obtained by palpation of the skin sites monthly after the third month to estimate the volume of each lesion, and finally by autopsy (8). Rats with lepromatous lesions in sub- and intracutaneous sites were maintained as long as possible, since the scratch sites were slow in developing signs of disease and often failed to develop into lepromas. Each rat which became lepromatous in four or more sites was scored as an immunization failure and removed from the experiment.

Experiment 2.—This experiment was designed in accordance with Experiment 1, with the following exceptions: (*a*) the much more susceptible Wiersing family of rats was employed; (*b*) the experimental groups contained 17-20 animals each; (*c*) Group B received 3 mgm. moist weight of heat-killed *M. leprae murium* in 3 cc. of sterile olive oil, divided into four subcutaneous sites of 0.75 cc. each; (*d*) Group C received 0.15 mgm. of the attenuated strain R1Rv of tubercle bacilli instead of BCG;[8] (*e*) Group D received 0.5 mgm. of heat-killed *M. leprae murium* in the 0.15 mgm. dose of R1Rv; (*f*) all antigens were injected once only; (*g*) the final challenge was delayed 7 months from the date of the immunizing injections; (*h*) challenge was limited to the two most severe methods: 2 sites subcutaneous and 2 intracutaneous.

The foregoing changes in design were made for several reasons: (1) No other laboratory was known to work with rats as resistant as the Wistar family employed in Experiment 1; (2) Dr. Aronson and Dr. Suter had suggested that the R1Rv strain of tubercle bacilli might persist more successfully than BCG in rats;[9] (3) it was desired to learn if killed *M. leprae murium* could enhance resistance after a single dose of antigen, i. e., in accordance with the proposals for Groups C and D; (4)

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[6] We are indebted to Dr. J. D. Aronson, Henry Phipps Institute, Philadelphia, Pa.; to Dr. A. Q. Wells, Sir William Dunn School of Pathology, Oxford, England; and to Dr. W. McDermott and Dr. R. M. McCune, Cornell University Medical School, New York City, for advice in this matter; particularly to the last-named for their inquiries to other workers and for a careful search of the literature.

[7] The inadvisability of exposing *M. leprae murium* to blood or serous exudates has been demonstrated and discussed (11).

[8] We are again indebted to Dr. Suter for an actively growing suspension.

[9] The persistence of R1Rv was studied in a special subgroup of rats set aside for periodic autopsies. Viable tubercle bacilli were demonstrated by platings from skin sites, lymph nodes, liver and spleen after 1 month; from skin sites and lymph nodes after 3 months; and in small numbers from lymph nodes as late as 5 months.

final challenge was delayed until there might be full maturation, or possibly waning, of the incited immune response. Aside from employing a tubercle bacillus which might possibly be superior to BCG, and the incorporation of killed *M. leprae murium* in olive oil,[10] this experiment was designed to stack the cards against any easy successes which might not be reproducible in other laboratories.

RESULTS

The pertinent results of Experiment 1 at 10 months following challenge are summarized in Table 1 in order to show the effects produced by the three methods of immunization as revealed by three methods of challenge in each rat. This interval is chosen because 100 per cent of the nonimmunized controls had been lepromatous for 3-6 months and could be maintained no longer; also because the number of rats per group had fallen to approximately 10.

TABLE 1.—*Criteria employed to assess the protective effect of the antigens employed in Experiment I and the results after 11 months.*

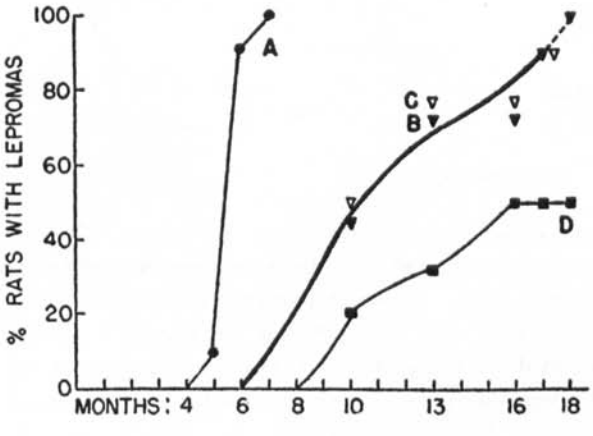
	Rat groups			
	A Control	B Mlm	C BCG	D Mlm-BCG
No. of rats per group	8	11	10	9
No. of immunizing injections:	0	2	1	1
Total antigen (mgm. moist weight) a) Mlm		14	0	1.0
Total antigen (mgm. moist weight) b) BCG		0	0.165	0.165
Incidence of disease:				
Rats with leprosy, per cent	100	45	50	22
Lesions positive, per cent[a]				
Subcutaneous	100	41	40	11
Intracutaneous	100	41	50	22
Scratch	88	23	25	11
Severity of disease[b]				
Subcutaneous lepromas	2.7	1.2	1.0	0.2
Intracutaneous lepromas	1.1	0.5	0.3	0.1
Scratch sites lepromatous	50%	0	10%	0

a Based on 2 subcutaneous, 2 intracutaneous, and 2 scratch sites per rat.

b Severity of disease is indicated by the average weight of leproma-site inoculated; also by the percentage of scratch sites which had become lepromatous.

[10] Vegetable oils were known to be water-wettable and digestible, and inferior to mineral oil as adjuvant depots (6). Mineral oils, however, produce permanent "vaselinomas" which encapsulate antigen in subcutaneous tissues. The alternative would have been to use the intramuscular route of administration (20).

It is evident that the rats exhibited three levels of resistance: (1) the usual susceptibility of nonimmunized rats; (2) an enhanced resistance in rats stimulated by means of killed *M. leprae murium* alone or by BCG alone; and (3) a more effective protection in those which had received a small dose of killed *M. leprae murium* combined with BCG. This conclusion is supported by each comparison: the percentages of rats infected, the numbers of lesions produced by the different methods of challenge, the time of onset and the severity of infection as judged by estimation of lesion weights, and the incidence of scratch sites which became lepromatous.



TEXT-FIG. 1. The rates at which murine leprosy lesions developed in Wistar rats challenged 63 days after initiation of immunization.

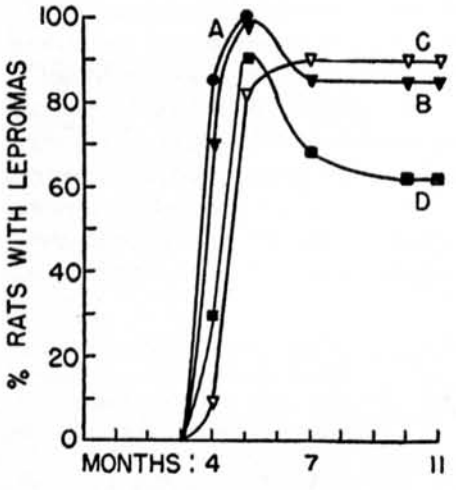
A = Nonimmunized controls. B = Animals receiving heat-killed *M. leprae murium*, as described in text. C = BCG animals. D = Animals given the mixed suspension of *M. leprae murium* and BCG. Solid lines indicate palpable lepromas. Because of overlapping, the B and C curves have been drawn as a single line.

It will be seen in Text-fig. 1 that the "immunized" animals continued one by one to develop the disease. At 17 months approximately 90 per cent of survivors stimulated by means of killed *M. leprae murium* alone or living BCG alone were lepromatous. None was found free from infection when autopsied after 18 months. However, four of the eight survivors (50%) in the group which received killed *M. leprae murium* combined with BCG exhibited no evidence of disease at autopsy.

The results of Experiment 2, conducted with the more susceptible Wiersing rats, as shown in Text-fig. 2, define the merits of the three immunizing procedures in the order described above.

Results differ in other respects: (*a*) the onset of disease in the control rats occurred sooner, all being lepromatous in all sites of challenge two months earlier; (*b*) the latency induced by the antigen administration was not as striking; (*c*) the incidence of disease in the antigen-treated rats was higher at the end of 10-11 months; and (*d*) healing of lesions (7th to 11th months) occurred only in those more resistant individuals which had received specific antigen (Groups B and D).

No minimal lesions were discovered at autopsy. Although the disease in these animals ran a more rapid course, it seems to have been suppressed or healed in the more resistant individuals by the acquisition of an effective immunity.



TEXT-FIG. 2. The incidence of murine leprosy disease in Wiersing rats challenged seven months after immunization.

A = Nonimmunized controls. B = Animals receiving heat-killed *M. leprae murium* in olive oil. C = Animals receiving the living R1Rv tubercle bacillus. D = Those receiving the mixed suspension of *M. leprae murium* and the R1Rv tubercle bacillus.

DISCUSSION

The results of the present experiments illustrate a series of considerations which have not been incorporated into existing concepts of immunization against leprotic infections. Present comment, however, will be limited to two propositions: (1) the importance of specific modification of immune response, and (2) means for the enhancement of immunity while avoiding the liabilities created by large and repeated doses of mycobacterial antigen.

Under the conditions employed the specific antigens in heat-killed *M. leprae murium* provided about the same degree and permanency of protection as the cross-resistance incited by the attenuated tubercle bacilli. It may be noted, however, that the phenomenon of healing of established lesions was seen only in animals which received specific antigen (Text-fig. 2). As in human leprosy, a response to specific antigen appears to be a factor of ultimate significance to resistance.

Although the protective effects of *M. leprae murium* antigen were produced by one or two injections, it may be questioned whether the amount of antigen administered was optimal. Our original conviction of the futility of attempting to increase immune response by repeated or massive doses of antigen was based on the following considerations: (*a*) earlier attempts to protect rats, and many attempts to incite positive Mitsuda reactions in humans exhibiting poor response, have not produced the desired results; and (*b*) the immunologic difficulty in lepromatous leprosy is not due to lack of antigen but to lack of adequate response.

The results of a recent study by Weiss and Dubos (21) substantiate this view, and throw light on one reason why an excess of mycobacterial antigen may be a liability. These workers demonstrated that a major obstacle to immunization of mice with killed tubercle bacilli is the narrow margin between optimal and excessive antigen dosages. Maximal pro-

tection was obtained with 2 mgm. of phenol-killed bacilli; 3 mgm. did not induce greater resistance, but caused instead a serious loss of weight. In view of the slow destruction of the two types of killed leprosy bacilli in the tissues of rats and nonresponsive humans, the liabilities created by these bacilli possibly are not demonstrable by measuring toxicity. Nevertheless, a state of antigen saturation or paralysis may result from antigen excess. Because of the association between poor immune response and high susceptibility, this hazard is greatest in those individuals who are particularly in need of protection. It appears that this dilemma can be resolved only by more effective excitation of response.

Interest centers, therefore, in the fact that mixing *M. leprae murium* antigen with the attenuated tubercle bacilli provides greater protection than 6-14 times this amount of *M. leprae murium* antigen alone. The basis of this superior response lies in the fact that certain mycobacteria are not merely excellent antigens by themselves. They also possess the capacity to enhance immunologic responses to an accompanying antigen. An exaggeration of tuberculin-type hypersensitivity toward extraneous protein antigens injected into tuberculous lesions was first demonstrated by Dienes and Schoenheit (3) and confirmed by Hanks (7) and others. Raffel (19) has shown that the waxes are the chemical component of the tubercle bacillus primarily responsible for enhancement of sensitization, antibody production and "adjuvant" effects in general. The excitation of immune response to antigens which accompany killed tubercle bacilli or *M. butyricum* in water and oil emulsions has found wide application in immunologic investigations (6). Since induced resistance to mycobacterial infection cannot be attributed to antibody (15), and since rats acquire enhanced resistance in the absence of tuberculin, Koch or Mitsuda reactions (12, 17), it is evident that these measures modify also the fundamental physiological basis of resistance.

The merit of combining the antigens of killed murine leprosy bacilli with attenuated tubercle bacilli in the present work may, therefore, be summarized as follows: The tubercle bacilli are great stimulators of immune response, but their shortcoming for enhancement of resistance to the leprotic infections is that their antigen spectrum does not duplicate that in the causative agents of leprosy. *M. leprae murium*, although providing poor stimulus in the rat, possesses the required specific determinants. When these determinants are included with attenuated tubercle bacilli, the specific response is increased.

Since humans behave as though more resistant to leprosy than the rat to the murine infection, it seems likely that this principle has significant implications for prophylaxis or therapy in human leprosy. The fact that many individuals are sensitive to BCG and/or tuberculin, and that there is not available sufficient *M. leprae* antigen for extensive campaigns, is no deterrent to certan crucial types of inquiry.

SUMMARY AND CONCLUSIONS

The importance of specific immune response, and a means of enhancing specific protection against leprotic infections, have been investigated. Rats were challenged with *M. leprae murium* after having received the following antigens: attenuated tubercle bacilli of the BCG and R1Rv strains, heat-killed suspensions of *M. leprae murium*, and the attenuated tubercle bacilli accompanied by smaller amounts of *M. leprae murium* antigen. The results indicate that specific antigen plays a distinctive role in protection and that superior protection is afforded by small amounts of specific antigen combined with the tubercle bacilli as stimulators of immune response.

Significant protection was achieved by single administrations of antigen, on a basis adopted to practical application. The liabilities which may attend large and repeated doses of mycobacterial antigens have been emphasized as peculiar to those individuals most in need of protection. The theoretical basis of enhancing response to modest amounts of specific antigen has been indicated.

RESUMEN Y CONCLUSIONES

Esta investigación versó sobre la importancia de la inmunirreacción específica y sobre un medio de realzar la protección especfica contra las infecciones leprosas. Ratas fueron provocadas con *M. leprae murium* después de haber recibido los siguientes antígenos: bacilos tuberculosos atenuados de las cepas BCG y R1Rv, suspensiones matadas al calor de *M. leprae murium* y bacilos tuberculosos atenuados acompañados de cantidades más pequeñas de antígeno de *M. leprae murium*. Los resultados indican que un antígeno específico desemepeña un papel sobresaliente en la protección y que se obtiene protección superior con pequeñas cantidades del antígeno especifico combinado con los bacilos tuberculosos como excitadores de la inmunirreacción.

Se logró protección significativa con una sola administración de antígeno, usando un método adaptado a la aplicación práctica. Se recalcan los riesgos que pueden acompañar a las dosis grandes y repetidas de antígenos micobacterianos, como peculiares de los individuos que más necesitan protección. Se indica la base teórica en que se asienta la intensificación de la reacción a modestas cantidades de antígeno específico.

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