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ICRC Vaccine-induced Changes in *M. leprae*-specific Cell-mediated Immunity in Langur (*Presbytis entellus*) Monkeys[1]

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Vaccination, which acts by enhancing the host's protective immunity, is an established modality for the control of infectious diseases. The available clinical, epidemiological, and experimental evidence shows that cell-mediated immunity (CMI) is the dominant form of host protective immunity in leprosy; antibody formation has very little role ([5, 10, 11]). Among laboratory parameters, skin reaction and the *in vitro* lymphocyte blast transformation test (LTT) with antigens of *Mycobacterium leprae* have been widely used to assess CMI.

Skin tests for delayed hypersensitivity generally show maximum responses by 48–72 hours. The late lepromin (Mitsuda) reaction, which is elicited by particulate antigens of *M. leprae,* is a unique test, in the sense that there is hardly any response in the first week. However, by the end of the second week a small induration, which reaches a peak between 3–4 weeks, is observed in responsive individuals.

Five years ago, we developed an antileprosy vaccine from ICRC bacilli, which are cultivable mycobacteria that exhibit antigenic crossreactivity with *M. leprae* ([8]). The vaccine induces lepromin conversion in about 60% and 95% of lepromatous patients and lepromin-negative healthy subjects, respectively ([7]). Some patients even develop a reversal reaction with "up-grading" of the tissue response associated with bacillary clearance ([11]).

Very few species of laboratory animals exhibit the Mitsuda type of reaction ([12]). Recently, naturally occurring leprosy has not only been identified in sooty mangabey monkeys, but the disease has also been induced experimentally in a few other species of monkeys ([13, 21]). Like leprosy in man, in monkeys the dominantly affected tissues are also skin, mucous membranes, and peripheral nerves ([21]). Being phylogenetically closer to man and because some species develop leprosy in the natural state, we thought that monkeys might exhibit a Mitsuda reaction similar to that observed in man. This contention was substantiated by our preliminary studies. There was hardly any reaction to the administration of lepromin at 72 hours, but three weeks later a small induration was noted. It was, therefore, felt that the monkey could serve as a good laboratory model to study ICRC vaccine-induced alteractions in the two widely used parameters of CMI, namely, the skin test (Mitsuda reaction) and the LTT. Attempts were also made, initially, to estimate the peripheral blood lymphocytes and their subsets in vaccinated monkeys. However, this study had to be abandoned because, in preliminary experiments, it became evident that lymphocytes of langurs (*Presbytis entellus*), the species used in this study, formed very poor E and EAC rosettes, and the results were inconsistent.

MATERIALS AND METHODS

Animals. The experiments were conducted on 12 adult male Hanuman langur monkeys (*Presbytis entellus*) which live chiefly in northern India. They weighed between 6–8 kg and were individually housed in cages made of galvanized iron bars. Commercially available monkey diet (Hindustan Lever Ltd., India) and water were provided *ad libitum.* The animals were kept under observations for six weeks before experimen-

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THE TABLE. *Cell-mediated immunity in vaccinated monkeys.*[a]

Monkey no.	Lepromin (Mitsuda)[b] reaction		PHA (25 μg/ml)		ConA (5 μg/ml)		ICRC (2×10^7/ml)		*M. leprae* (2×10^7/ml)	
	I[c]	II	I	II	I	II	I	II	I	II
					Vaccinated					
1	−	++	1.620	2.279	1.511	0.635	1.213	1.419	1.783	1.427
2	−	++++	2.023	1.468	1.737	1.777	1.017	0.988	1.544	1.212
3	−	−	1.006	2.442	ND	1.263	9.110	0.927	1.307	1.454
4	+	+++	0.943	0.838	1.447	4.438	0.872	1.238	0.628	1.187
5	+	+++	8.351	1.233	5.759	5.480	1.025	1.184	5.465	1.317
6	−	++	3.681	1.740	2.325	1.982	2.070	0.928	10.552	2.301
7	+	++	1.901	0.928	2.306	4.359	1.072	6.213	11.739	5.174
8	−	++	1.867	0.927	1.975	0.721	0.867	1.591	0.998	1.550
		Mean	1.867	1.55	2.44	2.11	2.15	1.27	4.25	1.44
		±S.E.	±0.84	±0.21	±0.57	±0.66	±1.0	±0.12	±1.60	±0.15
					Nonvaccinated					
9	−	+	1.000	0.888	3.221	1.350	0.714	1.627	0.986	2.119
10	−	−	1.280	1.034	1.222	0.737	0.928	0.918	1.098	0.825
11	+	+	2.281	1.434	1.005	0.557	0.937	1.918	0.550	0.799
12	−	−	0.636	3.181	0.560	0.525	1.912	0.947	0.992	1.428
		Mean	1.30	1.63	1.50	0.76	1.12	1.35	0.910	1.30
		±S.E.	±0.35	±0.53	±0.59	±0.20	±0.27	±0.25	±0.12	±0.31

[a] Counts in the control (unstimulated) cultures varied between 1000–3000 cpm. Results given as stimulation index (SI).

[b] Grade + = 2–3.9 mm; ++ = 4–5.99 mm; +++ = 6–7.9 mm; ++++ = >8 mm diameter induration.

[c] Roman numerals I and II refer to values of the tests performed before and 8–10 weeks after vaccination, respectively.

tation. All laboratory tests were performed before and repeated 8–10 weeks after vaccination.

Skin tests. The tuberculin reaction was performed using 2 IU of PPD (BCG Laboratory, Guindy, Madras, India) administered under the skin of the eyelid. The reaction was read at 48 hr and 72 hr. The lepromin test was performed using 0.1 ml of Mitsuda antigen containing 4×10^7 armadillo-grown *M. leprae*/ml. Lepromin was obtained through the kind courtesy of Dr. W. F. Kirchheimer, National Hansen's Disease Center, Carville, Louisiana, U.S.A., with the assistance of the World Health Organization. The antigen was given intradermally in the forearm and the reaction (local induration), if any, was measured at 48–72 hr and thereafter weekly up to four weeks. Because of the dark color of the skin, it was not possible to record erythema. Four animals exhibited a weakly positive Mitsuda test (grade +).

Lymphocyte transformation test. Heparinized blood was collected from the femoral veins of the monkeys. Lymphocytes were separated on a Ficoll-Hypaque density gradient (4), washed three times with 0.9% saline, and suspended at 1×10^6 cells per ml of RPMI 1640 supplemented with 10% human AB group serum, glutamine, nonessential amino acids and antibiotics (streptomycin 100 μg/ml; penicillin 100 U/ml); 200 μl of culture (2×10^5 cells) were plated in triplicate with mitogens PHA (Burroughs Wellcome, U.K.) and concanavalin A (ConA; Sigma Chemical Co., St. Louis, Missouri, U.S.A.) for 72 hr and integral Dharmendra antigens of ICRC and *M. leprae* for six days. The mitogens PHA and ConA were added in concentrations of 25 μg per ml and 5 μg per ml of the media, respectively. Both of the mycobacterial antigens were used at the level of 2×10^7 bacilli/ml. Control cultures received no mitogen/antigen. The lectins as well as the antigens were used in optimal concentrations determined by preliminary dose response experiments. Eighteen to 24 hr prior to harvesting, 1 μCi ^{3}H-thymidine (Bhabha Atomic Research Centre, Bombay; specific activity 6 Ci/mM) was added to each well. The cultures were harvested on Whatman glass fiber filter discs and counts taken on the Rackbets liquid

scintillation counter (LKB). Results were expressed as a stimulation index (SI), which is the ratio of the mean counts per minute (cpm) of antigen/mitogen-stimulated cultures and the control (unstimulated) cultures.

Biopsies. Skin biopsies of the Mitsuda reaction were performed in representative animals before and after vaccination. They were fixed in 10% neutral buffered formalin. Paraffin sections cut at 4–5 μm were stained with hematoxylin and eosin (H&E) and Fite-Faraco.

ICRC antileprosy vaccine. Animals were divided into two groups. Out of the total of 8 animals in group I, 3 were Mitsuda positive and 5 were negative. Each animal in this group received intradermally, in the thigh, 0.1 ml of vaccine containing 1×10^8 bacilli inactivated by 250 Krad gamma irradiation. This dose is one fifth of that used in healthy human volunteers ([7]) and was chosen in view of the smaller body size of monkeys as compared to man. Details of the preparation of the vaccine have been described elsewhere ([8]). Each of the 4 animals in group II was given intradermally 0.1 ml of normal saline which was used as the vehicle in the vaccine preparation. One of these monkeys was Mitsuda positive and the other 3 were negative.

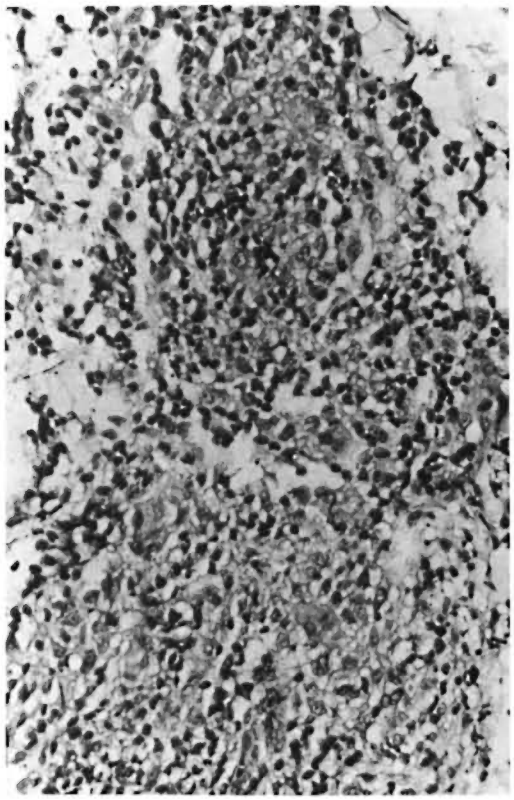

FIG. 1. Microphotograph of the pre-vaccination lepromin reaction in monkey no. 2. The inflammatory exudate consists of foamy macrophages and moderate number of lymphocytes (H&E ×240).

RESULTS

No significant local or systemic reaction was observed in the vaccinated animals in the first 3–4 days. Thereafter, the animals developed induration at the vaccination site associated with enlargement of regional lymph nodes. No other untoward reaction was seen, and the animals were healthy throughout the study.

Skin reactions. Lepromin conversion was observed in 4 out of 5 lepromin-negative animals after vaccination (The Table). The reaction became stronger in all 3 of the monkeys that had exhibited a weak positive response before vaccination. Biopsies obtained from the latter group (grade + pre-vaccination) showed granulomas consisting predominantly of foamy macrophages, a few lymphocytes, and an occasional ill-formed giant cell (Fig. 1). The morphologic picture resembled the features observed in borderline leprosy. A biopsy of the post-vaccination lepromin reaction revealed a lymphocyte-rich granuloma containing abundant numbers of lymphocytes, epithelioid cells, well-formed giant cells, and a central area of necrosis (Fig. 2). A few plasma cells and neutrophils were also seen. The latter were restricted predominantly to the central necrotic area. Except for the neutrophilic infiltration, which could be an acute response to the necrosis, the morphological picture of the post-vaccination, lepromin-induced granuloma was similar to that of the granuloma of tuberculoid leprosy. No changes were observed in the lepromin reaction in the unvaccinated control group except in monkey no. 11, in which a weak (grade +) positive response was observed with the second lepromin skin test. The first lepromin test was negative in this animal. Vaccination had no effect on the tuberculin reactions which remained negative throughout the study period.

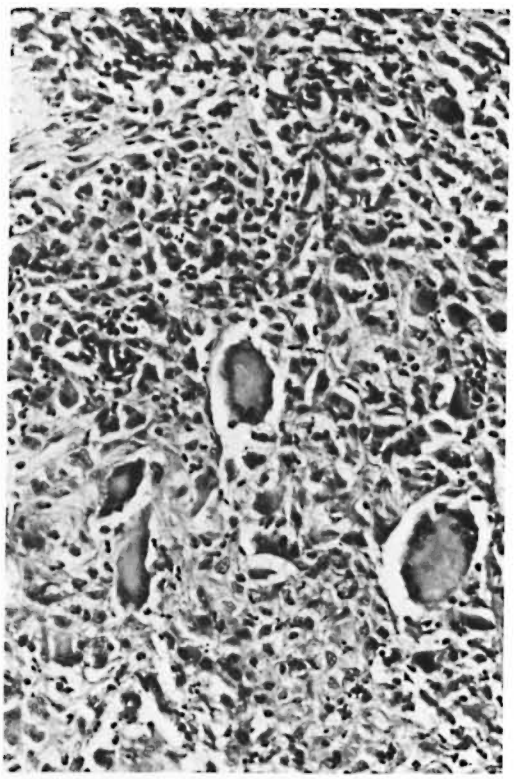

FIG. 2. Post-vaccination lepromin reaction in the same animal showing a lymphocyte-rich granuloma containing well-formed giant cells and histiocytes (H&E ×240).

Lymphocyte transformation test. The mitogenic responses of lymphocytes both to the lectins (PHA and ConA) and to the mycobacterial antigens were very poor and were unaffected by vaccination (The Table).

DISCUSSION

In this study, administration of the ICRC vaccine brought about lepromin (Mitsuda) conversion in a number of negative monkeys. There was not only an increase in the size of the response but the lepromin-induced granuloma also exhibited a change consistent with an "up-grading" of immunity. In the absence of a corresponding change in antigen-specific, *in vitro* lymphocyte response, it could be argued that the vaccine-induced lepromin conversion could be due to a nonspecific stimulation of CMI. However, the skin response to antigens of other mycobacteria, such as BCG, was not affected and all vaccinated animals remained PPD negative. The conversion also could not be due to the pre-vaccination lepromin tests since the controls, which did not receive the vaccine, exhibited no significant conversion.

The late lepromin (Mitsuda) reaction, which was first described more than 50 years ago, is a local response of tissues to integral antigen(s) of heat-killed *M. leprae.* The lepromin-induced granuloma in positive individuals resembles the lesion observed in tuberculoid leprosy and is characterized by a lymphocyte-rich epithelioid cell lesion containing well-formed giant cells. Within the leprosy spectrum, the reaction is strongly positive in the paucibacillary tuberculoid patients. On the other hand, in lepromatous patients, whose tissues are laden with *M. leprae,* the reaction is consistently negative and remains so despite prolonged drug therapy ([5, 17]).

As mentioned earlier, the Mitsuda reaction is a peculiar and unique test. The maximum induration is not observed until 3–4 weeks after administration of the antigens. Its mechanism is still poorly understood. Interestingly, even individuals living in leprosy-free areas exhibit a positive response ([16]). This is believed to be due to exposure of these individuals to environmental mycobacteria that crossreact antigenically with *M. leprae* ([16]). Alternatively, the intradermal administration of lepromin might act as a sort of micro-vaccination, and a positive Mitsuda test could be a manifestation of delayed-type hypersensitivity to locally persistent antigen ([18]). There are also other explanations ([16]).

If Mitsuda reactions were considered to be basically a hypersensitivity response to micro-vaccination, the negative individuals would then represent a population incapable of mounting immunity. For this reason, such a population would be susceptible to the disease. This appears to be the case. The pioneering work of Dharmendra and Chatterjee ([9]) has shown that Mitsuda-negative healthy subjects in endemic areas run a high risk of contracting multibacillary forms of leprosy which are hardly ever seen in Mitsuda-positive individuals. The negative subjects presumably represent a group that has remained nonresponsive despite a long exposure to *M. leprae.*

Leprosy has been experimentally trans-

mitted to armadillos ([14]). However, it has been observed that all animals inoculated with live *M. leprae* do not develop the disease. Recently, Job, *et al.* ([12]) have shown that armadillos exhibit a Mitsuda reaction similar to that observed in man. Further, Mitsuda-positive animals are relatively resistant to the disease ([13]).

In the study on experimental transmission of leprosy in monkeys, it was shown that the animals that developed lepromatous leprosy were lepromin negative and their sera contained antibodies to mycobacterial antigens 2, 5 and 7; a pattern similar to that observed in leprosy patients ([11]). One female monkey failed to develop the lesions; interestingly, it was strongly lepromin positive and had no circulating antibodies to the above-mentioned antigens ([20, 21]).

The data on the LTT to lectins should not be surprising because lymphocytes of a number of monkey species respond poorly to lectins ([18]). *M. leprae,* as such, is a poor mitogen. Further, there is also a wide variation in the LTT from animal to animal. Also, it is well known that the LTT response in the same individual shows marked fluctuations. These factors could mask minor alterations, if any, induced by the vaccine. On the other hand, it is well known that the LTT may show discordance with skin tests ([3]). This may be true of the present study where no correlation was observed between the LTT and the Mitsuda conversion. It may be mentioned that, in man, the status of the LTT as a predictive test for immunity in leprosy is not firmly established ([2]). In that respect, the evidence presented above indicates that the Mitsuda test is perhaps superior to the LTT.

Global attempts are being made to develop a vaccine effective against leprosy. Prevention of the disease in healthy subjects will be, of course, the ultimate test of the efficacy of the vaccine. Leprosy has a long incubation period. Before undertaking such trials, which would need several years for completion, it would be essential to show that a "candidate" vaccine brings about immune alterations consistent with protective immunity.

Availability of a laboratory model would facilitate screening of microbes and their antigenic components. Monkeys exhibit a Mitsuda reaction somewhat similar to that observed in man. Further, animals that develop the disease are lepromin negative ([20]). These observations suggest that, as in man, the Mitsuda test may be an expression of cellular immunity in monkeys. This view is further strengthened by the data of this study, in which it has been shown that a vaccine known to induce Mitsuda conversion in man is also able to do so in monkeys. For these reasons, we feel, that the monkey could be used as a laboratory model to screen immunogenic organisms and to identify purified antigens that are responsible for the Mitsuda test. The latter would be an important step in the development of a synthetic antileprosy vaccine.

SUMMARY

The effects of the administration of ICRC antileprosy vaccine on skin reactions and lymphocyte transformation tests (LTT) to antigens of *Mycobacterium leprae* have been investigated in Hanuman langur monkeys (*Presbytis entellus*) which live native to north India. In a majority of these monkeys, the vaccine brings about lepromin conversion associated with a change in tissue response consistent with "ugrading" of immunity. However, no concomitant changes were observed in the LTT. The significance of these observations is discussed. It is proposed that the langur monkey could be used as a laboratory model to screen "candidate" antileprosy vaccines.

RESUMEN

Se investigaron los efectos de la administración de la vacuna antileprosa ICRC sobre las reacciones en piel y sobre las pruebas de transformación de linfocitos (LTT) inducidas con antígenos del *Mycobacterium leprae,* en monos langur (*Presbytis entellus*), habitantes nativos del norte de la India. En la mayoría de los monos, la vacuna indujo una conversión positiva a la lepromina asociada a un cambio en la respuesta tisular sugerente de un incremento en la inmunidad. Sin embargo, no se observaron cambios en la prueba de transformación de linfocitos. Se discute el significado de estos hallazgos y se propone que los monos langur pueden ser usados como modelo de laboratorio para evaluar los preparados vacunales propuestos para su uso como vacunas contra la lepra.

RÉSUMÉ

On a étudié chez une espèce de singes qui vit à l'état naturel dans le Nord de l'Inde (*Presbytis entellus*), les effets de l'administration du vaccin antilépreux ICRC sur les réactions cutanées et sur les épreuves de transformation lymphocytaire (LTT) aux antigènes de *Mycobacterium leprae*. Chez la majorité de ces singes, le vaccin entraîne un virage de la lépromine, qui est associé avec une modification dans la réponse tissulaire qui correspond à un renforcement de l'immunité. Toutefois, aucune modification de ce genre n'a été observée pour l'épreuve de transformation lymphocytaire. La signification de cette observation est discutée. On propose que cette espèce de singes soit utilisée comme modèle de laboratoire passer au crible les vaccins antilépreux éventuels.

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