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Soluble Antigen of *M. leprae* Coupled with Liposomes Elicits Both "Early" and "Late" Delayed Hypersensitivity Skin Reactions[1]

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Lepromin is a preparation of killed *Mycobacterium leprae* of human or armadillo origin, and it is used extensively for the assessment of delayed-type hypersensitivity (DTH) against *M. leprae*. After intradermal injection of lepromin, two types of skin DTH responses are seen in people who are sensitized: an "early" (Fernandez) reaction which peaks at 24–48 hr and a "late" (Mitsuda) reaction which peaks at 3–4 weeks ([17]). The late lepromin reaction has shown a strong correlation with immunity to mycobacterial infections in both experimental animals ([2]) and humans ([5]). However, the integrity of bacterial morphology in a lepromin preparation appears to be essential for the development of a late reaction. Ultrasonically disrupted lepromin or the soluble extract of *M. leprae* (leprosin) elicit only the early reaction ([22, 23]). There are speculations that the soluble antigen(s) may also produce a late reaction, provided the antigen is presented in the same manner as that of intact *M. leprae* ([17, 22]). To investigate this possibility, we have used soluble antigen(s) of *M. leprae* ([23]) coupled with liposome as a skin-test antigen, hoping that such a system would mimic the antigen presentation by intact bacilli.

MATERIALS AND METHODS

All of the chemicals and reagents used in this study were of the highest purity available. Egg lecithin was prepared as described earlier ([21]). Gangliosides were isolated from buffalo brain using the known method ([13]). Cholesterol was purchased from Centron Research Laboratory, Bombay, India, and crystallized three times from methanol. Sodium cyanoborohydride was from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Sodium ^{125}I-iodide (carrier-free) was bought from Bhabha Atomic Research Centre, Trombay, India.

***M. leprae* soluble antigen(s).** Soluble antigen(s) of *M. leprae*, prepared by the described method ([23]), was kindly provided by Dr. R. J. W. Rees, Clinical Research Centre, Harrow, U.K. Briefly, *M. leprae* purified from infected armadillo tissues ([25]) were ultrasonically disrupted before ultracentrifugation (105,000 × *g* × 30 min). The supernatant, termed "leprosin-A," was used after protein estimation ([14]).

^{125}I-labeling of the soluble antigen(s). Fifty μg protein of the soluble antigen(s) (in 50 μl) was labeled with ^{125}I using the "Iodogen" technique ([7]). More than 95% of the radioactivity was incorporated in the protein peak recovered after Sephadex G-25 gel filtration.

Preparation of liposomes. Unilamellar liposomes were prepared from egg lecithin (45 μM), cholesterol (45 μM), and gangliosides (9 μM) in 2.0 ml borate-buffered saline (10 mM borate containing 60 mM NaCl, pH 8.4) by probe-type sonication and fractionated by ultracentrifugation, as described earlier ([9]).

Covalent coupling of liposomes with antigen(s). The soluble protein of *M. leprae* was covalently coupled to the liposome surface according to the method of Heath, *et al.* ([10]). Briefly, liposomes were oxidized with sodium periodate (2 hr, 25–30°C, dark). The excess reagent was removed by gel filtration, and the liposome fractions, after pooling together, were concentrated to about 1 ml (5–

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THE TABLE. *Early (48 hr) and late (3 weeks) skin reactions with different antigen preparations in borderline tuberculoid leprosy patients.*

Patient	Reaction	LA[a]	L[b]	L1[c]	L2[c]	L3[c]	C[d]
1	Early	29[e]	28	30	28	28	0
	Late	0	10	12	10	10	0
2	Early	20	25	32	26	27	<5
	Late	0	7	9	9	7	0
3	Early	28	22	ND[f]	46	32	<5
	Late	0	0	ND	0	0	0
4	Early	0	0	ND	13	15	<5
	Late	0	6	ND	9	0	0
5	Early	23	18	ND	27	30	<5
	Late	0	0	ND	0	0	0
6	Early	12	12	ND	15	13	0
	Late	0	0	ND	0	0	0
7	Early	15	12	ND	14	12	0
	Late	0	5	ND	8	5	0
8	Early	26	24	ND	23	27	0
	Late	0	12	ND	10	6	0
9	Early	30	22	ND	20	19	0
	Late	0	5	ND	10	10	0
10	Early	18	16	ND	16	14	0
	Late	0	5	ND	7	6	0

[a] LA = leprosin-A (10 μg/ml).
[b] L = Dharmendra lepromin (10^7 bacilli/ml).
[c] L1–3 = liposomized leprosin-A at 40, 20, and 10 μg protein/ml.
[d] C = control liposomes.
[e] Indurations in mm.
[f] ND = not determined.

7 μM lipid), as described earlier ([20]). To it were added the unlabeled *M. leprae* antigen(s) (about 8 mg), ^{125}I-labeled antigen(s) (≃50,000 cpm), and sodium cyanoborohydride (2 M, 10 μl/ml). The mixture was incubated at 25–30°C for 14–16 hr. It was chromatographed on a Sepharose 6B column (1.4 × 35 cm) using phosphate-buffered saline (10 mM phosphate containing 150 M NaCl, pH 7.4; PBS) as the eluant. The liposomes were recovered in the void volume. Under these conditions, 18–20% (≃1.5 mg unlabeled protein or ≃10,000 cpm) of the protein initially added got cross-linked to the liposome surface. The liposome-rich fractions were pooled together, dialyzed against sterile PBS, and filtered through a Millipore membrane (0.2 μm). A preparation of control liposomes was also subjected to the above-mentioned antigen coupling steps, in the absence of antigen.

Testing of antigen-coupled liposomes. The antigen-coupled liposomes were diluted to 10, 20, and 40 μg protein/ml sterile PBS and used for skin testing. Simultaneously, control liposomes (free of antigen), Dharmendra lepromin ([19]), and leprosin-A ([23]) were also tested. All of the preparations were coded and tested by a clinician (GR) in a double-blind manner. Ten borderline tuberculoid (BT) and five lepromatous (LL) leprosy patients ([18]) attending the outpatient department and wards of Central JALMA Institute of Leprosy (CJIL), Agra, India, were selected for the study. The test preparations were injected intradermally (0.1 ml each) into the back of every patient. The diameters of the skin indurations (mean of two readings at right angles to each other) were recorded in millimeters with the help of a vernier scale at 48 hr and 21 days. An induration of 5 mm or more was regarded as positive in the case of the 48-hr (early) reaction and that of 4 mm or more was regarded as positive for the 21-day (late) reaction ([11]). Biopsy specimens representing the early (48 hr) and late (21 day) skin reactions to the liposome-coupled antigen were fixed in Zenker-formol saline, processed, and embedded in paraffin. Five-μm sections were stained with hematoxylin and eosin (H&E). The sections were examined by a pathologist without referring to the case notes to avoid personal bias.

RESULTS

The Table depicts the skin reactions in 10 BT patients at 48 hr (early) and at 21 days (late) elicited by the different antigen preparations and controls. While leprosin-A (LA) elicited only the early reaction, Dharmendra lepromin (L) elicited early, late, or both early and late reactions. In two patients tested initially, all three concentrations of liposomized leprosin elicited strong reactions. In subsequent patients, the use of the highest concentration (L1) was omitted. In all of these patients, liposomized antigen elicited early and late skin reactions akin to those elicited by Dharmendra lepromin, except in one patient who was early-reaction negative with Dharmendra lepromin but positive with liposomized antigen. Control liposomes induced insignificant reactions at 48 hr in four patients.

The 48-hr skin reaction elicited by liposomized leprosin showed a histological pic-

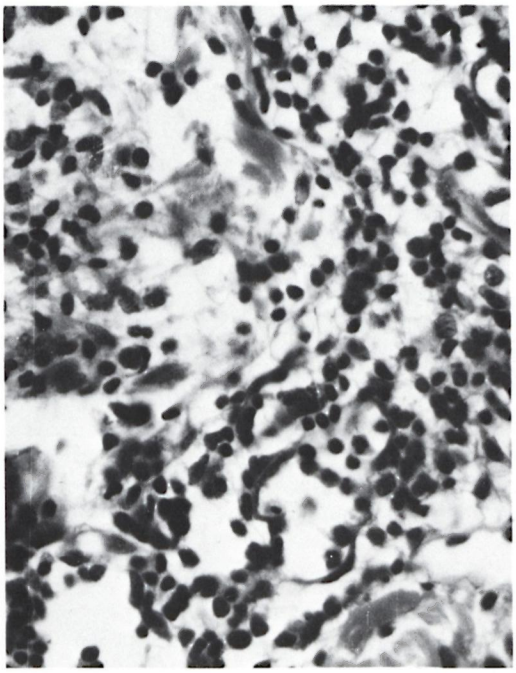

FIG. 1. Photomicrograph of 48-hr reaction of a BT case showing lymphocyte and polymorphonuclear infiltration (H&E ×1200).

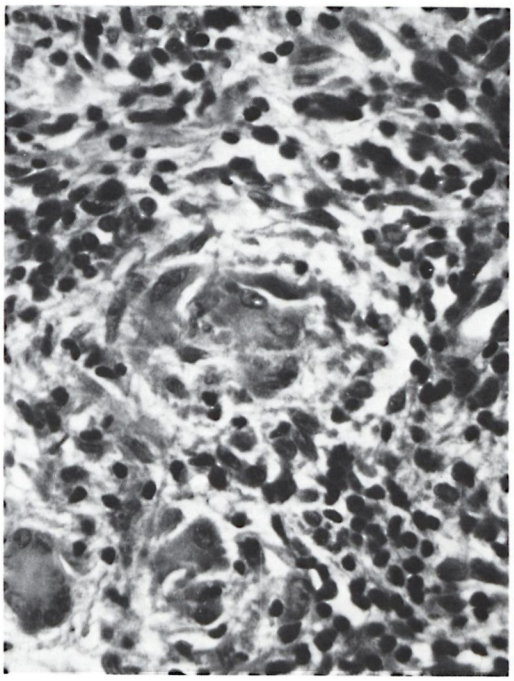

FIG. 2. Photomicrograph of 21-day reaction of a BT case showing lymphocytes, epithelioid cells, and giant cells in the infiltrate (H&E ×1200).

ture of DTH, with a predominant lymphocytic infiltration. Polymorphonuclear cells were present to a lesser extent (Fig. 1). The 21-day reaction to the liposomized antigen showed a histological picture resembling a tuberculoid granuloma with focal collections of epithelioid and giant cells (Fig. 2). None of the five LL patients showed any positive skin reaction to these antigens.

DISCUSSION

Several studies have shown that the immunogenicity of antigens is improved by incorporating them in liposomes ([8]). It has also been demonstrated that this action of liposomes is further enhanced when antigen is covalently attached on their surface ([24]). These observations prompted us to covalently couple soluble *M. leprae* antigen(s) (leprosin) to liposomes, and then to determine whether the liposome-coupled antigen(s) could elicit both the early and late skin DTH responses.

The results of this study support the working hypothesis that the presentation of eliciting antigen(s) in a specific orientation is required for the development of late reactions. In the method of Heath, *et al.* ([10]) which we used, protein molecules are covalently linked with the liposome surface due to a reaction between the amino groups of the proteins and the aldehyde groups of the ganglioside via the formation of Schiff's bases. In the present situation, it seems that such a linkage does not adversely affect the antigenicity of the attached proteins. Apparently the liposomes presented surface-bound protein antigens to the host cells in the specific manner of intact *M. leprae* and thus activated the lymphoid cell precursors which were responsible for the development of the late skin-test reaction.

The cells may not require the continued presence of the stimulus for the development of late reactions, since we know that liposomes are rapidly disintegrated *in vivo* ([1]). For the same reason, the adjuvant effect of liposomes may not be operational in the development of the late reaction. Moreover, in a separate study, when the liposome-entrapped leprosin was used for skin testing of BT patients it did not produce the late reaction although the early reaction was elicited (data not shown). Both early and

late reactions to the liposome-coupled antigen showed histological pictures corresponding to the reactions elicited by integral lepromin ([4]).

The early reaction elicited by control liposomes in some of the cases is not significant, since an early reaction of <5 mm is not regarded as positive ([11, 23]).

On the basis of the present study, it is not possible to define the molecule(s) involved in the development of the DTH reactions. The decision to use whole (unfractionated) soluble antigen was made because studies so far have led to diverse conclusions regarding the physicochemical properties of the DTH-eliciting antigens of *M. leprae* or even any other mycobacterium ([3]). Moreover, studies on *M. leprae* are limited due to non-availability of the organisms in sufficient quantities. The major source of *M. leprae* remains the experimentally infected nine-banded armadillo ([12]). Recently, isolation of molecularly defined *M. leprae* antigens has been made possible with the help of monoclonal antibodies ([6]) and gene cloning ([26]). Such preparations would be those of choice for future work using liposomes.

There is experimental evidence to suggest that the late (21 day) DTH reaction is a marker for protective immunity against multibacillary mycobacterial infections ([2, 5]). Since the *M. leprae* antigen covalently linked with liposomes is capable of eliciting the late reaction, liposome may serve as a carrier and immunopotentiator of activity of molecularly defined, cell-mediated immunity-inducing antigens of *M. leprae* which are presently being evolved ([14, 15]).

SUMMARY

The soluble antigen(s) of *Mycobacterium leprae* was(were) coupled to liposomes and used for skin testing of leprosy patients, hoping that this mode of antigen presentation would be identical to that of integral lepromin. The liposomized antigen(s) elicited both early (24–48 hr) and late (3–4 weeks) delayed-type hypersensitivity reactions, true to the nature of lepromin, unlike the soluble antigen(s) alone which elicit(s) only the early reaction.

RESUMEN

Se incorporaron antígenos solubles de *Mycobacterium leprae* a liposomas y se usaron para pruebas en piel en pacientes con lepra esperando que este modo de presentación del antígeno pudiera ser idéntico al de la lepromina integral. Los antígenos incluidos en los liposomas, indujeron tanto las reacciones de hipersensibilidad retardada tempranas (24–48 h) como las tardías (3–4 semanas), en tanto que los antígenos solubles solos nada más indujeron la reacción temprana.

RÉSUMÉ

On a couplé l'antigène ou les antigènes de *Mycobacterium leprae* à des liposomes pour pratiquer des épreuves cutanées chez des malades de la lèpre, dans l'espoir de démontrer que ce mode de présentation antigénique serait identique à celui de la lépromine intégrale. L'antigène ou les antigènes ont entraîné des réactions précoces après 24 à 48 heures, et des réactions tardives du type d'hypersensibilité retardée après 3 et 4 semaines, comme c'est le cas après injection de lépromine, alors que l'antigène ou les antigènes solubles administrés seuls n'ont entraîné qu'une réaction précoce.

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