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Use of Synthetic Peptides Corresponding to Sequences of *Mycobacterium leprae* Proteins to Study Delayed-type Hypersensitivity Response in Sensitized Guinea Pigs[1]

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Despite the efforts of several research groups ([1, 5, 24, 28]), it has not been possible to identify individuals at risk to become infected, or individuals who have already encountered but overcome infection, with *Mycobacterium leprae* or to identify patients actually incubating the bacilli. Therefore, the diagnosis and the epidemiological studies of leprosy would greatly benefit from the availability of a species-specific skin-test reagent.

In leprosy as in other intracellular infections, cell-mediated immunity (CMI) rather than antibodies provides protection ([25]). In general, protective immunity to mycobacterial infections, as manifested by CMI, is closely related to delayed-type hypersensitivity (DTH) reaction to mycobacterial antigens, with a few exceptions as demonstrated by Youmans ([32]) in mice.

Experimental trials using new antileprosy vaccines have been performed and are still being conducted ([8, 12, 21]). In these trials it is expensive and time consuming to assess the protective immunity imparted by such vaccines in human populations. Thus, it would be more convenient to evaluate the sensitization conferred by such vaccines in terms of DTH to mycobacteria skin-test antigens.

Induction of DTH to *M. leprae* is well documented ([28]), and has been achieved in guinea pigs ([17]), mice ([23]), and humans ([7, 28]). These studies show that DTH reaction can be produced with soluble extracts from killed *M. leprae* bacilli grown in armadillos, indicating that this preparation may be a candidate for a skin-test reagent ([28]). A safer and more reliable, specific skin-test reagent than those existing today ([15, 29, 31]) could be prepared by mixing a limited and defined number of antigens from the leprosy bacillus.

The complete amino acid sequences of several proteins from *M. leprae* have been obtained ([4, 6, 17, 30]), opening the possibility for prediction, synthesis and assay of fragments of these proteins identified as T-cell epitopes. Among the antigenic proteins are the 65-kDa, 28-kDa, 28-kDa superoxide dismutase (SOD), and 18-kDa proteins recently proposed by World Health Organization (WHO) experts (Dr. D. B. Young, personal communication) to be named antigen number: 2L, 9L, 4L, and 12L, respectively.

In the present communication we describe the selection, synthesis, and assay of eight peptides corresponding to segments of these *M. leprae* proteins that have been shown to induce T-cell proliferation in order to assess their ability to induce DTH responses in guinea pigs. Additionally, two other putative T-cell epitopes, predicted using a computer program ([3]), were also studied. Guinea pigs were chosen as animal models since they have a central position in the biological characterization of myco-

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bacterial antigens, due to the high degree of DTH which develops subsequent to their immunization with mycobacteria. Preliminary results of this work were presented during the leprosy meeting held in Caracas (Estrada-G., *et al.*, 1991, unpublished).

MATERIAL AND METHODS

Synthetic peptides. Peptides were synthesized by the manual Merrifield's solid phase method ([19]), using BOC-amino acids and chloro-methyl polystyrene resins (Peninsula Laboratories, Belmont, California, U.S.A.). Only analytical grade reagents were used. Methylene chloride (Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A.) used as solvent and dicyclohexylcarbodiimide (Aldrich) as coupling reagent. Trifluoroacetic acid (TFA) was used for removing the protecting tert-butyloxycarbonyl groups (t-BOC) and triethyl ammonium for neutralization. After synthesis, the peptides were cleaved by fluorhydric acid (Mateson Co. Inc., obtained through RAEL International, San Pedro, California, U.S.A.) and normally recovered in 5% acetic acid, occasionally in 1 M KOH solution, depending on the solubility characteristics of the given peptide. Crude peptides were purified by high-performance liquid chromatography (HPLC) using a dual pump system (Beckman Instruments, Inc., Fullerton, California, U.S.A.) equipped with a Vydac (Hesperia, California, U.S.A.) octadecyl silane column (10 × 250 mm) under the following conditions: mobil phase A (0.12% TFA), B (0.1% TFA in acetonitrile) with a linear gradient from 0–60% B in 60 min and a flow rate of 2 ml/min. Further purification included rechromatography of peptides in the same column under isocratic gradient conditions. The amino acid composition of the peptides was confirmed by two methods: a) amino acid analysis of samples hydrolyzed with 6 N HCl at 110°C during 20 hr ([2]), and b) amino acid sequence by automatic Edman degradation ([9], see also Acknowledgment). All experiments with guinea pigs were performed using pure peptides.

Animals. Outbred, Hartley strain, female guinea pigs weighing 350 g–500 g were sensitized in groups of four animals, except for sensitization against synthetic peptides, in which case we used three animals per experimental group.

Mycobacteria. We used either ^{60}Cobalt-irradiated (2.5 Mrad) or autoclaved *M. leprae* bacilli, both derived from nine-banded armadillos. The first was provided by Dr. R. J. W. Rees, National Institute for Medical Research, Mill Hill, London. The second source of *M. leprae* was obtained from our armadillo farm. *M. bovis* BCG was provided by the Instituto Nacional de Higiene, Mexico; *M. phlei* was grown in Nutrient Tween and *M. tuberculosis* in Dubos media as previously described ([10]).

Soluble extracts. Soluble extracts were prepared for *M. leprae* (MLSE), *M. tuberculosis* (MTSE), *M. bovis* BCG (BCGSE), and *M. phlei* (MPSE). In all cases, the mycobacteria were first sonicated and then centrifuged at 12,500 × *g* for 15 min from which the supernatants were recovered as soluble extracts. Before skin testing, the soluble extracts were adjusted to a final protein concentration of 2 mg/ml.

Vehicles. All antigens used to elicit DTH were diluted in saline. Incomplete Freund's adjuvant (IFA) was the vehicle when MLSE and synthetic peptides were used for sensitization of guinea pigs. Saline was used as the vehicle for sensitization with *M. leprae* bacilli.

Sensitization. Two different schemes were used for the sensitization of the guinea pigs:

Scheme I. The animals were sensitized by injecting intradermally (i.d.) 5×10^9 of either ^{60}Co-irradiated or autoclaved *M. leprae* distributed in five different sites at the base of the neck. For soluble extracts (MLSE), a single dose containing 500 μg/guinea pig was chosen, according to Mehra, *et al.* ([18]). In experiments aimed at verifying specificity, the animals were immunized with 5×10^9 autoclaved *M. bovis* BCG bacilli.

Scheme II. Guinea pigs were given four injections each, the first three were weekly intramuscular (i.m.) injections of MLSE suspended in IFA. Each injection contained 500 μg of protein. All animals received a fourth i.d. injection 1 week after the final i.m. injection, following the protocol of Minden, *et al.* ([20]). Control groups of animals were also injected with IFA or saline.

Peptide immunogenicity. Groups of three animals were immunized with 500 μg/guinea pig of each of the synthetic peptides, i.m., in five different sites on both flanks. After

TABLE 1. *Proteins from* M. leprae *and corresponding amino acid sequence of fragments chosen for the synthesis of antigenic peptides.*

From 65-kDa protein (antigen 2L)	
P1 (from 195–219)	KGYISGYFVTDAERQEAVLEEPYIL
P2 (from 201–219)	YFVTDAERQEAVLEEPYIL
P3 (from 65–85)	YEKIGAELVKEVAKKTDDVAG
P4 (from 422–437)	PALDKLKLTGDEATGA
From 28-kDa protein (antigen 9L)	
P5 (from 24–48)	PCAYFLVYEPTASAKPAAKHYEFKQ
P6 (from 181–205)	VNDLLKVANQLGASQVMDLIKGVVM
From 18-kDa protein (antigen 12L)	
P7 (from 8–23)	FRELDRFAEQVLGSTA
P8 (from 131–148)	GNNGHQTINKTAHEIIDA
From 28 SOD protein (antigen 4L)	
[a]P9 (from 46–60)	KLDEARAKDDHSAIF
[a]P10 (from 100–117)	DETFGSFDKFRAQFSAAA

[a] These peptides were chosen with the MSEQ program ([2]).

4 weeks skin tests were performed in these animals using 250 μg and 100 μg of the synthetic peptides. P4 and P8 were tested only with 100 μg, due to sample limitations.

Skin tests. After sensitization the guinea pigs were tested with 50 μg, 5 μg, and 0.5 μg of soluble extracts from each sample of MLSE, MTSE, BCGSE, and MPSE. Synthetic peptides were tested at 250 μg, 100 μg and 0.05 μg for each group. All the antigens were injected in 0.1 ml intradermally (i.d.) on both flanks. Diameters of induration of the test sites were measured at 4 hr, 24 hr, and 48 hr. Unless otherwise indicated, the data represent readings taken 24 hr after skin testing. It is worth mentioning that in our experimental conditions the readings at 24 hr and 48 hr were very similar. Positive reactions were considered when indurations were equal to or greater than 5 mm. For experiments using *M. leprae*- or BCG-sensitized guinea pigs, groups of 12 animals were used. As negative controls for skin tests, 12 nonsensitized animals were used. The controls were chosen from the same batch of animals and were injected with saline alone or with IFA in order to rule out the possibility of background sensitization to mycobacterial antigens.

RESULTS

Synthetic peptides. Table 1 shows the amino acid sequences of the peptides synthesized. In Figure 1 are two examples of peptide separation by HPLC. Figure 1a shows the purification of P2, in which the asterisk indicates the peptide with the best fitted (expected) amino acid composition. This fraction was rechromatographed in isocratic gradient, showing a main purified component (Fig. 1b). The same is shown in Figure 1c and 1d for peptide P7. All ten peptides used in this work were similarly prepared and purified. An example of an amino acid analysis is given for peptide 6 (protein 28 kDa, residues 181–205), found (expected): Asp 3.89 (4), Glu 1.80 (2), Ser 1.05 (1), Gly 2.40 (2), Ala 2.20 (2), Val 4.85 (5), Met 2.10 (2), Ile 0.71 (1), Leu 3.50 (4), Lys 2.20 (2). These analyses were performed after a 20-hr hydrolysis in HCl, and integration was obtained by HPLC of carbamyl-derivatives of the amino acids. Some amino acids had variations between found/expected, but these are due to the method of analysis ([2]), as shown later by amino acid sequencing.

The peptides (P2, P5 and P7) which gave the most promising results in terms of specific DTH response, as discussed below, have had their sequences confirmed (see Acknowledgment). These peptides gave the exact expected sequence with a confidence interval higher than 95%. Hence, on this basis we are reasonably sure that our peptides correspond to the amino acid sequences chosen.

Comparison of sensitization of guinea pigs with intact *M. leprae* or MLSE. The initial series of studies compared the ability of in-

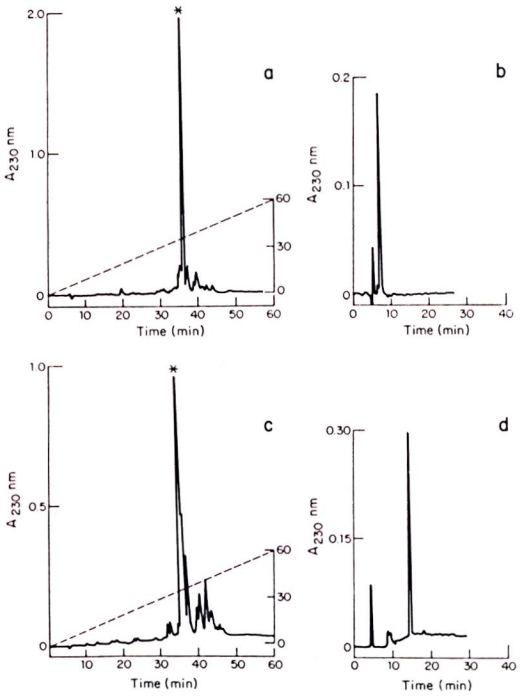

FIG. 1. HPLC separations of synthetic peptides. Fifty μg of peptide P2 (**a**) and 50 μg of peptide P7 (**c**) were fractionated in a C18 reverse-phase column with a linear gradient of 0.12% TFA (solution A) and 0.10% TFA in acetonitrile (solvent B) run during 1 hr from 0 to 60% B at a flow rate of 2 ml/min. Expected peptides are indicated by asterisks; **b** and **d** are rechromatographic separations of partially purified peptides P2 and P7, respectively, using isocratic gradients of 30% solvent B for **b** and 32% of solvent B for **d.**

tact *M. leprae* (5×10^9) in saline (scheme I) and that of MLSE (500 μg) in IFA (scheme II) to induce DTH responses in guinea pigs. To assess the degree and specificity of sensitization, animals immunized according to both schemes were tested intradermally with MLSE, MTSE, MPSE and BCGSE in amounts of 50 μg, 5 μg, and 0.5 μg 4 weeks after sensitization. All reactions were measured at 4 hr, 24 hr, and 48 hr. No reactions were present at 4 hr after injection. Additionally, histopathological observations were conducted in skin-test sites of guinea pigs injected with MLSE, BCGSE, and MPSE. Infiltrations were due mainly to mononuclear cells, with few polymorphonuclear cells (data not shown). Readings at 4 hr showed no inflammatory signs. Figure 2 shows that both sensitization schemes were equally effective in inducing strong DTH responses.

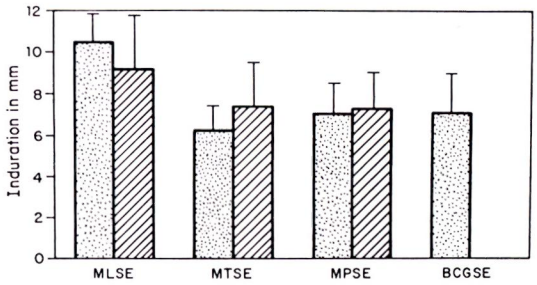

FIG. 2. Comparison of two different sensitization schemes. Groups of 16 animals were immunized with a single dose of 5×10^9 ^{60}Co-irradiated *M. leprae*/ guinea pig i.d. in 5 different sites (scheme I) or with 4 weekly injections of MLSE (scheme II). Four weeks after the sensitization, animals were tested i.d. with 5 μg/0.1ml of MLSE, MTSE, MPSE and BCGSE. Reactions were measured at 24 hr. Each bar represents the mean induration of 16 animals; ▩ = results with scheme I; ▨ = results with scheme II.

Our results indicate that animals sensitized with *M. leprae* are also sensitized against the other mycobacteria tested. As shown in Figure 2, both schemes of immunization induced crossreactive recognition of MTSE and MPSE. However, scheme I always gave stronger DTH responses to *M. leprae,* and the differences observed between these specific responses and those to MTSE and MPSE were greater than those observed with scheme II. BCG was only used to induce DTH in animals immunized with scheme I, and in all the animals tested we could also observe a crossreactive recognition. In scheme I, we sensitized with either ^{60}Co-*M. leprae,* autoclaved bacilli, or MLSE. No differences were observed among the different antigens (data not shown). Our experimental results indicate that the optimal procedure for sensitization of guinea pigs to *M. leprae* is a single intradermal, multiple-site inoculation of whole bacilli (irradiated or autoclaved) using saline as vehicle.

Induction of DTH with synthetic peptides. In order to assess the capability of synthetic peptides to induce a DTH response, groups of four animals were immunized with ^{60}Co-*M. leprae* using scheme I. After 4 weeks all peptides were injected i.d. using three different quantities of peptides: 250 μg, 100 μg, and 0.05 μg. MLSE and saline were also injected. In total, we used 12 *M. leprae*-sensitized guinea pigs. Table 2 shows the mean value of induration

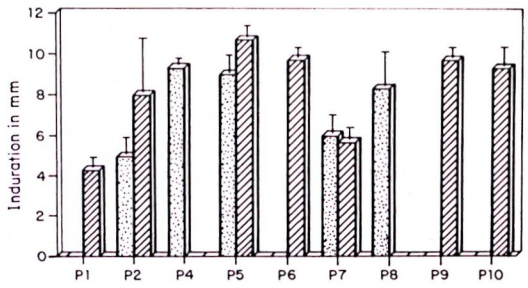

FIG. 3. DTH responses elicited by synthetic peptides in peptide-sensitized guinea pigs. Groups of 3 animals were sensitized with a single dose (500 μg) of each peptide. After 4 weeks each group was tested i.d. with MLSE (50 μg) and the corresponding homologous peptide (250 μg). Each bar is the mean induration of 3 animals read at 24 hr. Animals sensitized with P4 and P8 were tested at 100 μg (negative results). ▩ = mean value of induration for guinea pigs tested against MLSE; ▨ = mean value obtained by testing the homologous peptides. P3 was not used in these experiments.

(in mm) induced by the synthetic peptides. Positive reactions were considered to be those that gave an induration larger than or equal to 5 mm. Negative reactions were observed with both 250 μg and 100 μg of the peptides in this group of animals. It is important to note that all of the animals were sensitized to *M. leprae* because they demonstrate positive indurations when tested with MLSE. In order to verify specificity, 12 BCG-sensitized guinea pigs were used to test for DTH responses when challenged with all the peptides. The results are shown in Table 2. Only three of the synthetic peptides (P7, P10 and to a less extent P3) gave positive indurations.

Induction of DTH with MLSE in peptide-sensitized guinea pigs. Groups of three guinea pigs were immunized with a single does of 500 μg/guinea pig of each peptide emulsified in IFA. After 4 weeks each group was skin tested with the corresponding peptide using 250 μg and 100 μg, and two different quantities of MSLE—50 μg and 5 μg. No DTH reactions were observed when tested with 100 μg for any of the peptides (data not shown). The results for the induction of DTH with peptides at 250 μg doses are shown in Figure 3. Results with P4 and P8 at 250 μg were not included since they were only tested at 100 μg. Only those guinea pigs immunized with peptides P2, P4, P5, P7, and P8 gave positive DTH responses when skin tested with MLSE.

None of the guinea pigs sensitized with saline or IFA alone (control group) gave positive DTHs when tested with MLSE or any of the peptides. This confirmed that our experimental animals were not previously sensitized with mycobacterial antigens (MLSE, BCGSE, and MPSE).

DISCUSSION

Lepromin ([31]) and leprosin A ([15, 29]) are two preparations widely used as skin tests for leprosy ([7]). Both are relatively crude preparations made of heat-killed *M. leprae* or sonicates of irradiated and heat-killed bacilli. Both offer some disadvantages as skin tests ([26]). One important problem is the need for growing large quantities of bacilli which, in turn, are very selective because *M. leprae* only grows in certain animals (for example, the nine-banded armadillo). Both preparations are very complex mixtures of components imposing technical problems for standardization of the different batches of processed antigens. They are also very time-consuming processes and present a possibility of contamination with infectious organisms ([26]). Thus, the possibility of obtaining a simpler, reliable, specific and very well-defined antigen preparation for skin testing in leprosy is highly desirable. Alternative procedures are currently pursued by means of DNA recombinant techniques ([14]) and by means of synthetically prepared peptides, as we describe here.

It seems clear that diagnostic and epidemiological studies of leprosy would benefit greatly from the availability of species-specific skin-test reagents. A possible specific skin-test reagent could be prepared with a limited and defined number of antigens from the leprosy bacillus. Now that new technologies have been applied to the study of *M. leprae* antigens, evaluation of the contribution of individual antigens to immunity became feasible. Of major interest is the fact that both T and B epitopes have been mapped, allowing researchers to synthesize and test them in different immunological systems.

In this communication we report the synthesis of ten peptides containing 15 to 25

TABLE 2. *Induction of DTH responses with synthetic peptides in* M. leprae*-sensitized guinea pigs.*[a]

Synthetic peptides[b]	Positive DTH/Total (percentage)[c]		Mean induration ± S.D.[d]	
	M. leprae	BCG	*M. leprae*	BCG
P1	2/12 (17)	0/12 (0)	10.0 ± 0.0	0.0
P2	5/12 (42)	0/12 (0)	8.0 ± 3.4	0.0
P3	7/12 (58)	1/12 (8)	6.6 ± 1.8	5.0
P4	3/12 (25)	0/12 (0)	5.7 ± 0.9	0.0
P5	3/12 (25)	0/12 (0)	6.3 ± 1.9	0.0
P6	3/12 (25)	0/12 (0)	6.7 ± 2.4	0.0
P7	4/12 (33)	3/12 (25)	7.8 ± 2.8	5.0 ± 0.0
P8	5/12 (42)	0/12 (0)	7.2 ± 1.8	0.0
P9	3/12 (25)	0/12 (0)	6.3 ± 1.9	0.0
P10	3/12 (25)	3/12 (25)	7.7 ± 2.5	5.3 ± 0.5

[a] Animals were sensitized following scheme I with 5×10^9 autoclaved *M. leprae* or BCG bacilli (see Material and Methods). Positive responses were considered when induration was equal to or greater than 5 mm.
[b] DTH response induced with 0.05 μg of peptide.
[c] Number of guinea pigs with positive induration over total assayed (percentage of positives).
[d] Read 24 hr after injection.

amino acid residues which correspond to segments of the amino acid sequence of proteins from *M. leprae*. Purification of the peptides was obtained through HPLC in a C18 reverse-phase column eluted with an acetonitrile linear gradient followed by a second separation under isocratic conditions. Amino acid analysis of the separated peptides indicated the probable composition of the various peptides. Three of the most promising ones (P2, P5, P7) have had their amino acid sequence determined, confirming the results obtained by amino acid analysis. The purified peptides were used in the guinea pig model to assess delayed type hypersensitivity.

Two different immunization protocols ([17, 20]) were used to determine optimal conditions for the sensitization of guinea pigs with *M. leprae* antigens. Our results indicate that both schemes produce significant sensitization either specific with MLSE or crossreactive with MTSE and MPSE (Fig. 2). For continuation of our studies we have decided to use scheme I, since scheme II uses large amounts of antigen for sensitization of the animals and consumes a considerable amount of peptide.

Intact bacilli or soluble extracts induced the same degree of sensitization (data not shown), when using scheme I ([17]).

For initial testing of the ability of synthetic peptides to induce DTH responses in *M. leprae*-sensitized guinea pigs, two quantities of the peptides were used—250 μg and 100 μg. Contrary to what we expected, no indurations were obtained when challenging immunized guinea pigs with such amounts of peptides. Since these quantities of peptides were used previously for the induction of DTH in a similar system ([20]), we decided to investigate if the peptides were immunogenic by themselves. We have sensitized guinea pigs with each of the peptides and DTH responses were induced either with the homologous peptide or MLSE. Figure 3 shows that all peptides, except P1 (less than 5-mm induration), P4 and P8, gave strong DTH responses with the homologous peptide used at 250 μg. However, only P2, P4, P5, P7, and P8 were capable of sensitizing the guinea pigs to MLSE. In the case of P4 and P8, DTH responses were tested only with 100 μg, which might explain the lack of induration with these probes.

Since the lack of DTH responses induced by the peptides was not due to their lack of immunogenicity, lower concentrations of peptides were used subsequently to test induction of DTHs. As previously described by other research groups ([13]), proteins in amounts as small as nanograms were capable of inducing DTH. As shown in Table 2, induration was obtained after 24 hr (confirmed after 48 hr), when the peptides were tested at 0.05 μg (50 ng) in *M. leprae*-sen-

sitized guinea pigs. Peptides P2 and P3 (from 65 kDa), and P8 (from 18 kDa) were able to induce positive DTH in more than 40% of the animals; whereas other peptides, like P1, were able to induce DTH in 2 out of 12 guinea pigs. It is important to point out that all animals were strongly sensitized to *M. leprae,* thus eliminating the possibility of the lack of reactivity to *M. leprae* antigens. The variability in positive reactions is not surprising, because we have used an outbred strain of animals (Hartley) with a heterogeneous genetic background. More difficult to understand, however, is the fact that P1 did not induce similar positive results as the related peptide P2. The amino acid sequence of P2, which induced DTH in five animals, is included in the sequence of P1. It is tempting to speculate that the shorter peptide (P2) probably does not need to be processed by antigen-presenting cells; whereas an internal epitope of P1 is lost by cleavage when P1 is processed by the cells.

Also in Table 2, except for P7 and P10, all of the other peptides seem to be specific for *M. leprae* although they vary in immunogenicity. Induction of positive DTH responses varies from 17% to 58% of the animals assayed. P3 in particular (the peptide fragment corresponding to the sequence 65–85 of the 65-kDa protein) seems to be promising, with the highest percentage of positiveness among *M. leprae*-sensitized guinea pigs.

Of particular interest was the fact that animals within the same group did not responded equally well to the testing with a particular peptide. These findings again might reflect differences in terms of the genetic background of the outbred strain of guinea pigs we have used ([11]). The evidence for the implication of genetic factors in the host response to mycobacterial infection, as suggested by Skamene, has been reviewed ([27]). More recently the antibody response following immunization with a culture filtrate of *M. tuberculosis* was studied in 11 strains of inbred mice ([16]). A clear association of antibody response to their H-2 phenotype was demonstrated. This is also the case for CMI responses, as demonstrated by Haslov, *et al.* ([13]) who studied the immunological activity of five defined antigens of *M. tuberculosis* in seven inbred strains of guinea pigs. Thus, because we have used an outbred strain of guinea pigs, it is not surprising to have obtained a different pattern of DTH response. Concerning the fact of lack of reactivity when challenging immunized animals with high quantities of peptides (250 μg and 100 μg), we surmise that blockage of receptors occurred due to an excessive concentration of the ligands. This has been observed in other systems ([22]).

Skin-test experiments described in the present communication differ from previous reports ([20]) in that we have used single synthetic peptides (not polymerized or conjugated) in order to either induce DTH responses or to sensitize guinea pigs.

Because the ability to raise an immune response to a single mycobacterial antigen can be expected to be under genetic control ([13, 16, 27]), it will be important to assess this genetic factor with regard to the immunological activity of purified mycobacterial antigens or synthetic peptides. Future work should include the use of both inbred and outbred strains of guinea pigs.

SUMMARY

In this work we report the synthesis of 10 peptides (P1–P10) corresponding to one or several segments of the amino acid sequence of proteins from *Mycobacterium leprae*: 65 kDa, 28 kDa, 18 kDa, and 28 kDa superoxide dismutase, recently renamed antigens 2L, 9L, 12L, and 14L, respectively. They were assayed in the guinea pig model for the induction of a delayed-type hypersensitivity response in *M. leprae* and BCG-sensitized animals. To sensitize the animals two schemes were used: either a single dose of 5×10^9 irradiated or autoclaved whole bacilli, or four weekly intramuscular injections each containing 500 μg of soluble extract of *M. leprae* (MLSE) in incomplete Freund's adjuvant. Because the second scheme used far too much antigen, we decided to use the first scheme for the experiments we report here. DTH reactions of sensitized animals were induced after 30 days with intradermal injections of 5 μg of MLSE and with each of the 10 peptides at three different concentrations: 250 μg, 100 μg, and 0.05 μg. All *M. leprae*-sensitized guinea pigs gave indurations of 10 mm or more with MLSE, which indicates

that the animals were sensitized. None of them gave DTH indurations with 250 μg or 100 μg, but some of them had positive DTH reactions with the 0.05 μg doses of the synthetic peptides. This is most likely due to the fact that we have used an outbred strain of guinea pigs. The peptides were also tested at 0.05 μg in animals sensitized with BCG. P7 and P10 seem to be nonspecific peptides; the remaining peptides only induced DTH in the *M. leprae*-sensitized guinea pigs. P3 (segments 65–85 of the 65-kDa protein) induced a positive DTH in 58% of the tested animals.

In other experiments, guinea pigs were sensitized with a single injection (500 μg) of each of the synthetic peptides. All animals, except those sensitized with P4 and P8, had positive DTH responses when the homologous peptide was used. Those sensitized with P2, P4, P5, P7, and P8 were able to produce indurations when MLSE was used for the induction of the DTH reaction.

RESUMEN

En este trabajo reportamos la síntesis de 10 péptidos (P1–P10) correspondientes a uno o a varios segmentos de las proteínas del *Mycobacterium leprae*, 65 kDa, 28 kDa, 18 kDa, y 28 kDa (superoxido dismutasa), recientemente renombradas como antígenos 2L, 9L, 12L y 14L, respectivamente. Los péptidos se usaron para inducir respuestas de hipersensibilidad tardía (DTH) en cobayos sensibilizados con *M. leprae* o con BCG. Los animales se sensibilizaron con una sóla dosis de 5×10^9 bacilos completos irradiados o autoclaveados, o con cuatro inyecciones intramusculares de 500 μg de extracto soluble de *M. leprae* (MLSE) en adyuvante incompleto de Freund. Debido a que el segundo esquema requirió demasiado antígeno, decidimos usar el primero para los experimentos reportados aquí. Las reacciones de DTH en los animales sensibilizados se indujeron después de 30 días con inyecciones intradérmicas de 5 μg de MLSE o con cada uno de los 10 péptidos a 3 diferentes concentraciones: 250 μg, 100 μg, y 0.05 μg. Todos los cobayos sensibilizados con *M. leprae* dieron induraciones de 10 mm o más con el MLSE. Ninguno de ellos dio induraciones de DTH con 250 μg o con 100 μg de los péptidos sintéticos, aunque algunos de ellos dieron reacciones positivas con la dosis de 0.05 μg. Quizá esto se debió a que se usaron cepas abiertas de cobayos. Los péptidos también fueron probados a la concentración de 0.05 μg en los animales sensibilizados con BCG. P7 y P10 parecieron ser péptidos no específicos; el resto de los péptidos sólo indujeron reacciones DTH en los animales sensibilizados con *M. leprae*. P3 (segmentos 65–85 de la proteína 65-KDa) indugo una DTH positiva en el 58% de los animales probados.

En otros experimentos, los cobayos fueron sensibilizados con una sola inyección (500 μg) de cada péptido sintético. Todos los animales, excepto aquellos sensibilizados con P4 y P8, dieron respuestas DTH positivas cuando se usaron los péptidos homólogos. Aquellos sensibilizados con P2, P4, P5, P7, y P8 fueron capaces de producir induraciones cuando se usó MLSE en la inducción de la reacción de DTH.

RÉSUMÉ

Dans ce travail nous rapportons la synthèse de 10 peptides (P1–P10) correspondant à 1 ou plusieurs segments de séquences d'acides aminés de protéines de *Mycobacterium leprae*: 65 kDa, 28 kDa, 18 kDa, et al dismutase superoxyde de 28 kDa récemment renommés les antigènes 2L, 9L, 12L, et 14L, respectivement. Ils ont été testés chez le cobaye pour la production d'une réponse d'hypersensibilité de type retardé chez des animaux sensibilisés au *M. leprae* et au BCG. Deux protocoles distincts ont été suivis pour sensibiliser les animaux: soit une dose unique de 5×10^9 bacilles entiers irradiés ou autoclavés, soit 4 injections intramusculaires hebdomadaires contenant chacune 500 μg d'extrait soluble de *M. leprae* (MLSE) dans l'adjuvant incomplet de Freund. Du fait que le second protocole utilisait beaucoup trop d'antigènes, nous avons décidé d'utiliser le premier protocole pour les expérimentations que nous rapportons ici. Des réactions d'hypersensibilité retardée chez les animaux sensibilisés ont été produites après 30 jours avec des injections intradermiques de 5 μg de MLSE et avec chacun des 10 peptides à 3 concentrations différentes: 250 μg, 100 μg, et 0,05 μg. Tous les cobayes sensibilisés au *M. leprae* ont donné des indurations de 10 mm ou plus avec MLSE, ce qui indique que les animaux étaient sensibilisés. Aucun d'entre eux ne donna des indurations d'hypersensibilité retardée avec 250 μg ou 100 μg, mais certains d'entre eux avaient des réactions d'hypersensibilité retardée avec des doses de 0,05 μg des peptides synthétiques. Ceci est vraisemblablement dû au fait que nous avons utilisé une souche de cobaye d'élevage. Les peptides ont également été testés à 0,05 μg chez des animaux sensibilisés au BCG. Les peptides P7 et P10 semblent ne pas être spécifiques; les peptides restants n'induisaient une hypersensibilité retardée que chez les cobayes sensibilisés à *M. leprae*. Le peptide P3 (les segments 65–85 de la protéine de 65 k-Da) induisait und hypersensibilité retardée positive chez 58% des animaux testés. Dans d'autres expérimentations, des cobayes ont été sensibilisés avec une injection unique (500 μg) de chacun des peptides synthétiques. Tous les animaux à l'exception de ceux sensibilisés avec les peptides P4 et P8 avaient des réponses d'hypersensibilité retardée positive quand le peptide homologue était utilisé. Ceux sensibilisés avec P2, P4, P5, P7, et P8 étaient capables de produire des

indurations quand MLSE était utilisé pour produire la réaction d'hypersensibilité retardée.

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