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Lack of Tuberculin Activity of Synthetic Peptides

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We synthesized an octapeptide, H-Asp-Gly-Gly-Ser-Glu-Ser-Glu-Gly-OH, and a hexadecapeptide, H-Asp-Gly-Gly-Ser-Glu-Ser-Glu-Gly-Lys-Asn-Gly-Ser-Gln-Met-Arg-Leu-OH, which corresponded to amino acids 61 to 68 and 61 to 76, respectively, of the amino acid sequence of a crystalline protein reported to be tuberculin active. Authenticity and purity of the synthesized peptides were confirmed by high-pressure liquid chromatography, amino acid analysis, mass spectrometry, and protein sequencer analysis. Tuberculin activity of the synthesized peptides was examined in guinea pigs sensitized with *Mycobacterium tuberculosis* or *Mycobacterium bovis* BCG and in tuberculin-positive healthy humans. Neither the octa- nor the hexadecapeptide was as active as tuberculin skin-test antigen.

Purified protein derivative (PPD) is presently the most widely used tuberculin-active preparation, but it is far from homogeneous, being a complex mixture of proteins. Many attempts have been made to obtain a more potent and more homogeneous tuberculin-active preparation. In 1973, Kuwabara (3, 4) reported crystallization of a tuberculin-active protein which was purified from culture filtrates of *Mycobacterium tuberculosis* Aoyama B. He determined the amino acid sequence of this crystalline protein and reported that one of the tryptic digests (Asn70-Gly-Ser-Gln-Met-Arg75) of the protein was tuberculin active. Later, in 1980 and 1983, Šavrda (5, 6) synthesized three peptides according to the amino acid sequence proposed by Kuwabara and reported that the hexadecapeptide showed tuberculin activity.

We synthesized octa- and hexadecapeptides, the same as those synthesized by Šavrda, and examined their tuberculin activities in guinea pigs and humans.

MATERIALS AND METHODS

Synthesis of H-Asp-Gly-Gly-Ser-Glu-Ser-Glu-Gly-OH (Syn-P-8) by the solid-phase method. The octapeptide was synthesized by the solid-phase method of Ikota et al. (1, 2). An ethanol solution of *t*-butoxycarbonyl (BOC)-Gly-OH (1.10 g/8 ml) was diluted with water (2 ml), and the pH was adjusted to 7.0 with 10% CS_2CO_3. The solvent was evaporated under reduced pressure, and the residue was dissolved in benzene (5 ml), and then the solvent was evaporated again. This procedure was repeated three times. After drying over P_2O_5 in vacuo, the final residue was dissolved in dimethylformamide (DMF) (72 ml), chloromethyl resin (12 g) was added, and the mixture was treated at 50°C for 24 h with gentle agitation. The resin was collected by filtration and washed with DMF, DMF-H_2O (9:1), and DMF and ethanol (60 ml each) successively and then dried. The yield of BOC-Gly-resin was 12.3 g (BOC-Gly, 0.301 mmol/g as determined by Porath's method [1, 2]).

With BOC-Gly-resin (4.0 g) as the starting material, Syn-P-8 was synthesized with a solid-phase synthesis apparatus by the procedure shown in Table 1. Coupling of BOC-amino acids to the glycyl resin at step 8 in Table 1 was carried out successively under the conditions shown in Table 2.

After the completion of the coupling, trifluoroacetic acid (TFA) (84.4 ml), thioanisol (9.88 ml), and trifluoromethanesulfonic acid (7.44 ml) were added to the resin, and the mixture was stirred for 1 h at 0°C. The resin was filtered off and washed with TFA three times (24 ml each time). The filtrate and the washings were combined and chilled to 0°C, and ethyl ether (200 ml) was added. Crystalline precipitates thus formed were collected by centrifugation and washed with ethyl ether. After treatment with an IRA-68 column

TABLE 1. General procedure for diethyl phosphorocyanidate (DEPC) coupling in solid-phase peptide synthesis.

Step	Reagents and operations[a]	Mix time (min)
1	Washing with CH_2Cl_2 (three times)	5 each
2	Treatment with 50% TFA in CH_2Cl_2	30
3	Washing with CH_2Cl_2 (three times)	5 each
4	Washing with ethanol (three times)	5 each
5	Washing with DMF (three times)	
6	Treatment with 10 equivalents of triethylamine in DMF	10
7	Washing with DMF (six times)	5 each
8	Treatment with BOC-amino acid in DMF[b]	5
9	Treatment with DEPC[c] in DMF	5
10	Treatment with triethylamine[c] in DMF	120
11	Washing with DMF (three times)	5 each
12	Washing with ethanol (three times)	5 each

[a] The amount of each solvent used was 25 ml for Syn-P-8 and 70 ml for Syn-P-16-S.
[b] See Tables 2 and 3.
[c] Amounts of DEPC and triethylamine at steps 9 and 10, respectively, were equivalent to the amount of BOC-amino acid in the preceding step 8.

TABLE 2. Conditions for DEPC coupling in the solid-phase synthesis of Syn-P-8

Step	Reactant[a]	Equivalent	Reaction time (h)[b]
1	BOC-Glu (OBzl)-OH	3	2
2	BOC-Ser (Bzl)-OH	3	2
3	BOC-Glu (OBzl)-OH	4	2
4	BOC-Ser (Bzl)-OH	4	2
5	BOC-Gly-OH	5	2
6	BOC-Gly-OH	5	2
7	BOC-Asp (OBzl)-OH	6	2

[a] Bzl, Benzyl.
[b] The reaction was carried out 0°C for the initial 30 min and then at room temperature.

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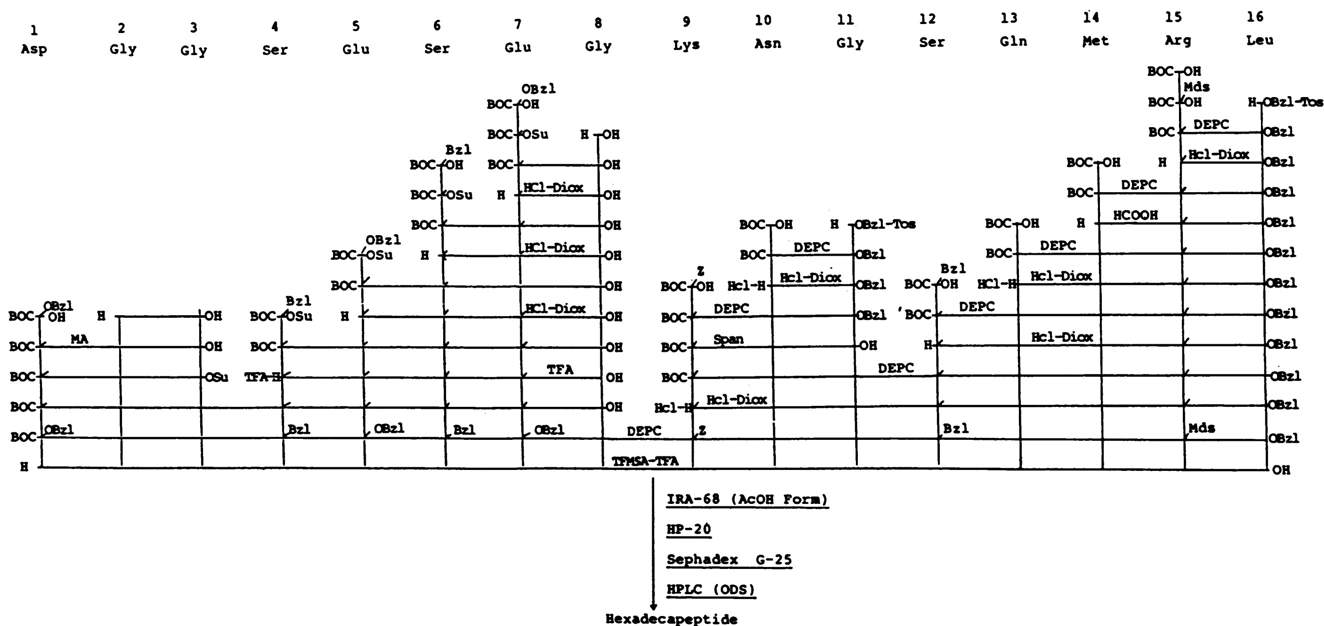

FIG. 1. Synthesis scheme for Syn-16-P-L. Bzl, Benzyl; Z, benzyloxycarbonyl; DEPC, diethyl phosphorocyanidate; Tos, tosyl(*p*-toluene sulfonyl); TFMSA, trifluoromethanesulfonic acid; Mds, *p*-methoxy-*O*-*O*-dimethylbenzene sulfonyl; Osu, succimido-oxy; AcOH, acetic acid; ODS, Zorbax ODS; Diox, dioxane.

(acetate form; 10 by 200 mm; 1 M acetate), the eluate was lyophilized. The crude Syn-P-8 thus obtained was further purified by column chromatography with Diaion HP-20 (3.3 by 100 mm) and Sephadex G-15 (26 by 860 mm), and then lyophilized. The yield was 288 mg.

Synthesis of H-Asp-Gly-Gly-Ser-Glu-Ser-Glu-Gly-Lys-Asn-Gly-Ser-Gln-Met-Arg-Leu-OH (Syn-P-16-S) by the solid-phase method. BOC-Leu-resin was prepared with BOC-Leu-OH · H_2O (1.745 g), chloromethyl resin (12.0 g), and DMF (70 ml) in the same way as BOC-Gly-resin described above. The yield was 13.2 g (BOC-Leu, 0.391 mmol/g as determined by Porath's method [1, 2]).

With BOC-Leu-resin (7.0 g) as the starting material, Syn-P-16-S was synthesized by the same procedure as

TABLE 3. Conditions for DEPC coupling in the solid-phase synthesis of Syn-P-16-S

Step	Reactant[a]	Equivalent		Reaction time (h)	
		Coupling 1	Coupling 2[b]	Coupling 1	Coupling 2[b]
1	BOC-Arg (Mts)-OH	3		2	
2	BOC-Met-OH	3		2	
3	BOC-Gln-OH	4		2	
4	BOC-Ser (Bzl)-OH	4		2	
5	BOC-Gly-OH	5		3	
6	BOC-Asn-OH	5		3	
7	BOC-Lys (Z)-OH	3	3	2	2
8	BOC-Gly-OH	3	3	2	2
9	BOC-Glu (OBzl)-OH	4	3	2	2
10	BOC-Ser (Bzl)-OH	4	3	2	2
11	BOC-Glu (OBzl)-OH	4	3	3	2
12	BOC-Ser (Bzl)-OH	4	3	3	2
13	BOC-Gly-OH	4	3	4	2
14	BOC-Gly-OH	4	3	4	2
15	BOC-Asp (OBzl)-OH	4	3	5	3

[a] Mts, 2-Mesitylenesulfonyl.
[b] Double coupling was done only at and after step 7.

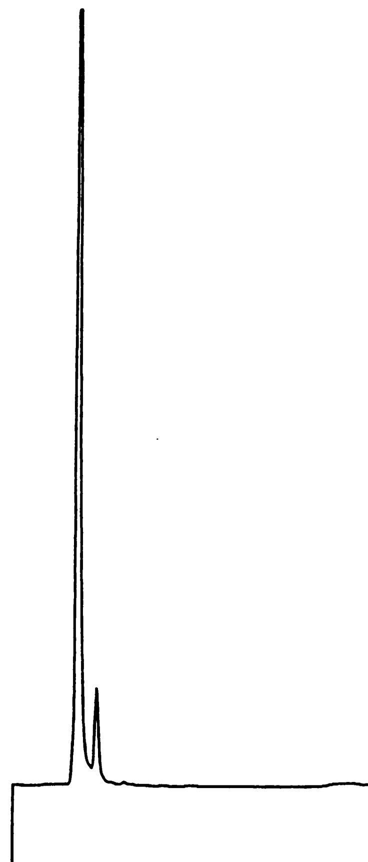

FIG. 2. HPLC of Syn-P-8. Sample, Lot no. K 5905A; column, Zorbax ODS (4.6 by 250 mm); eluent, CH_3CN–12.5 mM KH_2PO_4 (1.25:98.75); flow rate, 0.5 ml/min; detection, 210 nm. Syn-P-8 was eluted with a retention time of 5.09 min. Purity, 91.4%.

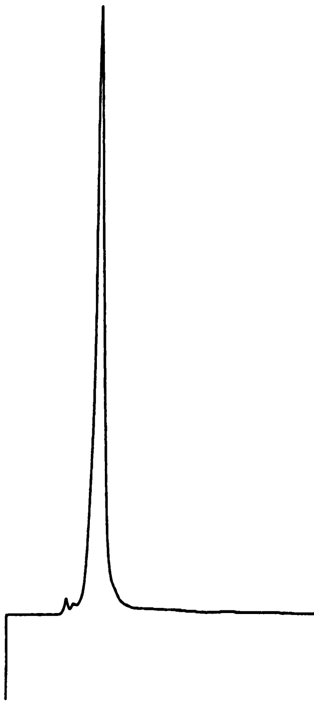

FIG. 3. HPLC of Syn-P-16-S. Sample, Lot no. K 5905A; column, Zorbax ODS (4.6 by 250 mm); eluent, CH_3CN-H_2O-TFA (30:70:0.08); detection, 210 nm. Syn-P-16-S was eluted with a retention time of 7.13 min. Purity, 99.0%.

Syn-P-8 shown in Table 1. BOC-amino acids were successively coupled with the leucyl-resin under the conditions shown in Table 3. After the coupling with BOC-Lys (benzyloxycarbonyl)-OH (step 7), the resin was treated with acetic anhydride (10 equivalents) and triethylamine (3 equivalents) in DMF (40 ml) at room temperature for 1 h. After the completion of all coupling procedures, the resin was washed with CH_2Cl_2 three times (40 ml each time), and then TFA (123 ml), thioanisol (14.46 ml), and trifluoromethanesulfonic acid (10.90 ml) were added. The mixture was stirred at 0°C for 1 h and at room temperature for an additional 1 h. The resin was collected by filtration and washed with TFA three times (30, 20, and 15 ml, respectively). The filtrate and the washings were combined and chilled to 0°C, and then ethyl ether was added under stirring. Crystalline precipitates thus formed were collected by centrifugation, washed with ethyl ether, dissolved in 1 M acetic acid (10 ml), and then treated with IRA-68 (acetate form; 10 by 500 mm; 1 M acetic acid)

TABLE 4. Amino acid analyses of Syn-P-16-S

Amino acid	Calculated	Found by:	
		Acid hydrolysis	Aminopeptidase digest
Asp	1	2.07	0.97
Asn	1		1.09
Ser	3	2.58	3.00
Glu	2	3.05	1.97
Gln	1		0.98
Gly	4	3.87	3.83
Met	1	1.02	1.03
Leu	1	1.06	1.08
Lys	1	0.91	0.98
Arg	1	1.00	1.03

and Diaion HP-20 (3.3 by 170 mm; H_2O). The eluate was lyophilized and further purified by gel filtration with Sephadex G-15 (26 by 860 mm) and Sephadex G-25 (superfine; 26 by 860 mm) and then lyophilized. The yield was 1.6168 g.

Synthesis of the hexadecapeptide (Syn-P-16-L) by the liquid-phase method. The hexadecapeptide was also synthesized by the conventional liquid-phase (solution) method as illustrated schematically in Fig. 1.

Characterization of the products. Amino acid analyses were carried out with a reaction-type Hitachi 638-50 high-pressure liquid chromatograph. Mass spectra were measured with JMS-OISG 2, JMS-D 300, and Hitachi M-80A mass spectrometers. Amino acid sequencing analyses were performed with the Applied Biosystems model 470-A gas-phase protein sequencer. High-pressure liquid chromatography (HPLC) was carried out with a Hitachi 655 high-pressure liquid chromatograph.

Sensitization and skin testing of guinea pigs. *M. tuberculosis* H37Rv and *Mycobacterium bovis* BCG (Tokyo) were cultured on Sauton synthetic liquid medium for 3 weeks at 37°C. Harvested cells were suspended in saline by shaking with glass beads. A portion of each bacterial suspension was heated in a boiling water bath for 30 min and, after cooling to room temperature, was emulsified with equal amount of Freund incomplete adjuvant (FIA).

Female guinea pigs (Hartley strain, 300 to 400 g) were sensitized by an intramuscular injection of either saline suspension of live bacteria or water-in-oil emulsion of heat-killed bacteria, either *M. tuberculosis* or *M. bovis* BCG. After 3 or 4 weeks, animals were skin tested by injecting PPD (2.5 or 5.0 μg in 0.1 ml) and synthetic peptides (50 μg in 0.1 ml) intradermally. Diameters of induration were measured 24 and 48 h after the injections.

Skin testing of humans. PPD (0.05 μg in 0.1 ml) and

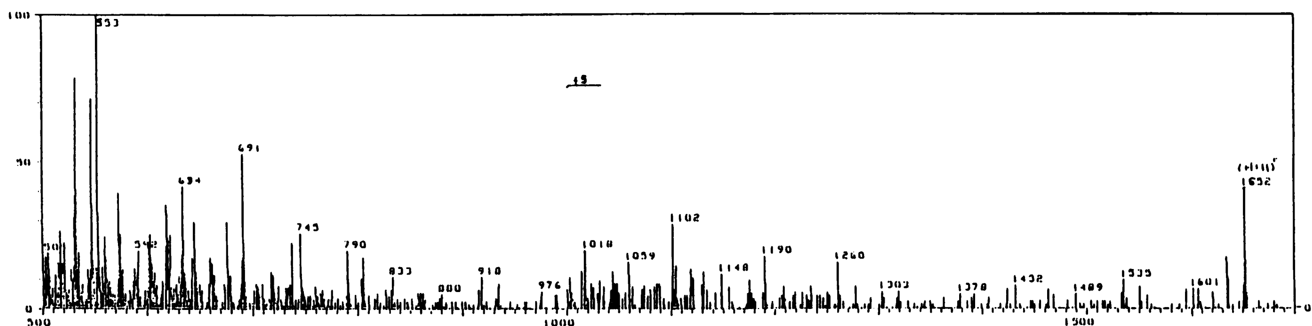

FIG. 4. Secondary-ion mass spectrometry of Syn-P-16-S.

TABLE 5. Skin reactions with Syn-P-16-S, Syn-P-8, and PPD in sensitized guinea pigs

Sensitization	Animal no.	Diam (mm) of induration[a]					
		PPD (5 μg)		Syn-P-8 (50 μg)		Syn-P-16-S (50 μg)	
		24 h	48 h	24 h	48 h	24 h	48 h
None	1	4 by 4	2 by 3	2 by 2	0	0	0
	2	3 by 3	3 by 3	0	0	0	0
	3	4 by 4	3 by 3	4 by 4	0	2 by 2	0
Killed BCG (0.5 mg) + FIA	1	24 by 15	31 by 16*	3 by 2	0	3 by 2	0
	2	21 by 14	37 by 28*	2 by 3	0	0	0
Live BCG (2 mg)	1	21 by 15	18 by 17	0	0	0	0
	2	0	14 by 13	0	0	0	0
	3	0	19 by 16	0	0	0	0
Killed H37Rv (0.25 mg) + FIA	1	30 by 22	25 by 26	0	0	0	0
	2	36 by 22	37 by 29	0	0	0	0
Live H37Rv (1 mg)	1	26 by 23	25 by 25	3 by 4	0	0	0
	2	45 by 35	35 by 29*	0	0	0	0
	3	47 by 22	22 by 20	0	0	0	0

[a] *, Accompanied by ulcer. Skin tests were carried out 4 weeks after the sensitization.

Syn-P-16-S (5 μg in 0.1 ml) were injected intradermally on each forearm of two healthy humans (one female and one male), and diameters of induration and erythema were measured after 3, 24, and 48 h.

Chemicals. Chloromethyl resin (Cl, 0.87 mmol/g; 200/400 mesh; divinylbenzene [DVB] 1%) and aminopeptidase M were purchased from the Protein Research Foundation, Suita-shi, Osaka, Japan. FIA and PPD were obtained from Difco Laboratories, Detroit, Mich., and Japan BCG Laboratory, Chiyoda-ku, Tokyo, Japan, respectively. Unless otherwise stated, all optically active amino acids are of L configuration.

RESULTS

Characteristics of the synthesized peptides. (i) Syn-P-8. The HPLC pattern of Syn-P-8 revealed a satisfactorily high purity (Fig. 2). Amino acid analysis of an acid hydrolysate of Syn-P-8 gave the following: Asp 1.10:Glu 2.00:Gly 2.80:Ser 2.04, which well accorded with the expected ratio of 1:2:3:2. The characteristic peak was observed at m/z = 737 ($[M + H]^+$) in a field-desorption mass spectrum of Syn-P-8. The primary structure of Syn-P-8 was determined with a gas-phase protein sequencer to be H-Asp-Gly-Gly-Ser-Glu-Ser-Glu-Gly-OH.

(ii) Syn-P-16-S. Reverse-phase HPLC of Syn-P-16-S revealed a high purity (Fig. 3). The main peak fraction was collected from the HPLC column and used for further chemical analyses and biological assay. Results of amino acid analyses of Syn-P-16-S after acid hydrolysis with 6 N HCl and enzymatic digestion with aminopeptidase M were in good agreement with the expected value (Table 4). The characteristic signal was observed at m/z = 1,652 ($[M + H]^+$) in secondary-ion mass spectrometry (Fig. 4). The primary structure of Syn-P-16-S was examined with the gas-phase protein sequencer and confirmed to be H-Asp-Gly-Gly-Ser-Glu-Ser-Glu-Gly-Lys-Asn-Gly-Ser-Gln-Met-Arg-Leu-OH.

(iii) Syn-P-16-L. Reverse-phase HPLC of Syn-P-16-L also revealed high purity, and the main peak fractions were collected from HPLC and used for further chemical analyses and biological assay. Amino acid analysis of an acid hydrolysate of Syn-P-16-L gave the following: Arg 0.91:Asp 2.16:Glu 3.17:Gly 3.69:Leu 1.02:Lys 1.06:Met 0.90:Ser 3.00.

TABLE 6. Skin reactions with Syn-P-16-S, Syn-P-16-L, and PPD in sensitized guinea pigs

Sensitization	Animal no.	Diam (mm) of induration[a]					
		PPD (2.5 μg)		Syn-P-16-S (50 μg)		Syn-P-16-L (50 μg)	
		24 h	48 h	24 h	48 h	24 h	48 h
Killed BCG (2.5 mg) + FIA	1	40 by 28*	32 by 18*	0	0	0	0
	2	28 by 16	18 by 15	3 by 3	5 by 2	2 by 2	3 by 3
	3	32 by 22	29 by 18*	0	0	0	0
Live BCG (5 mg)	1	26 by 18	22 by 18	3 by 3	2 by 2	2 by 2	1 by 1
	2	28 by 18	24 by 17	8 by 8	3 by 3	7 by 7	3 by 3
Killed H37Rv (0.25 mg) + FIA	1	28 by 17	19 by 12	3 by 3	2 by 2	4 by 4	2 by 2
	2	30 by 17	18 by 13	5 by 5	3 by 3	3 by 3	3 by 3
	3	27 by 18	17 by 12	2 by 2	2 by 2	3 by 3	2 by 2
Live H37Rv (0.5 mg)	1	38 by 16*	27 by 12*	0	0	0	0
	2	38 by 27*	27 by 18*	5 by 5	2 by 2	2 by 3	0

[a] *, Accompanied by bleeding. Skin tests were carried out 25 days after the sensitization.

TABLE 7. Skin reaction with Syn-P-16-S in PPD-positive healthy humans

Subject	Sex	Skin reaction antigen (μg)	Skin reaction[a]		
			3 h	24 h	48 h
1	Female	PPD (0.05)	0/14 by 11	25 by 22/36 by 28	25 by 25/40 by 38
		Syn-P-16-S (5)	0/7 by 6**	0/5 by 5	0/5 by 5
2	Male	PPD (0.05)	0/10 by 10	19 by 19/29 by 26	18 by 18*/54 by 39
		Syn-P-16-S (5)	8 by 10**/12 by 13	0/9 by 9	0/9 by 9

[a] Figures show the diameters (millimeters) of induration/erythema. *, Accompanied by a blister; **, accompanied by pain and itching.

The characteristic signal was observed at m/z = 1,652 ([M + H]$^+$) in secondary-ion mass spectrometry. Amino acid sequencing analysis of Syn-P-16-L with the gas-phase protein sequencer gave the same result as that of Syn-P-16-S, as expected.

Tuberculin activities of the synthesized peptides in guinea pigs. Tuberculin activities of Syn-P-8, Syn-P-16-S, and Syn-P-16-L were examined in guinea pigs sensitized with *M. tuberculosis* or *M. bovis* BCG and compared with that of PPD. None of the synthesized peptides elicited a tuberculin-type delayed skin reaction even with a dose as high as 50 μg (Tables 5 and 6). On the contrary, 5 or 2.5 μg of PPD elicited a very strong delayed-type hypersensitive skin reaction in all sensitized animals.

Tuberculin activity of Syn-P-16-S in humans. Tuberculin activity of Syn-P-16-S was examined in two healthy humans. A 5-μg dose of Syn-P-16-S evoked a purple swelling accompanied by itching and pain soon after the injection, but the reaction reached its peak within 2 or 3 h and then diminished gradually, with only residual pigmentation remaining after 24 and 48 h. In contrast, 0.05 μg of PPD elicited very strong delayed-type skin reactions in both subjects (Table 7; Fig. 5).

DISCUSSION

PPD has been used worldwide as a skin-test antigen to detect mycobacterial infection both in clinics and in tuberculosis control programs, but it is far from homogeneous and consists of a complex mixture of proteins and polysaccharides. Many attempts have been made to obtain a more homogeneous tuberculin-active protein without remarkable success. In 1973, Kuwabara (3, 4) reported that he had succeeded in crystallizing a tuberculin-active protein from culture filtrates of *M. tuberculosis* ahd had determined its amino acid sequence. He also reported that one of tryptic digests of this crystalline protein, Asn70-Gly-Ser-Gln-Met-Arg75, was tuberculin active. Although his purification and crystallization procedures have not been confirmed by other investigators, Šavrda (5, 6) synthesized three peptides according to the amino acid sequence proposed by Kuwabara and reported that a hexadecapaptide which corresponded to amino acids 61 to 76 of the amino acid sequence of Kuwabara was tuberculin active in guinea pigs sensitized with *M. tuberculosis*. A heptapeptide synthesized by Šavrda which corresponded to the tuberculin-active tryptic digest of Kuwabara was inactive as a tuberculin skin-test antigen.

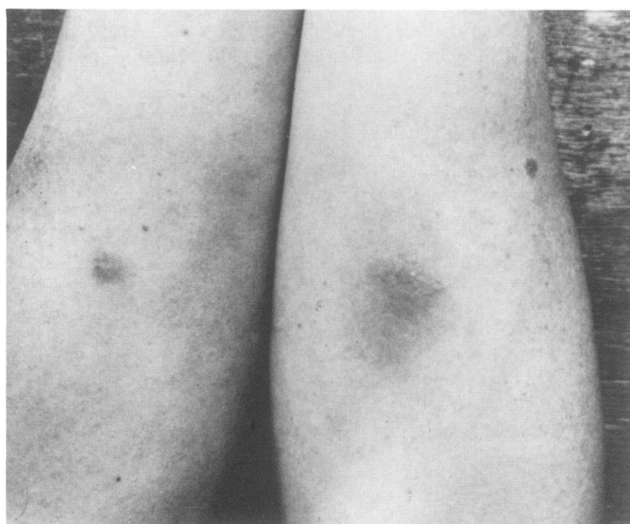

FIG. 5. Skin reaction with Syn-P-16-S in PPD-positive healthy humans. Skin reactions 48 h after intradermal injections of 5 μg of Syn-P-16-S (left forearm) and 0.05 μg of PPD (right forearm).

We also synthesized octa- and hexadecapeptides which corresponded to amino acids 61 to 68 and 61 to 76, respectively, of the amino acid sequence of Kuwabara. These peptides were synthesized by a solid-phase method as well as a liquid-phase method in the case of the latter peptide. The authenticity and purity of our synthesized peptides were confirmed by HPLC, amino acid analysis, mass spectrometry, and amino acid sequencing analysis.

Tuberculin activity of the synthesized peptides was examined in guinea pigs sensitized with *M. tuberculosis* and *M. bovis* BCG and also in PPD-positive healthy humans. Neither Syn-P-8 nor Syn-P-16-S were active as an antigen for the tuberculin skin test even with a dose as high as 50 μg in guinea pigs and 5 μg in humans. These doses were, respectively, 10 to 20 times and 100 times the dose of PPD that elicited very intense delayed-type skin reactions in guinea pigs and humans.

From these results, Syn-P-8 and Syn-P-16 are not likely to be antigenic determinants of the tuberculin skin-test antigen, although there still remains a possibility that they may be active on a carrier protein or in an aggregated form. Further efforts to obtain homogeneous tuberculin-active preparations and to establish their amino acid sequence should be continued.

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