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Synthesis of Deacetyl-thymosin β_{10} and Examination of Its Immunological Effect on T-Cell Subpopulations of a Uremic Patient with Tuberculosis[1]

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Deacetyl-thymosin β_{10} was synthesized by a conventional solution method, by assembling seven peptide fragments followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole in trifluoroacetic acid. The synthetic deacetyl-thymosin β_{10} increased the entire peripheral T-cell population and a helper T-cell subset when incubated *in vitro* with blood obtained from a uremic patient with tuberculosis, but a suppressor/cytotoxic T-cell subset was unaffected under these conditions.

Keywords——deacetyl-thymosin β_{10}; trifluoromethanesulfonic acid deprotection; T-cell subpopulation; uremic patient; tuberculosis; β,β,β-trichloroethyloxycarbonylhydrazide; thioanisole-mediated deprotection

Thymosin β_{10} is composed of 42 amino acid residues with acetyl-alanine at the N-terminus, and shows 75% homology with thymosin β_4.[2] As shown in Fig. 1, the amino acid sequence of thymosin β_{10} differs from that of thymosin β_9, the corresponding peptide in calf thymus, at positions 6 and 10, where thymosin β_9 contains Leu and Asn, respectively. It also differs at the COOH terminus, which is Gln–Ala–Lys–OH in thymosin β_9.[3] Several recent studies have also demonstrated that thymosin β_4 is important for maturation and functioning of the immune system in man and animals.[4-6] Thymosin β_4 has been chemically synthesized by the solid-phase procedure.[7,8]

In 1984, we reported[9] the synthesis of deacetyl-thymosin β_4 and showed that the synthetic tritetracontapeptide could increase the entire T-cell population and a helper T-cell subset when incubated *in vitro* with blood from a patient with chronic renal failure. In 1982, we had reported[10] the synthesis of calf thymosin β_9; the synthetic hentetracontapeptide could

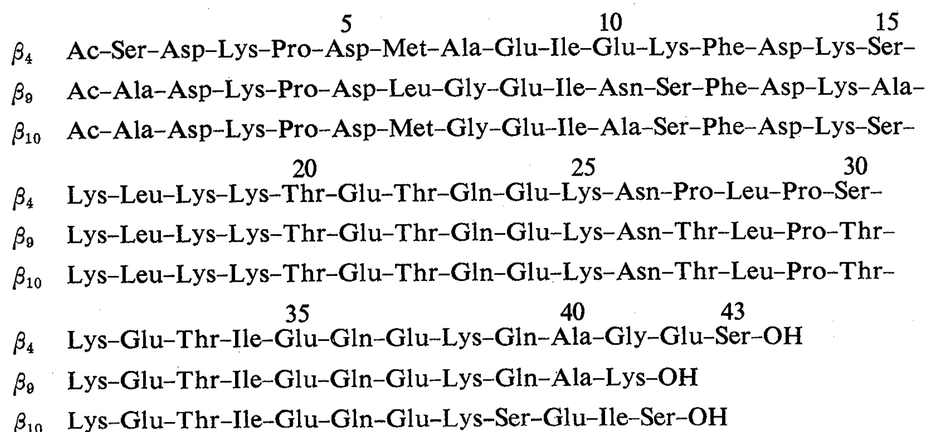

Fig. 1. Comparison of Amino Acid Sequences of Thymosins β_4, β_9 and β_{10}

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increase the peripheral E-rosette-forming cells when incubated *in vitro* with blood from a lupus nephritis patient. Therefore, we concluded that the acetyl group at the N-terminal Ser residue of thymosin β_4 is not required for increasing the activity of helper T-cells in cases of chronic renal failure.[9]

The life expectancy of chronic renal failure patients has been prolonged by recent advances in hemodialysis therapy. However, the complication of infectious diseases in chronic hemodialysis patients still poses a grave problem in terms of their prognosis. According to Sasaki *et al.*,[11] the incidence of tuberculosis in such patients is high, and is 6—16 times greater than that in the general population. It is also an accepted fact that there is a decrease in immunity, particularly cell-mediated immunity, in uremic patients.[12] Patients with chronic uremia may have thymic atrophy.[13] The thymus may show a marked reduction in lymphoid elements and extensive replacement, as well as cystic degradation, may be seen. These observations suggested that the cell-mediated immune abnormalities seen in chronic uremia might be attributable to thymic hormone deficiency.

We describe here the synthesis of deacetyl-thymosin β_{10} by the solution method and the *in vitro* effect of this peptide on the impaired T-cell subsets in a uremic patient with

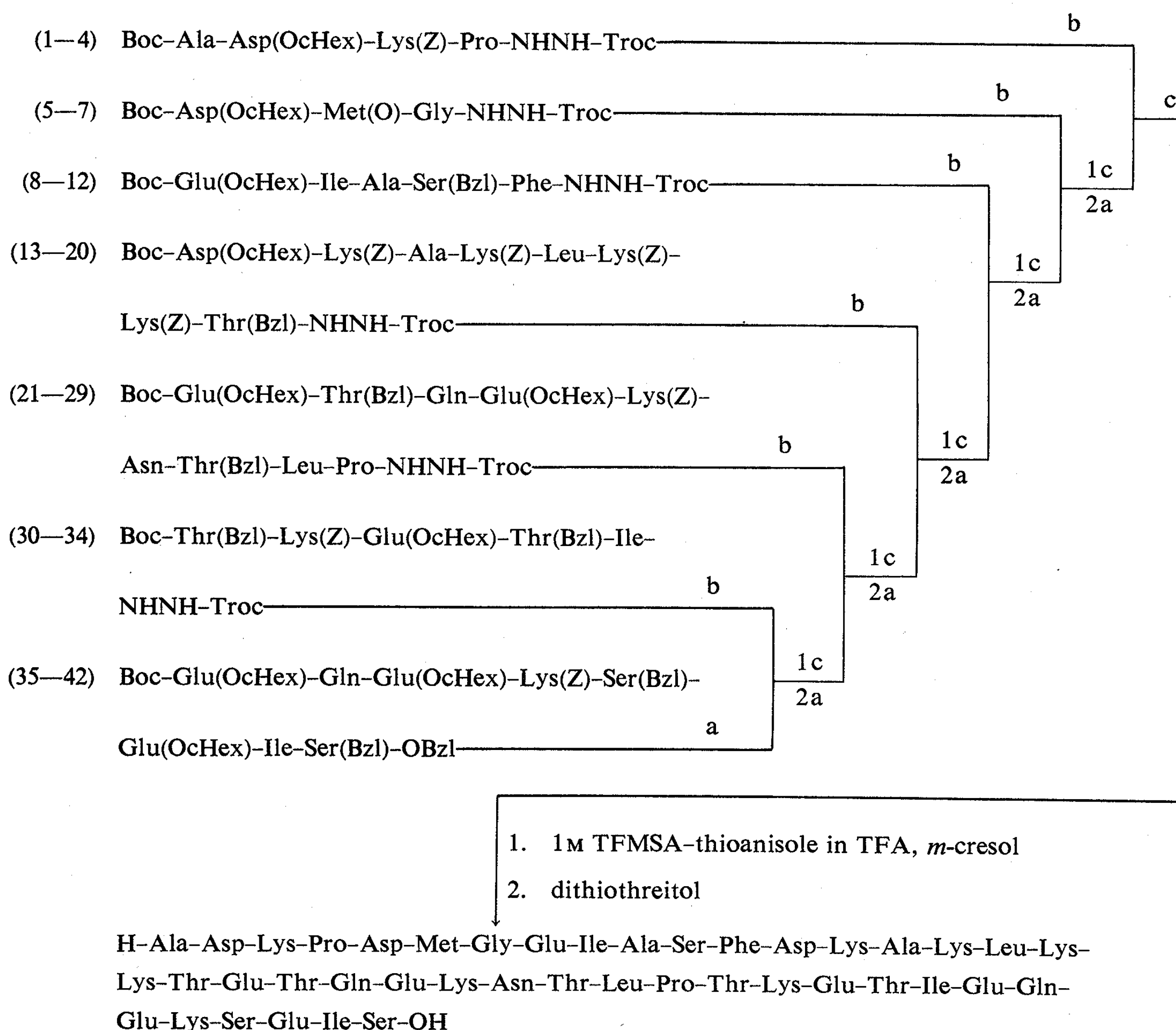

Fig. 2. Synthetic Route to Deacetyl-thymosin β_{10}

a: TFA–anisole. b: Zn–AcOH. c: azide.

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tuberculosis.

Several improvements have been made in the present synthesis, as illustrated in Fig. 2, compared with our previous syntheses of thymosins.[9,10,14] The thioanisole-mediated TFMSA deprotecting procedure[15,16] was employed. Besides, Lys(Z), Thr(Bzl), Met(O), Ser(Bzl) and Ser(Bzl)–OBzl, two new amino acid derivatives bearing protecting groups removable by 1 M TFMSA–thioanisole in TFA were employed, *i.e.*, Glu(OcHex) and Asp(OcHex).[17] Asp(OcHex) was employed to minimize aspartimide formation during the synthesis of Asp-containing peptide.[17] Glu(OcHex) was also employed to suppress base-catalyzed pyrrolidone formation.[18] These protecting groups survive mostly intact under careful TFA treatment for removal of the Boc group, employed as a temporary α-amino protecting group.

As shown in Fig. 2, seven fragments were selected as building blocks to construct the entire peptide backbone of deacetyl-thymosin β_{10}. Each fragment was synthesized by the known amido-forming reactions. The procedure using azide[19] was applied to condense these fragments successively, because of the low risk of racemization involved in this procedure, as compared to other amido-forming reactions.

Throughout the syntheses of these intermediates and fragments, the purity of every fragment and intermediate was confirmed by TLC, amino acid analysis of acid hydrolysates and elemental analysis. The analytical results were within $\pm 0.4\%$ of theoretical values in all cases.

First, the C-terminal fragment, Boc–(35—42)–OBzl, was prepared stepwise starting from Boc–Ser(Bzl)–OBzl by the HOBT–WSCI procedure,[20] except for the introduction of the Gln residue, which was introduced by the NP active ester procedure.[21] For the preparation of the six fragments containing Glu(OcHex) or Asp(OcHex), Boc–(30—34)–NHNH–Troc, Boc–(21—29)–NHNH–Troc, Boc–(13—20)–NHNH–Troc, Boc–(8—12)–NHNH–Troc, Boc–(5—7)–NHNH–Troc and Boc–(1—4)–NHNH–Troc, we employed a substituted hydrazide, Troc–$NHNH_2$,[22] the protecting group of which is known to be cleaved by Zn[23] in AcOH without affecting side chain protecting groups such as Boc, Z, cHex and Bzl. Thus, these fragments were prepared without exposing the corresponding methyl or ethyl esters to hydrazide. The six fragments, Boc–(30—34)–NHNH–Troc, Boc–(21—29)–NHNH–Troc, Boc–(13—20)–NHNH–Troc, Boc–(8—12)–NHNH–Troc, Boc–(5—7)–NHNH–Troc and Boc–(1—4)–NHNH–Troc, were also prepared stepwise by the HOBT–WSCI procedure[20] except for the introduction of Asn and Gln residues, which were introduced by the NP active ester procedure.[21] Boc groups of intermediates were removed by treatment with TFA–anisole prior to the next coupling reaction.

The seven fragments thus obtained were assembled successively according to Fig. 2 by the procedure of Rudinger and Honzl using azide.[19] Each protected product was purified either by precipitation from DMF or DMSO with an appropriate solvent, such as MeOH or EtOH, or by gel-filtration on Sephadex LH-60 using DMF or DMSO in the presence of 5% H_2O as an eluent. Throughout this synthesis, satisfactory incorporation of each fragment in condensation reactions was confirmed by comparison of the recovery of Ile or Leu with those of newly incorporated amino acids.

Moreover, we attempted to take advantage of the well-known fact that D-allo-Ile or D-allo-Thr is easily separable from the parent amino acid, Ile or Thr, by using an amino acid analyzer to detect the degree of racemization during the coupling reactions by amino acid analysis.[24] For this purpose, we chose the two synthetic fragments, Boc–(30—34)–$NHNH_2$ and Boc–(13—20)–$NHNH_2$, bearing Ile and Thr in the C-terminus, respectively. Fortunately, after coupling of these fragments, we could not detect any D-allo-Ile or D-allo-Thr in the resulting peptides.

Boc–(30—34)–NHNH–Troc was treated with Zn in AcOH and MeOH to remove the Troc group, and the last trace of zinc acetate was removed by treatment with EDTA to give

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the pentapeptide hydrazide in analytically pure form. The hydrazine test on TLC and the elemental analysis data were consistent with homogeneity of the desired product. The Boc group of Boc–(35—42)–OBzl was removed by the usual TFA–anisole treatment and the corresponding free amine was condensed with Boc–(30—34)–$NHNH_2$ (1.5 eq) by the azide procedure to give Boc–(30—42)–OBzl (I), which was purified by column chromatography on Sephadex LH-60 with DMF containing 5% H_2O. The homogeneity of the peptide was assessed by elemental analysis, TLC and amino acid analysis of the acid hydrolysate with 6 N HCl. The solubility of protected intermediates in DMF decreased remarkably with chain elongation. Consequently, mixtures of DMF–DMSO had to be employed for subsequent fragment condensation reactions. Next, after removal of the Troc group of Boc–(21—29)–NHNH–Troc by treatment with Zn in AcOH and DMF, the resulting nonapeptide hydrazide (2 eq) was condensed with H–(30—42)–OBzl by the azide procedure to yield Boc–(21—42)–OBzl (II), which was purified by repeated precipitation from DMF with MeOH. The homogeneity of the peptide was assessed by elemental analysis, TLC and amino acid analysis of the acid hydrolysate. Then, the Boc group of Boc–(21—42)–OBzl (II) was removed and the corresponding free base was condensed with Boc–(13—20)–$NHNH_2$ (2 eq), by the azide procedure in the same manner as described above to give Boc–(13—42)–OBzl (III), which was purified by column chromatography on Sephadex LH-60 with DMSO containing 5% H_2O. The homogeneity of the peptide was assessed by elemental analysis, TLC and amino acid analysis of the acid hydrolysate. The Boc group of Boc–(13—42)–OBzl (III) was removed and the corresponding free base was condensed with Boc–(8—12)–$NHNH_2$ by the azide procedure. This condensation reaction was performed using 2 eq of acyl component, Boc–(8—12)–$NHNH_2$, followed by addition of further azide (1 eq) after 36 h to yield Boc–(8—42)–OBzl (IV), which was purified by repeated precipitation from DMSO with MeOH. The homogeneity of the peptide was assessed by elemental analysis, TLC and amino acid analysis of the acid hydrolysate. The Boc group of Boc–(8—42)–OBzl (IV) was removed and the corresponding free base was condensed with Boc–(5—7)–$NHNH_2$ by the azide procedure. This condensation reaction was performed using 3 eq of acyl component, Boc–(5—7)–$NHNH_2$, followed by addition of further azide (1 eq) after 36 h to yield Boc–(5—42)–OBzl (V), which was purified by repeated precipitation from DMSO with EtOH. The homogeneity of the peptide was also assessed by elemental analysis, TLC and amino acid analysis of the acid hydrolysate. The Boc group of Boc–(5—42)–OBzl (V) was removed and the corresponding free base was condensed with the N-terminal subunit, Boc–(1—4)–$NHNH_2$, by the azide procedure. This condensation reaction was performed using 3 eq of acyl component, Boc–(1—4)–$NHNH_2$, followed by addition of further azide (1 eq) after 36 h to yield the protected dotetracontapeptide VI corresponding to the entire amino acid sequence of calf thymosin β_{10}, which was purified by repeated precipitation from DMSO with MeOH. The homogeneity of the peptide was assessed by elemental analysis, TLC and amino acid analysis of the acid hydrolysate. In the final step, the protected dotetracontapeptide thus obtained was treated with 1 M TFMSA–thioanisole in TFA in the presence of *m*-cresol and ethanedithiole at 0 °C for 3 h to remove all protecting groups employed, except Met(O). Met(O) is known to be partially reduced in this thioanisole-mediated process. The deprotected peptide was treated with 1 N NH_4OH (pH 8.0, 0 °C, 30 min) to reverse any possible N→O shift[25] and *m*-cresol was used as an additional cation-scavenger to suppress a side reaction.[26] This product was incubated with dithiothreitol (60 °C, 36 h) to ensure complete reduction of Met(O). The reduced product, after gel-filtration on Sephadex G-25, was purified by ion-exchange chromatography on DEAE-Sephadex A-25 using gradient elution with 0.12 M NH_4HCO_3 buffer (pH 7.6). The main product was rechromatographed on the DEAE-Sephadex A-25 column as described above. After being desalted by repeated lyophilization, the product was further purified by column chromatography on cellulose powder using Walley's solvent

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TABLE I. Yields and Physical Constants of Protected Intermediates

Peptide	Yield (%)	mp (°C)	$[\alpha]_D^{21}$ (c=1.0, DMF)	TLC[a) Rf^1	Rf^2
Boc-(41—42)-OBzl	92	70—73	−7.9	0.72	0.70
Boc-(40—42)-OBzl	77	74—77	−8.2	0.73	0.74
Boc-(39—42)-OBzl	66	117—123	−14.8	0.70	0.69
Boc-(38—42)-OBzl	99	136—143	−10.3	0.69	0.68
Boc-(37—42)-OBzl	93	152—158	−6.7	0.70	0.72
Boc-(36—42)-OBzl	76	154—163	−31.6	0.62	0.65
Boc-(35—42)-OBzl	80	146—152	−20.3	0.72	0.74
Boc-(33—34)-NHNH-Troc	86	72—75	−34.8	0.63	0.65
Boc-(32—34)-NHNH-Troc	81	112—115	−15.2	0.74	0.76
Boc-(31—34)-NHNH-Troc	85	128—133	+12.4	0.71	0.73
Boc-(30—34)-NHNH-Troc	92	120—127	+5.1	0.74	0.76
Boc-(30—34)-$NHNH_2$	85	163—174	+25.4	0.48[b)	0.50[b)
Boc-(27—29)-NHNH-Troc	65	81—84	−26.5	0.72	0.75
Boc-(26—29)-NHNH-Troc	83	168—175	−7.9	0.62	0.65
Boc-(25—29)-NHNH-Troc	82	120—127	−16.7	0.72	0.77
Boc-(24—29)-NHNH-Troc	79	98—105	−12.3	0.72	0.76
Boc-(23—29)-NHNH-Troc	86	113—119	−14.3	0.65	0.62
Boc-(22—29)-NHNH-Troc	82	124—130	−5.9	0.77	0.78
Boc-(21—29)-NHNH-Troc	68	140—146	−9.1	0.70	0.72
Boc-(21—29)-$NHNH_2$	96	191—203	−21.8	0.52[b)	0.54[b)
Boc-(15—20)-NHNH-Troc	93	114—121	−11.2	0.67	0.72
Boc-(14—20)-NHNH-Troc	91	104—112	−7.2	0.74	0.72
Boc-(13—20)-NHNH-Troc	84	132—144	−5.2	0.71	0.77
Boc-(13—20)-$NHNH_2$	94	184—193	−15.9	0.55[b)	0.52[b)
Boc-(11—12)-NHNH-Troc	86	90—94	−8.7	0.75	0.76
Boc-(10—12)-NHNH-Troc	79	119—124	−5.6	0.72	0.73
Boc-(9—12)-NHNH-Troc	84	92—96	+15.1	0.75	0.78
Boc-(8—12)-NHNH-Troc	82	113—117	+4.6	0.74	0.75
Boc-(8—12)-$NHNH_2$	88	173—181	+19.3	0.48[b)	0.44[b)
Boc-(6—7)-NHNH-Troc	61	139—144	−30.3	0.52	0.55
Boc-(5—7)-NHNH-Troc	71	121—127	+10.4	0.69	0.71
Boc-(5—7)-$NHNH_2$	90	140—146	+19.8	0.40[b)	0.42[b)
Boc-(2—4)-NHNH-Troc	65	139—144	−15.2	0.72	0.74
Boc-(1—4)-NHNH-Troc	71	142—148	−29.6	0.70	0.71
Boc-(1—4)-$NHNH_2$	82	129—136	−32.6	0.40[b)	0.41[b)

a) The protected peptides were deblocked with TFA unless otherwise mentioned. Fluorescamine-positive spot. *b)* Hydrazine-positive spot.

system[27)] as an eluant. The product thus obtained was then subjected to a Sephadex G-25 column chromatography as described above. The product thus purified gave a single spot on TLC in two different solvent systems and behaved as a single band on disk isoelectrofocusing (Pharmalyte, pH 3—10). Acid hydrolysis gave the amino acid ratios predicted by theory. The purity of this product was further confirmed by enzymatic digestion. However, the purity of the product as estimated by HPLC was only about 95%. The minor impurity (approximately 5%) was found to be the Met(O) derivative, since the main product was converted to this minor component by excess H_2O_2 treatment.

The *in vitro* effect of the synthetic deacetyl-thymosin β_{10} on the impaired T-cell subsets in a uremic patient with tuberculosis is summarized in Table II. For this immunological analysis, we used monoclonal antibodies against the cell-surface antigens of helper (T4) and suppressor/cytotoxic (T8) T-cell subsets and against a common T-cell antigen (T3) defining all

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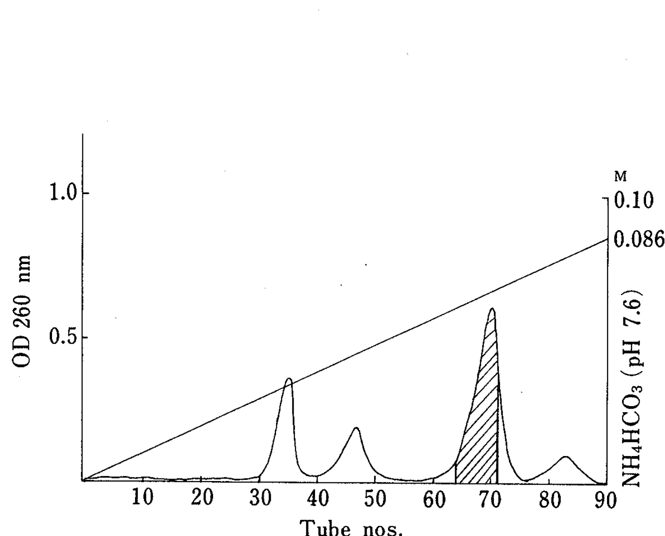

Fig. 3. Purification of Synthetic Deacetyl-thymosin β_{10} on a Column of DEAE-Sephadex A-25

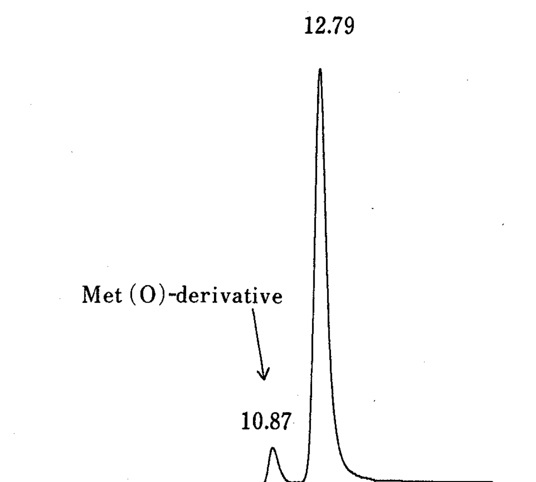

Fig. 4. HPLC of Synthetic Deacetyl-thymosin β_{10}

TABLE II. Effect of the Synthetic Deacetyl-thymosin β_2 on Impaired T-Cell Subsets of a Uremic Patient with Tuberculosis

Peptide	Dose (μg/ml)	Reactivity with monoclonal antibodies[d] (%)		
		Anti-T3	Anti-T4	Anti-T8
—[a]	—	63.0 ± 6.8	33.8 ± 8.0	21.3 ± 5.4
—[b]	—	39.6 ± 8.8[e]	18.4 ± 7.9[e]	22.6 ± 5.7
Deacetyl-thymosin β_{10}[b,c]	1	48.3 ± 7.2[f]	24.8 ± 7.6[f]	20.4 ± 5.8
Deacetyl-thymosin β_{10}[b,c]	10	58.1 ± 7.6[f]	28.9 ± 7.7[f]	21.4 ± 5.5

a) Normal persons. *b*) A uremic patient with tuberculosis. *c*) Incubation was carried out for 60 min at 37 °C. *d*) Each value represents the mean ± S.D. of triplicate measurements. *e*) Significantly different from normal persons at a *p* value of 0.05 or less. *f*) Significantly different from the uremic patient at a *p* value of 0.01 or less.

peripheral T-cells.[28,29] In contrast to normal persons, we found that the uremic patient with tuberculosis had reduced percentages of helper T-cells and all peripheral T-cells, but the percentage of suppressor/cytotoxic T-cells was at a normal level. Statistical analysis of the data for percentages of T-cell subsets in peripheral blood incubated with or without the synthetic deacetyl-thymosin β_{10} showed that, in the case of the patient investigated, the synthetic deacetyl-thymosin β_{10} restored to nearly normal values the percentages of helper T-cells and all peripheral T-cells at a dose of 10 μg/ml, but this peptide did not change the percentage of suppressor/cytotoxic T-cells under the same conditions.

These results suggest that the synthetic deacetyl-thymosin β_{10} has activity to restore the defect of helper T-cells *in vitro*, and the acetyl group at the N-terminal Ala residue of thymosin β_{10} is not required for increasing activity of helper T-cells in a uremic patient with an infectious disease (tuberculosis).

Experimental

General experimental procedures used in this paper are essentially the same as described in the previous papers.[9,14] Azides were prepared according to Honzl and Rudinger[19] with isoamyl nitrite. Unless otherwise mentioned, products were purified by one of the following two procedures. Procedure 1: For purification of protected

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peptides soluble in EtOAc, the extract was washed with 1 N citric acid, H_2O, 5% $NaHCO_3$ and H_2O, then dried over $MgSO_4$ and concentrated. The residue was precipitated or recrystallized from appropriate solvents. Procedure 2: For purification of protected peptides almost insoluble in EtOAc, the reaction mixture was poured into ice-chilled 1 N citric acid with stirring. The powder thereby formed was washed with 1 N citric acid, H_2O, 5% $NaHCO_3$ and H_2O. The dried product was recrystallized or precipitated from appropriate solvents. General procedure for obtaining protected peptide hydrazides from Troc-derivatives: Troc-derivatives in DMF or MeOH were treated with Zn dust in the presence of AcOH for 8—12 h. The solution was filtered, the filtrate was concentrated and the residue was treated with 3% EDTA. The resulting powder was washed with 5% $NaHCO_3$ and H_2O and precipitated from appropriate solvents. Boc–Lys(Z)–Pro–NHNH–Troc, Boc–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–NHNH–Troc and Boc–Leu–Pro–NHNH–Troc were the same intermediates as used for our previous syntheses of deacetylthymosin β_4[9] and thymosin β_8.[14]

Melting points are uncorrected. Rotations were measured with an Atago Polax machine (cell length: 10 cm). The amino acid compositions of acid and enzymatic hydrolysates were determined with a Hitachi type 835-50 amino acid analyzer. Solvents were concentrated in a rotary evaporator under reduced pressure at a temperature of 30—40 °C. Boc groups of the protected peptides were removed by TFA–anisole treatment. The resulting amino components were chromatographed on silica gel plates (Kieselgel G, Merck) and *Rf* values refer to the following solvent systems: Rf^1, the Partridge system[30]; Rf^2, the Walley system.[27] Troc–$NHNH_2$ was purchased from the Kokusan Chemical Works Ltd., Japan. LAP (Lot. 14F-8115) was purchased from Sigma Chemical Co. Monoclonal antibodies (Ortho Diagnostic Systems K. K., N. J., U.S.A.) used were OKT3 (all peripheral T-cells), OKT4 (helper T-cells) and OKT8 (suppressor/cytotoxic T-cells). Preparations of protected intermediates were repeated several times in order to obtain sufficient quantities for the next step. Venous blood was obtained from a uremic patient with tuberculosis. Venous blood samples from three healthy donors were used as a control. HPLC was conducted with a Shimadzu LC-3A apparatus coupled to a Cosmosil $5C_{18}$ column (4.6 × 150 mm).

Boc–Thr(Bzl)–Lys(Z)–Glu(OcHex)–Thr(Bzl)–Ile–Glu(OcHex)–Gln–Glu(OcHex)–Lys(Z)–Ser(Bzl)–Glu(OcHex)–Ile–Ser(Bzl)–OBzl (I)——Boc–Glu(OcHex)–Gln–Glu(OcHex)–Lys(Z)–Ser(Bzl)–Glu(OcHex)–Ile–Ser(Bzl)–OBzl (680 mg) was treated with TFA–anisole (7 ml : 1.4 ml) in an ice-bath for 40 min, then dry ether was added. The resulting powder was washed with dry ether, dried over KOH pellets *in vacuo* for 2 h and then dissolved in DMF (6 ml) containing NMM (0.05 ml). The azide [prepared from 662 mg of Boc–Thr(Bzl)–Lys(Z)–Glu(OcHex)–Thr(Bzl)–Ile–$NHNH_2$ (1.5 eq)] in DMF (5 ml) and NMM (0.12 ml) were added to the above ice-chilled solution and the mixture, after being stirred at −10 °C for 36 h, was neutralized with AcOH and evaporated *in vacuo*. The residue was treated with 1 N citric acid and the precipitate thereby formed was washed successively with 1 N citric acid and H_2O. The resulting powder was purified by gel-filtration on Sephadex LH-60 (3 × 98 cm) with DMF containing 5% H_2O. The ultraviolet (UV) absorption at 260 nm was determined in each fraction (5 ml) and the desired fractions containing a substance of Rf^1 0.60 were combined. The solvent was removed by evaporation and the residue was treated with ether to afford a powder; yield 849 mg (80%), mp 182—190 °C, $[\alpha]_D^{21}$ −13.0° (c = 1.0, DMF), Rf^1 0.60, Rf^2 0.64, single fluorescamine-positive spot. *Anal.* Calcd for $C_{143}H_{198}N_{16}O_{33} \cdot 8H_2O$: C, 61.05; H, 7.67; N, 7.97. Found: C, 61.14; H, 7.85; N, 7.72. Amino acid ratios in a 6 N HCl hydrolysate: Ile 2.00, Thr 1.85, Ser 1.83, Glu 4.89, Lys 2.07 (recovery of Ile 84%).

Boc–Glu(OcHex)–Thr(Bzl)–Gln–Glu(OcHex)–Lys(Z)–Asn–Thr(Bzl)–Leu–Pro–Thr(Bzl)–Lys(Z)–Glu(OcHex)–Thr(Bzl)–Ile–Glu(OcHex)–Gln–Glu(OcHex)–Lys(Z)–Ser(Bzl)–Glu(OcHex)–Ile–Ser(Bzl)–OBzl (II)——The above Boc–(30—42)–OBzl (I) (703 mg) was treated with TFA–anisole (7 ml : 1.4 ml) as described above and the N^{α}-deprotected peptide was dissolved in DMF–DMSO (1 : 1, 6 ml) containing NMM (0.03 ml). The azide [prepared from 826 mg of Boc–Glu(OcHex)–Thr(Bzl)–Gln–Glu(OcHex)–Lys(Z)–Asn–Thr(Bzl)–Leu–Pro–$NHNH_2$ (2 eq)] in DMF–DMSO (1 : 1, 6 ml) and NMM (0.08 ml) were added to the above ice-chilled solution and the mixture, after being stirred at −10 °C for 36 h, was neutralized with AcOH and evaporated *in vacuo*. The residue was poured into ice-chilled MeOH with stirring. The precipitate thereby formed was washed successively with MeOH, H_2O, 1 N citric acid and H_2O. The dried product was reprecipitated from DMSO with MeOH; yield 729 mg (70%), mp 178—186 °C, $[\alpha]_D^{21}$ −12.8° (c = 1.0, DMSO), Rf^1 0.64, Rf^2 0.69, single fluorescamine-positive spot. *Anal.* Calcd for $C_{221}H_{308}N_{28}O_{52} \cdot 11H_2O$: C, 60.50; H, 7.58; N, 8.94. Found: C, 60.45; H, 7.66; N, 8.92. Amino acid ratios in a 6 N HCl hydrolysate: Leu 1.00, Ile 2.08, Pro 0.92, Thr 3.79, Ser 1.80, Asp 1.01, Glu 7.85, Lys 3.02 (recovery of Leu 84%).

Boc–Asp(OcHex)–Lys(Z)–Ala–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–Glu(OcHex)–Thr(Bzl)–Gln–Glu(OcHex)–Lys(Z)–Asn–Thr(Bzl)–Leu–Pro–Thr(Bzl)–Lys(Z)–Glu(OcHex)–Thr(Bzl)–Ile–Glu(OcHex)–Gln–Glu(OcHex)–Lys(Z)–Ser(Bzl)–Glu(OcHex)–Ile–Ser(Bzl)–OBzl (III)——The above Boc–(21—42)–OBzl (II) (548 mg) was treated with TFA–anisole (6 ml : 1.2 ml) and the N^{α}-deprotected peptide ester, isolated as usual, was dissolved in DMF–DMSO (1 : 1, 6 ml) containing NMM (0.014 ml). The azide [prepared from 457 mg of Boc–Asp(OcHex)–Lys(Z)–Ala–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–$NHNH_2$ (2 eq)] in DMF–DMSO (1 : 1, 5 ml) and NMM (0.033 ml) were added to the above ice-chilled solution and the mixture was stirred at −10 °C for 36 h. Additional azide [prepared from 1 eq of hydrazide] in DMF–DMSO (1 : 1, 3 ml) and NMM (0.016 ml) were added and stirring was continued for an additional 24 h. After neutralization with a few drops of AcOH, the solution was concentrated and the residue was treated with 1 N citric acid. The resulting powder was washed with 1 N citric acid and

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H_2O and precipitated from DMSO with MeOH. The crude product was dissolved in DMSO containing 5% H_2O (3 ml) and the solution was applied to a column of Sephadex LH-60 (3 × 93 cm), which was eluted with the same solvent. The fractions with Rf^1 0.52 were combined and the solvent was removed by evaporation. Treatment of the residue with ether afforded a powder; yield 504 mg (69%), mp 204—213 °C, $[\alpha]_D^{21}$ −10.4° (c=1.0, DMSO), Rf^1 0.52, Rf^2 0.53, single fluorescamine-positive spot. *Anal.* Calcd for $C_{307}H_{424}N_{40}O_{71} \cdot 16H_2O$: C, 60.46; H, 7.54; N, 9.19. Found: C, 60.35; H, 7.80; N, 9.22. Amino acid ratios in a 6 N HCl hydrolysate: Ala 1.04, Leu 2.00, Ile 2.02, Pro 0.95, Thr 4.78, Ser 1.89, Asp 2.07, Glu 7.92, Lys 7.03 (recovery of Lue 83%).

Boc–Glu(OcHex)–Ile–Ala–Ser(Bzl)–Phe–Asp(OcHex)–Lys(Z)–Ala–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–Glu(OcHex)–Thr(Bzl)–Gln–Glu(OcHex)–Lys(Z)–Asn–Thr(Bzl)–Leu–Pro–Thr(Bzl)–Lys(Z)–Glu(OcHex)–Thr(Bzl)–Ile–Glu(OcHex)–Gln–Glu(OcHex)–Lys(Z)–Ser(Bzl)–Glu(OcHex)–Ile–Ser(Bzl)–OBzl (IV)——The above Boc–(13—42)–OBzl (III) (436 mg) was treated with TFA–anisole (5 ml : 1 ml) and the N^{α}-deprotected peptide obtained as described above was dissolved in DMF–DMSO (1 : 2, 5 ml) containing NMM (0.08 ml). The azide [prepared from 127 mg of Boc–Glu(OcHex)–Ile–Ala–Ser(Bzl)–Phe–$NHNH_2$ (2 eq)] in DMF–DMSO (1 : 2, 3 ml) and NMM (0.02 ml) were added to the above ice-chilled solution and the mixture was stirred at −10 °C for 36 h. Additional azide [prepared from 1 eq of hydrazide] in DMF–DMSO (1 : 2, 3 ml) and NMM (0.01 ml) were added and stirring was continued for an additional 32 h. After being neutralized with a few drops of AcOH, the solution was concentrated and the residue was treated with MeOH. The resulting powder was washed successively with MeOH, H_2O, 1 N citric acid and H_2O. The dried product was reprecipitated from DMSO with MeOH; yield 308 mg (64%), mp 239—247 °C, $[\alpha]_D^{21}$ −17.4° (c=1.0, DMSO), Rf^1 0.52, Rf^2 0.59, single fluorescamine-positive spot. *Anal.* Calcd for $C_{340}H_{473}N_{45}O_{79} \cdot 18H_2O$: C, 60.28; H, 7.51; N, 9.30. Found: C, 59.96; H, 7.48; N, 9.42. Amino acid ratios in a 6 N HCl hydrolysate: Ala 2.10, Leu 2.00, Ile 3.05, Phe 0.97, Pro 0.92, Thr 4.83, Ser 2.91, Asp 1.98, Glu 9.01, Lys 7.03 (recovery of Leu 86%).

Boc–Asp(OcHex)–Met(O)–Gly–Glu(OcHex)–Ile–Ala–Ser(Bzl)–Phe–Asp(OcHex)–Lys(Z)–Ala–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–Glu(OcHex)–Thr(Bzl)–Gln–Glu(OcHex)–Lys(Z)–Asn–Thr(Bzl)–Leu–Pro–Thr(Bzl)–Lys(Z)–Glu(OcHex)–Thr(Bzl)–Ile–Glu(OcHex)–Gln–Glu(OcHex)–Lys(Z)–Ser(Bzl)–Glu(OcHex)–Ile–Ser(Bzl)–OBzl (V)——The above Boc–(8—42)–OBzl (IV) (225 mg) was treated with TFA–anisole (4 ml : 0.8 ml) and the N^{α}-deprotected peptide obtained as described above was dissolved in DMF–DMSO (1 : 2, 4 ml) containing NMM (0.004 ml). The azide [prepared from 55 mg of Boc–Asp(OcHex)–Met(O)–Gly–$NHNH_2$ (3 eq)] in DMF–DMSO (1 : 2, 2 ml) and NMM (0.02 ml) were added to the above ice-chilled solution and the mixture was stirred at −10 °C for 36 h. Additional azide [prepared from 1 eq of hydrazide] in DMF–DMSO (1 : 2, 2 ml) and NMM (0.006 ml) were added and stirring was continued for an additional 32 h until the solution became ninhydrin-negative. After being neutralized with a few drops of AcOH, the solution was concentrated and the residue was treated with 1 N citric acid. The resulting powder was washed successively with 1 N citric acid and H_2O, and precipitated from DMSO with EtOH; yield 121 mg (51%), mp 269—282 °C, $[\alpha]_D^{21}$ −11.6° (c=1.0, DMSO), Rf^1 0.69, Rf^2 0.74, single fluorescamine-positive spot. *Anal.* Calcd for $C_{357}H_{500}N_{48}O_{85}S \cdot 19H_2O$: C, 59.57; H, 7.53; N, 9.34. Found: C, 59.46; H, 7.81; N, 9.20. Amino acid ratios in a 6 N HCl hydrolysate: Gly 1.02, Ala 2.06, Leu 2.00, Met 0.79, Ile 3.01, Phe 0.92, Pro 0.89, Thr 4.80, Ser 2.84, Asp 3.06, Glu 8.86, Lys 6.90 (recovery of Leu 84%) (Met(O) was not calculated).

Boc–Ala–Asp(OcHex)–Lys(Z)–Pro–Asp(OcHex)–Met(O)–Gly–Glu(OcHex)–Ile–Ala–Ser(Bzl)–Phe–Asp(OcHex)–Lys(Z)–Ala–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–Glu(OcHex)–Thr(Bzl)–Gln–Glu(OcHex)–Lys(Z)–Asn–Thr(Bzl)–Leu–Pro–Thr(Bzl)–Lys(Z)–Glu(OcHex)–Thr(Bzl)–Ile–Glu(OcHex)–Gln–Glu(OcHex)–Lys(Z)–Ser(Bzl)–Glu(OcHex)–Ile–Ser(Bzl)–OBzl (VI)——Boc–(5—42)–OBzl (V) (90 mg) was treated with TFA–anisole (2 ml : 0.4 ml) and the N^{α}-deprotected peptide obtained as described above was dissolved in DMF–DMSO (1 : 2, 3 ml) containing NMM (0.001 ml). The azide [prepared from 30 mg of Boc–Ala–Asp(OcHex)–Lys(Z)–Pro–$NHNH_2$ (3 eq)] in DMF–DMSO (1 : 2, 2 ml) and NMM (0.004 ml) were added to the above ice-chilled solution and the mixture was stirred at −10 °C for 36 h. Additional azide [prepared from 1 eq of hydrazide] in DMF–DMSO (1 : 2, 2 ml) and NMM (0.001 ml) were added and stirring was continued for an additional 32 h until the solution became ninhydrin-negative. After being neutralized with a few drops of AcOH, the solution was concentrated and the residue was treated with MeOH. The resulting powder was washed successively with MeOH, H_2O, 1 N citric acid and H_2O, and precipitated from DMSO with MeOH; yield 68 mg (69%), mp 204—216 °C, $[\alpha]_D^{21}$ −15.3° (c=0.4, DMSO), Rf^1 0.69, Rf^2 0.74, single fluorescamine-positive spot. *Anal.* Calcd for $C_{389}H_{545}N_{53}O_{93}S \cdot 22H_2O$: C, 59.29; H, 7.53; N, 9.42. Found: C, 59.11; H, 7.61; N, 9.40. Amino acid ratios in a 6 N HCl hydrolysate: Gly 1.03, Ala 3.02, Leu 2.00, Met 0.77, Ile 3.04, Phe 0.99, Pro 1.87, Thr 4.84, Ser 2.80, Asp 3.98, Glu 9.02, Lys 7.94 (recovery of Leu 81%) (Met(O) was not calculated).

H–Ala–Asp–Lys–Pro–Asp–Met–Gly–Glu–Ile–Ala–Ser–Phe–Asp–Lys–Ala–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–Thr–Leu–Pro–Thr–Lys–Glu–Thr–Ile–Glu–Gln–Glu–Lys–Ser–Glu–Ile–Ser–OH (Corresponding to Deacetyl-thymosin β_{10}) (VII)——The above protected dotetracontapeptide (50 mg) was treated with 1 M TFMSA–thioanisole in TFA (2 ml) in the presence of *m*-cresol (0.05 ml) in an ice-bath for 3 h, then dry ether was added. The powder thus formed was dissolved in H_2O (5 ml). The solution was treated with Amberlite CG-4B (acetate form, approximately 2 g) for 30 min, and filtered by suction. The filtrate was adjusted to pH 8.0 with 1 N NH_4OH and stirred

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in an ice-bath for 30 min to reverse a possible N→O shift at the Ser and Thr residues. The pH of the solution was adjusted to pH 6.5 with a few drops of AcOH and the solution was lyophilized. The residue was dissolved in H_2O (6 ml). The solution, after addition of dithiothreitol (20 mg), was incubated at 60 °C under N_2 gas for 36 h. The solvent was evaporated off *in vacuo* and the residue was dissolved in a small amount of 1% AcOH and then applied to a column of Sephadex G-25 (2.6 × 96 cm), which was eluted with the same solvent. Individual fractions (4 ml each) were collected and the absorbancy at 260 nm was determined for each fraction. The fractions corresponding to the front peak (tube Nos. 59—66) were combined and the solvent was removed by lyophilization. The residue was dissolved in H_2O (2 ml) and the solution was applied to a column of DEAE-Sephadex A-25 (2.3 × 62 cm), eluted with a linear gradient formed from 250 ml each of H_2O and 0.12 M NH_4HCO_3 buffer at pH 7.6. Individual fractions (4 ml each) were collected and the absorbancy at 260 nm was determined. A main peak present in the gradient eluates (tube Nos. 64—71) was collected. The solvent was evaporated off and the residue was rechromatographed on a DEAE-Sephadex A-25 column as described above. Next, the residue was dissolved in Walley's solvent system[27] (1 ml) and the solution was applied to a column of cellulose powder (2.3 × 91 cm), which was eluted with the same solvent system. Each fraction was examined by means of the ninhydrin test. The fractions which exhibit a ninhydrin-positive single spot (Rf^2 0.09) on TLC were combined and the solvent was evaporated off. The residue was dissolved in 1% AcOH. The solution was then subjected to Sephadex G-25 column chromatography as described above; yield 5.8 mg (19%), $[\alpha]_D^{21}$ −78.6° (c=0.3, 1% AcOH), Rf^1 0.03, Rf^2 0.09, single ninhydrin-positive spot. The synthetic peptide exhibited a single band in disk isoelectrofocusing on 12.5% polyacrylamide gel (0.5 × 6.3 cm) containing Pharmalyte (pH 3—10): mobility, 1.8 cm from the origin toward the cathodic end of the gel, after running at 200 V for 4 h (stained with Coomassie Brilliant Blue G-250, Sigma). The purity of the final product was estimated by HPLC to be about 95% (Fig. 4). It was difficult to remove the *ca.* 5% contamination with the Met(O)-derivative of deacetyl-thymosin β_{10} by means of the open-column systems which we employed for the final purification procedures. The synthetic peptide exhibited two peaks on HPLC using a Cosmosil $5C_{18}$ column (4.6 × 150 cm) at retention times of 10.87 min (5%) and 12.79 min (95%), when eluted with a gradient of acetonitrile (30 to 45% in 20 min) in 0.1% TFA at a flow rate of 1.0 ml per min (Fig. 4). Amino acid ratios in a 6 N HCl hydrolysate: Gly 1.01, Ala 3.05, Leu 2.00, Met 0.77, Ile 3.07, Phe 0.97, Pro 1.87, Thr 4.81, Ser 2.83, Asp 3.89, Glu 8.89, Lys 8.01 (recovery of Leu 83%) (Met(O) was not calculated). Amino acid ratios in LAP digestion: Gly 1.02, Ala 3.04, Leu 2.00, Met 0.83, Ile 3.01, Phe 0.96, Pro 1.83, Thr 4.93, Ser 2.91, Asp 2.94, Glu 6.89, Lys 8.03; Asn and Gln were not determined (recovery of Leu 82%).

Distribution of T-Cell Subsets in a Uremic Patient with Tuberculosis and Effect of Synthetic Deacetyl-thymosin β_{10}: Analysis with Monoclonal Antibodies——A 5 ml aliquot of venous blood was drawn into a syringe containing 25 U/ml heparin and was incubated with the synthetic peptide for 60 min at 37 °C, then the T-cells were isolated from venous blood by Ficoll–Hypaque density centrifugation as described elsewhere.[31] T-cells obtained on the Ficoll–Hypaque density centrifugation were examined for membrane antigens by indirect immunofluorescence assay with murine monoclonal antibodies (OKT3, OKT4 and OKT8) according to Hoffman[32] and Janossy *et al.*[33] The isolated T-cells were incubated with OKT3, OKT4 or OKT8 monoclonal antibodies (Ortho Diagnostic Systems, Roritan, N. J.) (1×10^6 mononuclear cells in 0.2 ml of minimum essential medium with 5% fetal calf serum in 10 μl of antibody) at 4 °C for 30 min. After being washed twice, the cells were allowed to react with goat anti-mouse IgG antibody labelled with fluorescamine, and incubated at 4 °C for 30 min. The cells were washed three times and resuspended in 1.0 ml of minimum essential medium supplemented with 5% fetal calf serum and 3 mM EDTA. Labelled cell counting was done under a Nikon UFD-TR fluorescence microscope. The reactivity of antisera was evaluated concomitantly with determination of the phase-contrast morphology of the cells. Two hundred lymphocytes were observed under a fluorescence microscope to calculate the percentage of cells.

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References and Notes

1) The amino acid residues mentioned in this paper are of L-configuration except for glycine. The abbreviations used to denote amino acid derivatives and peptides are those recommended by the IUPAC-IUB Joint Commission on Biochemical Nomenclature: *European J. Biochem.*, **138**, 9 (1984); *Int. J. Peptide Protein Res.*, **24**, No. 1 (1984). Other abbreviations used: DMF, dimethylformamide; DMSO, dimethylsulfoxide; WSCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBT, 1-hydroxybenzotriazole; Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl; NP, *p*-nitrophenyl; OBzl, benzyl ester; Bzl, benzyl; Troc, β,β,β-trichloroethyloxycarbonyl; OcHex, cyclohexyl ester; NMM, *N*-methylmorpholine; DEAE, diethylaminoethyl; TFA, trifluoroacetic acid; AcOH, acetic acid; MeOH, methanol; EtOH, ethanol; EtOAc, ethyl acetate; EDTA, ethylenediaminetetraacetic acid; E-rosette, a rosette with sheep erythrocytes; TFMSA, trifluoromethanesulfonic acid; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; LAP, leucine aminopeptidase; IgG, immunoglobulin G.

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