[Chem. Pharm. Bull. 27(12)3171—3175(1979)]

UDC 547.466.1.04.09 : 615.357.011.5.015.4

The Effect of a Synthetic Thymosin α_1 Fragment on the Inhibition of E-rosette Formation by the Serum of a Patient with Nephrotic Syndrome[1)]

Takashi Abiko, Ikuko Onodera, and Hiroshi Sekino

Kidney Center, Sendai Insurance Hospital[2)]

(Received May 31, 1979)

The decapeptide corresponding to the C-terminal amino acid sequence (residues 19—28) of bovine thymosin α_1 was synthesized using HONB-WSCI as a coupling reagent. After incubation of lymphocytes with various amounts of the decapeptide from 10 to 100 μg/ml in the presence of serum from a patient with nephrotic syndrome, recovery of E-rosette formation was observed.

Keywords——thymosin α_1 fragment; nephrotic syndrome; E-rosette formation; HONB-DCC method; lymphocytes

Human peripheral blood lymphocytes are surrounded *in vitro* by an E-rosette formation.[3,4)] These RFC are thymus-derived cells (T cells).[5)] The key role of the thymus in the development and maintenace of cellular immune competence animals and man is well established. Subsequently, Goldstein, *et al.*[6,7)] prepared from bovine thymus a heat-stable acidic molecule composed of 28 amino acid residues, called thymosin α_1. Bovine thymosin α_1 administered to neonatally thymectomized mice has been shown to decrease the incidence of wasting disease,[8)] and to increase the development of cell-mediated immune responses such as the capacity of host cells to elicit a normal graft host reaction.[9)] On the other hand, there are studies showing that sera from patients with nephrotic syndrome inhibit E-rosette formation.[10)] An extract of calf thymus, thymosin, induces some recovery of E-rosette formation when incubated *in vitro* with human peripheral blood lymphocytes and such serum.[10)]

On the other hand, it is known that the shorter chain peptides eledoisin and physalaemin possess *in vitro* contractile activities much higher than those of the parent peptides.[11)] We

1) The amino acid residues are of the L-configuration. The abbreviations used to denote amino acid derivatives and peptides are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochem. Biophys. Acta*, **263**, 205 (1972). Other abbreviations: DMF=dimethylformamide, TFA=trifluoroacetic acid, WSCI=water-soluble carbodiimide, EDTA=ethylenediamine tetraacetic acid, E=sheep erythrocytes, FCS=fetal calf serum, GVB^{2+}=gelatin veronal buffer, HONB=N-hydroxy-5-norbornene-2,3-dicarboxyimide, RFC=rosette-forming cell, PBS=phosphate-buffered saline, DCC=dicyclohexylcarbodiimide.

2) Location: *Tsutsumimachi 3-16-1, Sendai, 980, Japan.*

3) J.F. Bach, J. Dormont, M. Dardenne, and H. Balner, *Transplantation*, **8**, 265 (1969).
4) P. Brain, J. Gordon, and W.A. Willets, *Clin. Exp. Immunol.*, **6**, 681 (1970).
5) J. Wybran, M. C. Carr, and H.H. Fudenberg, *J. Clin. Invest.*, **51**, 2537 (1972).
6) A.L. Goldstein, A. Guha, M.M. Zatz, M.A. Hardy, and A. White, *Proc. Natl. Acad. Sci. USA*, **69**, 1800 (1972).
7) A.L. Goldstein, T.L.K. Low, M. Mcadoo, J. Mcclure, G.B. Thurman, J. Rossio, C. Lai, D. Chang, S. Wang, C. Harvey, A.H. Ramel, and J. Meinhofer, *Proc. Natl. Acad. Sci. USA*, **74**, 725 (1977).
8) Y. Asanuma, A.L. Goldstein, and A. White, *Endocrinology*, **86**, 600 (1970).
9) L.W. Law, *Nature* (London), **219**, 1391 (1968).
10) S. Tomisawa, S. Suzuki, M. Oguri, and T. Kuroume, Abstracts of Papers, "20th Annual Meeting of the Nephrological Society of Japan, Nagoya," December, 1977, p. 157.
11) L. Bernardi, G. Bosisio, Chillemi, G. deCaro, R. Castiglione, V. Erspamer, A. Glasser, and O. Gooredo, *Exp.*, **20**, 306 (1964).

describe here the synthesis of a decapeptide with an amino acid sequence corresponding to positions 19—28 (H–Lys–Lys–Glu–Val–Val–Glu–Glu–Ala–Glu–Asn–OH) of thymosin α_1 and report the results of immunological assay of the synthetic peptide. The synthetic route for the decapeptide is illustrated in Fig. 1. The DCC condensation of suitable peptide fragments was performed in the presence of a racemization suppressor, such as HONB.[12]

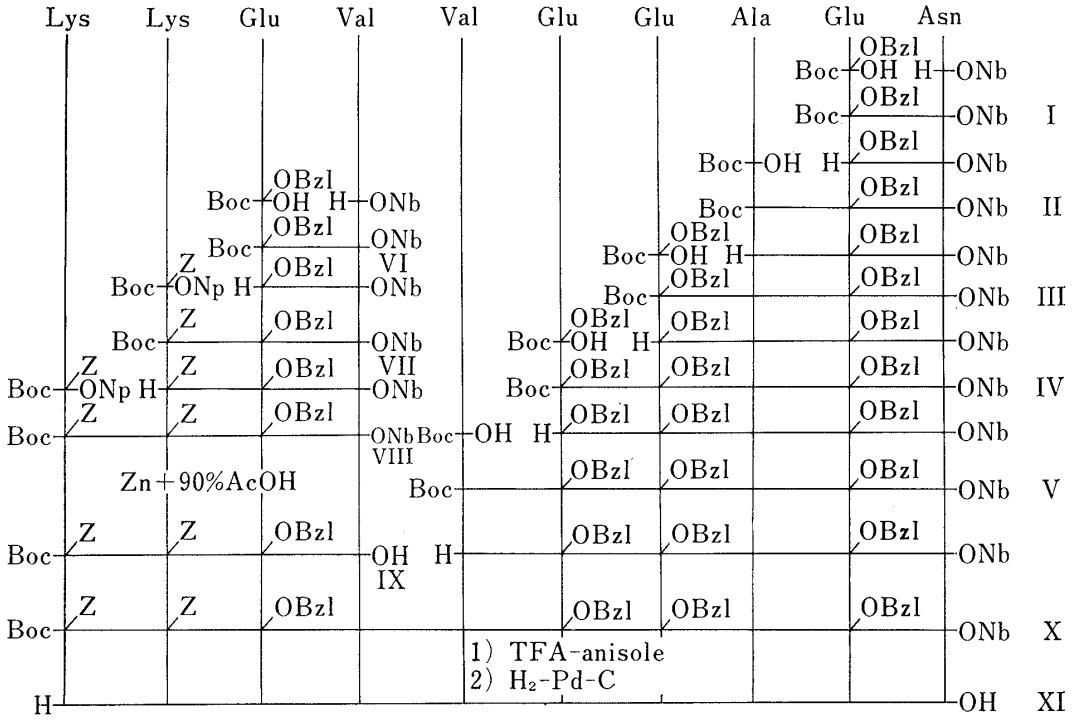

Fig. 1. Synthetic Scheme for the Thymosin α_1 Fragment (residues 19—28)

Z–Asn–ONb was treated with HBr in AcOH–anisole to remove the Z group and the resulting product was condensed with Boc–Glu(OBzl)–OH by the HONB–DCC method,[12] giving Boc–Glu(OBzl)–Asn–ONb (I). I was treated with TFA-anisole and the resulting product was coupled with Boc–Ala–OH by the HONB–DCC method to give Boc–Ala–Glu-(OBzl)–Asn–ONb (II). II was treated with TFA–anisole and the resulting product was condensed with Boc–Glu(OBzl)–OH by the HONB–DCC method to give Boc–Glu(OBzl)–Ala–Glu(OBzl)–Asn–ONb (III). The Boc group of III was removed and the corresponding free base was condensed with Boc–Glu(OBzl)–OH by the HONB–DCC method to yield Boc–Glu(OBzl)–Glu(OBzl)–Ala–Glu(OBzl)–Asn–ONb (IV). The Boc group of IV was removed and the corresponding free base was condensed with Boc–Val–OH by the HONB–DCC method to obtain Boc–Val–Glu(OBzl)–Glu(OBzl)–Ala–Glu(OBzl)–Asn–ONb (V). The Z group of Z–Val–ONb was removed and the corresponding free base was condensed with Boc–Glu(OBzl)–OH by the HONB–DCC method to give Boc–Glu(OBzl)–Val–ONb (VI). The Boc group of VI was removed and the corresponding free base was condensed with Boc–Lys(Z)–ONp[13] to yield Boc–Lys(Z)–Glu(OBzl)–Val–ONb (VII). The Boc group of VII was removed and the corresponding free base was condensed with Boc–Lys(Z)–ONp to obtain Boc–Lys(Z)–Lys-(Z)–Glu(OBzl)–Val–ONb (VIII), from which the *p*-nitrobenzyl ester group was removed by treatment with Zn in 90% AcOH and DMF.[14] The last traces of metal contamination were

12) M. Wakimasu and M. Fujino, *Chem. Pharm. Bull.* (Tokyo), **26**, 1522 (1978).
13) K. Suzuki, *Chem. Pharm. Bull.* (Tokyo), **14**, 909 (1966).
14) K. Suzuki, N. Endo, K. Nitta, and Y. Sasaki, "Proceedings of the 14th Symposium on Peptide Chemistry," Hiroshima, 1976, p. 45.

removed by treatment with EDTA. The Boc group of hexapeptide V was removed and the corresponding free base was condensed with the N-terminal moiety Boc–Lys(Z)–Lys-(Z)–Glu(OBzl)–Val–OH (IX) by the HONB–DCC method to yield Boc–Lys(Z)–Lys(Z)–Glu-(OBzl)–Val–Val–Glu(OBzl)–Glu(OBzl)–Ala–Glu(OBzl)–Asn–ONb (X). After removal of the Boc group of X with TFA, the resulting decapeptide ester was hydrogenated over 10% Pd-C in AcOH solution overnight. The hydrogenated product was purified by column chromatography on Sephadex G-15. Analysis of the main fraction by paper chromatography using Partridge's[15] and Waley's[16] solvent systems revealed the presence of one major ninhydrin-positive spot and one minor spot. The crude decapeptide was further purified by DEAE-Sephadex A-25 chromatography. The decapeptide (XI) thus obtained was found to be pure by paper chromatography using two different solvent systems. The amino acid ratios in the acid hydrolysate of XI and its aminopeptidase (AP)–M digest agreed with the theoretical values. Incubation of lymphocytes with serum from a patient with nephrotic syndrome decreased the proportions of T-cell rosettes from 78% to 45%. After incubation of cell suspensions with various amounts of the decapeptide from 10 μg/ml to 100 μg/ml, recovery of T-cell rosette formation was observed (Table I). Our data establish that the key residues involved in the active site of thymosin α_1 are present within our synthetic decapeptide.

Table I. Effect of the Synthetic Thymosin α_1 Fragment (Residues 19—28) on the Action of Inhibiting Factor of Serum from a Patient with Nephrotic Syndrome

Peptide	Dose (μg/ml)	E-rosette formation (%)
——[a,b]		78
——[a,c]		45
Gly–Gly–His[a,c,d]	10	44
	100	42
Lys–Lys–Glu–Val–Val–Glu–Glu–Ala–Glu–Asn[a,c]	10	51
	100	76

a) Normal lymphocytes.
b) Control serum: incubation was carried out for 30 min at 37° at a concentration of 30%.
c) Patient's serum: incubation was carried out for 30 min at 37° at a concentration of 30%.
d) Control peptide (purchased from the Protein Research Foundation, Minoh, Osaka).

Experimental

Melting points are uncorrected. Unless otherwise mentioned, Z groups of the protected amino acids and peptides were deblocked with HBr in AcOH, and Boc groups with TFA. The resulting amino components were chromatographed on filter paper, Toyo Roshi No. 51, at room temperature. Rf^1 values refer to the Partridge system[15] and Rf^2 values refer to BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24).[16] Rotations were determined with an Atago Polax. Amino acid analyses were performed with a JEOL JLC-8AH amino acid analyzer. Evaporations were carried out in a rotary evaporator under reduced pressure at a temperature of 35—40°. Serum samples were obtained from a patient with nephrotic syndrome. Control serum were obtained from healthy persons. The blood was centrifuged and the separated serum was kept at −20° until use.

Boc-Glu(OBzl)-Asn-ONb (I)——Z–Asn–ONb (5.0 g) was dissolved in AcOH (20.0 ml), anisole (1.3 ml) and 25% HBr in AcOH (20.0 ml). After 40 min at room temperature, the reaction mixture was shaken vigorously with dry ether. The precipitate was washed with dry ether and dried over KOH pellets *in vacuo*. HONB (2.3 g),[12] Boc–Glu(OBzl)–OH (4.3 g) and WSCI (2.1 g) were added to an ice-cold solution of H–Asn–ONb·HBr in DMF (25.0 ml), followed by addition of N-methylmorpholine[17] to keep the solution slightly alkaline. After 24 hr at −10°, the reaction mixture was poured into 1 N $NaHCO_3$, with stirring. The precipitate thus formed was washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O. The

15) S.M. Partridge, *Biochem. J.*, **42**, 238 (1948).
16) S.G. Waley and G. Watson, *Biochem. J.*, **55**, 328 (1953).
17) G.W. Anderson, F.M. Callahan, and J.E. Zimmerman, *J. Am. Chem. Soc.*, **89**, 178 (1967).

precipitate was reprecipitated from DMF and 1 N citric acid. Yield 4.4 g (60%), mp 93—96°, $[\alpha]_D^{23}$ −23.6° (c=1.0, DMF), *Anal.* Calcd. for $C_{28}H_{34}N_4O_{10}$: C, 57.33; H, 5.84; N, 9.55. Found: C, 56.94; H, 6.12; N, 9.66. Rf^1 0.53, Rf^2 0.68, single ninhydrin-positive spot.

Boc-Ala-Glu(OBzl)-Asn-ONb (II)——The protected dipeptide ester I (2.400 g) was dissolved in TFA (4.0 ml) in the presence of anisole (0.7 ml) and the solution was kept at room temperature for 20 min, then ether was added. The precipitate formed was dried over KOH pellets *in vacuo*. The resulting dipeptide ester was condensed with Boc–Ala–OH (0.823 g) in the presence of HONB (0.789 g) and WSCI (0.682 g) essentially as described for the preparation of I. EtOAc was added to the mixture and the EtOAc solution was washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O. The solution was dried over $MgSO_4$ and concentrated to a small volume, then petroleum ether was added. The precipitate was reprecipitated from EtOAc and petroleum ether was added to the residue. The precipitate was reprecipitated from EtOAc and petroleum ether. Yield 1.8 g (67%), mp 142—144°, $[\alpha]_D^{23}$ −35.0° (c=1.0, DMF), *Anal.* Calcd. for $C_{31}H_{39}N_5O_{11}$: C, 56.61; H, 5.98; N, 10.65. Found: C, 56.81; H, 5.99; N, 10.36. Rf^1 0.68, Rf^2 0.79, single ninhydrin-positive spot.

Boc-Glu(OBzl)-Ala-Glu(OBzl)-Asn-ONb (III)——II (1.000 g) was treated with TFA (2.0 ml) as described above. The resulting tripeptide ester was condensed with Boc–Glu(OBzl)–OH (0.571 g) in the presence of HONB (0.304 g) and WSCI (0.263 g) essentially as described for the preparation of I. The reaction mixture was extracted with EtOAc and washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O. The solution was dried over $MgSO_4$ and concentrated to a small volume, then petroleum ether was added to the residue. The precipitate was reprecipitated from EtOAc and ether. Yield 0.785 g (60%), mp 135—145°, $[\alpha]_D^{23}$ −32.8° (c=1.0, DMF), *Anal.* Calcd. for $C_{43}H_{52}N_6O_{14}$: C, 58.89; H, 5.98; N, 9.59. Found: C, 58.64; H, 6.11; N, 9.36. Rf^1 0.64, Rf^2 0.79, single ninhydrin-positive spot.

Boc-Glu(OBzl)-Glu(OBzl)-Ala-Glu(OBzl)-Asn-ONb (IV)——III (439 mg) was treated with TFA (1.0 ml) as described above. The resulting tetrapeptide ester was condensed with Boc–Glu(OBzl)–OH (176 mg) in the presence of HONB (99 mg) and WSCI (86 mg) essentially as described for the preparation of II. Yield 405 mg (71%), mp 121—123°, $[\alpha]_D^{23}$ −31.9° (c=1.0, DMF), *Anal.* Calcd. for $C_{55}H_{65}N_7O_{17}\cdot 2H_2O$: C, 58.34; H, 6.14; N, 9.66. Found: C, 58.31; H, 6.36; N, 9.46. Rf^1 0.71, Rf^2 0.83, single ninhydrin-positive spot.

Boc-Val-Glu(OBzl)-Glu(OBzl)-Ala-Glu(OBzl)-Asn-ONb (V)——IV (365 mg) was treated with TFA (1.0 ml) as described above. The resulting pentapeptide ester was condensed with Boc-Val-OH (92 mg) in the presence of HONB (66 mg) and WSCI (57 mg) essentially as described for the preparation of II. Yield 300 mg (75%), mp 93—108°, $[\alpha]_D^{23}$ −26.4° (c=1.0, DMF), *Anal.* Calcd. for $C_{60}H_{74}N_8O_{18}$: C, 60.29; H, 6.24; N, 9.38. Found: C, 60.42; H, 6.39; N, 8.91. Rf^1 0.73, Rf^2 0.79, single ninhydrin-positive spot.

Boc-Glu(OBzl)-Val-ONb (VI)——The compound was prepared from Z–Val–ONb (3.9 g), Boc–Glu(OBzl)–OH (3.6 g), HONB (2.0 g) and WSCI (1.8 g) essentially as described for the preparation of I. The reaction mixture was extracted with EtOAc and washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O. The solution was dried over $MgSO_4$ and concentrated to a small volume, then petroleum ether was added to the residue. Yield 4.0 g (60%), mp 65—70°, $[\alpha]_D^{23}$ −19.4° (c=1.0, DMF), *Anal.* Calcd. for $C_{29}H_{37}N_3O_9\cdot H_2O$: C, 59.07; H, 6.67; N, 7.13. Found: C, 59.43; H, 6.88; N, 6.91. Rf^1 0.80, Rf^2 0.91, single ninhydrin-positive spot.

Boc-Lys(Z)-Glu(OBzl)-Val-ONb (VII)——VI (2.3 g) was treated with TFA (3.0 ml) as described above. Boc–Lys(Z)–ONp (1.7 g) was added to a solution of this product in DMF (20.0 ml), followed by N-methylmorpholine to keep the solution slightly alkaline. After 24 hr at room temperature, the reaction mixture was diluted with 1 N NH_4OH (3.0 ml) with stirring. After 1 hr, the mixture was extracted with EtOAc and washed successively with 1 N NH_4OH, H_2O, 1 N citric acid and H_2O. The solution was dried over $MgSO_4$, concentrated to a small volume, and petroleum ether was added to the residue. The precipitate was reprecipitated from EtOAc and ether. Yield 2.5 g (72%), mp 76—80°, $[\alpha]_D^{23}$ −30.0° (c=1.0, DMF), *Anal.* Calcd. for $C_{43}H_{55}N_5O_{12}$: C, 65.71; H, 7.05; N, 8.91. Found: C, 66.02; H, 7.28; N, 8.85. Rf^1 0.53, Rf^2 0.64, single ninhydrin-positive spot.

Boc-Lys(Z)-Lys(Z)-Glu(OBzl)-Val-ONb (VIII)——The compound was prepared from VII (2.1 g) and Boc–Lys(Z)–ONp (1.1 g) essentially as described for the preparation of VII. The product was recrystallized from hot MeOH. Yield 1.6 g (55%), mp 155—156°, $[\alpha]_D^{23}$ −24.6° (c=1.0, DMF), *Anal.* Calcd. for $C_{57}H_{73}N_7O_{15}$: C, 62.45; H, 6.71; N, 8.95. Found: C, 62.34; H, 7.01; N, 8.99. Rf^1 0.65, Rf^2 0.71, single ninhydrin-positive spot.

Boc-Lys(Z)-Lys(Z)-Glu(OBzl)-Val-OH (IX)——Zn dust (327 mg) was added to a solution of VIII (1100 mg) in DMF–90% AcOH (7 ml–7 ml) and the mixture was stirred at room temperature for 3 hr. The solution was filtered, the filtrate was condensed *in vacuo* and the residue was treated with 2% EDTA (60 ml). The gelatinous mass formed on standing in a refrigerator overnight was collected by filtration, washed with 1 N citric acid and H_2O and then crystallized twice from hot MeOH–H_2O (2: 1). Yield 501 mg (55%), mp 110—116°, $[\alpha]_D^{23}$ −38.6° (c=1.0, DMF), *Anal.* Calcd. for $C_{50}H_{70}N_7O_{13}$: C, 61.15; H, 7.22; N, 10.05. Found: C, 61.46; H, 7.46; N, 9.78. Rf^1 0.44, Rf^2 0.46, single ninhydrin-positive spot.

Boc-Lys(Z)-Lys(Z)-Glu(OBzl)-Val-Val-Glu(OBzl)-Glu(OBzl)-Ala-Glu(OBzl)-Asn-ONb (X)——V (219 mg) was treated with TFA (1.0 ml) as described above. The resulting hexapeptide ester was condensed with IX (235 mg) in the presence of HONB (40 mg) and WSCI (62 mg) essentially as described for the preparation

of I. The product was recrystallized twice from EtOAc. Yield 339 mg (83%), mp 117—123°, $[\alpha]_D^{23}$ −40.1° (c=1.0, DMF), *Anal.* Calcd. for $C_{105}H_{132}N_{14}O_{28}$: C, 61.87; H, 6.53; N, 9.62. Found: C, 61.48; H, 6.63; N, 9.81. Rf^1 0.83, Rf^2 0.92, single ninhydrin-positive spot.

H-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-OH (XI)——The protected decapeptide X (200 mg) was treated with TFA (1.0 ml) in the presence of anisole (0.1 ml) at room temperature for 20 min, then dry ether was added. The resulting powder was washed with ether and dried over KOH pellets *in vacuo*. The de-Boc peptide ester was hydrogenated in 50% AcOH (12 ml) over 10% Pd-C for 20 hr. The catalyst was removed using aid of cellite. The filtrate was evaporated to dryness and the residue was dried over KOH pellets *in vacuo*. The hydrogenated product thus obtained was dissolved in 1% AcOH (3.0 ml) and applied to a column of Sephadex G-15 fine (2.8×60.0 cm), eluting with the same solvent. Individual fractions (4.0 ml each) were collected and the absorbancy at 230 nm was determined. Fractions corresponding to the main peak (tube Nos. 41—53) were combined and the solvent was removed by lyophilization; yield 108 mg. Analysis by paper chromatography revealed the presence of two ninhydrin-positive spots with Rf^1 0.10 (major), 0.31 (minor) and Rf^2 0.18 (major), 0.38 (minor). The product was then dissolved in a small amount of H_2O and the solution was applied to a column of DEAE-Sephadex A-25 (2.8×30.0 cm), eluting with a linear gradient formed from H_2O (150 ml) in the mixing chamber to 0.1 M NH_4OAc buffer (pH 6.50, 150 ml) in the reservoir. Individual fractions (4 ml each) were collected and the absorbancy at 230 nm was determined. Fractions corresponding to the main peak (tube Nos. 43—51) were combined and the solvent was evaporated off *in vacuo*. The product was lyophilized. Yield 65 mg (57%), mp 180—191°, $[\alpha]_D^{23}$ −76.3° (c=1.0, H_2O), Rf^1 0.10, Rf^2 0.18, single ninhydrin-positive spot. Amino acid ratios in the acid hydrolysate: Glu 3.69, Asp 0.86, Val 2.01, Ala 0.98, Lys 1.88. Amino acid ratios in the AP-M digest: Glu 3.63, Asn 0.89, Val 1.96, Ala 1.02, Lys 1.96.

E-rosette Formation——Peripheral blood lymphocytes were isolated in a Hypaque-Ficoll gradient[18] for T cell rosette formation. Isolated lymphocytes were adjusted to 5×10^5 cells/ml with PBS. Contamination by monocytes and polymorphonuclear cells amounted to less than 5%. Sheep erythrocytes were washed with PBS and a suspension (1×10^6/ml) was prepared. Lymphocytes were suspended in GVB^{2+} or FCS (1.0 ml) and incubated for 30 min at 37° with the patient's serum (0.3 ml) and the thymosin α_1 fragment. Lymphocytes were washed with GVB^{2+} and centrifuged for 10 min at 1500 rpm, then suspended in GVB^{2+} or FCS (1.0 ml). The suspension was mixed with sheep erythrocytes (0.5 ml) and incubated for 18 hr at 4°. The mixture was then centrifuged for 5 min at 900 rpm. Triplicate wet-cell preparations were checked by phase contrast microscopy. For each preparation, 200 lymphocytes were counted, and the proportion binding more than three sheep erythrocytes was determined. Monocytes or polymorphonuclear cells forming rosettes were excluded (Table I).

Acknowledgement The authors thank the staff of the Central Analysis Room of the Pharmaceutical Institute, Tohoku University, for elemental analysis.

18) R. Harris and E.O. Ukaejiofo, *Brit. J. Haematol.*, **18**, 229 (1970).