Stabilized Analogs of Thymopentin. 3. Evaluation of Ketomethylene Pseudopeptides for Antiarthritic Properties

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Received November 1, 1995⊗

This study analyzed the role of ketomethylene pseudopeptides of thymopentin as potential agents for the treatment of arthritis. The analogs were tested *in vivo* using assessment of inflammation and antibody production in the mouse type II collagen arthritis model and the rat adjuvant arthritis model. The compounds were also tested for immune-potentiating activity *in vitro* using induction of the lymphocyte marker, Thy-1.2, in mouse spleen cells and stimulation of T-cell proliferation. The results show that certain of the compounds exhibit disease-remitting properties for arthritis as evidenced by reduction of paw swelling in the mouse and rat models and decreased incidence of disease in the mouse model. The active compounds were dose specific and represented a range in efficacy. In spite of effects on arthritis, type II collagen antibody levels were not altered in the mouse model. Selected compounds also exhibited immune potentiating properties as evidenced by induction of Thy-1.2 expression and stimulation of T-cell proliferation. The absence of effect of the compounds on type II collagen antibody production suggests that the antiarthritic activity of the effective compounds results from alteration of cell-mediated immunity.

Introduction

In rheumatoid arthritis, modulation of immune system components continues as an attractive approach for therapeutic intervention. The thymopoietin family of molecules remains a select group of target compounds that upregulate immune system activity by increasing numbers of immune competent cells. The thymopoietin family acts through recruitment of immature thymus-derived cells that eventually mature into a diverse range of helper and suppressor T-cell subsets. In this third paper of a series[1,2] describing the synthesis and biological properties of analogs of thymopentin, a pentapeptide derived from thymopoietin, we have examined the compounds for antiarthritic properties and, in some cases, for immune-potentiating activity by examining effect on induction of the lymphocyte marker, Thy-1.2, in spleen cells and as an inducer of T-cell proliferation, with and without addition of interleukin-1.

Thymopentin (TP-5) functions as an immunomodulatory pentapeptide[3−5] with demonstrated clinical efficacy in treatment of rheumatoid arthritis.[6−9] TP-5 corresponds to residues 32−36 (Arg-Lys-Asp-Val-Tyr) of the parent polypeptide hormone, thymopoietin (**3**). Thymopoietin arises in the epithelium of the thymus and exhibits pleiotropic functions including generation of prothymocytes.[10−12] Although primarily thymic in origin, thymopoietin has been isolated in skin.[13] Unlike interleukin-1 (IL-1) or interleukin-2 (IL-2), thymopoietin appears to enhance T-cell subsets through a process of recruitment and maturation of prothymocytes, rather than direct stimulation of cell proliferation.[14]

TP-5 exhibits distinct patterns of immune-potentiating activity in a variety of *in vitro* systems[15−17] and has been tested for activity in a number of immune-related diseases.[18−20] The efficacy of TP-5 in the treatment of rheumatoid arthritis typically centers on clinical manifestations of the disease. In one study, prolonged intravenous (iv) infusion (10 min) of TP-5 resulted in an improvement of all clinical parameters evaluated except grip strength.[21] In a multicenter study, slow iv infusion of TP-5 (50 mg) three times a week for three weeks improved clinical parameters including the Ritchie index and the sum score of swollen joints whereas lab parameters were unchanged.[22]

The clinical efficacy of TP-5 treatment appears dependent on slow iv infusion of the compound. Analysis of degradation profiles for TP-5 in serum show a half-life in human plasma of about 1 min primarily due to peptidase activity.[23] The half-life of TP-5 in serum could be increased by inhibition of proteases or by removal of divalent cations.[24] These observations suggest that alterations in the primary structure of TP-5 to yield resistance to peptidases could prolong serum half-life and improve drug efficacy.

In the previous two papers of this series,[1,2] we have reported the synthesis of pseudopentapeptide analogs of thymopentin designed to improve stability to serum peptidases. These compounds featured insertion of noncleavable ketomethylene moieties at the 1,2-, 3,4-, and 4,5-peptide bonds in the thymopentin structure and are represented in formulas **1**−**3** below. In the accompanying papers we described the chemistry and measurements of serum stability and *in vitro* binding to a thymic-related receptor for these analogs.[1,2] In this paper we report their evaluation for antiarthritic effects in animal models and other immune-related properties.

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⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1997.

S0022-2623(95)00805-3 CCC: $14.00

R_1-R_2-R_3-R_4(k)R_5

1a: Arg-Lys-Asp-Val(k)Phe
b: Arg-Pro-Asp-Val(k)Phe
c: Arg-Nle-Asp-Val(k)Phe
d: Arg-Leu-Asp-Val(k)Phe
e: Arg-Ala-Asp-Val(k)Phe
f: Arg-D-Lys-Asp-Val(k)Phe
g: Arg-Nle-Asp-Ala(k)Phe
h: Arg-Nle-Asp-Val(k)Val
j: Ac-Arg-Pro-Asp-Val(k)Phe
k: Ac-Arg-Nle-Asp-Val(k)Phe
l: Arg-NMe-Nle-Asp-Val(k)Phe
m: Arg-NMe-Nle-Asp-Val(k)Val
n: Arg-Nle-Asp-Val(CH)Phe

R_1(k)R_2-R_3-R_4-R_5

2a: Arg(k)Nle-Asp-Val-Phe
b: Arg(k)Nle-Asp-Val-PheNH_2
c: Arg(k)Nle-Asp-D-Val-Phe

R_1-R_2-R_3(k)R_4-R_5

3a: Arg-Lys-Asp(k)Val-Phe
b: Arg-Nle-Asp(k)Val-Phe
c: Arg-Pro-Asp(k)Val-Phe
d: Arg-NMe-Lys-Asp(k)Val-Phe
e: Arg-Lys-Asp(k)Ala-Phe
f: Arg-Lys-Asp(k)Val-Tyr
g: Arg-Pro-Asp(k)Val-PheNH_2

Biological Evaluation of Antiarthritic Activity

The animal model selected for primary screening of the TP-5 analogs was the mouse type II collagen assay as developed by Courtenay et al.[25] We chose the collagen-induced model because it represents an inflammatory process that more closely resembles the rheumatoid arthritis condition in humans. It was expected that the inflammatory process would encompass the inductive effect of the antibody to foreign type II collagen and the amplifying action of T-cells in the inflamed region. In this procedure, DBA/1 mice, a genetically conserved strain, are injected with bovine type II collagen to induce disease. In our modification of the Courtenay procedure, a booster dose of antigen was injected at day 21 in order to ensure that virtually all animals develop significant inflammation. Inflammation was usually observed by days 23−30 and became maximal about days 37−44. In addition to the mouse collagen II assay the standard rat adjuvant assay was performed on the more interesting analogs. In both *in vivo* models efficacy was assessed by determining disease onset and inhibition of disease severity.

Analysis of Immune Potentiating Activity. Immune function of the analogs was analyzed with respect to induction of Thy-1.2 expression on nu/nu mice spleen cells and with respect to effects on anti-collagen IgG titers in the animals treated for arthritis. In addition, the effects of the thymopentin analogs as inducers of T-cell proliferation in nonadherent spleen cells of nu/nu mice was quantified.

Methods

Type II Collagen Arthritis Model. Type II collagen arthritis was induced in DBA/1 Lac mice (Jackson Labs, Bar Harbor, ME) using bovine type II collagen emulsified in Freund's complete adjuvant.[25] The primary injections of the collagen (100 μg) were given in four to six sites on the back, and the animals were boosted with a single ip injection (100 μg) on day 21 post primary immunization. Disease onset was scored by detection of erythema and edema in the limbs. The extent of inflammation was quantified using constant tension calipers to measure both hind paws; the swelling typically increased the normal paw thickness from 2.1 mm to a range from 3.0 to 4.5 mm by day 40 post primary immunization. Radiographic analysis was carried out to document the appearance of bony erosion in late stage disease (day 40).

TP-5 Analog Treatment of Type II Collagen Arthritis. Mice immunized with type II collagen were treated with TP-5 and the TP-5 analogs using intravenous injection. The compounds were dissolved in sterile saline immediately prior to use. Treatment was begun at day 19, 2 days prior to the collagen boost. Treatment was administered on every second day and continued through day 40. Each compound was tested initially at three concentrations, 0.1, 0.01, and 0.001 mg/kg; evidence of analog efficacy was investigated by expanding the dose-ranging experiments to determine maximum or minimum effects on disease parameters.

Rat Adjuvant Arthritis. Adjuvant arthritis was induced in Lewis rats using a suspension of *Mycobactericum tuberculum* cell walls (10 mg/mL in mineral oil) injected at the base of the tail (1 mg in 0.1 mL).[26] Onset of disease was marked by presence of erythema and edema; the extent of the disease was quantified by measurement of hind paw swelling as above. TP-5 and TP-5 analogs were applied by iv injection; treatment regimens followed the protocol used for the mouse type II collagen arthritis model except that treatment began 2 days prior to administration of the adjuvant/antigen challenge.

Lymphocyte Proliferation. Whole spleens were collected asceptically from BALB/c or nu/nu mice, minced, and pooled in minimal essential medium (MEM).[27] The cells were collected by centrifugation and resuspended in MEM. The cells were adjusted to a concentration of 1×10^8 cells/mL in 5% fetal bovine serum (FBS) in MEM, and the cell suspension was passed through a 1 mL nylon wool column which was maintained at 37 °C for 1 h. The nonadherent cells were subsequently eluted from the column, centrifuged, and resuspended in MEM. The cell concentration was adjusted to 2×10^7/mL in 10% FBS and MEM. The cells were then dispersed into individual wells of a 96-well plate and stimulated with thymopentin or thymopentin analogs at final concentrations of 100, 10, 1, and 0.1 μg/mL with and without concanavalin A (Con A) or phytohemagglutinin (PHA). Controls included cells without additions and cells exposed to Con A and PHA alone. The cells were maintained for 48 h, and then tritiated thymidine was added at a final concentration of 2.5 μCi/mL. After 18 h, the cells were collected onto filter paper, and thymidine radioactivity was determined by scintillation counting.

Thy-1 Expression. Analysis of Thy-1.2 induction by TP-5 was carried out using the fluorescent cell sorter or by microscopic analysis.[28] Analysis of induction of Thy-1 antigen was carried out using spleen cells of nu/nu mice as a source of prethymocytes. The spleen cells were suspended at a concentration of 1×10^7 cells/mL and fractionated using Ficoll/Hypaque separation. The isolated spleen cells were then adjusted to a final concentration of 1×10^6 cells/mL and exposed to TP-5 analogs for 4.5 h at 37 °C. The cells were then washed in phosphate-buffered saline containing 0.1% azide and treated with FITC-labeled antibody at 4 °C for 30 min. The cells were then washed by centrifugation through heat-inactivated fetal bovine serum and then fixed for analysis with the fluorescence-activated cell sorter. In an alternate procedure, the experimental protocol consisted of analyzing nu/nu mouse spleen cells following exposure of the cells to TP-5 alone and TP-5/IL-1 for a time period of 4 days. The spleen cells were recovered from the culture wells after dissociation with *N*-acetylgalactosamine. The cells were then stained with FITC-antibody specific for Thy-1.2 prepared from rat serum. Controls included blocking the cells with nonimmune serum to test for stain specificity.

Type II Collagen Antibody Titers. An ELISA assay was used for the analysis of effect of TP-5 analogs on the humoral response of the mice to the type II collagen. The assay included coating Immulon II microtiter wells at 4 °C overnight using 100 μL of a 10 μg/mL solution of bovine type II collagen. The plates were then washed with buffered saline containing the detergent Tween 20 at a concentration of 0.05%. Nonspecific binding sites were blocked by addition of a 2% solution of bovine serum albumin in buffered saline. The plates were then washed, and the mouse serum dilutions were added to the wells. Recognition of antibody binding was carried out using peroxidase conjugated goat anti-mouse IgG antibody. Enzyme-dependent color development was quantified by reading the optical density at 490 nm using an automated ELISA reader.

Table 1. Effect of TP-5 Analogs on Onset and Incidence of Arthritis in the Mouse Type II Collagen Model

		incidence of disease[b]			
compd	dose[a] (mg/kg)	day 30	day 37	day 40	day 44
1a-A	0.5	5/12	7/12	8/12	7/12
1b-A	0.01	8/12	9/12	9/12	9/12
1b-B	1.0	6/12	9/12	10/12	11/12
1c-A	0.01	5/12	9/12	10/12	8/12
1c-B	1.0	8/12	11/12	11/12	12/12
1g-A	1.0	8/12	11/12	11/12	12/12
1h-A	1.0	7/12	10/12	10/12	10/12
1h-B	1.5	6/12	7/12	ND	9/12
1k-A	1.0	8/12	11/12	11/12	12/12
1l-A	0.001	7/12	7/12	7/12	8/12
1m-A	1.0	3/12	6/12	7/12	7/12
1m-B	1.0	8/12	11/12	11/12	12/12
2a-B	0.001	3/12	7/12	7/12	8/12
3a-A	0.03	5/12	8/12	ND	9/12
3a-B	1.0	8/12	11/12	12/12	12/12
3d-A	1.0	6/12	4/12	8/12	8/12
3e-A	1.0	5/12	9/12	11/12	12/12
3e-B	1.0	10/12	11/12	10/12	12/12
3f-A	1.0	8/12	11/12	12/12	12/12
TP-5	1.0	8/12	11/12	11/12	12/12
untreated		8/12	11/12	11/12	12/12

[a] All compounds were tested initially at doses that ranged from 1.0, 0.1, and 0.01, to 0.001 mg/kg; with apparent efficacy, the dose range was extended either higher or lower as appropriate. Each dose was tested in a group of 12 animals. Viability was 100%. [b] The onset and incidence of arthritis was scored positive as a result of erythema or edema being present on any digit, paw, or appendage.

Table 2. Extent of Paw Swelling following Treatment with TP-5 Analogs in the Mouse Type II Collagen Arthritis Model

	dose[a]	% of paw swelling[b]			
compd	(mg/kg)	day 30	day 37	day 40	day 44
1a-A	0.5	100	100	93	53
1b-A	0.01	62	72	60	47
1b-B	1.0	66	59	42	53
1c-A	0.01	66	69	70	63
1c-B	1.0	100	100	100	100
1g-A	1.0	100	100	100	100
1h-A	1.0	95	75	66	63
1h-B	1.5	58	17	54	49
1k-A	1.0	100	100	100	100
1l-A	0.001	90	43	ND	68
1m-A	1.0	60	100	73	58
1m-B	1.0	100	100	100	100
2a-B	1.0	100	100	100	100
3a-A	0.03	30	30	70	73
3a-B	1.0	100	100	100	100
3d-A	1.0	100	46	53	53
3e-A	1.0	88	57	73	75
3e-B	1.0	100	68	77	77
3f-A	1.0	100	100	100	100
TP-5	1.0	100	100	100	100
untreated		100	100	100	100

[a] All compounds were tested initially at doses that ranged from 1.0, 0.1, and 0.01, to 0.001 mg/kg; with apparent efficacy, the dose range was extended either higher or lower as appropriate. Each dose was tested in a group of 12 animals. [b] The extent of inflammation was scored by direct caliper measurement of each hind paw of each mouse in a group of twelve animals at the day indicated. The extent of paw swelling is expressed as a percentage of that observed in the untreated animals as determined by the following formula: [*(test compound paw swelling (mm)) − (normal paw (mm))*]/[(untreated paw swelling (mm)) − (normal paw (mm))] × 100. The range of paw swelling for the untreated animals at day 44 was between 3.0 and 3.4 mm. The normal paw values of age-matched negative control animals ranged between 2.07 and 2.14 mm.

Results

Effect of TP-5 Analogs on Mouse Type II Arthritis. A number of TP-5 analogs reduced the time of the onset and overall incidence of arthritis in the mouse model (Table 1). The scoring included an analysis of a time course for disease onset that quantified the number of animals exhibiting disease at days 30, 37, 40, and 44. Some compounds exhibited persistent effects and prevented disease onset at day 44, 4 days after cessation of treatment. Most of the compounds that were assayed outperformed TP-5, which was inactive at all doses administered over the range of 0.001−1.0 mg/kg. The two most interesting analogs in this assay with respect to onset/incidence were **1m-A** and **3d-A**. Both showed particularly strong ability to suppress visual evidence of disease at the 30 and 37 day observations. Other compounds with good activity in these parameters were **1a-A**, **1h-B**, **1l-A**, and **2a-B**. Compounds **1b-A**, **1c-A**, and **3a-A** were moderately effective in this regard.

Only three compounds were able to strongly inhibit the extent of inflammation (paw swelling) over the duration of the assay (Table 2). These were, namely, **1b-B**, **1h-B**, and **3d-A** which suppressed the swelling by about 50% or better over the course of the important 37−44 day period. Compound **1b-A** was also fairly effective and actually showed its greatest effect at the day 44 observation point when drug effect would have been expected to decline. The most active compounds overall were **3d-A** and **1h-B** when one considers the combined effect on onset/incidence and inhibition of paw swelling. Compounds **1l-A** and **1m-A** were also of interest in terms of their combined effect. Compound **3a-A** which showed a strong effect on suppression of inflammation at the early data points had clearly begun to lose effect by days 40−44.

It was of interest to compare the performance of the analogs with strong binding to the T-cell receptor and only minimal improvement in serum stability against those with relatively poor binding, but with much enhanced stability as reported in the accompanying papers of this series.[1,2] Of the three compounds (**3a-A**, **3a-B**, **1c-A**) evaluated *in vivo* that possessed strong binding characteristics and moderate stability improvement in mouse serum, **3a-A** and **1c-A** had a moderate to good effect in the collagen II model. However, **3a-B**, the isomer of **3a-A**, gave a poor showing for antiarthritic action. Compounds **1l-A**, **1m-A**, and **3d-A** were all poor binders, but were essentially nondegraded in serum because of the presence of an NMe-lysine or NMe-norleucine residue at the number 2 position. These analogs showed good to strong antiarthritic effects *in vivo*, which tends to suggest that increased serum stability is a key factor in obtaining a strong and durable effect. Compound **2a-B** with an Arg(k)Nle at the 1,2-bond would be expected to have excellent stability (similar to that of **2a-A**), but this analog fared poorly in the arthritis model. Compound **1b-A** showed good binding and enhanced stability because of the less susceptible arg−pro bond and the Val(k)Phe noncleavable bond and gave moderate to good results in the arthritis model.

Rat Adjuvant Arthritis. In this model, compounds **1m-A**, **1h-B**, **3a-A**, and **3d-A** exhibited inhibitory effects on the extent of inflammation at day 19 (Table 3); days 17−19 correspond to the time of maximal inflammation in untreated animals. Although these compounds decreased paw swelling, some evidence of disease was present as determined by the presence joint erythema.

Table 3. Extent of Paw Swelling following Treatment with TP-5 Analogs in the Rat Adjuvant Arthritis Model

compd	dose[a] (mg/kg)	% of paw swelling[b] day 12	day 16	day 19
1c-A	0.050	0	100	100
1h-B	0.075	0	100	70
1k-A	0.050	0	100	100
1m-A	0.050	0	80	45
1m-B	0.05	0	100	100
3a-A	0.50	0	100	39
3d-A	0.50	0	80	52

[a] All compounds were tested initially at doses that ranged from 0.05 to 0.50 mg/kg; with apparent efficacy, the dose range was extended either higher or lower as appropriate. Each dose was tested in a group of eight animals. [b] The extent of inflammation was scored by direct caliper measurement of each hind paw of each rat in a group of eight animals at the day indicated. The extent of paw swelling is expressed as a percentage of that observed in the untreated animals as determined by the following formula: [*(test compound paw swelling (mm)) − (normal paw (mm))*]/[(untreated paw swelling (mm)) − (normal paw (mm))] × 100. The range of paw swelling for the untreated animals at day 19 was between 5.2 and 5.6 mm. The normal paw values of age-matched negative control animals ranged between 3.7 and 3.9 mm.

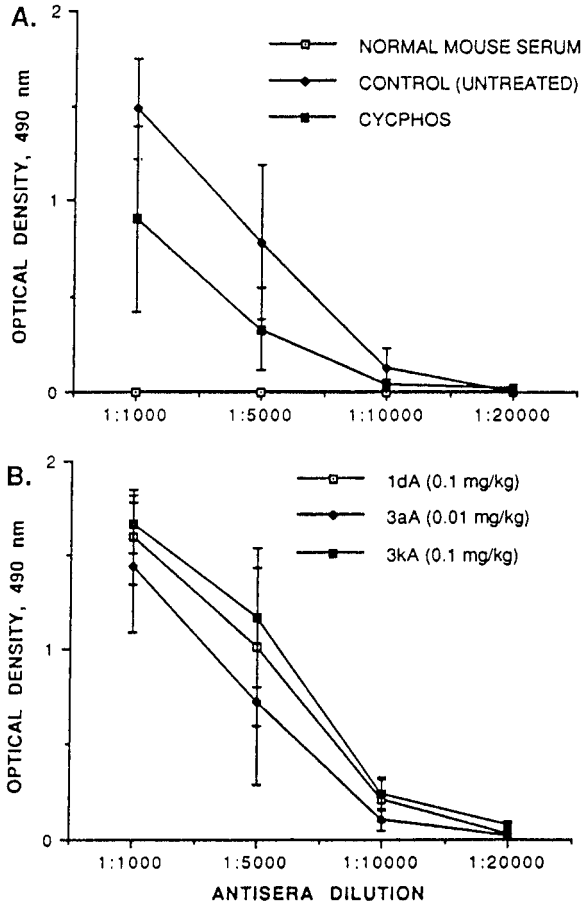

Figure 1. Type II collagen antibody titers in untreated animals and in animals treated with TP-5 pseudopeptides. The data represent the ELISA determination of anti-type II collagen antibodies present (A) in the serum of untreated and cyclophosphamide treated animals and (B) in animals treated with selected TP-5 analogs.

Type II Collagen Antibody Titers. The results for selected TP-5 analogs on type II collagen antibody titers as determined by ELISA analysis are shown in Figure 1. The arthritic mice show significantly increased titers of anti-type II collagen antibodies that reached levels greater than 10000:1 (Figure 1A). Cyclophosphamide treatment served as a positive control and decreased antibody titers by 2.5-fold relative to the untreated controls. Antibody titers in animals with disease were not decreased by treatment with TP-5 or selected TP-5 analogs (Figure 1B).

Table 4. Induction of Thy-1 Expression in nu/nu Mouse Spleen Cells[a]

compd tested	Thy-1 expression
TP-5	+
1a-A	±
1b-A	+
1c-A	++
3f-A	++

[a] Cells were stained with antibody following fixation in paraformaldehyde as described in the Methods. The staining was ranked according to the strength of signal presentation in 10 independent assessed fields of view; all ranking was carried out independently by two observers (RLS and LD) using double-blinded methods.

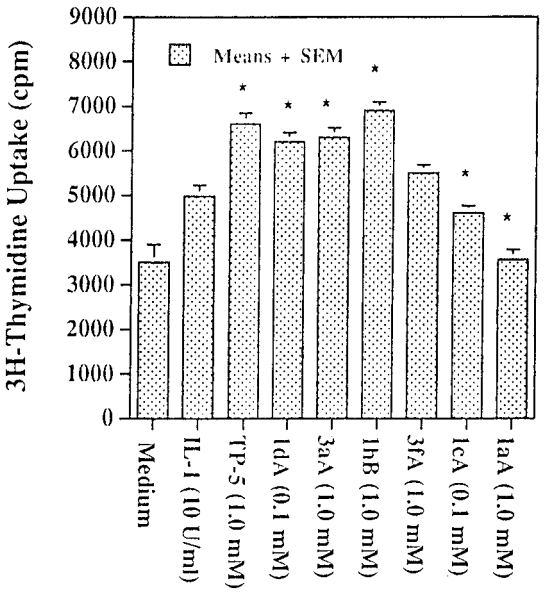

Figure 2. TP-5 and TP-5 analog stimulation of nu/nu mouse spleen cell proliferation: TP-5 and selected compoounds were added to nu/nu mouse spleen cells alone and in combination with interleukin-1 (IL-1). TP-5-stimulated cell proliferation when added alone at 1 mM and when added in combination with IL-1 (10 units/mL). Each analog was tested with IL-1 at 10 units/mL. Statistical significance was determined by one-way analysis of variance (ANOVA) and by Student's two-sample *t*-test (two-tailed). Significance at $p < 0.05$ is indicated by the asterisk.

Thy-1.2 Expression. Analysis of Thy-1.2 expression using fluorescent cell sorting of the athymic nu/nu mouse spleen cells showed that TP-5 and **1c-A** at concentrations of 10^{-3} M increased Thy-1 positive cells to 38% and 46%, respectively, from 25% in untreated controls. Analogs **1b-A** and **3f-A** also induced Thy-1 expression to similar levels. The effect of TP-5 and selected TP-5 analogs on induction of Thy-1.2 expression was confirmed using fluorescence microscopy to assess anti-Thy-1.2 binding to cells. Compounds **1a-A**, **1b-A**, **1c-A**, and **3f-A** were as effective for induction of the Thy-1.2 expression as the parent compound, TP-5 (Table 4).

TP-5/IL-1 Dependent Spleen Cell Proliferation. Induction of Thy-1.2 expressing cells in the nu/nu spleen cell population confirmed that a small number of cells, possibly prothymocytes, were increased following treatment with TP-5. This observation suggested that co-stimulation of the nu/nu spleen cells with TP-5 and a

T-cell cytokine, such as interleukin-1, would result in an enhanced proliferative response the hypothesis being that TP-5 would recruit IL-1 responsive cells. This hypothesis was tested by treating dissociated nu/nu spleen cells with IL-1 alone and in combination with TP-5. A small but significant increase in spleen cell proliferation was observed with TP-5 with and without IL-1. Further analysis of spleen cell proliferation showed that selected TP-5 analogs added together with IL-1 (10 units/mL) also exhibited statistically significant ($p < 0.05$) dose-specific effects on cell proliferation (Figure 2). Analog **1b-A** was stimulatory at 10^{-4} M; analogs **3a-A** and **1h-B** were stimulatory at 10^{-3} M. Analog **3f-A** had no effect on spleen cell proliferation beyond induced by IL-1 alone. Analogs **1a-A** and **1c-A** were both inhibitory to cell proliferation.

In the studies described in this series of three papers we have shown that stable analogs of thymopentin can be prepared which have improved binding to T-cell receptors and/or much improved stability in serum. Some of the analogs showed potential for use as antiarthritic agents in both the mouse and rat models and seem to have an effect on T-cell-related processes as a probable mechanism of action. Their antiarthritic effects in animal models was not exceptionally strong when compared to powerful cytotoxic immunosuppressive drugs such as methotexate or cyclophosphamide. However, the pseudopeptide analogs clearly outperformed the pure pentapeptide, thymopentin, in the animal models, suggesting that they may have a clinical potential greater than thymopentin itself.

Acknowledgment. This work was sponsored by Japan Tobacco Ltd.

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JM950805V