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Immunomodulatory Activity of Hexapeptides Related to Proline Rich Peptide from Colostrum

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Abstract—Twelve analogues of an immunomodulatory hexapeptide YVPGFP (**I**) derived from Proline rich peptide (from colostrum) have been synthesized with modifications at positions 2, 4 and 6. In MLR assay one of the analogues exhibited approx 50% inhibition at 0.1 μg/mL concentration in contrast to prednisolone and **I** which caused around 70 and 20% suppression respectively, at the same concentration.

The powerful influence of peptides on the immune system has been well documented. Several small peptides of diverse structures have been synthesized and evaluated as immunomodulators. Examples include early work on muramyl dipeptides and lauryl tetrapeptide of microbial origin,[1,2] and peptides[3,4] derived from immunoglobulin G (tuftsin) and thymopentin (TP-5). Other potent low molecular weight peptides reported with immunomodulatory properties include: peptides derived from immunoregulatory proteins,[5] CD4 D4 domain[6] and human leukocyte antigen 1.[7] Peptides derived from food proteins such as human milk casein (hexapeptide and tripeptide)[8,9] and ovine colostrum[10] (hexapeptide) have also been reported to be endowed with immunomodulatory activity.

Out of these peptides, the milk casein fragments and peptides derived from colostrum are of considerable importance since it is the first mammalian food and, being intravital could be devoid of the toxic side effects normally associated with the peptides of microbial origin. Recently we reported structure activity relationship studies on human casein fragments and found that structural modifications in fragment resulted in congeners with increased immunostimulant activity.[11,12] This encouraged us to further examine the role of peptides, derived from other food protein colostrum, in immunomodualtion.

Janusz et al.[10] for the first time reported immunomodulatory properties in a peptide isolated from ovine colostrum called proline rich peptide (PRP) beacuse of a high number of Pro residues. Later, Siemion and coworkers[13] reported immunosuppressive activity in a hexapeptide YVPGFP (**I**) derived from YVPLFP, a minimal fragment in PRP with immunostimulant activity. Since there is an ongoing requirement to develop immunosuppressive drugs with novel mode of action and with an improved ratio of desired activity to toxic effects, we decided to synthesize analogues of **I** with the view to develop superactive congeners with immunosuppressor activity. In the present paper immunomodulatory activity of twelve novel analogues related to YVPGFP is described.

Chemical Synthesis

Chemical synthesis of peptides was carried out on Cl-trityl chloride resin (1.2 mmol/gm) using standard Fmoc/Bu^t strategy.[14] After the loading of the first amino acids, the remaining peptide chain was assembled by sequential coupling of Fmoc-protected amino acids (3 equiv) in the presence of diisopropyldicarbodiimide (3 equiv) and 1-hydroxybenzotriazole (3 equiv) in DMF for 3 h. The completeness of each coupling was verified by the Kaiser test.[15] The Fmoc group was removed by treatment with piperidine (20% v/v in DMF). The final peptide was liberated from the resin by treatment with reagent K.[16] The crude peptide was then purified on a semipreparative reverse phase column using linear gradient of A: 0.1% TFA in water and B; 0.1% TFA in acetonitrile, from 80 to 50% A over 45 min. The final peptides were characterized using FABMS and amino acid analysis. The physicochemical characteristics of compounds **II** to **XII** have been given in Table 1.

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Immunomodulatory Activity

The synthetic peptides were tested for their immunomudulatory activity in vitro using lymphocyte transformation test (LTT) and mixed lymphocyte reaction (MLR).

For LTT Splenocytes from normal Swiss mouse were cultured for 72 h in the presence of concanavalin-A at different concentrations (10, 1, 0.1 and 0.01 μg/mL) of test compounds in a total volume of 200 μL in CO_2 using standard protocol. On the other hand for mixed lymphocyte reaction (MLR), cells from two genetically different strains of mice (Swiss and Fawn C3H strains) were used. The results have been expressed as stimulation index i.e., the ratio of the DPM in experimental wells to that in control (untreated wells). The data were statistically analyzed by student '*t*' test.

Results and Discussion

The results of the lymphocyte transformation test have been summarized in Table 2. As is evident, the analogues exhibited moderate to marked order of immunostimulant and immunosuppressant activity depending on the concentration used.

Analogue **X** with Sarcosine (Sar) at position 6 and Gly at poistion 4 resulted in significant stimulatory effect with 93% increase in the stimulation index ($P < 0.001$) at 1 μg/mL. At lower concentration (0.1 μg/mL) however, it exhibited significant decrease in the stimulation index value. Thus, it is interesting to note that analogue **X** with Sar at position 6 and Gly at position 4 exhibited both stimulatory as well as inhibitory effect in a dose dependent manner. A possible explanation for this observation awaits further investigation. In contrast analogue **IX** with Sar at position 6 and Leu at position 4 instead of Gly as in analogue **X** had no affect on stimulation index. Similarly replacement of amino acids at positions 3 and 4 with Sar in analaogues **XI** and **IV** had no affect on the stimulation index.

In the case of analogue **VI** with Leu at position 2 and Gly at position 4, strong depression in lymphocyte proliferation was observed. The stimulation indices exhibited nearly 40% inhibition at all concentrations except at 0.01 μg/mL where the inhibition was of the order of 25%. Thus, replacement of Val at position 2 in the lead peptide **I** by Leu and presence of Gly at position 4 resulted in an analogue with potent immunosuppressive effect at low concentrations. This gets further support from the fact that analogue **V** with Norvaline (Nval) at position 2 and Gly at position 4 exhibited no improvement in the biological profile. These studies suggest that significant immunosuppressor effect exhibited by analogue **VI** may be attributed to hydrophobicity and steric/branching associated with the side chain of Leu at position 2 and to the presence of Gly at position 4.

Further structural modifications, in which L-Pro at position 3 and 6 was replaced by D-Pro in analogues **II**, **III**, **VII** and **VIII** and by Pro-NH_2 in analogue **XII** had no favorable effect on biological response.

This was followed by evaluation of compounds **III, VI, X** and the lead peptide **I** for their immunosuppressive activity in mixed lymphocyte culture. The results have been compared with the immunosuppressive effects of prednisolone, a standard immunosuppressant and summarized in Table 3. Compound **III** with no significant activity in LTT assay was included in this assay with the view to

Table 2. Effect of peptides on lymphocyte proliferation (LTT)

Peptides	Stimulation index (μg/mL concentration)[a,b]			
	10.00	1.00	0.10	0.01
Lead peptide **I**	0.75±0.12	1.02±0.14	1.05±0.10	1.13±0.53
II	0.82±0.09	0.88±0.15	1.08±0.34	1.10±0.18
III	1.40±0.37	1.17±0.24	1.29±0.0	0.97±0.10
IV	0.96±0.27	1.37±0.39	1.21±0.37	1.41±0.47
V	0.73±0.23	1.15±0.44	1.06±0.49	0.86±0.38
VI	0.59±0.06	0.62±0.16*	0.64±0.13**	0.75±0.16
VII	1.05±0.34	0.96±0.33	1.02±0.4	0.89±0.08
VIII	0.95±0.35	1.27±0.52	0.86±0.22	0.78±0.12
IX	1.20±0.50	1.21±0.52	0.86±0.14	0.90±0.28
X	1.60±0.15***	1.93±0.4	0.61±0.13**	0.83±0.25
XI	1.12±0.25	1.24±0.4	0.97±0.5	0.88±0.28
XII	0.84±0.16	1.10±0.51	0.85±0.4	0.83±0.12

[a]Values less than 1 indicate suppression effect.
[b]Values expressed as ±S.D. of the experiments (in triplicate). *$P < 0.02$; **$P < 0.01$; ***$P < 0.001$.

Table 1. Physicochemical characteristics of peptides **II** to **XII**

Peptides	FABMS	Retention time[b] t_R (min)	Amino acid analysis[a]
Tyr-Val-Pro-Leu-Phe-D-Pro **(II)**	735 (M + H)	21.83	Y 0.97 (1), V 1.04 (1), P 2.06 (2), L 0.98 (1), F 1.05 (1)
Tyr-Val-D-Pro-Gly-Phe-Pro **(III)**	679 (M + H)	15.85	Y 0.94 (1), V 1.09 (1), P 2.05 (2), G 1.03 (1), F 0.98 (1)
Tyr-Val-Pro-Sar-Phe-Pro **(IV)**	693 (M + H)	16.96	Y 0.96 (1), V 0.97 (1), P 1.93 (2), Sar 0.98 (1), F 1.02 (1)
Tyr-Nval-Pro-Gly-Phe-Pro **(V)**	680 (M + H)	12.93	Y 0.97 (1), P 2.09 (2), G 0.97 (1), F 0.98 (1)
Tyr-Leu-Pro-Gly-Phe-Pro **(VI)**	693 (M + H)	18.01	Y 1.06 (1), L 0.98 (1), P 2.10 (2), G 0.98 (1), F 1.09 (1)
Tyr-Val-Pro-Gly-Phe-D-Pro **(VII)**	701 (M + Na)	16.31	Y 0.94 (1), V 1.01 (1), P 2.06 (2), G 0.96 (1), F 1.01 (1)
Tyr-Val-D-Pro-Leu-Phe-Pro **(VIII)**	757 (M + Na)	21.21	Y 0.95 (1), V 0.97 (1), P 2.09 (2), L 0.97 (1), F 1.04 (1)
Tyr-Val-Pro-Leu-Phe-Sar **(IX)**	709 (M + H)	21.32	Y 0.96 (1), V 1.07 (1), P 0.95 (1), L 1.02 (1), F 0.98 (1), Sar 0.97 (1)
Tyr-Val-Pro-Gly-Phe-Sar **(X)**	675 (M + Na)	16.45	Y 1.01 (1), V 0.98 (1), P 0.98 (1), G 1.03 (1), F 1.04 (1), Sar 1.05 (1)
Tyr-Val-Sar-Leu-Phe-Pro **(XI)**	709 (M + H)	21.53	Y 1.06 (1), V 0.96 (1), P 0.97 (1), Sar 0.98 (1), L 0.98 (1), F 1.07 (1)
Tyr-Val-Pro-Gly-Phe-Pro-NH_2 **(XII)**	678 (M + H)	22.07	Y 0.97 (1), V 1.01 (1), P 2.06 (2), G 1.03 (1), F 0.98 (1)

[a]A single letter code has been used to denote amino acids, data for Norvaline (Nval) in analogue **V** has not been shown.
[b]t_R Denotes retention time of pure peptides on HPLC.

Table 3. Effect of peptides I, III, VI and X on mixed lymphocyte reaction

	Stimulation index (μg/mL concentration)			
Peptides	10.00	1.00	0.10	0.01
Lead peptide **I**	1.00±0.42	0.85±0.23	0.79±0.18	0.72±0.10
III	1.00±0.14	1.31±0.63	0.90±0.25	0.96±0.25
VI	1.24±0.05	1.20±0.20	0.50±0.17	0.63±0.12
X	0.92±0.40	0.72±0.19	0.80±0.17	0.71±0.15
Prednisolone	—	—	0.30±0.22	—

have a negative control. Out of the four compounds tested, only peptide **VI** exhibited high order of immunosuppressive activity at lower concentrations. It exhibited approximately 50% inhibition at 0.1 μg/mL concentration in contrast to prednisolone which caused around 70% suppression at the same concentration. The lead peptide **I**, and one of its analogues, **X,** however, expressed only 20% suppression whereas analogue **III** did not show any significant suppression at 0.1 μg/mL. Thus, in the MLR assay, peptide **VI** was found to be approximately over two times more active than the lead peptide **I** and slightly less active than prednisolone.

Thus our studies led to the identification of peptides **VI** and **X** as lead molecules for the development of potent immunomodulators. Strong immunosuppressor activity exhibited by analogue **VI** may be attributed to hydrophobicity and steric/branching associated with the side chain of Leu at position 2 and presence of Gly at position 4. Since commercially available immunosuppressive drugs (reviewed in ref 17) Ciclosporin A, FK 506, corticosteroids etc. are known to be associated with several toxic side effects, peptide **VI** derived from food protein, lays a foundation for the design of more potent peptides or peptidomimetics.

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