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IMMUNOSUPPRESSANT ACTIVITY IN HUMAN β-CASEIN FRAGMENT ANALOGS[$]

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Abstract: Human β-casein fragment (54-59) having the amino acid sequence Val-Glu-Pro-Ile-Pro-Tyr, has been shown potent immunostimulant activity. Several analogs of this hexapeptide have been synthesized with modification in the N-terminal region and tested for their immunomodulatory activity. Intrestingly, two hexapeptides have shown significant immunosuppressant activity.

Since the discovery of muramyl dipeptide (MDP), the smallest fragment of bacterial peptidoglycan, as powerful immunostimulant, efforts are on to develop chemically defined and low molecular weight substances as immunomodulating agents. As a result a number of highly potent compounds have been identified[1] and some of these are under clinical trials[2] too. However, most of them have microbial origin and are therefore associated with some toxic side effects[1]. Parker and associates in 1984 reported a hexapeptide corresponding to fragment 54-59 of human β-casein capable of exerting strong immunostimulating activity including resistance to certain bacterial infections[3]. By virtue of being derived from the food protein it may be devoid of unwanted side effects associated with the substances of microbial origin. In view of this, we synthesized several analogs of this hexapeptide and evaluated for their immunostimulant activity as well as for the enhancement of non-specific resistance to infectious agents[4,5]. In continuation of these efforts, we have synthesized few more analogs having modifications at N-terminal region leading to enzymatically resistant analogs and evaluated for immunomodulating activity. Following compounds have been synthesized. Val-Glu-Pro-Ile-Pro-Tyr **Fragment(54-59)**, Val-γ-Abu-Ile-Pro-Tyr (**1**), Val-Glu-Acc-Ile-Pro-Tyr (**2**), Val-D-Glu-Pro-Ile-Pro-Tyr (**3**), Val-Gly-Gly-Ile-Pro-Tyr (**4**), Ala-Glu-Pro-Ile-Pro-Tyr (**5**) and Ala-D-Glu-Pro-Ile-Pro-Tyr (**6**). In this communication synthesis and immunosuppressant activity is being reported in human β-casein fragment (54-59) analogs. This is the first report describing immunosuppressant activity in this class of compounds.

Synthesis of peptides

Synthesis of human β-casein fragment and its analogs **1-6** were carried out by step wise chain elongation using solution phase method of peptide synthesis. Boc group[6] and the benzyl group were employed for the

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protection of α-amino and carboxyl functions respectively except Val and Ala at position 1 where Z group[7] was used for α-amino protection in order to achieve simultaneous removal of Z and benzyl group by catalytic hydrogenation[8] in the last step of the synthesis. DCC/HOBt[9] was used as coupling reagent for preparation of peptide bond. Boc group was removed by treating the peptide derivatives with HCl/dioxane in the presence of thioanisole. Z and benzyl groups were removed by catalytic hydrogenation. The peptides were characterized by spectroscopic methods[10]. Homogeneity was established by TLC and reversed phase HPLC prior to bioevaluation. The physicochemical data of final peptides are presented in Table 1.

Table-1 : Physicochemical characteristics of the Peptides

Peptide	m. p. °C	Yield(%)	$[\alpha]_D^{25}$ (MeOH)	Rf(B)	FAB MS [M+H] calc.(found)
1	159-160	86	-16	0.66	576(576)
2	198(d)	77	-36	0.76	731(731)
3	201	86	-100	0.73	717(717)
4	200-203	74	-38	0.60	605(605)
5	210	90	-90	0.68	689(689)
6	185-187	95	-84	0.72	689(689)

B = n Butanol : Acetic acid : Water (4:1:5)

Immunomodulatory activity :

The synthetic peptides were tested for their immunomodulatory activity *in vitro* by lymphocyte transformation test (LTT) and mixed lymphocyte reaction (MLR). The details are as follows;

Lymphocyte Transformation Test (LTT) :

Splenocytes from normal Swiss mouse were prepared in RPMI medium containing glutamine (2 mM), HEPES (10 mM), penicillin (100 μg/ml), streptomycin (100 μg/ml) and gentamycin (40 μg/ml). Red blood cells were lysed by treatment with the lytic buffer i.e 0.83% ammonium chloride in 10 mM Tris buffer (pH 7.2). After proper washing with the medium, the cells were finally suspended in complete RPMI medium (RPMI fortified with 10% fetal calf serum) to the strength of $5x10^6$ cells/ml. 100 μl of the cells were cultured for 72 hr in presence of a mitogen (PHA-P) at different concentrations (10, 1, 0.1, and 0.01 μg/ml) of test compounds in a total volume of 200 ml in a 96 well flat bottom culture plate in a CO_2 incubator at 37°C. The cultures were pulsed with [^{3}H] thymidine and were harvested after 18 hrs of extended incubation. Radioactivity

incorporated by the cells was determined by a β scintillation counter (LKB Rackbeta-1209, Wallac, Finland). The results have been expressed as stimulation index i.e. ratio of the DPM in experimental wells to that in control (untreated) wells (Table 2).

Mixed Lymphocyte Reaction Test (MLR):

Spleen cells from two genetically different strains of mice (Swiss and Fawn C3H strain) were prepared, washed and suspended as described above for LTT. Spleen cells from Fawn mice were treated with 25 μg/ml of mytomycin C for 30 min at 37°C, washed four times with the medium and finally suspended in complete RPMI to a density of $1X10^7$ cells/ml. Equal number of splenocytes ($5x10^5$) of both the mice were cocultured with different concentrations of the standard and test compounds in a total volume of 200 μl in a 96-well flat bottom culture plate in a humid CO_2 incubator. After 72 hrs, the cultures were pulsed, harvested and counted for radioactivity as described above.

Results and Discussion

The results of lymphocyte transformation test (LTT) are presented in Table 2. It is apparent that none of the analogs **1-6** showed stimulatory effect better to that expressed by the native fragment (54-59). Even compouds **5** and **6** which exhibited some stimulation had activity similar to that posssed by the native peptide. However, it is intersting to note that some of the peptides particularly **1** and **2**, exhibited inhibition. Peptide **1** was found to be the most active expressing approx 45% inhibition (at 0.01 μg/ml). Mixed Lymphocyte Reaction (MLR) exhibited suppressive effect by all the six compounds (Table 3). However, for peptides **3**, **4** and **6**, the transformation indices remained 0.9 or above at one or the other concentration. This indicated for the weak immunosuppressive nature of these peptides. The remaining three *i.e.* peptides **1**, **2** and **5** are considered as good suppressors.. Nevertheless, when the data from both MLR and LTT are analyzed together, only peptides **1** and **2** qualify as lead molecules for the optimization towards the search for the potential immunosuppressors.

Modulation of immune system of the host by chemically defined low molecular weight substances has been an important area of investigation. Human casein fragments have been reported with interesting biological properties[11]. Identification of a hexapeptide fragment of human casein as immunostimulant provided a new lead for obtaining a potent and non-toxic immunostimulant. Initially Parker and coworkers reported that macrophages are the main target for the activity of this hexapeptide[3]. We have carried out structure-activity relationship studies and demonstrated that replacement of Pro residue at position 3 and/or 5 lead to compounds which are capable to exerting better immunostimulatory effects involving both macrophages and T-lymphocytes[4,5]. Our earlier results indicated that C-terminal Tyr is very essential for the activity. Therefore, for

Table 2: Effect of Peptides on Lymphocyte Proliferation (LTT)

Peptide No.	Stimulation index (μg/ml concentration)			
	10.0	1.0	0.1	0.01
Fragment (54-59)	1.45±0.35	1.50±0.28	1.43±0.42	1.27±0.36
1	0.88±0.03	0.69±0.26	0.73±0.11	0.55±0.21
2	1.10±0.18	1.09±0.09	0.70±0.08	0.69±0.31
3	1.18±0.22	1.22±0.17	1.08±0.06	0.82±0.05
4	1.22±0.08	1.15±0.27	1.03±0.25	0.93±0.15
5	1.22±0.14	1.40±0.01	1.57±0.07	1.17±0.23
6	1.32±0.41	1.30±0.17	1.27±0.12	1.32±0.18

Values expressed as ±SD of the experiments, each run in triplicate

Table 3: Effect of Peptides on Mixed Lymphocyte Reaction (MLR)

Peptide No.	Stimulation index (μg/ml concentration)		
	10.0	1.0	0.1
Fragment (54-59)	0.90±0.32	0.90±0.20	1.00±0.24
1	0.74±0.14	0.77±0.43	0.67±0.32
2	0.62±0.04	0.86±0.02	0.65±0.17
3	0.77±0.10	0.75±0.03	0.93±0.13
4	0.88±0.18	0.95±0.14	0.66±0.19
5	0.72±0.16	0.82±0.16	0.74±0.34
6	0.75±0.05	0.89±0.28	0.84±0.21
Prednisolone	-	-	0.30±0.22
Cyclosporin	-	0.18±0.07	-

Values expressed as ±SD of the experiments, each run in triplicate

the present study, modifications are made at the N-terminal part only. In peptide **1** both Glu^2 and Pro^3 were replaced by γ-amino butyric acid which can be a close isostere to a dipeptide and the resulting pentapeptide will be resistant to proteolytic cleavage. Similarly, in peptide **2**, Pro^3 has been replaced by a cyclic amino acid 1-amino-cyclopentane-1-carboxylic acid (Acc) which is also known to induce enzymatic resistance. In peptides **3** and **6**, Glu at position 2 was replaced with D-Glu for similar reasons while in peptide **4**, position 2 and 3 were

replaced with Gly-Gly residues. The results indicated that this type of changes introduced in the human β-casein fragment (54-59), which are metabolically stable, provided compounds with noticeable immunosuppressant activity (analogs **1** & **2**). A possible explanation for this interesting observation awaits further exploration. It however appears that the enzymatically resistant analogs prepared as above, are affecting the immune system with a different mechanism as does the natural sequence and hence elicit suppressive effect.

In summary, the immunosuppressant activity in human β-casein fragment (54-59) analogs is an important lead towards the development of safe and effective immnunosuppressants which are in high demand. Currently available immunosuppressive drugs *viz.* FK-506, cyclosporin etc. are known to exert several toxic side effects.

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References and Notes:

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1. Adam, A. and Lederar, E. *Med. Res. Rev.* **1984**, *4*, 111. and Werner, G.H., Floc'h, F., Migliore-Samour, D., Jolles, P. *Experientia*, **1986**, *42*, 521.
2. Information update, *Drugs of future* **1997**, *22*, 313.
3. Parker, F., Migliore-Samour, D., Floc'h, F., Zerial, A., Werner, G. H., Jolles, J., Casarett, M., Zahn, H., Jolles, P. *Eur. J. Biochem.* **1984**, *145*, 677.
4. Sharma, P., Anuradha, Sharan, R., Haq, W., Kundu, B., Katiyar, J.C., Mathur, K.B. *Protein and Peptide Letters* **1996**, *3*, 261.
5. Sahai, R., Puri, A., Saxena, R.P., Sharan, R., Haq, W., Kundu, B., Mathur, K.B. *Immunopharmacol. Immunotoxicol.* **1996**, *18*, 511.
6. Moroder, L., Hallett, A., Wunsch, E., Keller, O., Wersin, G. *Z. Physiol. Chem.* **1976**, *357*, 1651.
7. Bergman, M. and Zervas, L., *Ber.* **1932**, *65*, 1192.
8. Schwyzer, R., Sieler, P., *Helv. Chim. Acta* **1959**, *42*, 972.
9. Konig, W. and Geiger, R., *Chem. Ber.* **1970**, *103*, 788.

10. NMR data of the peptides **1-6**, ^{1}H NMR (DMSO, δ, ppm): Peptide **1**: 0.78 (t, 3H, δ-CH_3-Ile), 0.75 (d, 3H, γ-CH_3-Ile), 1.01-1.17 (2d, 6H, γ-CH_3-Val),1.2-2.25 (m, 12 H, β & γ-CH_2-Pro, β-CH & γ-CH_2-Ile, β-CH-Val, β & γ-CH_2-Abu), 2.85 (m, 2H, β-CH_2-Tyr), 3.5-4.25 (m, C^{α} H^{s}), 6.6 (d, 2H, Ar-$C^{3,5}$ H-Tyr), 6.9 (d, 2H, Ar-$C^{2,6}$ H-Tyr), 7.75-8.75 (NHs)., Peptide **2**: 0.68 (t, 3H, δ-CH-Ile), 0.71 (d, 3H, γ-CH_3-Ile), 0.87, 0.90 (2d, 6H, γ-CH_3-Val), 1-2.5 (m, 16H, β & γ-CH_2-Pro, β-CH & γ-CH_2-Ile, β-CH-Val, β-CH_2-Glu, β, γ-CH_2-Acc), 2.8 (m, 2H, β-CH_2-Tyr), 3.45-4.3 (m, C^{α} H^{s}) 6.7 (d, 2H, Ar-$C^{3,5}$ H-Tyr), 7.0 (d, 2H, Ar-$C^{2,6}$ H-Tyr), 7.65-8.5 (NHs)., Peptide **3**: 0.75 (t, 3H, δ-CH_3-Ile), 0.82 (d, 3H, γ-CH_3-Ile), 1.10-1.22 (2d, 6H, γ-CH_3-Val), 1.25-2.25 (m, 16H, β & γ-CH_2-Pro, β-CH & γ-CH_2-Ile, β-CH-Val, b, γ-CH-Glu), 2.8 (m, 2H, β-CH_2-Tyr), 3.48-4.50 (m, C^{α} H^{s}), 6.7 (d, 2H, Ar-$C^{3,5}$ H-Tyr), 7.0 (d, 2H, Ar-$C^{2,6}$ H-Tyr), 7.75-8.70 (NHs)., Peptide **4**: 0.73 (t, 3H, δ-CH_3-Ile), 0.75 (d, 3H, γ-CH_3-Ile), 0.89,0.92 (2d, 6H, γ-CH_3-Val), 1.25-2.25 (m, 8H, β & γ-CH_2-Pro, β-CH & γ-CH_2-Ile), 2.9 (m, 2H, β & γ-CH_2-Tyr), 3.4-4.5 (m, C^{α} H^{s}), 6.75 (d, 2H, Ar-$C^{3,5}$ H-Tyr), 7.0 (d, 2H, Ar-$C^{2,6}$ H-Tyr), 7.75-8.6 (NH protons) Peptide **5**: 0.75 (t, 3H, δ-CH_3-Ile), 0.83 (d, 3H, γ-CH_3-Ile), 1.4 (d, 3H, CH_3-Ala), 1.75-2.5 (m, 15H, β & γ-CH_2-Pro, β-CH & γ-CH_2-Ile, ,β & γ-CH_2-Glu), 2.75 (m, 2H, β-CH_2-Tyr), 3.45-4.30 (m, C^{α} H^{s}), 6.7 (d, 2H, Ar-$C^{3,5}$ H-Tyr), 6.9 (d, 2H, Ar-$C^{2,6}$ H-Tyr) 7.8-8.15 (NHs)., Peptide **6**: 0.74 (t, 3H, δ-CH_3-Ile), 0.76 (d, 3H, γ-CH_3-Ile), 1.4 (d, 3H, β-CH_3-Ala), 1.75-2.5 (m, 15H, β & γ-CH_2-Pro, β-CH & γ-CH_2-Ile, β, γ-CH_2-Glu), 2.8 (m, 2H, β-CH_2-Tyr), 3.45-4.30 (m, C^{α} H^{s}), 6.7 (d, 2H, Ar-$C^{3,5}$ H-Tyr), 6.9 (d, 2H, Ar-$C^{2,6}$ H-Tyr) 7.8-8.15 (NHs).

11. Teschmacher, H., Koch, G., Brantl, V. *Biopolymers (Peptide Science)*, **1997**, *43*, 99. and Meisel, H., ***ibid*** **1997**, *43*, 119