IMMUNOSTIMULANT ACTIVITY OF PEPTIDES RELATED TO HUMAN β-CASEIN FRAGMENT Gly-Leu-Phe

Sushma SHUKLA[a], Anju PURI[b], Ragini SAHAI[b], Bijoy KUNDU[a1,*], Lalit M. TRIPATHI[b] and Vishwa M. L. SRIVASTAVA[b]

[a] *Division of Biopolymers, Central Drug Research Institute, Lucknow 226001, India; e-mail: [1] root@cscdri.ren.nic.in*

[b] *Division of Biochemistry, Central Drug Research Institute, Lucknow 226001, India*

Received April 15, 1998
Accepted August 15, 1998

Twenty novel analogues of β-casein fragment (Gly-Leu-Phe) have been synthesised and evaluated in mouse for their impact on macrophage migration and humoral response to sheep red blood cells. As an outcome of the present investigation, compound **1** has been found to be very promising.

Key words: Peptides; Tripeptides; Macrophage activation; Humoral response; Sheep red blood cells; Immunomodulators.

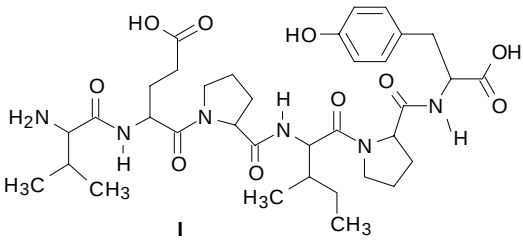
The immune system comprises of a heterogenous group of lymphoid cells involved in providing protection against foreign invading substance: bacteria, virus or fungi *etc*. The immune system's response to an antigenic challenge is diverse and results in a cascade that leads to the production of either specifically activated effector cells or humoral products, or both. An immunomodulatory drug should be able to directly influence a specific immune reaction or to modify one or more components of the immune network to accomplish an indirect effect on specific immunological functions. Major efforts are underway to develop drugs that can modulate the host natural defense and restore impaired immune functions[1–5].

The powerful influence of peptides on the immune system has been well documented. Several small peptides of diverse structures have been synthesised and evaluated as immunostimulants. Examples include early work on muramyl peptides and lauryl tetrapeptide of microbial origin[6,7], and those[8,9] derived from immunoglobulin G and thymopoietin. Other potent low molecular weight peptides that have been reported include: pidotimod, FK 565, IMREG 1 (structure not yet established) and ubenimex[10].

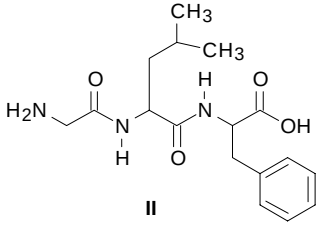
* The author to whom correspondence should be addressed.

Peptides derived from food protein such as soyabean globulin and human milk casein have been also reported to be endowed with immunostimulant activity[11–13].

Out of these peptides, the milk casein fragments appear to be of considerable importance since it is the first human food and, being intravital, it may be devoid of the toxic effects usually associated with the immunostimulants of microbial origin. Recently, we reported structure–activity relationship studies on human casein fragment **I** (Leu-Glu-Pro-Ile-Pro-Tyr) and stimulation of non-specific resistance by **I** and its analogue against the *L. donovani* infection[14,15]. This prompted us to undertake structure–activity relationship studies of the second fragment, Gly-Leu-Phe (**II**) isolated from the enzymatic digest of milk casein with the aim of improving its biological activity.



I



II

In the present paper, immunostimulant activity of twenty novel analogues related to Gly-Leu-Phe has been described. The modifications were introduced at all the three positions in order to identify the steric, electronic and lipophilic/hydrophilic requirements essential for the biological response. The compounds synthesised for this purpose are illustrated in Table I.

EXPERIMENTAL

Synthesis of Peptides

Synthesis of all the peptides except **20** was carried out by a well established procedure in solution phase. They were synthesised either in a stepwise manner or by a 2 + 1 fragment condensation. The latter strategy was used in order to avoid diketopiperazine formation from the C-terminal dipeptide ester during deblocking. Methyl and benzyl groups were empoyed for the protection of carboxyl functionalities. The side chains of Asp and Ser were protected by benzyl groups. For the protection of amino function, Boc group was generally employed. However, in some cases, Z group was used to protect the α-NH_2 function of the N-terminal amino acid, as it could be cleaved from the fully protected tripeptide in a single step along with the benzyl group used for blocking carboxyl functionalities. The method adopted for peptide bond formation involved the use of DCC/HOBt, mixed anhydride and active ester. However, for the acylation of *N*-methylamino acids, PyBrOP was empoyed as the coupling reagent[16]. Compound **20** was synthesised by the solid phase method using Merrifield's resin followed by disulfide bond formation with the iodine/methanol method[17]. The crude peptides were purified on a semipreparative reverse phase column chromatography using a linear gradient of A: 0.1% TFA in water and B: 0.1% TFA in acetonitrile from 80% A to 50% A over 45 min. All the peptides were characterised by FAB MS and elemental analysis (see Table I).

TABLE I
Analytical data of peptides **1**–**20**

Compound	$[\alpha]_D$ (*c*)	FAB MS	Formula	Calculated/Found		
				% C	% H	% N
1 (D-Ala-Leu-Phe)	+44.9 (0.15)	350	$C_{18}H_{27}N_3O_4 \cdot HCl$	56.03 55.97	7.31 7.02	10.89 10.74
2 (βAla-Leu-Phe)	–17.2 (0.14)	350	$C_{18}H_{27}N_3O_4 \cdot HCl \cdot H_2O$	53.53 53.46	7.49 7.40	10.40 10.50
3 (D-Phe-Leu-Phe)	+45.8 (0.15)	426	$C_{24}H_{31}N_3O_4 \cdot HCl$	62.40 62.43	6.98 6.97	9.10 9.13
4 (Pro-Leu-Phe)	–32.5 (0.13)	376	$C_{20}H_{29}N_3O_4 \cdot HCl \cdot 2\ H_2O$	53.63 53.65	7.65 7.60	9.38 9.42
5 (Ser-Leu-Phe)	–3.78 (0.13)	366	$C_{18}H_{27}N_3O_5 \cdot HCl \cdot 2\ H_2O$	49.37 49.41	7.37 7.42	9.60 9.75
6 (Asp-Leu-Phe)	–3.16 (0.13)	394	$C_{19}H_{27}N_3O_6 \cdot HCl \cdot H_2O$	50.95 51.08	6.75 6.59	9.38 9.24
7 (Octanoyl-Gly-Leu--Phe)	–14.4 (0.07)	484	$C_{25}H_{39}N_3O_5$	65.04 65.01	8.45 8.35	9.11 9.01
8 (Gly-D-Leu-Phe)	–19.5 (0.11)	336	$C_{17}H_{25}N_3O_4 \cdot HCl$	54.91 54.93	7.05 7.07	11.30 11.11
9 (Gly-Ile-Phe)	–14.3 (0.10)	336	$C_{17}H_{25}N_3O_4 \cdot HCl \cdot 3\ H_2O$	47.94 47.98	7.57 7.63	9.87 10.02
10 (Gly-Pro-Phe)	–24.26 (0.11)	320	$C_{16}H_{21}N_3O_4 \cdot HCl$	54.01 54.05	6.23 6.24	11.81 12.01
11 (Gly-Leu-D-Phe)	–5.69 (0.11)	336	$C_{17}H_{25}N_3O_4 \cdot HCl \cdot H_2O$	52.37 52.42	7.24 7.30	10.78 10.94
12 (Gly-Leu-Gly)	–29.18 (0.07)	246	$C_{10}H_{19}N_3O_4 \cdot HCl \cdot 2\ H_2O$	37.80 37.83	7.61 7.68	13.22 13.38
13 (Gly-Leu-Pro)	–59.5 (0.17)	286	$C_{13}H_{23}N_3O_4 \cdot HCl \cdot H_2O$	45.95 45.99	7.71 7.79	12.37 12.52
14 (Gly-Leu-Phe-OMe)	–23.0 (0.15)	350	$C_{18}H_{27}N_3O_4 \cdot HCl$	56.03 56.12	7.31 7.30	10.89 11.01
15 (Gly-Leu-Phe-NH_2)	–34.5 (0.13)	335	$C_{17}H_{26}N_3O_4 \cdot HCl$	55.06 55.11	7.34 7.35	15.11 15.20
16 (Sar-Leu-Phe)	–12.0 (0.12)	350	$C_{18}H_{27}N_3O_4 \cdot HCl \cdot H_2O$	53.53 53.55	7.49 7.57	10.40 10.49

TABLE I
(*Continued*)

Compound	$[\alpha]_D$ (*c*)	FAB MS	Formula	Calculated/Found		
				% C	% H	% N
17 (MeAla-Leu-Phe)	–16.61 (0.13)	364	$C_{19}H_{29}N_3O_4 \cdot HCl \cdot H_2O$	54.61 54.63	7.66 7.71	10.06 10.12
18 (Gly-MeLeu-Phe)	–49.0 (0.13)	350	$C_{18}H_{27}N_3O_4 \cdot HCl \cdot 2\ H_2O$	51.25 51.30	7.59 7.64	9.96 10.01
19 (Gly-Leu-MePhe)	–42.1 (0.13)	350	$C_{18}H_{27}N_3O_4 \cdot HCl \cdot H_2O$	53.53 53.66	7.43 7.30	10.41 10.49
20 (cyclo[Cys-Gly--Leu-Cys-]NH_2)	–25.5 (0.15)	539	$C_{23}H_{34}N_6O_5S_2 \cdot HCl$	48.04 48.11	6.09 6.14	14.62 14.71

Immunostimulant Activity

The tripeptides were examined *in vivo* for their capacity to alter macrophage migration and humoral response to sheep red blood cells (SRBC) in a mouse model. For this macrophage migration index (MI) and hemagglutinating antibody (HA) titre were examined by the methods described elsewhere[18,19]. In brief, male Swiss mice obtained from the CDRI animal house were divided into groups of 8 each. The test peptides were administered intraperitoneally to mice at the dose of 4 mg/kg on days –7 and –3. The controls were given PBS by the same route. On day 0, four animals from each group were sacrificed and peritoneal exudate cells (PEC) from them were collected for the determination of MI (ref.[19]). The remaining four animals from each group were injected $1 \cdot 10^8$ SRBC by i.p. route. Six days later, serum was collected from the retroorbital plexus for the assay of HA titre[20]. Two such experiments were performed for each compound. The data concerning immunostimulant activity of the tripeptides have been listed in Table II.

Macrophage migration index: Heparinised microhematocrit capillary was filled with PEC suspension. After sealing one end with plasticine, the cells were packed by centrifugation. The capillary was cut at the cell/liquid interface and the portion containing the cells was fixed at the closed end to the well. The wells were filled with RPMI-1640 medium containing 10% fetal calf serum. The plate was incubated at 37 °C in the humid chamber for 24 h. The area of PEC migration from the capillaries into the chamber was marked on Whatman filter paper No. 1 using camera Lucida. The paper of the marked area was cut and weighed.

$$\mathrm{MI} = \frac{\text{Weight of the paper of experimental set}}{\text{Weight of the paper of control set}}$$

Hemagglutinating antibody (HA) titre: HA titre was determined in the sera obtained from the immunised animals. 50 µl aliquots of two folds serial dilutions from (1 : 2 to 1 : 4 096) in phosphate buffer (PBS) were prepared in one row of a 96-well microtitre plate. An amount of 25 µl of 1% fresh SRBC suspension in PBS was dispensed into each well and mixed thoroughly. The plate was incu-

bated at room temperature for two hours and examined for agglutination. The reciprocal of the highest dilution of the test giving visible agglutination has been expressed as HA titre. The data for HA titre were analysed by Student t-test for significance.

RESULTS AND DISCUSSION

It is evident from the Table II that analogues **2**, **3** and **6** in which structural modifications were introduced at position 1 did not have a favourable effect on the biological activity. Similarly, analogues **12** and **15** having modification at position 3 had a detrimental effect. Interestingly enough, both the types of responses, humoral and macrophage activation, were considerably stimulated by the tripeptides **4** and **5** in which Gly at position 1 was replaced by Pro and Ser, respectively. The activity of these analogues,

TABLE II
Structure and biological activity data for compounds **1**–**20**

Compound	Sequence	MI[a]	HA titre[a]
Fragment **II**	Gly-Leu-Phe	2.49 ± 0.83	1 792 ± 256[b]
1	**D-Ala**-Leu-Phe	2.59 ± 0.59	2 867 ± 502[b]
2	**βAla**-Leu-Phe	1.03 ± 0.32	896 ± 128[b]
3	**D-Phe**-Leu-Phe	1.66 ± 0.33	640 ± 128[d]
4	**Pro**-Leu-Phe	2.26 ± 0.34	1 792 ± 256[b]
5	**Ser**-Leu-Phe	2.66 ± 0.31	1 664 ± 384[b]
6	**Asp**-Leu-Phe	0.90 ± 0.01	1 280 ± 256[b]
7	**Octanoyl-Gly**-Leu-Phe	1.41 ± 0.91	1 280 ± 256[b]
8	Gly-**D-Leu**-Phe	1.91 ± 0.44	786 ± 147[d]
9	Gly-**Ile**-Phe	2.07 ± 0.82	896 ± 128[c]
10	Gly-**Pro**-Phe	2.04 ± 0.93	1 152 ± 322[b]
11	Gly-Leu-**D-Phe**	1.25 ± 0.35	1 152 ± 644[b]
12	Gly-Leu-**Gly**	1.02 ± 0.23	640 ± 128[d]
13	Gly-Leu-**Pro**	1.96 ± 0.81	1 112 ± 48[b]
14	Gly-Leu-Phe-**OMe**	2.06 ± 0.71	1 152 ± 322[b]
15	Gly-Leu-Phe-**NH_2**	1.20 ± 0.80	768 ± 295[d]
16	**Sar**-Leu-Phe	1.10 ± 0.11	640 ± 128[d]
17	**MeAla**-Leu-Phe	1.38 ± 0.06	288 ± 80[b]
18	Gly-**MeLeu**-Phe	1.03 ± 0.32	1 024 ± 36[b]
19	Gly-Leu-**MePhe**	2.13 ± 0.69	640 ± 128[d]
20	cyclo(-**Cys**-Gly-Leu-Phe-**Cys**-)**NH_2**	1.75 ± 0.46	896 ± 128[b]
21	Control	1.0	640 ± 128

[a] The data are means ± s.d. of 8 animals; [b] $p < 0.001$; [c] $p < 0.005$; [d] not significant with respect to control.

however, could not exceed that of the parent compound. Analogues **7**, **8**, **9**, **10**, **11**, **13** and **14** although exhibited higher mean values of MI and HA titres than the control, stimulation in each case was however less pronounced than the lead peptide Gly-Leu-Phe.

The immunostimulant activity of conformationally constrained analogues **16**–**20**, prepared by either introducing *N*-methylamino acid or by cyclisation resulted in the loss of activity as far as the stimulation of both humoral and macrophage migration response is concerned. Compound **17** which markedly suppressed humoral response without stimulating MI to a noticeable degree, warrants deeper investigation. It is pertinent to add that MI exceeding 2.0 only is considered significant.

The most active compound of the series was compound **1** which has been obtained by the replacement of Gly at position 1 with D-Ala. Though it was equipotent with the lead tripeptide **II** in MI assay, the mean value for HA titre (2 867 ± 502) was much higher than the lead tripeptide (1 792 ± 256).

Thus, on the basis of the data presented in Table II, it can be inferred that the types of substitution introduced at positions 2 and 3 lead to inactive congeners. It appears that the side chain functionalities of the two amino acids Leu^2 and Phe^3 are essential for the expression of immunostimulant activity of the casein fragment. The most interesting finding, however, is that the immunostimulant activity of the tripeptide fragment increases considerably if Gly at position 1 is replaced by D-Ala. This may be attributed to the increased stability of the peptide towards aminopeptidases. Further studies with backbone-modified tripeptides and with dimeric peptides are in progress.

REFERENCES

1. Kekelj D., Pecar S., Kotnik V., Stalc A., Wraber-Herzog B., Simcic S., Ihan A., Klamfer L., Povsic L., Grahek R., Suhadolc E., Hocevar M., Honig H., Rogi-Kohlenprath R.: *J. Med. Chem.* **1998**, *41*, 530.
2. Georgiev V. St.: *Trends Pharmacol. Sci.* **1988**, *9*, 446.
3. Georgiev V. St.: *Med. Res. Rev.* **1990**, *10*, 371.
4. Georgiev V. St.: *Trends Pharmacol. Sci.* **1990**, *11*, 373.
5. Georgiev V. St.: *Med. Res. Rev.* **1991**, *11*, 81.
6. Duncan E. S. Stewart-Tull: *Prog. Drug Res.* **1988**, 305.
7. Migliore-Samour D., Bouchaudon J., Floch F., Zerial A., Ninet L., Werner G. H., Jolles P.: *Life Sci.* **1980**, *26*, 883.
8. Najjar V. A., Nishioka K.: *Nature* **1970**, *228*, 672.
9. Audhya T., Goldstein G.: *Int. J. Pept. Protein Res.* **1983**, *22*, 568.
10. Prous J. R., Ed.: *Year's Drug News*, p. 382. Prous Science, Barcelona 1995.
11. Yoshikawa M., Kishi K., Takahashi M., Watanabe A., Miyamura T., Yamazaki M., Chiba H.: *Ann. Acad. Sci (N.Y.)* **1993**, *685*, 375.
12. Parker F., Migloire-Samour M., Floch F., Zerial A., Werner G. H., Jolles J., Casaretto H., Zahn H., Jolles P.: *Eur. J. Biochem.* **1984**, *145*, 677.
13. Berthon J., Migliore-Samour D., Lifchitz A., Delattre J., Floch F.: *FEBS Lett.* **1987**, *218*, 55.

14. Sahai R., Puri A., Saxena R. P., Saran R., Haq W., Kundu B., Mathur K. B.: *Immunopharmacol. Immunotoxicol.* **1996**, *18*, 511.
15. Sharma P., Anuradha, Sharan R., Haq W., Kundu B., Katiyar J. C., Mathur K. B.: *Protein Pept. Lett.* **1996**, *3*, 261.
16. Castro J., Frerot E., Jouin P.: *Tetrahedron Lett*. **1991**, *32*, 1967.
17. Kamber B.: *Helv. Chim. Acta* **1971**, *54*, 927.
18. Puri A., Saxena R., Saxena R. P., Saxena K. C., Srivastava V., Tandon J. S.: *J. Ethnopharmacol*. **1994**, *42*, 31.
19. Saxena K. C., Puri A., Sumati, Saxena R., Saxena R. P.: *Immunol. Invest*. **1991**, *20*, 431.
20. Nelson D. A., Mildenhall P.: *Aust. J. Exp. Biol. Med. Sci*. **1967**, *45*, 113.