

Derivatives of human β -Casein fragments (54-59) exhibit highly potent immunosuppressant activity.

A. Puri^{1*}, M.Bhattacharya², L.M.Tripathi³ and W. Haq²,
Divisions of Biochemistry¹, Medicinal & Process Chemistry² and Parasitology³
Central Drug Research Institute, Lucknow-226001

*Corresponding author:

Dr. Anju Puri
Division of Biochemistry
Central Drug Research Institute
Lucknow -226001, INDIA.
Telephone-91-522-26212411-18 (Ext- 4342)
Fax-91-522-2623938, 2623405
Email- anjup4@rediffmail.com

Abstract

Human β -casein fragment (54-59) having the amino acid sequence Val-Glu-Pro-Ile-Pro-Tyr, has shown potent immunostimulant activity. Several analogs of this hexapeptide have been synthesized with modification at the N-terminal region and two analogs, viz. peptide **I** and peptide **II** have shown significant immunosuppressant activity *in-vivo* mouse model. Effect on cell mediated immunity (CMI) and humoral immunity was studied in mouse/SRBC model. Both the peptides failed to stimulate immune response *in vivo* and showed inhibition of CMI and humoral response to sheep red blood cells (SRBC). Peptides showed inhibition in alloantigen induced lymphocyte proliferation, i.e., mixed lymphocyte reaction (MLR) *in vitro*. Treatment with peptides inhibited the production of interferon- γ (IFN- γ), and increased the production of interleukin-4 (IL-4) as well as improved the skin graft survival. Cyclosporine a known immunosuppressant showed similar effect on mouse model. Present study thus provides a lead for the development of safe and effective immunosuppressant.

Keywords: human β -casein fragments, Immunosuppressant peptide, immunomodulator, Lymphocytes proliferation.

1. Introduction

Modulation of immune system is an important phenomenon as it forms the basis for the treatment of many pathological conditions such as organ rejection after transplantation, recovery from infectious diseases, treatment of cancer, autoimmune diseases and primary immunodeficiency. Schimutzler and coworkers have described the immunomodulation and according to them "Immunomodulation" has at least three therapeutic goals – Suppression, Stimulation and Restoration as physiological and therapeutic aspects of immunomodulators [1]. Bacterial products have always been considered to be very potent immunoregulators and have been studied intensively in relation to host's natural resistance to infections and to tumors. Muramyl dipeptide (MDP) is a minimal structure required for the immunomodulatory activity of Gram +ve bacterial cell wall peptidoglycans [2-7]. MDP and

its derivatives are known to stimulate the reticuloendothelial system [7, 8]; form epithelial granuloma [9]; protect against *Pseudomonas aeruginosa* or *Candida albicans* infection [10] as well as Staphylococcal infection [11]; and to show antitumor activity [12].

However MDP and related molecules, being microbial origin, are associated with some toxic side effect (13, 14). 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 (15). By virtue of being derived from the food protein it may be devoid of unwanted side effects associated with the substances of microbial origin. Having this view in mind we have synthesized several analog of this hexapeptide and evaluated them for immunomodulatory activity as well as enhancement of non-specific resistance to infectious agents (16-17). In continuation of these efforts, we have synthesized few more analogs having modification at N-terminal region leading to enzymatically resistant analogs. Among these two analogs viz. Val-D-Glu-Gly-Ile-Pro-Tyr (**I**) and Val-Glu-D-Pro-Ile-Pro-Tyr (**II**) showed immunosuppressive activity. We have designed peptide to enhance metabolic stability and therefore, these peptides were tested in animal model by oral route of administration. The present communication deals with the synthesis and immunosuppressant activity of these analogs via oral route of administration.

2. Materials and Methods

2.1 Synthesis of peptides

Synthesis of human β -casein fragment analogs **I** & **II** were carried out by step wise chain elongation using solution phase method of peptide synthesis as reported earlier [18]. Boc group and the benzyl group were employed for the protection of α -amino and carboxyl functions respectively [19] except Val at position 1 where Z group [20] was used for amino protection in order to achieve simultaneous removal of Z and benzyl group by catalytic hydrogenation in the last step of the synthesis. DCC/HOBt [21] 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 Mass spectroscopy. Homogeneity was established by TLC and reversed phase HPLC prior to bioevaluation

2.2 Animals

Male mice (20-22g) were obtained from the inbred colony of BALB/c strain maintained in the Institute's animal house by brother-sister mating.

2.3 Immunomodulatory effect of peptides

2.3.1 Administration of Peptides

For observing immunomodulatory response aqueous solution of the peptides was orally given to the mice (15 animals for each group) at a dose of 5mg/kg for 14 consecutive days. On day 15 the animals were studied for various parameters. Control animals were simultaneously maintained and given water instead of peptide solution.

2.3.2. Immune response to SRBC

Control and the peptide treated mice (10 from each group) were intraperitoneally administered with 10^8 sheep red blood cells (SRBC). Four days later blood and spleen from 5 animals of each group were collected for the determination of haemagglutinating antibody (HA) titer and plaque-forming cells (PFC) counts [22, 23]. HA titer has been expressed as the reciprocal of highest dilution of test serum giving visible agglutination. In remaining SRBC treated animals DTH response to SRBC was determined by the method of Saiki et al. [24]. The response was measured with the help of Schnelltaster (Kroplin, Germany) and expressed as difference in the thickness (mm) between the footpad injected with SRBC and that injected with phosphate buffer saline (PBS, pH-7.2).

2.3.3 Lymphocyte transformation test (LTT)

Splenocytes from normal and peptide treated mice were prepared in RPMI-1640 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 hypotonic treatment with 0.83% (w/v) ammonium chloride in 10mM Tris buffer (pH 7.2). After two washings with the medium, the cells were finally suspended in complete RPMI medium (RPMI supplemented with 10% fetal calf serum) to the strength of 4×10^6 cells/ml. 100µl of the cell suspension were transferred to each well of a 96-well culture plate. Concanavalin-A at a concentration of 0.01µg/ml was added to the wells in triplicate. The volume of each well was adjusted to 200 µl and the culture was allowed to run for 72 hrs at 37°C in a CO₂-incubator. The cells were pulsed with 0.5 µci of [³H] - thymidine and harvested onto glass fiber filters after 18 hrs of extended incubation. Radioactivity incorporated by the cells was determined with a b-scintillation counter (LKB Rackbeta – 1209, Wallac, Finland). The results have been expressed as stimulation index (SI) i.e. ratio between DPM in experimental wells to that in control (untreated) wells.

2.3.4 Mixed Lymphocyte Reaction(MLR) Test

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 mitomycinC for 30 min at 37°C, washed four times with medium and finally suspended in complete RPMI medium to a density of 1×10^7 cells/ml. Equal number of splenocytes (5×10^5) of both the mice were co cultured with 0.1µg/ml concentration of peptide **I**, **II** and cyclosporine in a total volume of 200µl in a 96-well flat bottom culture plate in a humid, CO₂ incubator. After 72 hrs, the cultures were pulsed, harvested and counted for radioactivity as described above and SI was calculated.

2.3.5 Cytokine generation

Splenocytes (4×10^5 cells/ml) were cultured in a 96-well culture plate (Costar, Cambridge, USA) with and without con-A as described above for LTT. After 48 hr the plate was spun at 900 X g for 10 min and the supernatant was collected separately into sterilized sample tubes. The cell supernatant was assayed for various cytokines (IL-2, IL-4, IL-6, IL-10 and IFN-γ) contents as per protocol given with the individual kits obtained from Perspective Diagnostic Inc., Cambridge, USA. The absorbance of the final color was recorded at 450 nm in an automicroplate reader (ELISA analyzer, ETY-98, Japan). Cytokine level was calculated from the standard plot drawn at the same time.

2.3.6. Skin graft survival

Male C3H (FAWN) mice (20-22g) were used as the skin donor and male Swiss mice of same weight as recipients. On day 0, tail skin was removed from C3H mice, cut into pieces of 5X10 mm, and each piece was randomly transplanted to the tail of 20 Swiss mice. The animals were divided into four groups of 5 animals each. To animals of group 1 and 2 were administered peptide **I** and **II** at a dose of 5mg/kg/day i.p. while to group 3 was given cyclosporine at 1mg/kg/day dose for 14 days starting from day one. Untreated animals of group 4 were used as controls.

2.4 Data analysis

Statistical analysis of the data was done by Student's, 't' test.

3. Results

3.1 Immunosuppressive effect in mouse

To study the effect of *in vivo* administration of peptides **I** & **II** on antibody response, mice were administered with these peptides (5 mg/kg) and 15 days later these were immunized with sheep RBC as described in Materials and Methods. Results in Table 1 show that these

peptide induced significant decrease, as compared to control, in humoral response to SRBC as evident by significant decrease in HA ($p < 0.001$) titer and plaque forming cell number ($p < 0.001$, < 0.02). Peptides **I** & **II** showed a suppression of 25 and 68 per cent in HA titer while of 20 and 60 per cent inhibition in plaque forming cells (PFC) respectively (Table 1, Fig 1).

Towards the antiproliferative effect of these peptides and cyclosporine on lymphocytes, mouse splenocytes were stimulated with mitogen in the presence of either peptides or cyclosporine at 0.1 $\mu\text{g/ml}$ concentration. Peptides **I** and **II** inhibited the proliferation of splenocytes by 65 and 70 per cent respectively. At same concentration the inhibition of splenocytes proliferation by cyclosporine was 62 per cent (Fig. 1).

We further examined whether these peptides could inhibit the proliferation response of lymphocytes to alloantigen. Splenocytes were isolated from two genetically different strains of mouse, mixed in one way MLR (mixed lymphocyte reaction). Stimulator cells were treated with mitomycin-C and cultured in presence of 0.1 $\mu\text{g/ml}$ peptides or cyclosporine for 72 hr. Peptides or cyclosporine treatment inhibited the alloantigen induced proliferation by more than 40 per cent (Fig. 2A). Both the peptides were equipotent to cyclosporine in inhibition of alloantigen induced proliferation (Table 2, Fig. 2A).

We have also studied the effect of these peptides on *in vitro* generation of cytokines by splenocytes. Supernatant from lymphocyte culture, in presence of mitogen, was collected at 48 hr and used for the secretion of cytokines. Peptide **I** and **II** treatment significantly decreased the production of $\text{INF-}\gamma$ by 36% ($p < 0.01$) and 42% ($p < 0.001$) respectively. Among the Th2 cytokines, peptide **II** treatment significantly increased the production of IL-10 and IL-4 (Table 2). IL-10 exhibited an increase by 72% (NS) and 376 % ($p < 0.001$) respectively with peptide **I** and **II** while IL-4 production showed a significant increase of 132% ($p < 0.001$) with peptide **I** only (Fig 2A, B). No effect was observed on the production of IL-2, and IL-6 by these peptides (Table 2, Fig. 2A, and B).

The effect of these peptides on skin graft survival indicated that both the peptides improved the skin graft survival. Mean skin graft survival in nonimmunosuppressant recipients (control) was 8.5 ± 2.4 days. Whereas the mean skin graft survival in peptide **I** and **II** recipients was 13.5 ± 4.4 and 14.75 ± 4.5 days respectively (Table 3).

Cyclosporine which was used as standard immunosuppressant showed almost similar effect, on each parameter, by these peptides (Figs 1, 2A & B, Table 1, 2 & 3).

4. Discussion

Modulation of immune system of the host by chemically defined low molecular weight substances has been an important area of investigation. Isolation and identification of a hexapeptide (Val-Glu-Pro-Ile-Pro-Tyr) corresponding to fragment (54-59) of human β -casein as immunostimulant [25, 26] provided a new lead for obtaining a potential and non-toxic immunostimulant. Initially Parker and coworker [15] reported that macrophages are the main target for the activity of this hexapeptide. On the basis of the structure of this hexapeptide we have carried out structure activity relationship studies and demonstrated that replacement of Pro residue at position 3 & 5 lead to compounds with better Immunostimulatory activity involving both macrophage and T-lymphocytes [16, 17]. Our work has also indicated that C-terminal tyrosine is very essential for the activity. Subsequently we made modifications at N-terminal part only and made such substitutions at various positions to make the peptides metabolically stable along with improved immunosuppressant activity (18). With a view to obtain a better immunosuppressant we have introduced modifications at position 2 and 3 with the view to make the hexapeptide more resistant to metabolic degradation. These peptides are therefore tested for immunosuppressant activity by oral route of administration. The peptides showed the suppression of HA-titer, PFC, mitogen and alloantigen induced lymphocyte

proliferation, production of IFN- γ . In order to further elaborate the scope of activity to clinical relevance the peptides were tested for resistance to graft transplantation. The peptides were administered at the dose of 5mg/kg for 14 days after graft transplantation. These peptides improved the survival of skin graft transplantation and increased the production of IL-4 and IL-10 by lymphocytes. These peptides showed different immunosuppressive properties compared to commonly used immunosuppressant cyclosporine-A and FK506 which acts by inhibiting production of IL-2 by lymphocytes [27]. These peptides rather act like 15-Deoxyspirogualin (DSG) which suppresses the PFC number, production of IFN- γ and prolongation of skin graft survival [28, 29]. The lack of effect of these peptides and DSG on IL-2 production indicates that these peptides must not interfere significantly with the early activation events of the immune response as IL-2 gene activation occurs at early phase of lymphocyte activation.

It is pertinent to mention here that peptides derived from different sources have been shown to possess immunosuppressant activity. Szewczuck and co-worker [30, 31] have shown that RGD containing nonapeptide fragments of HLA-DQ (164-172) strongly suppressed the cellular and humoral immune response. Based on the crystal structure of this peptide these workers have synthesized cyclic analogs which strongly suppressed both the humoral and cellular response [32, 33]. Aramburru and coworker [34] have identified a conserved sequence SPRIEIT motif of NFAT (Nuclear factor of activated T-cell) protein, which is a docking site for calcineurin. This conserved motifs necessary for the effective recognition and dephosphorylation of NFAT1 by calcineurin. Synthetic SPRIEIT inhibited calcineurin binding to NFAT and prevented its dephosphorylation. When expressed intracellularly the peptide inhibited NFAT dephosphorylation and nuclear translocation, and NFAT mediated gene expression in response to stimulation. The peptides are also assayed for their cytotoxic effect *in vitro* and safety index of both the peptides was above 950. Thus this peptide is a potential candidate immunosuppressant.

These studies along with our studies clearly indicate that peptides have lead enough to develop them as safe and nontoxic immunosuppressant.

Acknowledgement

The authors are thankful to the Director, Central Drug Research Institute for providing the necessary facilities and SAIF for providing analytical data.

References

- [1] Schmoltzer W, Bent ST, Drager A, Zwadlo-Klarwasser G. Pharmacological and therapeutic aspect of immunomodulators. *Allergologie* 1989;12:198-203.
- [2] Elloug F, Adam A, Ciorbarus R, Lederer E. Minimal structural requirements for adjuvant activity of bacterial peptidoglycan derivatives. *Biochem Biophys Res Commun* 1974;59:1317-25.
- [3] Chedid I, Audibert F, Lefraucier P, Choay J, Lederer E. Modulation of the immune responses by a synthetic adjuvant and analogs. *Proc Nat Acad Sci U S A* 1976;73: 2472-5.
- [4] Chedid I, Paraut M, Paraut F, Lefraucier P, Choay J, Lederer E. Enhancement of non specific immunity to *Klebsiella pneumoniae* infection by a synthetic immuno-adjuvant (N-acetyl-muramyl-L-alanyl-D-isoglutanoioine) and several analogs. *Proc Nat Acad Sci U S A* 1977;74:2089-93.
- [5] Kotani S, Watanabe Y, Kinoshita F, Schimono T, Morisaki I, Shiba T, et al. Immunoadjuvant activity of synthetic N-acetylmuramyl-peptide or amino acid. *Biochem J* 1975;18:105-11.

- [6] Lederer E. Synthetic immunostimulants derived from the bacterial cell wall. *J Med Chem* 1980;23:819-25.
- [7] Tanaka A, Nagao S, Nagao R, Kotani S, Shiba T, Kusumoto S. Stimulation of the reticuloendothelial system of mice by muramyl dipeptide. *Infect Immun* 1979;24:302-7.
- [8] Wahl SM, Wahl L M, Mc Carthy JB, Chedid L, Mergenlagen SE. Macrophage activation by microbacterial water soluble compound and synthetic muramyl dipeptide. *J Immunol* 1979;122:2226-31.
- [9] Tanaka A, Emori K. Epithelioid granuloma formation by a synthetic bacterial cell wall component, muramyl dipeptide (MDP). *Am J Pathol* 1980;98:733-41.
- [10] Fraser-Smith EB, Matthews TR. Protective effect of muramyl dipeptide analogs against infection of *Pseudomonas aeruginosa* or *Candida albicans* in mice. *Infect Immun* 1981;26:676-83.
- [11] Grabchenko NI, Karpov AV, Chirva VIa, Tkachikova L, Spivak NIa. Effect of synthetic muramyl dipeptide derivatives on staphylococcal infection in mice. *Zh. Mikrobial Epidemiol Immunobiol* 2001;3:50-3.
- [12] Dzierzbicka K, Kolodziejczyk AM. Synthesis and antitumor activity of conjugates of muramyl dipeptide or nor muramyl dipeptide with hydroxyacridine/acridone derivatives. *J Med Chem* 2003;46:183-9.
- [13] Adam A, Lederer E. Muramyl peptides: immunomodulators, sleep factors and vitamins. *Med Res Rev* 1984;4:111-52.
- [14] Werner GH, Floch F, Migliore-Samour D, Jollès P. Immunomodulating peptides. *Experientia* 1986;42:521-31.
- [15] Parker F, Migliore-Samour D, Floch F, Zerial A, Werner GH, Jolles J, Casaretto M, Zahn H, Jolles P. Immunomodulating hexapeptide from human casein: amino acid sequence, synthesis and biological properties. *Eur J Biochem* 1984;145: 677-82.
- [16] Sharma P, Anuradha, Sharma R, Haq W, Kundu B, Katiyar JC, Mathur KB. Stimulation of non-specific resistance by human casein fragment (54-59) and its synthetic analogs against *L. donovani* infection. *Prot Pep Lett* 1996;3:261-66.
- [17] Sahai R, Puri A, Saxena RP, Saran R, Haq W, Kundu B, Mathur KB. Synthesis and immunostimulant activity of novel analogs of human casein fragment (54-59). *Immunopharmacol Immunotoxicol* 1996;18:512-28.
- [18] Bhattacharya M, Puri A, Sahai R, Haq W, Tripathi LM, Srivastava VML. Immunosuppressant activity in human β -casein fragment analogs. *Bioorg Med Chem Lett* 1998;8:327-32.
- [19] Schwyzer R, Sieler P. t-Butyloxycarbonylamino acids and their use in peptide synthesis. *J Am Chem Soc* 1957;79:6180-83.
- [20] Bergman M, Zervas L. A general procedure for the synthesis of peptides. *Ber B* 1932;65:1192-201.
- [21] König W, Geiger R. A new method for synthesis of peptides: activation of the carboxyl group with dicyclohexylcarbodiimide using 1-hydroxybenzotriazoles as additives. *Chem Ber* 1970;103:788-98.
- [22] Puri A, Rizvi SY, Haq W, Kundu B, Saxena RP, Guru PY, et al. Immunostimulant activity of novel lipopeptide and its protective action against *L. donovani*. *Immunopharmacol Immunotoxicol* 1993;15:539-56.
- [23] Jerne NK, Nordin AA. Plaque formation in agar by single antibody producing cells. *Science* 1963;140:405-7.
- [24] Saiki I, Tanio Y, Yamawaki M, Uemiya M, Kabayashi S, Fukuda T, et al. Adjuvant activity of Quinonyl-N-acetyl muramyl dipeptides in mice and guinea pigs. *Inf Imm* 1981;31:114-21.

- [25] Teschmacher H, Koch G, Brantl V. Milk protein derived opioid receptor ligands. *Biopolymers (Peptide Science)* 1997;43:99-117.
- [26] Meisel H. Biochemical proteins of regulatory peptides derived from milk proteins. *Biopolymers (Peptide Science)* 1997;43:119-28.
- [27] Nussler AK, Thomson AW. Immunomodulatory agents in the laboratory and clinic. *Parasitol* 1992;105:S5-S23.
- [28] Maeda K, Umeda Y, Saino, T. Synthesis and background chemistry of 15-deoxyspergualin. *Ann New York Acad Sci* 1993;685:123-35.
- [29] Holcombe H, Mellaman I, Janeway CA, Bottomly K, Dittel, BN. The immunosuppressive agent 15-deoxyspergualin functions by inhibiting cell cycle progression and cytokine production following native T cell activation. *J Immunol*. 2002;169:4982-9.
- [30] Szewczuk Z, Siemion IZ, Wieczorek Z. Immunological properties of the thymopentin-like fragments of HLA-DQ. *Mol Immunol* 1996a;33:903-8.
- [31] Szewczuk Z, Wieczorek Z. Stefanowicz P, Wilczynski A, Siemion IZ. Further investigations on thymopentin-like fragments of HLA-DQ and their analogues. *Arch Immunol Exp Ther* 1997;45:335-41.
- [32] Szewczuk Z, Stefanowicz P, Wilczynski A, Siemio, IZ, Cierniewski CS, Kralisz U, Wieczorek Z. The immunosuppressive and adhesive mini-region of the human major histocompatibility protein, HLA-DQ. *Biopolymers* 1996b;40:517-83.
- [33] Szewczuk Z, Buczek P, Stefanowicz P, Krajewski K, Wieczorek Z. Siemion IZ. Immunosuppressive activity of the cyclodimeric peptide with RGD-sequences. *Acta Biochimica Polonica* 2001;48:121-30.
- [34] Aramburu J, Garcia-Cozar F, Raghavan, A, Okamura H, Rao A, Hogan PG. Selective inhibition of NFAT activation by a peptide spanning the calcineurin targeting site of NFAT. *Molec Cell* 1998;1:627-37.

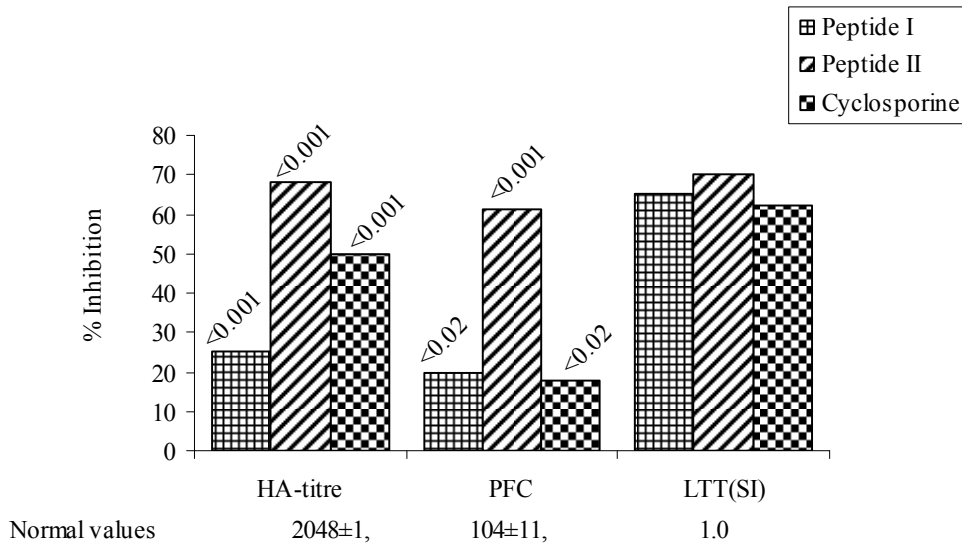


Figure: 1

Legend for fig 1

Fig.1 (Graphical representation in terms of percent inhibition of parameters in Table1). Percent change in specific and non specific immune response-HA; haemagglutinating antibody titre, PFC; plaque forming cell and LTT; lymphocyte transformation test, SI; simulation index, against sheep RBC's in a group of BALB/c mice treated with peptide I & II at a dose of 5 mg/kg/day, p.o. (oral) and cyclosporine 25 mg/kg for 14 consecutive day then administered with SRBC's; i.p. on day 15. Mice treated with water are taken as control.

Inhibition values were calculated with mean of experimental values with respect of mean control values.

Significance with respect to control is calculated by Student's 't' test. LTT is index value therefore significance value is not mentioned.

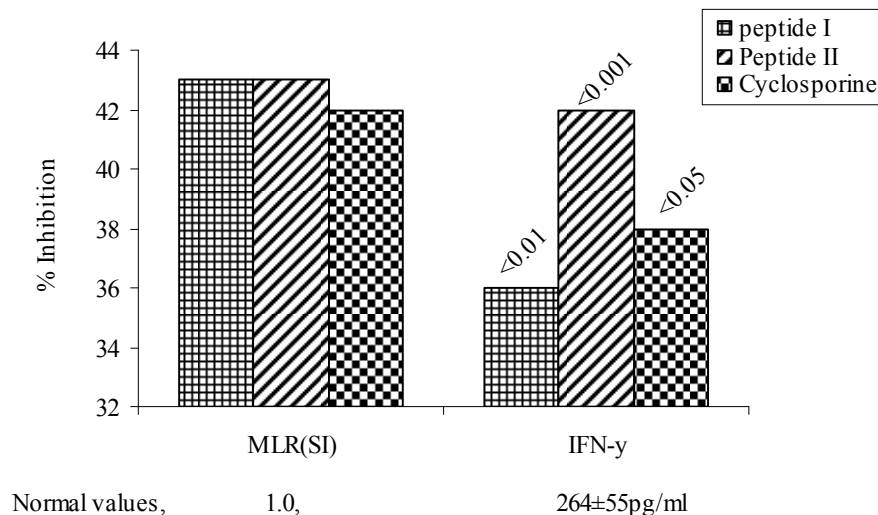


Fig: 2A

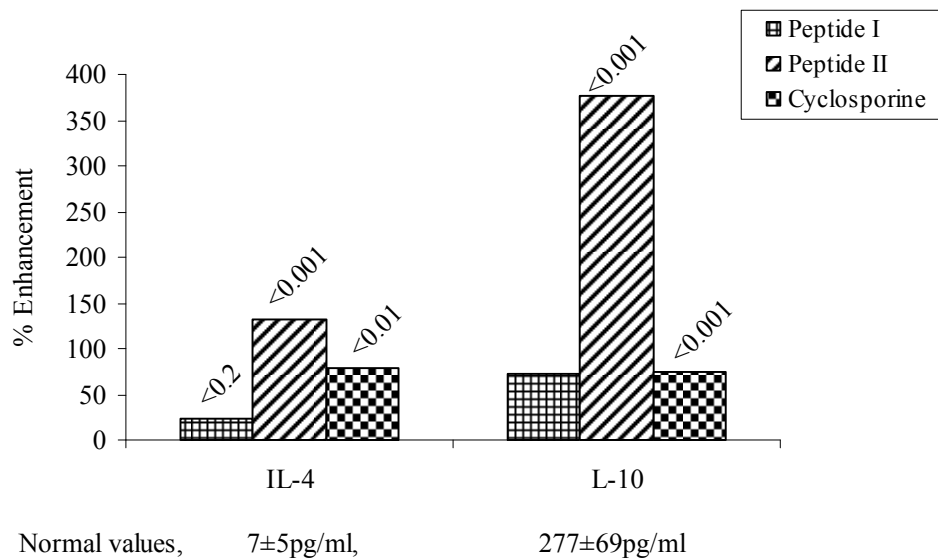


Fig.2 B

Figure: 2

Legend for fig 2

Fig. 2 (Graphical representation in terms of percent inhibition of parameters in Table 2).

A: Percent inhibition value of MLR; mixed lymphocyte reaction and IFN- γ . (SI; stimulation index).

B: Percent enhancement in Interleukin-4 (IL-4) and Interleukin-10 (IL-10) values.

Percent change in non-specific immune response in a group of BALB/c mice treated with peptide I & II at a dose of 5 mg/kg/day, p.o. (oral) and cyclosporine 25 mg/kg for 14 consecutive days.

Percent values were calculated with mean of experimental values with respect of mean control values. Significance with respect to control is calculated by Student's 't' test.

Note: MLR is in an index value therefore significance value is not mentioned.

Interleukin-2 (IL-2) and Interleukin-6 (IL-6) showed non-significant changes therefore not presented in graphs (Table 2).

Table 1

Specific and nonspecific immune response against sheep RBC in a group of BALB/c mice treated with peptide I and peptide II at the dose of 5 mg/kg/day and cyclosporine 25 mg/kg/day p.o.(oral) for 14 consecutive days then administered with SRBC's, i.p. on day 15.

Parameter	Control	peptide I treated	peptide II treated	cyclosporine
PFC/10 ⁵ splenocytes	104±11	84±22 ^a	41±9 ^b	85±21 ^a
HA titer	2048±1	1536±295 ^a	656±231 ^a	1024±1 ^a
DTH response(mm)	0.23±0.15	0.26±0.11 ^{ns}	0.20±0.15 ^{ns}	0.24±0.1 ^{ns}
LTT (SI)	1.0	0.35±0.20	0.30±0.12	0.38±0.21

Mice treated with water are taken as control.

PFC; plaque forming cell, HA; haemagglutinating antibody, DTH; delayed type hypersensitivity test, LTT; lymphocyte transformation test, SI; stimulation index.

Data based on two separate experiments with 5 animals in each group.

The values are mean ± SD of triplicate determinations.

Significance difference from control is calculated by student's 't'-test

^a $p < 0.02$.

^b $p < 0.001$.

^{ns} Not significant.

Table 2

In-vitro effect on lymphocytes from genetically different population (MLR- mixed lymphocyte reaction), and generation of cytokines by splenocytes of mice treated with peptides I and peptides II at the dose of 5 mg/kg/day and cyclosporine 25 mg/kg p.o.(oral) for 14 consecutive days .

Group (Treatment)	MLR (SI) <i>In vitro</i> *	Cytokines (pg/ml)				
		T _H 1 type		T _H 2 type		
		IL-2	INF- γ	IL-4	IL-6	IL-10
None(control)	1	333 \pm 179	264 \pm 55	7.3 \pm 5	576 \pm 6	277 \pm 69
Peptide I	0.57 \pm 0.01	336 \pm 161 ^{ns}	171 \pm 84 ^a	9.1 \pm 3 ^{ns}	571 \pm 6 ^{ns}	478 \pm 320 ^{ns}
Peptide II	0.57 \pm 0.01	348 \pm 160 ^{ns}	154 \pm 53 ^c	17.0 \pm 3 ^c	570 \pm 14 ^{ns}	1319 \pm 329 ^c
Cyclosporine	0.58 \pm 0.02	371 \pm 142 ^{ns}	163 \pm 74 ^b	13.0 \pm 5 ^c	568 \pm 26 ^{ns}	485 \pm 110 ^c

Mice treated with water are taken as control.

MLR; mixed lymphocyte reaction, SI; stimulation index.

Data based on 10 animals in each group.

The values are mean \pm SD of duplicate determinations.

Significance difference from control is calculated by student's 't'-test

^a $p < 0.01$.

^b $p < 0.005$.

^c $p < 0.001$.

^{ns} Not significant.

* *in vitro* concentration of sample is 0.1 μ g/ml.

Note: Significance for MLR test can not be calculated since it is a stimulation index value where control value has been taken as 1.

Table3

Effect of peptides I and II on skin graft survivals in C3H (FAWN) to Swiss mice skin transplantation.

Treatment	Mean survival time (days)
Peptide I (5mg/kg/day X 14days)	13.5 ± 4.4
Peptide II (5mg/kg/day X 14days)	14.75 ± 5.4
Cyclosporine (1mg/kg/day X 14days)	12.3 ± 6.0
None (Control)	8.5 ± 2.4

Skin pieces (5x10 mm) from C3H (FAWN) mice were transplanted to twenty Swiss mice and divided into 4 groups of 5 animals each. Animals of group 1 and 2 were treated with Peptide I and Peptide II at 5 mg/kg/day i.p.; group 3 were treated with cyclosporine at 1 mg/kg/day i.p for 14 days while animals of untreated group were used as controls.