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

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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: 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 HC1/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 μ g/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 $4x10^6$ 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 CO_2-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 $1X10^{7}$ cells/ml. Equal number of splenocytes ($5X10^{5}$) 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, CO_2 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 ($4x10^{5}$ 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 μg/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 μg/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 INF-γ 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 microphages 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 phage 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.

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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) *In vitro**	Cytokines (pg/ml)				
		T_H1 type		T_H2 type		
		IL-2	INF-γ	IL-4	IL-6	IL-10
None(control)	1	333±179	264±55	7.3±5	576±6	277±69
Peptide I	0.57±0.01	336±161[ns]	171±84[a]	9.1±3 [ns]	571±6 [ns]	478±320 [ns]
Peptide II	0.57±0.01	348±160 [ns]	154±53[c]	17.0±3[c]	570±14 [ns]	1319±329[c]
Cyclosporine	0.58±0.02	371±142 [ns]	163±74[b]	13.0±5[c]	568±26 [ns]	485±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 ± 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 0.1µg/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.

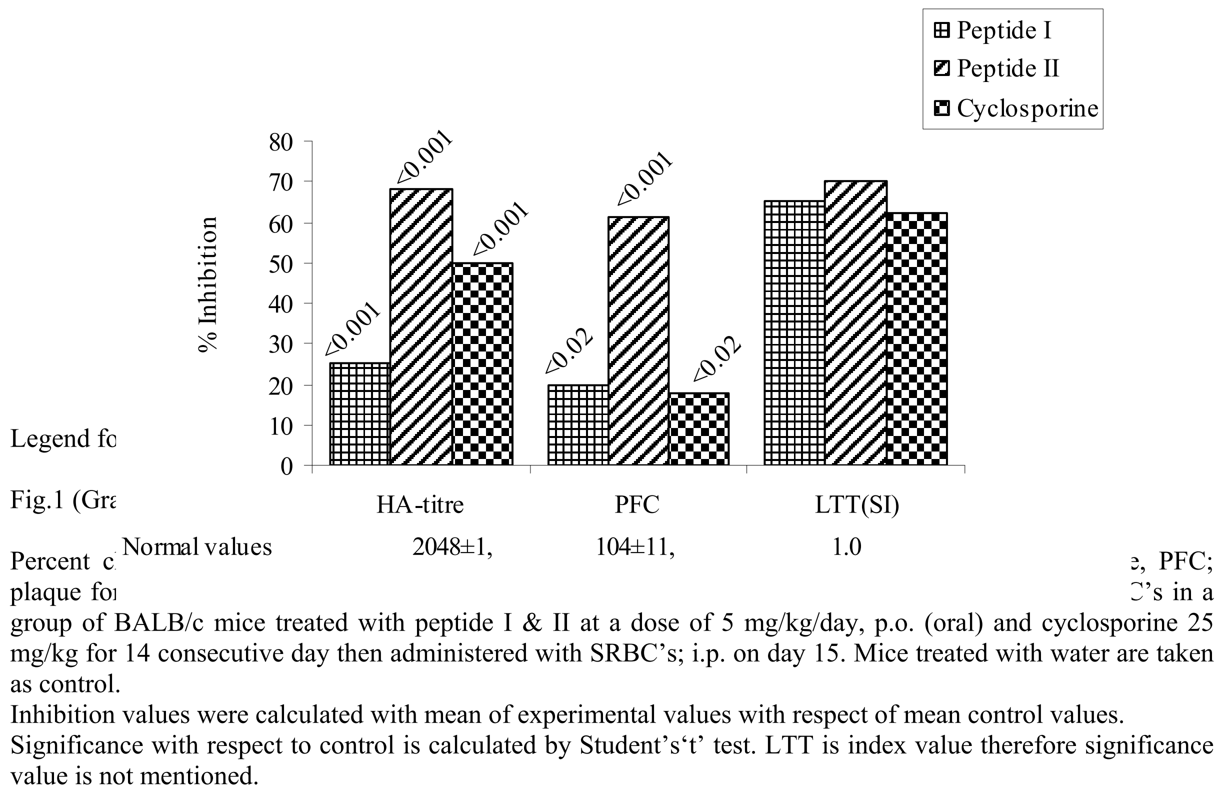

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Fig.1 (Gra

Percent c ... e, PFC;
plaque fo ... C'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.

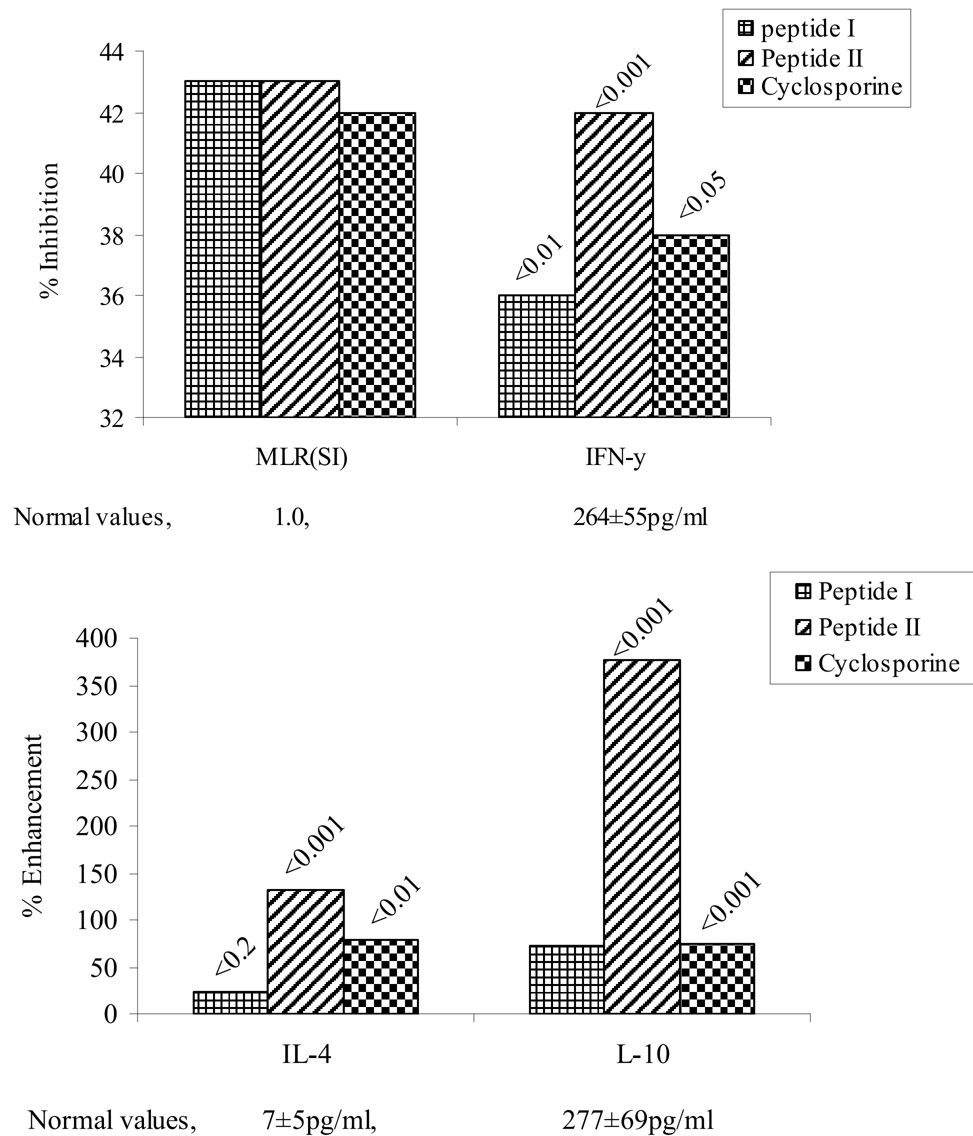

Fig.2 B

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).