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STIMULATION OF IL-2 PRODUCTION AND CD2R EXPRESSION BY SPLENOPENTIN ANALOGS

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Abstract—Splenopentin (SP-5), is a pentapeptide corresponding to the amino acid sequence 32–36 (Arg–Lys–Glu–Val–Tyr) of the splenic hormone splenin. Its synthetic analogs: Lys–Lys–Glu–Val–Tyr (**1**) and D-Lys–Lys–Glu–Val–Tyr (**2**) have been evaluated for active T-cell rosette (CD2R), total T-cell rosette (CD2), interleukin-2 (IL-2) stimulation and effect on antibody production. SP-5 as well as both the analogs stimulated CD2R. Analogs (**1**) and (**2**) were also found to stimulate IL-2 production. These observations suggest that *in vitro* human NK cell augmentation with analogs (**1**) and (**2**) reported earlier may be due to enhanced IL-2 production.

Keywords: CD2R, immunomodulators, interleukin-2, splenopentin

INTRODUCTION

Thymopoietin, a 49 amino acid polypeptide hormone of thymus discovered by its effect on neuromuscular transmission, was later shown to induce T-cell differentiation and to affect immunoregulatory balance (Goldstein, 1974). A radioimmunoassay for thymopoietin revealed a cross-reaction with another polypeptide found in spleen and lymph node but not in other tissues. The primary structure of this polypeptide called splenin, differs from thymopoietin only at position 34, Asp in thymopoietin and Glu in splenin. Synthetic pentapeptides corresponding to the region 32–36 of thymopoietin and splenin, called thymopentin (TP-5; Arg–Lys–Asp–Val–Tyr) and splenopentin (SP-5; Arg–Lys–Glu–Val–Tyr), respectively, reproduce biological activities of their parent polypeptides (Goldstein *et al.*, 1979). Thymopoietin and TP-5 affect neuromuscular transmission and induce the phenotypic differentiation of T-precursor cells *in vitro* while inhibiting phenotypic differentiation of B-cells. In contrast, Splenin and SP-5 do not affect neuromuscular transmission, and they induce both T- and B-cell precursors (Audhya *et al.*, 1984).

Immunomodulatory activity of TP-5 and its synthetic analogs has been well established and some of them are presently being evaluated clinically (Scheid *et al.*, 1978; Hadden, 1991). According to a report from Audhya *et al.* (1984), SP-5 exhibits a particularly interesting immunological profile. SP-5 is known to influence both T-cell and B-cell differentiation (Audhya *et al.*, 1984), to increase mitogen-induced interferon-gamma production *in vivo* (Diezel *et al.*, 1984) and to increase the number of antibody forming cells in mice after gamma irradiation (Diezel *et al.*, 1986). Stimulation of the recruitment of epidermal langerhans cells by SP-5 has also been demonstrated (Gruner *et al.*, 1990). SP-5 has been shown to provide 80% protection against foot-and-mouth disease virus (FMDV) when used in a conventional oil adjuvanted FMDV vaccine as compared to normal vaccine (Liebermann *et al.*, 1993). Long term treatment with natural and synthetic splenic peptides as well as cyclosporin A inhibited the development of antigen-

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Abbreviations: FCS = foetal calf serum; FMDV = foot and mouth disease virus; NK cells = natural killer cells; SP-5 = splenopentin; SRBC = sheep red blood cells; TP-5 = thymopentin.

induced arthritis in rats (Brauer *et al.*, 1993). SP-5 also acts as a co-stimulant for recombinant human granulocyte–macrophage colony-stimulating factor in the induction of human bone marrow cells derived colony formation *in vitro* (colony-forming units) (Diezel *et al.*, 1993). Continuous treatment with splenopentin significantly prevented symptoms of graft-vs-host reaction. SP-5 led to the reconstitution of antibody formation in immunosuppressed mice following lethal irradiation (Eckert *et al.*, 1989).

Impressed with the biological activities of SP-5 cited above, we undertook the synthesis of novel analogs of SP-5: Lys–Lys–Glu–Val–Tyr (**1**) and D-Lys–Lys–Glu–Val–Tyr (**2**) for studying the immunopotentiating activity. In case of analogs (**1**) and (**2**), the amino-acid Glu has been retained at position 3 and modifications have been introduced at position 1. We have demonstrated that analogs (**1**) and (**2**) significantly augment human natural killer (NK) cell activity *in vitro* (Rastogi *et al.*, 1993) and up regulate HLA class I gene transcription (Chatterjee-Kishore *et al.*, 1997). Here we show that analogs (**1**) and (**2**) stimulate CD2R expression and IL-2 production.

EXPERIMENTAL PROCEDURES

Synthesis of peptides

SP-5 and its analogs (**1**) and (**2**) were synthesized by the solution phase procedure using the 2+3 fragment condensation approach as described earlier (Rastogi *et al.*, 1993). In brief, deblocking of the protected pentapeptides Boc–Arg(NO_2)–Lys(Z)–Glu(OBzl)–Val–Tyr–OBzl, Z–Lys(Z)–Lys(Z)–Glu(OBzl)–Val–Tyr–OBzl and Z–D-Lys(Z)–Lys(Z)–Glu (OBzl)–Val–Tyr–OBzl, afforded SP-5 and its analogs in a pure state. SP-5 was characterized as its trihydrochloride and the analogs as triacetate salts with the help of elemental analysis, TLC, HPLC, NMR and FAB-MS.

T-cell rosette for CD2 and CD2R expression

T-cell rosettes were enumerated as described by Wybran *et al.*, 1979 with minor modifications (Bajpai *et al.*, 1997). In brief, 1×10^6 cells/ml of human peripheral blood mononuclear cell (PBMC) suspension was incubated with 10^{-4} M concentration of SP-5 or its analogs (1 and 2) at 37°C for 1 h in a CO_2 incubator. Later on the cells were washed with phosphate buffer saline and resuspended in foetal calf serum. For T-cell rosettes, human PBMC suspension in foetal calf serum was mixed with sheep red blood cells (SRBC) in saline to obtain SRBC and lymphocyte ratios of 10:1 and 1:1 for active (CD2R) and total T-cell rosettes (CD2), respectively. The tubes with cells were centrifuged gently at 200*g* for 5 min, resuspended and rosettes were counted after overnight incubation at 4°C. Lymphocytes with >3 SRBC were counted as rosettes. A minimum of 300 lymphocytes were enumerated and results are expressed as percentage of total PBMC.

Stimulation of IL-2

A suspension of human PBMC (0.5×10^6 cells/ml) was cultured in RPMI-1640 media (Life Technology, Grand Island, NY) for 24 h in the presence and absence of 10^{-4} M concentration of SP-5 and its two analogs. Phytohaemagglutinin (5 μg/ml) was used as a positive control. Supernatants were harvested and stored at −70°C until tested for IL-2.

IL-2 assay using ELISA

IL-2 assay was carried out as a solid phase enzyme immunoassay with ELISA kit (Genzyme Corporation, Cambridge, MA) based on multiple antibody sandwich principle. The ELISA plate wells precoated with mouse monoclonal antibody specific for human IL-2, were used to capture IL-2 present in standard and unknown samples. An anti-IL-2 rabbit polyclonal antibody which binds to captured IL-2 was then added. Next, peroxidase-conjugated goat anti-rabbit polyclonal antibody was added to each well followed by peroxide/TMB substrate solution. The substrate initiated a peroxide catalyzed color change which was stopped within 5 min by acidification with stop solution. The absorbance measured by ELISA reader (Titertek, Helsinki, Finland) at 450 nm was proportional to concentration of IL-2 present in the samples. A standard curve was obtained by plotting standard IL-2 concentration versus absorbance. The IL-2 concentration in experimental samples were determined from standard curve. Results are presented as OD ± SD as well as concentrations of IL-2 in pg/ml.

Antibody response against SRBC

Animals (4–6 weeks old Balb/c mice) were injected with SP-5/analogs (80 mg/kg body weight) intraperitoneally, and 24 h later, these mice were injected with 0.1 ml 1% suspension of SRBC. One week after immunization animals were bled from retro orbital venus plexus and sera samples were separated. Samples from 10 animals in a group were pooled for assay. Next day, animals were again immunized as before and bled 6 days later for secondary response. Haemagglutination assay was done as described earlier (Singh *et al.*, 1984).

Statistical analysis

Results are expressed as mean ± SD. Statistical significance was determined by Student's *t*-test. A p value of <0.05 was considered significant (Zar, 1984). Significant values found in the above test were further analysed by non-parametric tests, e.g. ANOVA and Student Newman–Keul test.

RESULTS

Effect of SP-5 and its analogs on human CD2 and CD2R expression

The effect of SP-5 and its analogs was investigated on CD2 which identifies total T-cells, and CD2R which identifies a sub-population of activated T-cells. The latter test is always done under sub-optimal conditions like low ratio of SRBC per lymphocytes, and a very short incubation period. Both the analogs (**1** and **2**) as well as SP-5 enhanced CD2R when PBMC were exposed to 10^{-4} M concentration of these peptides. The increase was found to be statistically significant (Table 1). These peptides had no effect on CD2.

Stimulation of IL-2 production by SP-5 and its analogs

Human PBMC were stimulated with 10^{-4} M concentration of SP-5 and its analogs (**1** and **2**) and supernatant samples were collected after 18 and 24 h. IL-2 estimation was carried out using sandwich ELISA. We found that SP-5 did not induce IL-2 production, whereas its analogs (**1** and **2**) stimulated the production of IL-2 at 18 and 24 h (Table 2). Level of IL-2 produced at 24 h was significantly higher as compared to control. Phytohaemagglutinin was used as control.

Table 1. Effect of SP-5 and its analogs on active (CD2R) and total T-cell rosette (CD2) formation *in vitro*

Treatment	Percent rosette formation	
	CD2R	CD2
Control	35 ± 4	52 ± 4
SP-5	52 ± 7 ($p<0.05$)	50 ± 4
Control	27 ± 5	53 ± 1
Lys–Lys–Glu–Val–Tyr (**1**)	70 ± 5 ($p<0.001$)	52 ± 2
d-Lys–Lys–Glu–Val–Tyr (**2**)	67 ± 1 ($p<0.001$)	51 ± 1

Dose of SP-5 and its analogs used was 10^{-4} M. Data represent mean ± SD of three different sets of *in vitro* experiments carried out separately with peripheral blood lymphocyte samples of three different individuals. The p values were calculated using Student's *t*-test in relation to the control (unstimulated cells). These values were also found to be significant by non-parametric tests, e.g. ANOVA and Student Newman–Keul test.

Table 2. IL-2 production in presence of SP-5 and its analogs

Treatment	IL-2 (OD + SD;pg/ml)	
	18 h	24 h
Control	0.004 ± 0.002	0.014 ± 0.001
Phytohemagglutinin	0.0175 ± 0.01 ($p<0.50$) (135 pg)	0.033 ± 0.01 ($p<0.05$) (203 pg)
SP-5	0.003 ± 0.02	0.012 ± 0.004
Analog (**1**)	0.008 ± 0.07 ($p<0.19$) (84.6 pg)	0.029 ± 0.004 ($p<0.01$) (187 pg)
Analog (**2**)	0.023 ± 0.01 ($p<0.10$) (165 pg)	0.029 ± 0.012 ($p<0.02$) (186 pg)

The dose of SP-5 and its analogs used was 10^{-4} M. ELISA was done in triplicate. The p value was calculated using Student's *t*-test in relation to control (supernatant from unstimulated cells). These values were also found to be significant by non-parametric tests, e.g. ANOVA and Student Newman–Keul test.

Effect of SP-5 and its analogs on antibody production

Results obtained with SRBC antigen in haemagglutination assay suggested that there was no difference between control and SP-5 or analogs treated animals in respect of primary antibody titer. Animals treated with SP-5 and analog (**1**) showed two fold higher secondary antibody against SRBC. Analog (**2**) did not show any difference with untreated control animals (data not presented).

DISCUSSION

SP-5 has been shown to augment the mitogen (concanavalin A) induced γ-interferon production (Diezel *et al.*, 1987). SP-5 treatment of sublethally irradiated mice results in an accelerated restitution of splenic plaque response against SRBC (Diezel *et al.*, 1986). We have observed elevated secondary antibody levels against SRBC by SP5 and analog (**1**). Similar effects of SP-5 treatment were established in syngeneic bone marrow grafted mice following lethal irradiation (Eckert *et al.*, 1986). Earlier we have demonstrated that both analogs (**1** and **2**) of SP-5 augment *in vitro* human NK cell cytotoxicity under conditions when SP-5 failed to do so (Rastogi *et al.*, 1993). The analogs did not show any effect on *in vitro* human T-cell proliferation response. Here we have shown that SP-5 as well as its analogs (**1** and **2**) enhance CD2R expression.

The three peptides, however, have no effect on CD2. Mazumder *et al.* (1993) have also demonstrated enhanced rosette forming T-cells on *in vitro* treatment with met-enkephalin, another small peptide used as immunomodulator. Enhanced CD2 and CD2R on treatment with met-enkephalin *in vitro* has also been reported by different groups (Wybran *et al.*, 1979; Plotnikoff *et al.*, 1985). Wybran (1985) observed enhancement of CD2R only. This is explained by the fact that the test for CD2R is performed under sub-optimal conditions for rosetting (low SRBC per lymphocyte) ratio and short incubation time between SRBC and lymphocytes. Therefore, CD2R is expected to be more easily influenced than the CD2. Assay of CD2 is performed under optimal conditions. Recently, we have also demonstrated that met-enkephalin and its two synthetic analogs stimulate CD2R expression (Bajpai *et al.*, 1997).

Both the analogs of SP-5 (**1** and **2**) stimulated IL-2 production as confirmed by ELISA. SP-5 did not induce IL-2 production. Our results suggest that splenopentin by itself did not have any effect on IL-2 production, but substitution of Arg at position 1 in SP-5 by Lys (analog **1**) or D-Lys (analog **2**) leads to peptides which induce production of IL-2 significantly. The induction of IL-2 by the two analogs could be important for the augmentation of NK cell activity reported by us earlier (Rastogi *et al.*, 1993). There is a report in literature that *in vivo* administration of TP-5 to immunocompromised aged subjects enhances the production of IL-2 (Meroni *et al.*, 1987), a factor well known for its capacity to modulate NK activity (Domzig *et al.*, 1983). SP-5, however, does not stimulate significant IL-2 production *in vitro*.

The findings of a single thymopoietin gene do not support earlier suggestions from protein sequencing that splenin is a distinct molecule (Harris *et al.*, 1994, 1995). Rather it would now seem that errors in interpretation of amino acid sequence data led to these conclusions and that the TP like material purified from spleen was thymopoietin itself. Nevertheless, SP-5 sequence was active in various immunological experiments (Brauer *et al.*, 1993; Diezel *et al.*, 1986, 1987, 1993; Eckert *et al.*, 1986, 1989; Liebermann *et al.*, 1993).

As part of our ongoing programme on development of small synthetic peptides as potent and non-toxic immunomodulators, we undertook the synthesis and evaluation of above two analogs of SP-5 (Bajpai *et al.*, 1995, 1997; Chatterjee-Kishore *et al.*, 1997; Puri *et al.*, 1993; Rastogi *et al.*, 1993; Sahai *et al.*, 1996; Sharma *et al.*, 1996; Singh *et al.*, 1991, 1994). It is important to note that SP-5 analogs capable of augmenting human NK cells *in vitro* can induce IL-2 production. Recently, we have shown that these two analogs up regulate transcription of the HLA-B7 gene in K562 cells which normally do not transcribe this gene. Electrophoretic mobility shift assays indicate that this transcriptional induction may be related to the appearance of novel HLA-B7 promoter binding factors in the nuclei of induced cells (Chatterjee-Kishore *et al.*, 1997). Such studies may generate new leads for designing analogs of other naturally occurring peptides as well so that peptides more suitable for pharmaceutical use are obtained. If these *in vitro* results are substantiated by *in vivo* studies, these peptides may provide a new tool for stimulation of innate host resistance, particularly against cancer and viral infections.

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