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Cytokine-inducing activity of a proline-rich polypeptide complex (PRP) from ovine colostrum and its active nonapeptide fragment analogs

Abstract

A complex of proline-rich polypeptides (PRP) was isolated from ovine colostrum in our laboratory and was shown to possess immunomodulatory properties and psychotropic activity, including beneficial effects in the treatment of Alzheimer's disease. A nonapeptide fragment (NP): Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro, isolated from the chymotryptic digestion products of PRP, and its C-terminal

fragment, a hexapeptide (HP): Tyr-Val-Pro-Leu-Phe-Pro also exhibited immunoregulatory activity. Although NP and HP expressed activity similar to that of PRP in studies on humoral and cellular immune responses, in other immune processes, e.g. induction of cytokines, they showed markedly lower activity than PRP. In the search for more active peptides, in the present study, we compared the cytokine-inducing ability of PRP, NP, HP, and linear oligomers of NP or HP. For this purpose, the induction of IFN, TNF-alpha, IL-6, and IL-10 in human whole blood cell cultures was measured. NP, HP, and their oligomers showed differential effects in the induction of cytokines, generally lower than that of PRP. Only the PRP complex showed a bell-shaped dose-response dependence suggesting regulatory properties. There were no distinct differences between monomeric forms of NP (NP1) or HP (HP1) and their oligomers in the induction of IFN and TNF-alpha (Th1 cytokines) but such differences were found in the induction of IL-6 and IL-10 (Th2 cytokines). Dimer (NP2) was less active than the monomeric NP1 nonapeptide in the induction of IL-6 and IL-10. On the other hand, oligomers: HP3 and HP4, showed a significantly higher ability to induce Th2 cytokines compared to HP1, HP2 or NP peptides. This was especially evident in the case of IL-10 induction, where the activity of HP4 surpassed the activity of PRP and approached the activity of LPS-PHA. The results obtained showed that some of the peptides studied, when used at higher concentrations (100 mug/ml) may replace the PRP complex as cytokine inducers. Our data also suggest the possibility of using certain oligomers for selective induction of particular cytokines.

Full text

INTRODUCTION

A proline-rich polypeptide (PRP) was isolated from ovine colostrum by Janusz *et al.* [1, 2]. The polypeptide showed immunoregulatory properties inducing maturation and differentiation of murine thymocytes, and affected humoral and cellular immune responses, both *in vivo* and *in vitro* [2-4]. PRP was able to reduce high, and to elevate low humoral immune responses to SRBC in mice [3]. The net effect of PRP depended on the actual state of the animals studied. PRP seems to restore balance in cellular immune functions. PRP is not species-specific and is active in mice [3, 4], humans [5], and rats [6]. The molecular weight of PRP, determined in SDS-PAGE, is about 6,000 [2], and it has been recently found that PRP is a complex of proline-rich polypeptides of molecular

weight up to 6,000 [Kruzel ML, Janusz M, Lisowski J, Fischleight RV, Georgiades JA: Towards an understanding of the biological role of Colostrinin peptides submitted]. PRP contains a high proportion of proline residues (25%) and hydrophobic amino acids (40%). An active nonapeptide fragment (NP): Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro was isolated from the chymotryptic digestion products of PRP. It showed in mice biological activity similar to the undigested PRP [2, 7]. Studies on the structure-activity relationship showed that the C-terminal fragment of NP, a hexapeptide (HP): Tyr-Val-Pro-Leu-Phe-Pro, also exhibited immunotropic activity similar to PRP and NP [8].

It was also found that the PRP complex, and to a lesser degree NP, is a modest cytokine inducer (IFN, TNF-alpha) in human whole blood cell cultures [5]. During our studies involving the possibility of using PRP as treatment for correcting immune disorders, we noticed that volunteers who received PRP showed signs of improvement in mood and cognitive abilities [5]. The immunoregulatory and psychoenhancing activity of PRP prompted us to investigate whether the polypeptide complex could be used for the treatment of Alzheimer's disease. In a double-blind, placebo-controlled study, we showed that PRP, in the form of orally administered tablets called Colostrinin®, improves the outcome of Alzheimer's disease patients [9]. The ability of PRP to induce secretion of IFN correlates well with the observation that this interleukin inhibits the formation of beta-amyloid deposits and might have a beneficial effects on Alzheimer patients [10-12]. PRP also has beneficial effects on cognitive functioning in aged rats [6].

In some immune processes NP and HP showed biological activity similar to that of the PRP complex, *e.g.* effect on the humoral or cellular immune responses or on the resistance of murine thymocytes to apoptosis induced by hydrocortisone [2]. However, NP and HP were inactive or showed significantly lower activity than PRP in the induction of cytokines in human pe-ripheral blood leukocytes [5] or in murine resident peritoneal cells [13]. Therefore, it was of interest to synthesize analogs of NP or HP which might show enhanced cytokine-inducing ability. For this purpose, NP and HP containing D-amino acids as N-terminal amino acids and covalent linear oligomers built of repeating units of NP or HP were synthesized:

NP_1: Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro

NP_2: $(Val\text{-}Glu\text{-}Ser\text{-}Tyr\text{-}Val\text{-}Pro\text{-}Leu\text{-}Phe\text{-}Pro)_2$

NP-D: D-Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro

HP_1: Tyr-Val-Pro-Leu-Phe-Pro

HP_2: $(Tyr\text{-}Val\text{-}Pro\text{-}Leu\text{-}Phe\text{-}Pro)_2$

HP_3: $(Tyr\text{-}Val\text{-}Pro\text{-}Leu\text{-}Phe\text{-}Pro)_3$

HP_4: $(Tyr\text{-}Val\text{-}Pro\text{-}Leu\text{-}Phe\text{-}Pro)_4$

HP-D: D-Tyr-Val-Pro-Leu-Phe-Pro

The aim of the present study was to examine whether linear oligomers and D-analogs of NP_1 or HP_1 could express improved cytokine-inducing activity compared to their monomeric analogs and PRP. The immunotropic activity of the peptides was evaluated by determination of the induction of interferons (IFN), tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6), and interleukin-10 (IL-10) in cultures of human whole blood cells.

MATERIALS AND METHODS

Reagents

A proline-rich polypeptide complex (PRP) was isolated from ovine colostrum in accordance with the method of Janusz *et al.* [1]. RPMI medium was obtained from the Laboratory of Biopreparations of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. Tissue culture plates were from Costar, USA. Leukoagglutinin (PHA), bacterial lipopolysaccharide (LPS) from *E. coli*, thiazolyl blue (MTT), actinomycin D, L-glutamine, sodium dodecyl sulfate (SDS), dimethylformamide (DMF) were obtained from Sigma, USA. Antibodies against IL-6 and IL-10, and recombinant IL-6 and IL-10 were purchased from PharMingen, USA. Polypeptides: NP, HP, and their linear covalent oligomers, NP and HP with D-amino acids at their N-terminal ends were synthesized by the solid-phase method using the Boc/Bzl procedure [14]. The peptides obtained were homogeneous on thin layer chromatography plates and their purity exceeded 95%, as judged by HPLC. They revealed the expected amino acid composition and molecular weight (MS-ESI).

Mice

Six week-old C57/BL/6 mice were purchased from the animal farm of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

Cytokine induction in human whole blood cell cultures

The experiments were performed according to the procedure described by Inglot *et al.* [5]. Blood samples were collected into syringes containing 10 U/ml of heparin. Within 2 hours after the collection, the blood was diluted 10-fold with RPMI-1640 medium supplemented with 100 units/ml penicillin, 100 mug/ml streptomycin, and 0.5 mg/ml L-glutamine. One ml portions of the cell suspensions were distributed in duplicates or triplicates into 24-well flat-bottomed tissue culture plates. The inducers, PRP and synthetic peptides, were added in volumes of 20 mul RPMI containing 1 mug, 10 mug, and 100 mug of the peptides. The reference of positive inducers were: 2 mug/ml of PHA plus 2 mug/ml of LPS. The control wells contained the culture medium only and were used to measure the spontaneous production of cytokines (negative control). The plates were incubated for 20 hours at 37° C in a 5% CO_2 atmosphere. After the incubation, plates were centrifuged at 1,000 rpm for 15 min at 4° C. The supernatants were collected and used for the determination of cytokines. The samples were stored for several weeks at - 20° C before the determination of cytokines.

Determination of cytokines

Interferons (IFN) and tumor necrosis factor-alpha (TNF-alpha) concentrations were measured using bioassays [5].

Interferon bioassay. IFN activity was measured in A549 cells (human lung adenocarcinoma, ATCC CCL 185). The confluent monolayers of A549 cells were prepared in 96-well microplates in Dulbecco's modified Eagle's minimum essential medium (DMEM) with 10% calf serum, L-glutamine, and antibiotics. IFN samples diluted on separate plates were added to the cell monolayers and incubated at 37° C for 24 hours at 5% CO_2 in humidified air. The cells were then washed and challenged with encephalomyocarditis virus (EMCV). The MTT method was used to determine the EMCV cytopathogenic effect and cytotoxicity of the peptides. The end-point of IFN titration in the MTT assay was

taken as 25% reduction of cell killing in comparison with control cells infected with 100 $TCID_{50}$ (tissue culture infective dose) of EMCV producing complete cytotoxicity. Laboratory standards of IFN were included in all assays.

Tumor necrosis factor-alpha (TNF-alpha) bioassay. The cytotoxic activity of TNF-alpha was measured in L929 cells. Samples serially diluted in culture medium supplemented with actinomycin D (5 mug/ml) were added to the monolayer of cells cultured in 96-well microplates. After incubation for 20 hours at 37° C, the cytotoxic effect was determined. The highest dilution causing the death of 50% of cells in the culture was defined as one unit of TNF-alpha activity. The standard preparation of recombinant TNF-alpha was used as a control.

IL-6 and IL-10 immunoassays. The interleukins were determined by microplate ELISA, using commercially available antibodies and recombinant interleukins from PharMingen according to the procedure recommended by the manufacturer.

Statistical analysis

Data are presented as means ± SD from at least 3 independent experiments performed in duplicate or triplicate. Data comparison were made with Student's t test for independent samples. Differences were considered significant when p was < 0.05.

RESULTS AND DISCUSSION

The cytokine induction was determined under *ex vivo* stimulation of human whole blood cell cultures with LPS and PHA, PRP, NP_1 or HP_1 and their analogs. This method was selected because it offers the great advantage of reproducing the natural microenvironment of immunocompetent cells and because it preserves the various intercellular communications between the different blood cell populations [15]. Using this method, it is possible to avoid cell activation related to isolation procedures [15, 16]. Cytokine production in whole blood cells can be used as an indicator of immune cellular status [17, 18]. Two types of cytokines were measured, IFN and TNF-alpha, secreted by Th1 cells and involved in the cell-mediated immunity, and IL-6 and IL-10, secreted by Th2 cells associated with the humoral immune response [18].

In all experiments, the cytokine-inducing ability of the PRP complex and of the other peptides was much lower than that of LPS-PHA. (Tables 1, 2, 3 and 4). However, PRP, at concentrations of 1-100 mug/ml, was able to induce the secretion of all cytokines studied (Tables 1, 2, 3 and 4). Maximal activity was observed at 10 mug/ml, with the exception of IL-6 induction which was the highest at 100 mug/ml (Tables 3 and 4).

The cytokine inducing activity of HP_1 was similar to the activity of NP_1. This resembles to the results of studies on the effect of NP_1 and HP_1 on the humoral and cellular immune responses, where both peptides showed similar effects [8]. These peptides were generally less effective in the induction of cytokines, compared to PRP, and their oligomerization showed different effects, depending on the cytokine induced.

In the case of induction of IFN, the activity of the peptides NP_1, HP_1, and their oligomers at 1 mug/ml and 10 mug/ml concentrations was lower compared to PRP, was similar to or even higher and at 100 mug/ml than the activity of PRP at 100 mug/ml. Generally, oligomerization of NP_1 and HP_1 did not increase, or even decreased, their IFN-inducing activity especially at lower concentrations (Tables 1 and 2).

The TNF-alpha-inducing ability of NP_1 and NP_2 (Table 1) was observed at concentrations of 100 mug/ml only, and the dimer NP_2 had the same activity as the monomeric NP_1. Oligomers HP_2, HP_3, and HP_4 showed the same activity as the monomeric HP_1 (Table 2). At concentrations of 100 mug/ml, HP peptides were more active than PRP at the same concentration.

In the case of induction of IL-6, NP_1 and NP_2, HP_1, HP_2, and HP_4 showed lower activity than PRP (Tables 3 and 4). The most active peptide was the oligomer HP_3, which activity was comparable to PRP.

IL-10-inducing ability of NP_1 and NP_2 was lower than that of PRP. Dimer (NP_2), was less active than the monomer NP_1 (Table 3). Interesting results were obtained in the case of HP_1 and its oligomers HP_2- HP_4 (Table 4). The peptides HP_1 and HP_2, at concentrations of 1 and 10 mug/ml, were less active than PRP, and at a concentration of 100 mug/ml, they showed similar activity to PRP at the same concentration. However, peptide HP_3, and especially HP_4, at concentration of 100 mug/ml, surpassed the activity of PRP and approached the activity of the

control inducers (LPS + PHA). In conclusion, trimerization or tetramerization of HP_1 markedly and selectively enhanced its IL-10-inducing ability.

Replacement of N-terminal L-amino acids by D-amino acids might increase the resistance of the peptides to aminopeptidases and so prolong their half-life in the organism. Therefore, analogs of the NP_1 and HP_1, containing D-amino acids (NP-D and HP-D), were obtained and their activity was determined (Tables 1, 2, 3 and 4). It was found however, that their cytokine-inducing activity was lower than that of NP_1 and HP_1. Introduction of D-amino acids may cause a change in the conformation of NP_1 and HP_1 and decrease their biological activity or the N-terminal amino acid residue may be essential for the activity.

The cytokine-inducing activity of the peptides studied indicates that they are bioavailable. However, the mechanism of the importation of these peptides into cells is, as yet, unresolved. Some of the peptides might behave as ligands and start transmission of signals into cells by as yet unidentified surface receptors, and activate the cells. On the other hand, it was found in other laboratories, that hydrophobic peptides containing proline residues, as in the case of PRP, NP, and HP, can freely penetrate cell membranes, enter and activate the cells and affect their effector functions [19-23]. The peptides can also be delivered into cells by peptide-transporting proteins which occur in all organisms to optimize utilization of the universal peptide pool [24, 25]. The internalized peptides can show free movement to cytoplasmic target proteins [20]. There is the possibility of their interaction with kinases, adapter proteins [26] and/or protein tyrosine phosphatases [27] that can cause an enhancement or attenuation of signals, and finally, the effector activity of cells. Interaction of PRP complex peptides with other proteins can be facilitated by the presence of block sequences of proline residues [1], which may be recognized by proteins containing SH3 domains [28]. Pro-Leu motifs, present in PRP, NP and HP may be recognized by proteins with WW domains [29]. The structure of the peptides studied suggests that they can also pass through the nuclear membrane and regulate expression of various genes [19].

CONCLUSION

In summary, the results obtained show that at higher concentrations (at least 100 mug/ml), NP, HP, and their oligomers can generally replace the PRP complex in the induction of cytokines in human whole blood cell cultures. However, only

the PRP complex showed a bell-shaped dose-response curve. Such a dependence indicating a regulatory activity of PRP was also observed in other assays, *e.g.* the effect of PRP on the release of antibodies from spleen cells [3]. This could be due to the fact that PRP is a complex of proline-rich polypeptides which could act in concert giving a resultant effect. NP, HP, and their oligomers showed a linear dose-response dependence.

The results presented here suggest the possibility of using a particular peptide, at a proper concentration, to induce a particular cytokine and so modulate the immune response. This was particularly observed in the case of Th2 cytokines, IL-6 and IL-10, where oligomers of HP were relatively strong inducers of the secretion of these cytokines.

We can assume that PRP acts like a "super-cytokine", initiating the cytokine cascade which help to keep the balance between the Th1 and Th2 cells responsible for the outcome of diverse immune processes [18, 30, 31]. Single cytokines can affect cells in a different way than a mixture of cytokines. They can mutually enhance or supress their effects on cells [30].

Recently, the involvement of the immune system in the pathogenesis of Alzheimer's disease and the role of cytokines in the regulation of neurodegenerative processes has been accepted [32-35]. The "super-cytokine"-like properties of the PRP complex shown in this paper may shed some light on the mechanism of the positive therapeutic effects of PRP in Alzheimer patients.

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Figures

Table 1
Induction of Th1 cytokines (IFN, TNF-alpha) by the PRP complex or by the nonapeptide (NP_1) and its analogs, in cultures of human whole blood cells

Results of experiments are expressed as means ± SD from at least 3 independent experiments. The data were analysed using Student's t test for independent samples. Differences between samples were assumed to be significant at $p < 0.05$. IFN and TNF-alpha were determined by bioassays.

Inducer	Concentration (mug/ml)	IFN (U/ml)	TNF-alpha (U/ml)
Control		7.9 ± 2.4	6.3 ± 2.9
LPS + PHA	2 + 2	214.1 ± 167.9*	182.7 ± 104.5*
PRP	1	18.1 ± 15.5*	27.1 ± 17.9*
	10	53.2 ± 47.5*	68.9 ± 25.6*
	100	20.7 ± 25.4	19.3 ± 19.9*
NP_1	1	13.5 ± 12.3	7.3 ± 4.0
	10	24.9 ± 33.5	11.7 ± 12.7
	100	30.1 ± 36.9*	37.5 ± 18.4*
NP_2	1	10.3 ± 3.4	5.0 ± 0.0
	10	10.6 ± 3.6	5.0 ± 0.0
	100	32.6 ± 21.2*	47.8 ± 49.1*
NP-D	1	7.5 ± 2.8	5.0 ± 0.0
	10	9.0 ± 0.0	5.5 ± 1.5
	100	16.3 ± 14.9	14.4 ± 12.1*

* Values compared with controls showed confidence level $p < 0.05$.

Table 2
Induction of Th1 cytokines (IFN, TNF-alpha) by the PRP complex or by the hexapeptide (HP_1) and its linear oligomers in cultures of human whole blood cells

Results of experiments are expressed as means ± SD from at least 3 independent experiments. The data were analysed using Student's t test for independent samples. Differences between samples were assumed to be significant at $p < 0.05$. IFN and TNF-alpha were determined by bioassays.

Inducer	Concentration (mug/ml)	IFN (U/ml)	TNF-alpha (U/ml)
Control		7.9 ± 2.4	6.3 ± 2.9
LPS + PHA	2 + 2	214.1 ± 167.9*	182.7 ± 104.5*
PRP	1	18.1 ± 15.5*	27.1 ± 17.9*
	10	53.2 ± 47.5*	68.9 ± 25.6*
	100	20.7 ± 25.4	19.3 ± 19.9*
HP_1	1	13.9 ± 11.9	6.4 ± 3.6
	10	21.0 ± 17.7*	7.2 ± 4.1
	100	40.1 ± 40.9*	32.3 ± 14.7*
HP_2	1	11.3 ± 4.2	5.0 ± 0.0
	10	11.6 ± 4.4	7.5 ± 4.6
	100	25.0 ± 19.5*	39.1 ± 28.4*
HP_3	1	10.3 ± 3.4	5.0 ± 0.0
	10	11.6 ± 4.4	10.0 ± 4.6*
	100	28.3 ± 20.3*	21.7 ± 7.9*
HP_4	1	10.1 ±3.2	5.0 ± 0.0
	10	12.4 ± 4.7	7.5 ±4.6
	100	35.4 ± 21.2	27.0 ± 13.7*
HP-D	1	6.7 ± 3.1	5.0 ± 0.0
	10	9.0 ± 0.0	5.0 ± 0..0
	100	11.0 ± 3.9	13.3 ± 8.9

* Values compared with controls showed confidence level $p < 0.05$.

Table 3
Induction of Th2 cytokines (IL-6, IL-10) by the PRP complex or by the nonapeptide (NP_1) and its analogs

Results of experiments are expressed as means ± SD from at least 3 independent experiments. The data were analysed using Student's t test for independent samples. Differences between samples were assumed to be significant at $p < 0.05$. IL-6 and IL-10 were determined by ELISA.

Inducer	Concentration (mug/ml)	IL-6 (ng/ml)	IL-10 (pg/ml)
Control		0.1 ± 0.13	1.2 ± 1.1
LPS + PHA	2 + 2	16.7 ± 9.9*	141.0 ± 35.3*
PRP	1	9.6 ± 3.9*	20.2 ± 18.1*
	10	13.1 ± 2.8*	51.0 ± 30.5*
	100	17.6 ± 3.6*	15.5 ± 17.6*
NP_1	1	2.8 ± 2.9*	3.1 ± 3.3*
	10	7.5 ± 6.1*	7.5 ± 10.1*
	100	12.9 ± 5.3*	36.6 ± 26.5*
NP_2	1	2.8 ± 2.6*	4.1 ± 2.8*
	10	3.0 ± 0.6*	4.6 ± 3.9*
	100	6.7 ± 3.4*	7.4 ± 5.3*
NP-D	1	1.0 ± 2.0*	2.2 ± 2.7
	10	5.6 ± 2.3*	8.2 ± 5.8*
	100	11.3 ±3.9*	19.2 ± 16.8*

* Values compared with controls showed confidence level $p < 0.05$.

Table 4
Induction of Th2 cytokines (IL-6, IL-10) by the PRP complex or by the hexapeptide (HP_1) and its linear oligomers in cultures of human whole blood cells

Results of experiments are expressed as means ± SD from at least 3 independent experiments. The data were analysed using Student's t test for independent samples. Differences between samples were assumed to be significant at $p < 0.05$. IL-6 and IL-10 were determined by ELISA.

Inducer	Concentration (mug/ml)	IL-6 (ng/ml)	IL-10 (pg/ml)
Control		0.1 ± 0.13	1.2 ± 1.1
LPS+PHA	2 + 2	16.7 ± 9.9*	141.0 ± 35.3*
PRP	1	9.6 ± 3.9*	20.2 ± 18.1*
	10	13.1 ± 2.8*	51.0 ± 30.5*
	100	17.6 ± 3.6*	15.5 ± 17.6*
HP_1	1	3.6 ± 3.2*	5.7 ± 4.3*
	10	4.3 ± 3.8	12.0 ± 10.8*
	100	7.1 ± 4.5*	25.6 ± 18.1*
HP_2	1	3.8 ± 1.8*	2.0 ± 2.1
	10	10.7 ± 5.2*	9.0 ± 8.8*
	100	12.7 ± 3.4*	17.1 ± 9.5*
HP_3	1	2.8 ± 0.4*	5.9 ± 3.6*
	10	10.7 ± 4.3*	23.0 ± 9.4*
	100	18.2 ± 2.9*	56.8 ± 44.3*
HP_4	1	2.4 ± 0.5*	2.0 ± 1.7
	10	3.8 ± 1.1*	11.9 ± 4.1*
	100	17.2 ± 8.9*	117.3 ± 47.0*
HP-D	1	0.2 ± 0.4*	5.8 ± 3.8*
	10	5.0 ± 2.2*	8.2 ± 12.6*
	100	15.8 ± 4.5*	13.4 ± 13.6*

* Values compared with controls showed confidence level $p < 0.05$.

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microsatellites, interleukin-10 promoter, multiplex-PCR, Caucasian, African.

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