# ORIGINAL PAPERS

Adv Clin Exp Med 2006, **15**, 4, 625–630
ISSN 1230-025X



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# A Proline-Rich Polypeptide Complex – Its Influence on Cytokine Induction in the Blood of Alzheimer's Patients

## Kompleks polipeptydowy bogaty w prolinę – wpływ na indukcję cytokin we krwi chorych na chorobę Alzheimera

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## Abstract

**Background.** The function of cytokines in neurodegenerative disorders has become a topic of intensive investigation. A proline-rich polypeptide complex (PRP) with immunomodulatory properties and psychotropic activity is a modest cytokine inducer. In the form of orally administered tablets it improves outcome in Alzheimer's patients.
**Objectives.** To further explore the role of PRP, the release of IL-1β, IL-6, IL-10, and TNF-α in cultured AD patient whole-blood samples was investigated. Control samples were collected from healthy persons of similar age.
**Material and Methods.** Blood samples taken from 20 AD patients (10 women and 10 men, mean age: 70 ± 7.9 years) and 10 healthy controls (6 women and 4 men, mean age: 68 ± 6.2 years) were investigated. LPS + PHA was used for cytokine induction. The interleukins were determined by microplate ELISA.
**Results.** In the absence of inducers, no statistically significant differences were found in the plasma of the controls and AD patients. In the presence of standard inducers (LPS + PHA) as well as in the presence of PRP (1–100 μg), secretion of IL-1β, IL-6, IL-10, and TNF-α was induced in both the control and AD blood samples. The level of induced cytokines increased with disease progression. Some modulatory effect of PRP added to the blood cell cultures simultaneously with or after LP induction was observed in the cases of IL-6 and IL-10.
**Conclusions.** The results presented here could shed some light on explaining the therapeutic effect of PRP in Alzheimer's disease (**Adv Clin Exp Med 2006, 15, 4, 625–630**).

**Key words:** Alzheimer's disease, whole-blood samples, cytokine induction, PRP.

## Streszczenie

**Wprowadzenie.** Cytokiny odgrywają ważną rolę w procesach neurodegeneracyjnych ośrodkowego układu nerwowego. Kompleks polipeptydowy bogaty w prolinę (PRP) o właściwościach immunomodulatorowych i psychotropowych jest induktorem wydzielania cytokin. Podawany pacjentom w postaci tabletek miał korzystne rezultaty terapeutyczne w przypadku choroby Alzheimera.
**Cel pracy.** W celu wyjaśnienia niektórych aspektów działania PRP określono wpływ na uwalnianie IL-1β, IL-6, IL-10 i TNF-α w hodowlach komórek pełnej krwi chorych. Kontrolę stanowiły próbki krwi pobrane od zbliżonej wiekiem grupy osób zdrowych.
**Materiał i metody.** Badaniami objęto 20 osób chorych z lekkim i średnim zaawansowaniem choroby (10 kobiet i 10 mężczyzn, średni wiek 70 ± 7.9 lat). Grupę kontrolną stanowiło 10 osób (6 kobiet i 4 mężczyzn w wieku 68 ± ± 6.2 lat). Stężenie cytokin oznaczano za pomocą handlowo dostępnych testów ELISA.
**Wyniki.** Przy braku czynników indukujących nie stwierdzono istotnych różnic w stężeniu badanych cytokin w osoczu chorych w odniesieniu do grupy kontrolnej. Pod wpływem induktorów LPS + PHA oraz PRP w dawkach 1–100 μg zarówno w krwi kontrolnej, jak i chorych indukowane jest wydzielanie IL-1β, IL-6, IL-10 i TNF-α. Ilość wydzielanych cytokin wzrasta wraz z progresją choroby. Pewien modulatorowy efekt PRP obserwowano w przypadku IL-6 i IL-10.
**Wnioski.** Uzyskane wyniki pozwalają tylko częściowo określić rolę PRP w regulacji wydzielania cytokin. Niemniej można sądzić, że PRP przez indukcję i modyfikację wydzielania cytokin może regulować procesy zachodzące w obrębie układu nerwowego (**Adv Clin Exp Med 2006, 15, 4, 625–630**).

**Słowa kluczowe:** choroba Alzheimera, hodowle komórek pełnej krwi, stężenie cytokin, PRP.

Alzheimer's disease (AD) is an irreversible neurodegenerative disorder. Despite advances in molecular biology and immunology, the cause of AD is still unknown. Several strategies for drug intervention in both the treatment and prevention of AD have been pursued, but up to now there has been no fully effective cure without side effects. Several lines of evidence indicate an immune-mediated pathophysiology of Alzheimer's disease. There has been increasing evidence that soluble mediators such as cytokines are able to modulate the growth and function of cells found within the central nervous system (CNS). Furthermore, glial cells, upon activation, can secrete immunoregulatory factors that influence lymphoid/mononuclear cells as well as the glial cells themselves. The bidirectional communication between lymphoid cells and glial cells within the CNS is, among others, mediated via cytokines. Their function and dysfunction in neurodegenerative disorders has become a topic of intensive investigation. Any substances which can affect the secretion of cytokines could exert therapeutic effects in neurodegenerative disorders such as AD [1–4].

It was found that proline-rich polypeptide complex (PRP), isolated from ovine colostrum and characterized by Janusz and Lisowski, is a modest cytokine inducer [5–8]. It was subsequently discovered that PRP improved mood and cognitive abilities in humans. In the form of orally administered tablets containing 100 μg of PRP it improves outcomes of Alzheimer's disease patients [7, 9, 10]. To further explore the role of PRP in the regulation of innate response in Alzheimer's disease patients, the release of IL-1β, IL-6, IL-10, and TNF-α in cultured whole-blood samples from AD patients was investigated.

## Material and Methods

The investigation was done on blood samples taken from 20 AD patients (10 women and 10 men, mean age: 70 ± 7.9 years) and 10 healthy controls (6 women and 4 men, mean age: 68 ± 7.2 years). All were outpatients and all met the DSM-III-R and NINCDS-ADRDA criteria for probable AD. Their primary health status was assessed by the Mini-Mental Status Examination (MMSE). Nine mildly (group I, MMSE: 16–24 points) and 11 moderately (group II, MMSE: < 16 points) affected patients were admitted to the study. None of the AD patients were under any immunomodulatory treatment on entry.

Blood samples were collected into heparinized tubes (Greiner Bio-One GmbH, Austria). Within two hours after the collection the blood was diluted 10-fold with RPMI-1640 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.5 mg/ml L-glutamine (obtained from the Laboratory of Biopreparates of the Institute of Immunology and Experimental Therapy, Wrocław, Poland). One-ml portions of the cell suspension were distributed in duplicate or triplicate into 24-well flat-bottomed tissue culture plates (Becton Dickinson, USA). For induction of cytokine generation, 2 μg LPS + 2 μg PHA (LP) or PRP in doses of 1, 10, or 100 μg were added to the 1-ml whole-blood cell cultures. PRP was prepared from ovine colostrum according to the procedure of Janusz et al. [5]. Lipopolysaccharide (LPS), *Escherichia coli* 055; B5, and Leucoagglutinin (PHA) were obtained from Sigma, USA.

After 22 hours of incubation (37°C, 5% $CO_2$ saturation), the plates were centrifuged for 5 min at 200 × *g* and the supernatants were kept for cytokine determination. When the modulatory effect of PRP on cytokine generation was investigated, PRP in doses of 1–100 μg was added simultaneously with or 6 hours after stimulation with LP. The interleukins were determined by microplate ELISA using commercially available antibodies and recombinant interleukins for IL-6, IL-10, and TNF-α from PharMingen, USA, and for IL-1β from Strathmann Biotech AG, Germany, according to the procedure recommended by the manufacturers.

Data comparisons were made with the program Statistica 6.0. The medians and standard deviations were calculated for each group. The hypothesis of the equality of means was verified using the non-parametric Wilcoxon test.

## Results

Twenty patients with mild (MMSE scores: 20–24) to moderately severe AD (MMSE scores: 16–20) were included to the study. The mean age of patients who entered the trial was 70 ± 7.9 and control group 68 ± 6.2. IL-10 and TNF-α were not detectable in the plasma of the control group or in either patient group. The plasma IL-1β and IL-6 levels in all the groups was 30–77 pg/ml; no significant differences between controls and patients of groups I and II were found. When the PRP effect on cytokine generation was investigated, the polypeptide complex was added in doses of 1, 10, or 100 μg simultaneously with or 6 hours after stimulation with LP. PRP, especially in doses 10 and 100 μg, as well as LP were potent cytokin inducers in the blood samples of the healthy controls (Table 1). IL-1β-, IL-6-, and TNFα-inducing activity comparable to that in the controls was

observed in the blood samples of the mild AD patients (Table 2). In the case of moderately severe AD, both LP and PRP induced IL-1β and IL-6 much more than in the controls and the patients of group I (Table 3). In the cases of TNF-α and IL-10, less significant increases with disease progression were observed. As presented in Tables 1–3, PRP added simultaneously with LP to the whole blood cell cultures did not change the LP-inducing activity in the control blood samples in a statistically significant manner. In the blood samples of group I, PRP showed some tendency to potentiate the stimulatory effect of inducing IL-1β, IL-6, and TNF-α when it was added simultaneously with LP. A statistically significant effect of PRP was observed in the case of IL-10 in control blood samples and in both groups of patients. A more pronounced effect of PRP was observed among the

**Table 1.** Induction of cytokines in blood cell cultures: healthy donors, mean MMSE score: 29

**Tabela 1.** Wydzielanie cytokin w hodowlach komórek dawców (średnia punktacja MMSE – 29)

| Sample (Próbka) | IL-1β (pg/ml) | IL-6 (ng/ml) | TNF-α (pg/ml) | IL-10 (pg/ml) |
|---|---|---|---|---|
| Control (Grupa kontrolna) | 10 ± 19 | 0 ± 0.346 | 66 ± 77 | 193 ± 57 |
| LP | 565 ± 318 | 2 ± 0.152 | 1040 ± 1430 | 1098 ± 287 |
| PRP 1 | 305 ± 314 | 1.0 ± 0.057 | 800 ± 309 | 700 ± 284 |
| PRP 10 | 460 ± 333 | 1.2 ± 0.264 | 940 ± 515 | 401 ± 159 |
| PRP 100 | 700 ± 309 | 1.1 ± 0.953 | 1180 ± 1178 | 580 ± 181 |
| PRP 1 + LP | 605 ± 341 | 2.2 ± 0.513 | 1100 ± 1463 | 1110 ± 287 |
| PRP 10 + LP | 600 ± 373 | 2.2 ± 0.200 | 1160 ± 1459 | 1058 ± 324 |
| PRP 100 + LP | 790 ± 275 | 2.2 ± 0.305 | 1160 ± 2006 | 850 ± 201 |
| PRP 1/6 h | 580 ± 308 | 2.0 ± 0.230 | 1060 ± 1580 | 700 ± 352* |
| PRP 10/6 h | 605 ± 268 | 2.2 ± 0.173 | 1670 ± 1374 | 860 ± 244 |
| PRP 100/6 h | 425 ± 509 | 2.2 ± 0.611 | 1169 ± 2216 | 778 ± 231* |

Whole blood cells suspensions were distributed into flat-bottomed tissue culture plates and incubated for 22 h at 37°C in the presence of the inducers: 2 μg LPS + 2 μg PHA (LP) or PRP at 1, 10, and 100 μg/ml. PRP was added simultaneously with (PRP+LP) or 6 hours after LP application (PRP/6h). Control samples were cultured in RPMI medium only. Supernatants were collected for cytokine determination by microplate ELISA. Data comparisons were made with the program Statistica 6.0. The medians and standard deviations were calculated for each group. The number of variation was taken from 9 to 11 experiments. A $p \leq 0.05$ was assumed to be significant. (*results statistically significant vs. LP induction).

**Table 2.** Induction of cytokines in blood cell cultures: patients with moderate AD, mean MMSE score: 17

**Tabela 2.** Wydzielanie cytokin w hodowlach komórek krwi pacjentów ze średnim zaawansowaniem choroby Alzheimera (średnia punktacja MMSE – 17)

| Sample (Próbka) | IL-1β (pg/ml) | IL-6 (ng/ml) | TNF-α (pg/ml) | IL-10 (pg/ml) |
|---|---|---|---|---|
| Control (Grupa kontrolna) | 0 ± 16 | 0 ± 0.44 | 50 ± 50 | 0 ± 0 |
| LP | 595 ± 383 | 2.2 ± 2.34 | 1720 ± 1412 | 1040 ± 486 |
| PRP 1 | 260 ± 285 | 1.5 ± 1.8 | 940 ± 473 | 352 ± 150 |
| PRP 10 | 480 ± 330 | 1.7 ± 2.56 | 1408 ± 984 | 432 ± 344 |
| PRP 100 | 900 ± 399 | 1.7 ± 2.52 | 1916 ± 1674 | 432 ± 607 |
| PRP 1 + LP | 845 ± 425 | 3.50 ± 2.53 | 1750 ± 1962 | 464 ± 499* |
| PRP 10 + LP | 925 ± 314 | 3.35 ± 2.88 | 1608 ± 2227 | 945 ± 627 |
| PRP 100 + LP | 760 ± 255 | 2.2 ± 3.27 | 2241 ± 2213 | 930 ± 595 |
| PRP 1/6 h | 615 ± 487 | 1.90 ± 2.76 | 1600 ± 1985 | 1088 ± 503 |
| PRP 10/6 h | 710 ± 366 | 2.00 ± 2.19 | 1875 ± 1963 | 810 ± 483* |
| PRP 100/6 h | 265 ± 542 | 2.05 ± 2.76 | 2025 ± 2164 | 120 ± 543 |

Details as described under Table 1.

Szczegóły według objaśnień pod tabelą 1.

**Table 3.** Induction of cytokines in blood cell cultures: patients with mild AD, mean MMSE score: 23

**Tabela 3.** Wydzielanie cytokin w hodowlach komórek krwi pacjentów z lekkim zaawansowaniem choroby Alzheimera (średnia punktacja MMSE – 23)

| Sample (Próbka) | IL-1β (pg/ml) | IL-6 (ng/ml) | TNF-α (pg/ml) | IL-10 (pg/ml) |
|---|---|---|---|---|
| Control (Grupa kontrolna) | 0 ± 0 | 0 ± 1.9 | 30 ± 119 | 0 ± 251 |
| LP | 1740 ± 585 | 3.45 ± 2.78 | 3870 ± 2025 | 592 ± 305 |
| PRP 1 | 560 ± 262 | 3.1 ± 1.24 | 1303 ± 1536 | 432 ± 343 |
| PRP 10 | 1300 ± 398 | 3.4 ± 2.01 | 1482 ± 1626 | 480 ± 262 |
| PRP 100 | 1600 ± 295 | 3.25 ± 2.66 | 1939 ± 1809 | 512 ± 201 |
| PRP 1 + LP | 1690 ± 599 | 4.05 ± 2.45 | 3300 ± 2412 | 480 ± 332 |
| PRP 10 + LP | 1600 ± 539 | 4 ± 2.93* | 3870 ± 1986 | 480 ± 432 |
| PRP 100 + LP | 2060 ± 638 | 4 ± 2.38 | 3815 ± 2038 | 592 ± 377 |
| PRP 1/6 h | 2060 ± 736 | 3.8 ± 1.06* | 3203 ± 2079 | 592 ± 345* |
| PRP 10/6 | 1220 ± 647 | 4.25 ± 4.72* | 3870 ± 2198 | 512 ± 291 |
| PRP 100/6 h | 1940 ± 666 | 3.85 ± 3.1 | 3690 ± 1967 | 432 ± 270 |

Details as described under Table 1.

Szczegóły według objaśnień pod tabelą 1.

patients of group II. Additionally, the increased IL-6 secretion induced by LP was observed in the presence of PRP (Table 3).

# Discussion

The beneficial effect of PRP in Alzheimer's disease could be explained by a modification of the sequence of pathogenic steps, beginning from the influence of APP proteolysis, diminution of Aβ aggregation, altered hyperphosphorylation of tau protein, up to microglial activation. Microglial activation affects the CNS's innate immunity, including the rapid activation of immune effector cells and release of cytokines, nitric oxide, and reactive oxygen species [11]. Therefore any substance which can regulate the secretory function of cells might exhibit a beneficial effect in AD. In previous results, an antioxidant effect of PRP was observed in cultures of human whole-blood cells and in the pheochromocytoma PC 12 cell line and the ECV 304 human endothelial cell line [12–14]. The cytokine-inducing activity of the PRP complex was shown in samples of whole blood of healthy blood donors and in human peripheral blood leukocytes [8, 15].

It was found that any substances which can affect cytokine secretion could exert a therapeutic effect in AD. It was interesting to compare the cytokine-inducing activity of PRP in blood samples of AD patients and healthy donors. Increased blood levels of IL-1β and IL-6 have been found during aging and in patients with late-onset AD [16, 17]. Higher levels of IL-1β and IL-6 were found by Licastro et al. [18] in 13% of controls and 53% of AD patients, respectively. Others, such as Angelis et al. [19], confirmed the increase in cytokine levels in AD blood samples. Presented results, similarly to the results of Licastro et al. [18], showed some increase in IL-1β and IL-6 levels both in elderly controls and in AD patients. Correlation between disease stage and cytokine secretion after LPS stimulation in the whole-blood culture system was found by Lombardi et al. [20]. As the authors showed previously, PRP is a modest cytokine inducer in whole-blood cultures and in human peripheral blood leukocytes [7]. PRP, in the form of orally administered tablets, exerts a therapeutic effect in AD when administered in a relatively low dose [9, 10]. Now increased cytokine-inducing ability of PRP correlating with AD stage has been shown. The mechanism of the beneficial effect of PRP in AD is not yet fully clarified. In the mechanism of PRP action, the hyporeactivity phenomenon in cytokine induction is observed. In the blood samples from patients treated with PRP tablets, tolerance or hyporeactivity developed: after the first induction, leukocytes or whole blood cells developed tolerance to the next induction [7]. The results presented in this paper indicate that cytokine secretion was induced in blood samples taken from healthy control donors and AD patients by both LPS+PHA and PRP. The induction depends on health status. The less pronounced dependency between AD patient status and induction of IL-10 compared with other cytokines may be connected with a different effect of PRP on $T_H1$ and $T_H2$ cytokines. The inhibitory

effect of PRP on LPS-induced IL-10 secretion suggests a control role of PRP in maintaining a balance in pro- and anti-inflammatory processes.

The cytokine network is complex and these molecules are biologically labile and rapidly disappear from the circulation. Therefore results from *in vitro* studies do not always fully reflect the situation *in vivo*. Nevertheless, the results presented here could shed some light on explaining the therapeutic effect of PRP in Alzheimer's disease.

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Conflict of interest: None declared

Received: 28.02.2006
Revised: 13.05.2006
Accepted: 26.05.2006

Praca wpłynęła do Redakcji: 28.02.2006 r.
Po recenzji: 13.05.2006 r.
Zaakceptowano do druku: 26.05.2006 r.