Activation effects of a prion protein fragment [PrP-(106–126)] on human leucocytes*

Luisa DIOMEDE†§, Silvano SOZZANI†, Walter LUINI‡, Marina ALGERI†, Luca DE GIOIA†, Roberto CHIESA†, Patricia M. J. LIEVENS‡, Orso BUGIANI‡, Gianluigi FORLONI†, Fabrizio TAGLIAVINI‡ and Mario SALMONA†

†Istituto di Ricerche Farmacologiche 'Mario Negri', Via Eritrea 62, 20157 Milan, Italy and the ‡Istituto Neurologico 'Carlo Besta', Via Celoria 11, 20133 Milan, Italy

Prion-related encephalopathies are characterized by the intracerebral accumulation of an abnormal isoform of the cellular prion protein (PrP^C) named scrapie prion protein (PrP^{Sc}). The pathological forms of this protein and its cellular precursor are not only expressed in the brain but also, at lower concentrations, in peripheral tissues. We recently showed that a synthetic peptide corresponding to residues 106–126 [PrP-(106–126)] of the human PrP is toxic to neurons and trophic to astrocytes *in vitro*. Our experiments were aimed at verifying whether PrP-(106–126) and other peptides corresponding to fragments of the amyloid protein purified from brains of patients with Gerstmann–Sträussler–Scheinker disease – namely PrP-(89–106), PrP-(106–114), PrP-(127–147) – were capable of stimulating circulating leucocytes. Native PrP expression in human lymphocytes, monocytes and neutrophils was first confirmed using PCR amplification of total RNA, after reverse transcription, and immunoblot analysis of cell extracts with anti-PrP antibodies. PrP-(106–126), but not the

other peptides, increased membrane microviscosity, intracellular Ca²⁺ concentration and cell migration in circulating leucocytes, and O₂^{•−} production in monocytes and neutrophils. Membrane microviscosity was determined by the fluorescence polarization technique, using diphenylhexatriene as a probe, 300 s after the addition of PrP-(106–126) to the cell suspension in the concentration range 5–50 µM. The increase in intracellular Ca²⁺ elicited by PrP-(106–126) was dose-dependent in the range 5–500 µM. PrP-(106–126) stimulated O₂^{•−} production in monocytes and neutrophils in a dose- (10–300 µM) and time- (5–30 min) dependent manner in the presence of 10 µM dihydrocytochalasin B. Both the increase in Ca²⁺ concentration and the O₂^{•−} production were partially sensitive to pertussis toxin. PrP-(106–126) stimulated leucocyte migration in a dose-dependent (30–300 µM) manner and, at the highest concentration used, this migration was comparable with that elicited by 2.5 nM interleukin 8 or 10 nM fMet-Leu-Phe peptide.

INTRODUCTION

Prion diseases are a group of transmissible neurodegenerative disorders that include kuru, Creutzfeld–Jakob disease and Gerstmann–Sträussler–Scheinker disease in humans, and scrapie and bovine spongiform encephalopathy in animals. They are characterized by cerebral accumulation of an altered form of the cellular prion protein (PrP^C), designated scrapie prion protein (PrP^{Sc}) [1,2]. PrP^C is a glycolipid-anchored cell surface protein that is expressed in the central nervous system [3,4] and in several peripheral tissues beginning early in embryonic development [5,6]. Although its physiological role has not been fully elucidated, at the level of the central nervous system, it may play a role in neural differentiation and synaptic transmission. In peripheral tissues, PrP^C, like other glycosylphosphatidylinositol-linked proteins, may act as a cell–cell adhesion molecule, membrane receptor transducer or activation-released soluble signal that modulates the activation of lymphoid subsets [5].

PrP^{Sc} is derived from PrP^C after a conformational modification that makes it partly resistant to protease digestion [7,8]. The conversion of PrP^C into PrP^{Sc} is considered to be the central pathological process in prion diseases [9]. Although PrP^{Sc} accumulates primarily in the brain, it has also been found at lower concentrations in some peripheral tissues, including circulating cells such as monocytes and lymphoid cells [6]. A vascular variant of PrP amyloidosis was described by Ghetti et al. [10], suggesting that the origin of brain PrP vascular amyloid

precursor may be derived, apart from the neuronal and cerebrovascular system, via the haematogenous route. Very recently Perini et al. [11] reported that platelets express PrP^C on their surface and that they shed it after stimulation with collagen.

The clinical signs are confined to the central nervous system, and no immune response seems to accompany the progression of the illness [9]. The molecular basis of neurological dysfunction in prion diseases is not known, although there is evidence that spongiform degeneration and death of neurons is related to the accumulation of PrP^{Sc}, which causes alterations in membrane receptors or ion channels functions, among other effects [12,13]. Kristensson et al. [14] reported that prion infection of cultured mouse neuroblastoma cells affected intracellular Ca²⁺ regulation, inducing abnormalities in intracellular Ca²⁺ release from internal stores and influx of extracellular Ca²⁺. Further evidence of specific biological effects of PrP^{Sc} on nerve and glial cells come from the observation that the exposure of primary neuronal and astroglial cultures to the protease-resistant core of PrP^{Sc} [i.e. PrP-(27–30)] caused neuronal death by apoptosis and hypertrophy and proliferation of astrocytes [15,16]. Identical effects on nerve and glial cells were obtained using a synthetic peptide homologous to residues 106–126 of the human prion protein, PrP-(106–126), suggesting that this amino acid sequence is a biological active site of the molecule [17–21]. These effects were accompanied by significant rigidification of the cell membranes [22] and, in astrocytes, by a rise in cytosolic Ca²⁺ concentration [Ca²⁺]_i [22]. Moreover, the neurotoxic effect of PrP-(106–126)

Abbreviations used: PrP, prion protein; PrP^{Sc}, scrapie prion protein; PrP^C, cellular prion protein; HBSS, Hanks balanced-salt solutions; FBS, fetal bovine serum; DPH, 1,6-diphenyl-1,3,5-hexatriene; fMLP, fMet-Leu-Phe peptide; PMA, phorbol 12-myristate 13-acetate; PT, pertussis toxin; dhCB, dihydrocytochalasin B; IL-8, interleukin 8.

* In memory of Professor Alfredo Leonardi, General Secretary of the Mario Negri Institute from its foundation until April 1995.

§ To whom correspondence should be addressed.

depends on the ability of nerve cells to express PrP^C. On this basis we investigated whether some of the biophysical and biochemical effects induced by PrP-(106–126) on nerve and glial cells can be found in circulating leucocytes as well.

EXPERIMENTAL

Peptides

The peptides PrP-(106–126) (Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly), scrambled PrP-(106–126) (Asn-Gly-Ala-Lys-Ala-Lys-Met-Gly-Gly-His-Gly-Ala-Thr-Lys-Val-Met-Val-Gly-Ala-Ala-Ala), PrP-(89–106) (Trp-Gly-Glu-Gly-Gly-Gly-Thr-His-Ser-Glu-Trp-Asn-Lys-Pro-Ser-Ser-Lys-Pro-Lys), PrP-(106–114) (Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly) and PrP-(127–147) (Gly-Tyr-Met-Leu-Gly-Ser-Ala-Met-Ser-Arg-Pro-Ile-Ile-His-Phe-Gly-Ser-Asp-Tyr-Glu-Asp) were synthesized as described in detail elsewhere [18] and purified by reverse-phase HPLC (model 243, Beckman Instruments Inc., Palo Alto, CA, U.S.A.) on a Delta-Pack C₁₈ column (19 mm × 300 mm; 30 nm pore size, 15 µm particle size; Nihon, Waters, Tokyo, Japan). Purity exceeded 95% for all peptides. All peptides contained less than 0.125 unit/ml endotoxin as checked by the *Limulus* amoebocyte lysate assay (Microbiological Associates, Walkersville, MD, U.S.A.).

Peripheral blood cells

Lymphocytes, monocytes and neutrophils from peripheral blood of healthy donors were obtained to greater than 95% purity by dextran sedimentation and Ficoll separation [23]. Cells were kept in RPMI 1640 medium with 10% fetal bovine serum (FBS) before experiments. Before use they were washed twice and suspended in Hanks balanced-salt solution (HBSS) containing 1.26 mM CaCl₂, pH 7.4. Cell viability, as assessed by erythrosin B dye exclusion, was greater than 97%.

Reverse transcription (RT)-PCR of PrP^C and β-actin transcripts

Total RNA was prepared from peripheral blood cells by a modification of the guanidinium isothiocyanate/CsCl method. PCR amplification was carried out from total RNA after reverse transcription using the gene AMP RNA kit (Cetus Perkin Elmer, Norwalk, CT, U.S.A.) according to the manufacturer's instructions. The following oligonucleotides were used: 5'-CTACAATGAGCTGCGTGTGG-3' for β-actin forward and 5'-ATAGCAACGTACATGGCTGG-3' for β-actin reverse primer [24], 5'-GTGCACGACTGCGTCAAT-3' for PrP forward and 5'-CCTTCCTCATCCACTATCAGG-3' for PrP reverse primer [24]. After 5 min denaturation at 95 °C, samples of β-actin and PrP^C were subjected respectively to 28 and 38 repeated cycles of denaturation at 95 °C for 1 min then exposed to primer annealing at 50 °C for 2 min and extension for 3 min at 72 °C. After amplification, samples were run on a 1.5% agarose gel and visualized by staining with ethidium bromide. The amplified sequences were verified by restriction analysis on the basis of the cDNA sequences (results not shown).

Immunoprecipitation and Western-blot analysis

Lymphocytes, monocytes and neutrophils were lysed in a buffer containing 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate and 20 mM Tris/HCl, pH 7.4, supplemented with protease inhibitors (5 mM EDTA, 0.5 mM PMSF, 1 µg/ml pepstatin, leupeptin and aprotinin). After centrifugation at 3000 g for 10 min, aliquots of supernatant equivalent to 20 × 10⁶ lymphocytes, 5 × 10⁶ monocytes and 32 × 10⁶ neutrophils were

immunoprecipitated as described by Lehmann and Harris [25], using 1 µl of monoclonal antibody 3F4 which recognizes an epitope corresponding to residues 109–112 of human PrP. Immunoprecipitated proteins were fractionated on SDS/12.5% polyacrylamide minigels, transferred to poly(vinylidene difluoride) membranes (Immobilon; Millipore) and probed with biotinylated 3F4 (1:50000). Immunoreactive bands were visualized by chemiluminescence (Amersham). Specificity of immunoreactions was verified in neutrophils by using the antibody 3F4 previously absorbed with a synthetic peptide corresponding to residues 106–126 of human PrP.

Membrane microviscosity

Membrane microviscosity was assessed in cell suspension (1 × 10⁶–2 × 10⁶ cells/ml) using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a fluorescent probe [26]. The reported fluorescence polarization (*p*) value is a function of the emission (420 nm), detected through an analyser oriented parallel (*p*₁) and perpendicular (*p*₂), to the direction of the polarization of the exciting light (365 nm), according to the equation $p = (p_2 - p_1) / (p_1 + p_2)$ [26]. The influence of time and peptide concentration on *p* was investigated in lymphocytes, monocytes and neutrophils. Membrane microviscosity (*η*, poise), was related to fluorescence polarization according to the formula $\eta = 2p / (0.46 - p)$ [26].

Intracellular Ca²⁺ concentration

[Ca²⁺]_i was measured as the change in Fura 2 fluorescence. Cells (1 × 10⁷/ml) were loaded with 1 µM Fura 2 acetoxymethyl ester in HBSS without Ca²⁺ at 37 °C for 30 min, washed twice and resuspended in HBSS containing 1.26 mM CaCl₂ at 5 × 10⁶ cells/ml, then used for the experiments. Dual excitation, alternating at 340 and 380 nm, was provided by a spectrofluorophotometer (model LS-50B, Perkin-Elmer, Beaconsfield, Bucks., U.K.) equipped with two excitation monochromators. The fluorescence signals were calibrated as described by Grynkiewicz et al. [27] using the relationship:

$$[Ca^{2+}]_i = K_d[R - R_{min}]/(R_{max} - R)b$$

where *R* is the measured fluorescence, *R*_{min} and *R*_{max} are the values of *R* at < 0.1 nM and > 1 mM [Ca²⁺]_i respectively, and *b* is the ratio of the emission intensities (at 480 nm) with excitation at 380 nm when the [Ca²⁺]_i is < 0.1 nM and > 1 mM. The Fura 2 *K*_d was taken as 224 nM. Triton X-100 and 50 mM EGTA were used to obtain the maximal and minimal Ca²⁺ levels respectively. The temperature was fixed at 37 ± 1 °C.

In some experiments, neutrophils (5 × 10⁶ cells/ml) in HBSS containing 1.26 mM CaCl₂ were incubated for 90 min at 37 °C with pertussis toxin (PT) (1.33 µg/ml). Control cells were incubated under the same conditions with an equal volume of HBSS containing 1.26 mM CaCl₂. During the last 30 min of incubation, cells were loaded with 1 µM Fura 2 acetoxymethyl ester. After being washed twice with HBSS without Ca²⁺, cells were resuspended in HBSS containing 1.26 mM CaCl₂, and the effect of 50 µM PrP-(106–126) or 2.5 nM interleukin 8 (IL-8), used as positive control, on [Ca²⁺]_i was measured in control or PT-pretreated cells. PT pretreatment did not alter cell viability (> 90%, evaluated by erythrosin B dye exclusion) nor the loading of the fluorescent probe (results not shown).

O₂^{•-} release

The release of O₂^{•-} from monocytes and neutrophils was measured as superoxide dismutase-inhibitable cytochrome *c*

reduction as previously described [28]. Briefly, cells (1×10^6 /ml) in HBSS containing 1.26 mM CaCl_2 were preincubated for 5 min at 37°C with or without $10 \mu\text{M}$ dihydrocytochalasin B (dhCB) and in the presence of $80 \mu\text{M}$ cytochrome *c*, then stimulated for 0–30 min with PrP peptides (0– $100 \mu\text{M}$). The reaction was stopped by immersion in a melting ice bath. Reaction mixtures were cleared by centrifugation ($200 g$; 10 min) and the amount of reduced cytochrome *c* in the supernatants was determined by measuring A_{550} in an automatic ELISA reader against a blank sample not exposed to neutrophils. The amount of $\text{O}_2^{\cdot-}$ released was expressed as nmol/time of incubation per 10^6 cells.

In some experiments, neutrophils (5×10^6 cells/ml) in HBSS containing 1.26 mM CaCl_2 were incubated for 90 min at 37°C with PT ($1.33 \mu\text{g}/\text{ml}$). Control cells were incubated under the same conditions with an equal volume of HBSS containing 1.26 mM CaCl_2 . At the end of the incubation (cell viability $> 90\%$) cells were washed twice in RPMI 1640/1% FBS, resuspended at 1×10^6 /ml in HBSS containing 1.26 mM CaCl_2 , preincubated for 5 min at 37°C with $10 \mu\text{M}$ dhCB in the presence of $80 \mu\text{M}$ cytochrome *c* then stimulated for 30 min with $100 \mu\text{M}$ PrP-(106–126) or fMLP ($1 \mu\text{M}$) and phorbol 12-myristate 13-acetate (PMA) ($100 \text{ ng}/\text{ml}$), used as positive and negative controls respectively. The amount of $\text{O}_2^{\cdot-}$ released was determined as described above.

Cell migration

Cell migration was evaluated using a microchamber technique (Nucleopore Corp. Pleasanton, CA, U.S.A.), as previously described [29,30]. PrP-(106–126) or scrambled PrP-(106–126) ($3\text{--}300 \mu\text{M}$) and the chemotactic agents IL-8 (2.5 nM) and fMLP (10 nM), diluted in RPMI 1640/1% FBS, were seeded in the lower compartment of the chemotaxis chamber and $50 \mu\text{l}$ of cell suspension (1.5×10^6 cells/ml) was seeded in the upper compartment. The two compartments were separated by $5 \mu\text{m}$ -pore-size polyvinylpyrrolidone-free polycarbonate (monocytes and neutrophils) or $5 \mu\text{m}$ nitrocellulose (lymphocytes) membranes, and chambers were incubated at 37°C in air with 5% CO_2 for 60 min (neutrophils), 90 min (monocytes) or 180 min (lymphocytes). At the end of the incubation filters were removed, fixed and stained. Migration of monocytes and neutrophils was evaluated as number of cells migrated in five high-power oil-immersion field. Lymphocyte chemotaxis was evaluated as leading-front migration [31].

Statistical analysis

Duncan's test for multiple comparisons and Student's *t* test were used.

RESULTS

Expression of PrP^C in peripheral blood cells

As shown in Figure 1(A), a PrP^C-specific band of the expected size was amplified using mRNA extracted from freshly isolated lymphocytes, monocytes and neutrophils. Although RT-PCR clearly demonstrated the PrP^C transcript in the three haematopoietic lineages, the mRNA levels were much higher in lymphocytes than in monocytes and neutrophils. In fact, lymphocytes were the only cell type in which significant amounts of PrP^C mRNA could be measured by Northern-blot analysis (results not shown). The expression of PrP in lymphocytes, monocytes and neutrophils was assessed by immunoblot analysis of cell lysates after immunoprecipitation with the monoclonal antibody 3F4, which recognizes an epitope corresponding to

Figure 1 Expression of PrP^C in leucocytes

(A) Total RNA from freshly isolated peripheral blood cells was reverse-transcribed and PCR amplified using oligonucleotide couples specific for PrP^C and β-actin. As a negative control, a blank sample (H_2O) was reverse-transcribed and PCR amplified exactly as above. The products of the amplification were run on a 1.5% agarose gel and visualized by staining with ethidium bromide. The expected size of the amplified products is 138 and 243 bp for β-actin and PrP^C respectively and is indicated on the right. (B) PrP from lymphocytes, monocytes and neutrophils was immunoprecipitated from cell lysates and analysed by immunoblot with the antibody 3F4 (3F4). The specificity of immunoreactions was verified in neutrophils by using the antibody previously absorbed with a synthetic peptide corresponding to residues 106–126 of human PrP (3F4 Abs). Protein bands that react specifically with the anti-PrP antibody have a molecular mass of 33–37 kDa. Molecular-mass markers are shown on the right.

residues 109–112 of human PrP (Figure 1B). The analysis showed that all these cells expressed 33–37 kDa peptides that were significantly labelled by the antibody 3F4.

Effect of PrP peptides on membrane microviscosity

The addition of PrP-(106–126) to neutrophils caused a significant increase ($P \leq 0.01$) in membrane microviscosity within 15 s, reaching a plateau after 5 min. The effect was dose-dependent and the changes became significant ($P \leq 0.01$) at $5 \mu\text{M}$ (Table 1). Scrambled PrP-(106–126), tested over the same range of concentrations, was inactive. The other peptides, which have been reported to be without biological effect on nerve and glial cells [20], were completely ineffective. PrP-(106–126) had similar dose-response rigidifying effects on lymphocytes and monocytes. In fact, $50 \mu\text{M}$ PrP-(106–126) caused, 300 s after its addition to the cuvette, an increase in the *p* value of lymphocytes from 0.256 to 0.362 and of monocytes from 0.264 to 0.385. As in neutrophils, the other PrP peptides did not modify the *p* value of lymphocytes and monocytes (results not shown).

Table 1 Effect of PrP peptides on the fluorescence polarization value of neutrophils

Cells (1×10^6 – 2×10^6 /ml) with DPH added ($2 \mu\text{M}$ in 5 mM phosphate buffer, pH 7.4) were investigated for fluorescence polarization before and 300 s after treatment with PrP peptides. Each value is the mean $\pm$ S.D. for three determinations at 25 °C. * $P \leq 0.01$ according to Duncan's test for multiple comparisons in relation to controls.

Peptide	Concentration (μM)	Fluorescence polarization value
None		0.261 ± 0.003
PrP-(106–126)	1.66	0.271 ± 0.002
	5	$0.284 \pm 0.003^*$
	10	$0.297 \pm 0.004^*$
	25	$0.329 \pm 0.006^*$
	50	$0.334 \pm 0.002^*$
Scrambled PrP-(106–126)	50	0.265 ± 0.004
PrP-(89–106)	50	0.270 ± 0.002
PrP-(106–114)	50	0.265 ± 0.001
PrP-(127–147)	50	0.272 ± 0.004

Effect of PrP peptides on $[\text{Ca}^{2+}]_i$

The addition of PrP-(106–126) to Fura-2-loaded neutrophils induced a fluorescence change characterized by a rapid rise in $[\text{Ca}^{2+}]_i$ (30–40 s), followed by slower decrease (Figure 2A). As reported in Figure 3, the rise in $[\text{Ca}^{2+}]_i$ was dose-dependent and

Figure 3 Dose-related effect of PrP-(106–126) or scrambled PrP-(106–126) on $[\text{Ca}^{2+}]_i$

Neutrophils loaded with Fura 2 as described in the Experimental section were resuspended at 5×10^6 cells/ml in HBSS containing 1.26 mM CaCl_2 . The increase in $[\text{Ca}^{2+}]_i$ (nM) caused by increasing concentrations (0–500 μM) of PrP-(106–126) (■) or scrambled PrP-(106–126) (□) was determined. Each value is the mean $\pm$ S.D. for five experimental values. * $P \leq 0.01$ according to Student's t test.

was about twice the basal value (75.6 ± 5 nM, $n = 7$) at the lowest effective concentration (10 μM) (139.5 ± 2 nM, $n = 5$) and five times the basal value at the highest concentration (500 μM) (419.6 ± 8 nM, $n = 3$). Scrambled PrP-(106–126) was

Figure 2 Effect of PrP-(106–126) on $[\text{Ca}^{2+}]_i$

Neutrophils were loaded with Fura 2 as described in the Experimental section and resuspended at 5×10^6 cells/ml in HBSS containing 1.26 mM CaCl_2 or not. The Figure shows the $[\text{Ca}^{2+}]_i$ rise (nM) caused by 50 μM PrP-(106–126) in the presence (A) or absence (B) of external Ca^{2+} . The effect of 5 mM EGTA (C) or 5 mM Ni^{2+} (D) is reported, in the absence of external Ca^{2+} , on the $[\text{Ca}^{2+}]_i$ rise induced by 50 μM PrP-(106–126).

Table 2 Regulation of agonist-induced increase in $[Ca^{2+}]_i$ by different agents

Cells loaded with $1 \mu M$ Fura 2 as described in the Experimental section were washed twice with HBSS without Ca^{2+} , and resuspended at $5 \times 10^6/ml$ in HBSS with or without $1.26 mM$ $CaCl_2$. The effect of $5 mM$ EGTA or $5 mM$ Ni^{2+} on the rise in $[Ca^{2+}]_i$ caused by $50 \mu M$ PrP-(106–126) was measured. Each value is the mean $\pm$ S.D. for three determinations. $\dagger P \leq 0.01$ according to Duncan's test for multiple comparisons in relation to the basal value measured in HBSS with $1.26 mM$ Ca^{2+} . $*P \leq 0.01$ according to Duncan's test for multiple comparisons in relation to the value of PrP-(106–126)-treated cells in HBSS with $1.26 mM$ Ca^{2+} .

Cell type	Addition	$[Ca^{2+}]_i$ (% of basal)	
		Basal	$50 \mu M$ PrP-(106–126)
Neutrophils	None in HBSS with $1.26 mM$ Ca^{2+}	100 ± 5	$162 \pm 6^\dagger$
	None in HBSS without Ca^{2+}	100 ± 5	$135 \pm 5^*$
	$5 mM$ Ni^{2+} in HBSS without Ca^{2+}	86 ± 5	$134 \pm 5^*$
	$5 mM$ EGTA in HBSS without Ca^{2+}	87 ± 3	$126 \pm 7^*$
	None in HBSS with $1.26 mM$ Ca^{2+}	100 ± 3	$171 \pm 5^\dagger$
Lymphocytes	None in HBSS without Ca^{2+}	100 ± 3	$143 \pm 6^*$
	$5 mM$ Ni^{2+} in HBSS without Ca^{2+}	85 ± 8	$141 \pm 7^*$
	$5 mM$ EGTA in HBSS without Ca^{2+}	88 ± 5	$137 \pm 5^*$
	None in HBSS with $1.26 mM$ Ca^{2+}	100 ± 5	$200 \pm 10^\dagger$
	None in HBSS without Ca^{2+}	100 ± 3	$172 \pm 5^*$
Monocytes	$5 mM$ Ni^{2+} in HBSS without Ca^{2+}	88 ± 3	$175 \pm 3^*$
	$5 mM$ EGTA in HBSS without Ca^{2+}	87 ± 5	$168 \pm 5^*$

Figure 4 Effect of PT on the $[Ca^{2+}]_i$ rise induced by PrP-(106–126)

Neutrophils, at 5×10^6 cells/ml in HBSS containing $1.26 mM$ $CaCl_2$, were incubated for 90 min at $37^\circ C$ with $1.33 \mu g/ml$ PT. Control cells were incubated in the same conditions with an equal volume of HBSS containing $1.26 mM$ $CaCl_2$. During the last 30 min of incubation, cells were loaded with $1 \mu M$ Fura 2. After being washed twice with HBSS without Ca^{2+} , cells were resuspended at $5 \times 10^6/ml$ in HBSS containing $1.26 mM$ $CaCl_2$, and the increase in $[Ca^{2+}]_i$ caused by $50 \mu M$ PrP-(106–126) (A) was measured in control cells (continuous line) or in PT-pretreated cells (dotted line). In order to verify that PT pretreatment was effective, the increase in $[Ca^{2+}]_i$ caused by $2.5 nM$ IL-8 (B) in control cells (continuous line) or PT-pretreated cells (dotted line) was measured.

Table 3 Effect of PrP peptides on $[Ca^{2+}]_i$ and O_2^{-} production in human neutrophils

Cells loaded with $1 \mu M$ fura 2, as described in the Experimental section, were washed twice with HBSS without Ca^{2+} , and resuspended at $5 \times 10^6/ml$ in HBSS with $1.26 mM$ $CaCl_2$. The $[Ca^{2+}]_i$ rise caused by each PrP peptide ($50 \mu M$) was measured. O_2^{-} production was measured in neutrophils ($1 \times 10^6/ml$) suspended in HBSS containing $1.26 mM$ $CaCl_2$, exposed for 30 min to each PrP peptide ($100 \mu M$). Each value is the mean $\pm$ S.D. for three determinations. $*P \leq 0.01$ according to Duncan's test for multiple comparisons in relation to the untreated sample. Under the same experimental conditions, $10 \mu M$ fMLP induced a $[Ca^{2+}]_i$ rise of $225 \pm 5\%$, and O_2^{-} production after 30 min of exposure was 58.70 ± 2 nmol/30 min per 10^6 cells.

Peptide	$[Ca^{2+}]_i$ (% of basal)	(nmol O_2^{-} production/30 min per 10^6 cells)
None	100 ± 5	6.34 ± 3
PrP-(89–106)	105 ± 6	7.53 ± 3
PrP-(106–114)	110 ± 5	7.54 ± 3
PrP-(106–126)	$174 \pm 6^*$	$63.05 \pm 5^*$
PrP-(127–147)	116 ± 5	4.51 ± 2

ineffective. Similar increases in $[Ca^{2+}]_i$ were obtained on exposing lymphocytes and monocytes to 5 – $500 \mu M$ PrP-(106–126) (results not shown). As reported in Table 2, $50 \mu M$ PrP-(106–126) caused an increase in $[Ca^{2+}]_i$ of $62 \pm 6\%$ ($n = 3$) in neutrophils, $71 \pm 5\%$ ($n = 3$) in lymphocytes and $100 \pm 10\%$ ($n = 3$) in monocytes.

To establish the contribution of Ca^{2+} influx in PrP-(106–126)-stimulated cells, Fura 2 fluorescence was measured in the absence of external Ca^{2+} , and in the presence of $5 mM$ EGTA or $5 mM$ Ni^{2+} [32]. As shown for neutrophils in Figures 2(B), 2(C) and 2(D), these conditions partially prevented the $[Ca^{2+}]_i$ increase caused by $50 \mu M$ PrP-(106–126), indicating that this rise is about 30% dependent on the influx of extracellular Ca^{2+} through plasma-membrane channels.

The effect of PT on the PrP-(106–126)-induced increase in $[Ca^{2+}]_i$ was also investigated. Pretreatment of neutrophils with $1.33 \mu g/ml$ PT for 90 min reduced the $[Ca^{2+}]_i$ rise in response to $50 \mu M$ PrP-(106–126) by $54.8 \pm 2\%$ ($n = 3$) (Figure 4A). In parallel experiments, the rise in $[Ca^{2+}]_i$ caused by $2.5 nM$ IL-8, used as positive control for a receptor-mediated mechanism [31], was reduced by $40.0 \pm 5\%$ ($n = 3$) after PT pretreatment (Figure 4B), suggesting that PrP-(106–126) partially increases $[Ca^{2+}]_i$ through a receptor-mediated mechanism.

The effect of PrP-(106–126) on Ca^{2+} transient appeared to be direct since no relevant cross desensitization was observed with leukotriene B₄ ($10 \mu M$), platelet-activating factor ($100 nM$), IL-8 ($2.5 nM$) or fMLP ($1 \mu M$) (results not shown). The direct effect of the other PrP peptides, PrP-(89–106), PrP-(106–114) and PrP-(127–147), on leucocyte $[Ca^{2+}]_i$ was also tested. None, at a concentration of $50 \mu M$, affected $[Ca^{2+}]_i$ in neutrophils (Table 3), lymphocytes or monocytes (results not shown).

Effect of PrP-(106–126) on O_2^{-} production

The effect of PrP-(106–126) on O_2^{-} production was investigated in intact monocytes and neutrophils in the presence of dhCB. In neutrophils, PrP-(106–126) induced a dose- (Figure 5A) and time- (Figure 5B) dependent increase in O_2^{-} production. After 30 min of incubation the effect was significant at $50 \mu M$ and increased at $100 \mu M$ PrP-(106–126) (Figure 5A). Scrambled PrP-

Figure 5 Neutrophil O_2^- release in response to PrP-(106–126) or scrambled PrP-(106–126)

O_2^- release, expressed as nmol/ 10^6 cells, was measured in neutrophils (1×10^6 cells) resuspended in HBSS containing 1.26 mM $CaCl_2$, and preincubated for 5 min at 37 °C with 10 μM dhCB in the presence of 80 μM cytochrome *c*. Dose-dependent O_2^- production was measured in neutrophils stimulated for 30 min with increasing concentrations (10–100 μM) of PrP-(106–126) (A). Time-dependent O_2^- production was measured by stimulating cells for different lengths of time (5–30 min) with 100 μM PrP-(106–126) (■) or scrambled PrP-(106–126) (□) (B). Each value is the mean $\pm$ S.D. for four experimental values. * $P \leq 0.01$ according to Student's *t* test.

(106–126) (Figure 5B) and the other PrP peptides examined (Table 3) did not affect basal O_2^- release. Similar results were obtained when monocytes were exposed to PrP-(106–126) or the other peptides (results not shown): exposure to 100 μM PrP-(106–126) for 30 min increased monocyte O_2^- production from 7.93 ± 0.5 to 65.3 ± 5 nmol/ 10^6 cells ($n = 3$).

Neutrophil O_2^- generation in response to 100 μM PrP-(106–126) was greatly decreased (about 50 %) in the absence of dhCB (15.05 ± 0.4 , 63.0 ± 2 , 6.5 ± 0.5 and 13.5 ± 0.3 nmol of O_2^- /30 min per 10^6 cells for control and stimulated neutrophils in the presence and absence of dhCB respectively).

As for the Ca^{2+} assay, neutrophils pretreated with PT were 64 ± 5 % ($n = 3$) less able to respond to 100 μM PrP-(106–126) in terms of O_2^- production (Table 4). Under the same experimental conditions the effect of exposure to fMLP was reduced by 47 ± 5 % and the effect of exposure to PMA was unaffected.

Effect of PrP-(106–126) on cell migration

The ability of 5×10^6 lymphocytes, monocytes and neutrophils to migrate in response to PrP-(106–126) or to its scrambled version

Table 4 Effect of PT on O_2^- release induced by PrP-(106–126) in neutrophils

Cells (1×10^6 /ml) in HBSS containing 1.26 mM $CaCl_2$ were incubated for 90 min at 37 °C with (1.33 μg /ml). Control cells were incubated under the same conditions with an equal volume of HBSS containing 1.26 mM $CaCl_2$. The release of O_2^- was measured, as described in the Experimental section, 30 min after stimulation with 100 μM PrP-(106–126), 1 μM fMLP or 100 ng/ml PMA. Each value is the mean $\pm$ S.D. for three determinations. * $P \leq 0.01$ in relation to control without PT; † $P \leq 0.05$ and ‡ $P \leq 0.01$ in relation to controls pretreated with PT; § $P \leq 0.01$ in relation to the corresponding cells not pretreated with PT, according to Duncan's test for multiple comparisons.

Stimulus	(nmol O_2^- production/ 30 min per 10^6 cells)
Control	8.35 ± 1
Control + PT	10.66 ± 1
PrP-(106–126)	$52.23 \pm 2^*$
PrP-(106–126) + PT	$26.38 \pm 2^{\dagger \ddagger}$
fMLP	$56.00 \pm 5^*$
fMLP + PT	$37.16 \pm 2^{\dagger \ddagger \S}$
PMA	$152.39 \pm 2^*$
PMA + PT	$136.78 \pm 3^{\dagger \ddagger}$

was investigated. PrP-(106–126) induced migration across polycarbonate filters in a dose-dependent manner starting at 30 μM , reaching a maximal effect at 100 μM (Figure 6). At this concentration the number of migrated cells was comparable with those elicited by an optimal concentration of IL-8 (2.5 nM) or fMLP (10 nM). Other PrP peptides [PrP-(89–106), -(106–114) and -(127–150)] had no effect (results not shown).

DISCUSSION

PrP^{Sc} derives from the native cellular protein PrP^C, the expression of which is not restricted to the brain but occurs also in peripheral tissues [5,6,11]. Cashman et al. [5] reported that normal human lymphocytes and lymphoid cells, but not granulocytes, express PrP^C mRNA and protein. Amplification of total mRNA extracted from freshly isolated peripheral blood cells and immunoblot analysis of cell extracts demonstrated the presence of PrP transcript and protein also in lymphocytes, monocytes and neutrophils. This indicates that PrP expression is not cell-type-specific.

If, on the one hand, the immune system does not provide any protection against prion infection [9], on the other its stimulation apparently enhances, rather than impedes, disease progression. In fact, activated lymphocytes express about four times more PrP^C on their surface than resting cells, thus providing a potential reservoir of PrP^{Sc} replication as well [5]. In Creutzfeldt-Jakob disease linked to a mutation at codon 200 of the PrP gene, monocytes and lymphocytes express not only PrP^C, but also altered PrP isoforms [6]. The latter are less resistant to proteinase K digestion than the PrP^{Sc} present in the brain, suggesting that the peripheral species correspond to intermediates lying between the normal and pathological PrP isoforms.

The role of PrP^C in peripheral tissues has still to be clarified. We analysed human leucocytes to investigate whether PrP peptides were able to stimulate immune cell functions. Since abnormalities in plasma-membrane properties [34] and ion-channel functions of nerve and glial cells [35] have been suggested as being important in the pathogenesis of prion diseases, we first investigated the ability of PrP peptides to modify membrane microviscosity and $[Ca^{2+}]_i$ in circulating cells. Of the PrP peptides tested, only PrP-(106–126) had any effect on cell membrane

Figure 6 Effect of PrP-(106–126) or scrambled PrP-(106–126) on cell migration

Neutrophils, monocytes and lymphocytes were exposed to increasing concentrations (3–300 μM) of PrP-(106–126) (■) or scrambled PrP-(106–126) (□) and the numbers of migrated cells (neutrophils and monocytes) or the migrated distance (μm, lymphocytes) were evaluated as described in the Experimental section. Each value is the mean ± S.D. for three experimental values. * $P \leq 0.01$ according to Student's t test.

microviscosity. Unlike the other PrP peptides, PrP-(106–126) has two distinct hydrophilic/hydrophobic moieties which mimic the transmembrane domain of proteins [18,19]. Thus the increase in membrane microviscosity caused by this peptide might depend on its capacity to enter the cell membrane [22]. The exposure of lymphocytes, monocytes and neutrophils to PrP-(106–126), but not to the other PrP peptides, was also accompanied by a rise in $[Ca^{2+}]_i$ that was partially sensitive to PT.

PrP-(106–126) modifies cell properties and functions of nerve

and glial cells *in vitro*. Nerve cell death by apoptosis and astrocyte proliferation after chronic exposure to the peptide were preceded by a significant rigidification of the cell membranes [22] and accompanied by an increase in $[Ca^{2+}]_i$ that was antagonized by an L-type Ca^{2+} -channel blocker [22]. Abnormalities in receptor-mediated Ca^{2+} response during scrapie infection have been observed by Kristensson et al. [14] in mouse neuroblastoma cells. In this model, prion infection reduced or abolished the increase in $[Ca^{2+}]_i$ by bradykinin, a neuronal mitogen that induces release of Ca^{2+} from intracellular stores and influx of Ca^{2+} across plasma-membrane channels. In the present study the exposure of neutrophils to PrP-(106–126) did not affect the Ca^{2+} response evoked by subsequent stimulation with fMLP, a prototype chemotactic agonist [23], or with leukotriene B₄, platelet-activating factor or IL-8, suggesting that the peptide effect on Ca^{2+} transient is direct. Thus PrP might interact with Ca^{2+} homeostasis at multiple levels, some of which could be dependent on cell lineage (i.e. neuroblastoma compared with circulating leucocytes) or related to the activation state of the cells (e.g. chronic infections compared with acute exposure).

Monocytes and neutrophils exposed to PrP-(106–126) exhibited increased $O_2^{\cdot -}$ production which was dependent on the presence of dhCB and was reduced by pretreatment with PT. In parallel, in the same concentration range, PrP-(106–126) was also able to induce directional migration of lymphocytes, monocytes and neutrophils. Thus PrP-(106–126) was able to activate the cytotoxic potential of phagocytic cells and acted in a very similar manner to a classical chemotactic agent (e.g. fMLP, C5a and IL-8). These results suggest a potential role for the PrP in modulating immune and inflammatory responses.

During prion infection, the immune system is thought to be 'blind' since it fails to activate the non-specific immune mediators that are normally induced during invasion by conventional pathogenic micro-organisms [9]. Our data indicate that the immune system is responsive to PrP-(106–126) since we can rule out the possibility that the leucocyte activation was a non-specific phenomenon due simply to the presence of fibrils acting as a particulate stimulus. In fact, PrP-(127–147), which was completely inactive under our experimental conditions, polymerized into either straight or twisted fibrils, having a diameter of 5–8 nm and a length ranging from 0.2 to 3.0 μm [17].

It is conceivable that in prion diseases the concentration of PrP^{Sc} in circulating leucocytes never reaches a critical threshold within these cells. Unlike brain cells, immune-system cells are continuously renewed and the expression of PrP on their surface is far lower. Nevertheless our data indicate that it cannot be ruled out that leucocyte PrP^{Sc} could participate in the progression of the disease by stimulating the production of mediators. A key role for immune-system mediators has been demonstrated in other disorders involving the accumulation of misfolded proteins [36,37].

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