Respiratory burst in peritoneal exudate cells in response to a modified tuftsin

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Abstract. Intraperitoneal administration of tuftsin-M [Thr – Lys – Pro – Arg – NH – $(CH_2)_2$ – NH – CO – $C_{15}H_{31}$] to Balb/C mice has been shown to induce a respiratory burst in the peritoneal exudate cells. The macrophages exhibited enhanced levels of O_2^-, H_2O_2, NADPH oxidase and myeloperoxidase, but the activities of superoxide dismutase, catalase and glutathione peroxidase remained virtually unchanged. The magnitude of the oxidative burst depended directly on the dose of tuftsin-M; higher activity was observed at higher doses of the peptide. Tuftsin-M enhanced the generation of both O_2^- and H_2O_2 under in vitro conditions, as did phorbol myristate acetate. These results suggest that tuftsin-M could enhance non-specific defence against infections by activating the macrophages.
Key words. Peritoneal exudate cells; tuftsin; respiratory burst; myeloperoxidase; NADPH oxidase; O_2^-; H_2O_2.

Tuftsin (Thr – Lys – Pro – Arg) is a natural killer peptide which specially recognizes and activates macrophages[1]. Earlier studies from this laboratory have shown that when this peptide is modified by the introduction of a hydrophobic residue (n-palmitoyl) through ethylene diamine at the C-terminus [Thr – Lys – Pro – Arg – NH – $(CH_2)_2$ – NH – CO – $C_{15}H_{31}$, tuftsin-M], it can be easily incorporated into the liposome bilayer[2]. Liposomes containing tuftsin-M provide significant protection against malarial and leishmanial infections[3,4]. To examine whether this biological activity of the peptide was due to the stimulation of the host's macrophages, the oxidative activity of the peritoneal exudate cells derived from animals pretreated with tuftsin-M was investigated.

Materials and methods

Chemicals and reagents. NADPH, cytochrome-c, superoxide dismutase, horseradish peroxidase (HRP), glutathione reductase, phorbol myristate acetate (PMA), medium RPMI-1640 and fetal bovine serum were obtained from Sigma Chemical Co., USA. All other reagents and chemicals used in this study were of the highest purity available. Tuftsin-M was prepared as described elsewhere[2].

Treatment and preparation of macrophages. Balb/C mice (18–20 g) were treated i.p. with tuftsin-M. The dose schedules used are shown in each table or figure. Control animals received equal amounts of Hank's balanced salt solution (HBSS).

Peritoneal exudate cells were isolated according to the method of Moiser[5]. The cells were washed twice with RPMI-1640 containing fetal bovine serum (5%) and finally suspended in HBSS. The viability of the recovered cells was estimated from their ability to exclude trypan blue. For enzyme estimation, a portion of the cell harvest was sonicated in KCl (1.15%), and homogenised. The membraneous portion was separated out by centrifugation at 9000 × g for 30 min at 4 °C and suspended in isotonic KCl.

Assay of O_2^- and H_2O_2. The rate of formation of O_2^- was measured by following the superoxide dismutase-inhibitable reduction of cytochrome-C at 550 nm[6]. H_2O_2 formation was measured by the phenol red method[7].

Enzyme assay. Superoxide dismutase, catalase, glutathione peroxidase and myeloperoxidase (MPO) were assayed in the cytosolic extract[8–11], while NADPH oxidase activity was determined in the membrane preparation[12]. Protein content was measured according to Lowry et al.[13]. For statistical evaluation, one-way ANOVA was performed for each group. On the evidence of a significant F-ratio, an individual comparison was done by Dunnett's test.

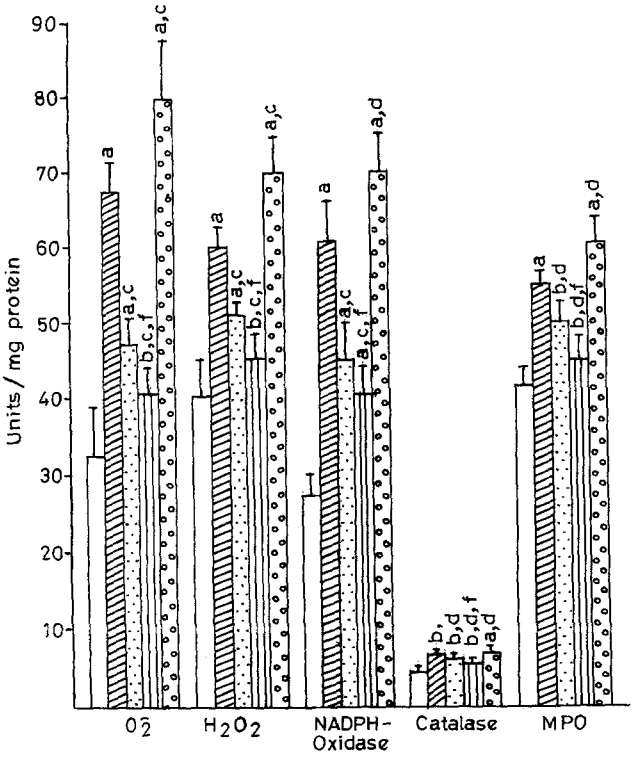
Results

Table 1 shows that tuftsin-M activates peritoneal exudate cells and stimulates production of O_2^- and H_2O_2. Also, the cells from treated mice showed greater activities of NADPH oxidase and MPO. The stimulation of O_2^- pro-

Table 1. Effect of tuftsin-M on the oxidant killing system of peritoneal exudate cells of Balb/C mice 24 h after a single i.p. dose

Parameter	Control	Dose: 25 µg/animal Activity	Change, %	Dose: 50 µg/animal Activity	Change, %
O_2^- production*	26.09 ± 2.91	40.09 ± 6.29[a]	+ 53.8	51.26 ± 4.90[a,c]	+ 97.1
H_2O_2 production*	46.09 ± 9.07	50.21 ± 6.09[b]	+ 9.1	81.40 ± 10.29[a,c]	+ 76.9
NADPH oxidase§	33.01 ± 9.01	39.22 ± 4.62[b]	+ 18.1	60.00 ± 10.66[a,c]	+ 81.8
Superoxide dismutase**	2.34 ± 0.06	3.00 ± 0.89[b]	+ 28.2	3.11 ± 0.99[b,d]	+ 32.9
Catalase§	4.00 ± 0.10	4.69 ± 0.39[a]	+ 17.2	4.78 ± 0.32[a,d]	+ 19.5
Glutathione peroxidase§	0.074 ± 0.007	0.070 ± 0.006[b]	− 5.5	0.060 ± 0.008[b,d]	− 19.0
Myeloperoxidase§	49.50 ± 4.05	56.49 ± 4.47[b]	+ 14.1	79.06 ± 6.09[a,c]	+ 61.2

* nmol/h/mg protein. § nmol/min/mg protein. ** units/mg protein (1 U = 50% inhibition of autooxidation of epinephrine at pH 10.2).
Control vs treated: a, significant ($p < 0.05$); b, insignificant ($p > 0.05$).
25 µg vs 50 µg: c, significant ($p < 0.05$); d, insignificant ($p > 0.05$).
The data are means ± SD of 5 experiments



Effect of time and liposomization on the efficacy of tuftsin-M to enhance respiratory burst in PEC after treatment of mice with 2 × 25 µg doses on two consecutive days. The data represent means ± SD of 5 experiments. Units are same as given under table 1.

□, Control; ▨, 1 day; ▩, 3 days; ▣, 7 days; and ▥, liposomized preparation on day 1

Control vs days, and liposomized: a, significant; b, insignificant
Day 1 vs day 3, 7 and liposomized: c, significant; d, insignificant
Day 3 vs day 7: e, significant; f, insignificant.

duction was substantial at doses of 25 µg and 50 µg/animal, but the other activities were enhanced only at a dose of 50 µg/animal.

Splitting of the dose showed only a marginal advantage. For instance, a single dose of 50 µg/animal enhanced O_2^- production by 97% (table 1), whereas if the peptide was given in two doses of 25 µg/animal it enhanced the radical production by 108% (fig.). The activation of peritoneal exudate cells by tuftsin-M exhibited a gradual decline with time. Thus, the rate of formation of O_2^-, which showed a 119% increase on day 1, showed only 45% and 25% enhancement on days 3 and 7, respectively. Formation of H_2O_2 and the activities of catalase and MPO registered a similar pattern. The effect on all the activities except that of NADPH oxidase became insignificant on day 7.

Tuftsin-M in vitro enhanced the production of O_2^- by peritoneal exudate cells in a concentration-dependent manner, but only upto 300 nM (table 2). Thereafter, the stimulation was reduced and at 1000 nM concentration it almost vanished. PMA, at a much lower concentration markedly enhanced the formation of both O_2^- and H_2O_2, and further addition of tuftsin-M had only little effect.

Discussion

Earlier studies have shown that treatment with tuftsin-M, a hydrophobic derivative of tuftsin, results in an inhibition of experimental malaria and leishmania infections[3,4]. Results of the present study suggest that this biological activity of the peptide may be due to the induction of the macrophage respiratory burst. Following i.p. administration in Balb/C mice, the peptide markedly enhances NADPH oxidase and thereby appreciably stimulates the production of O_2^- (table 1). The activated macrophages also produce greater amounts of H_2O_2 and exhibit a very high activity of MPO.

The unusual stoichiometry of O_2^- and H_2O_2 production may be due either to independence in production, or to the stimulation of one process by HRP. In support of the former possibility the work of Klimetzek and Schlumberger[14] may be cited. They reported that during in vitro culture of stimulated macrophages for 72 h, the amount of O_2^- generation decreased, but that of H_2O_2 increased with age. As far as the second possibility is concerned, type II HRP is reported to stimulate the formation of H_2O_2 by rat alveolar macrophages[15]. Accordingly, in the present study type I enzyme was used. Nevertheless, it is possible that this preparation also increases the peroxide production.

Both O_2^- and H_2O_2 are known to damage proteins, nucleic acids and membranes, sufficiently to kill the cell or even the whole organism. Nevertheless, for macrophages hypohalous acids, which are produced by the action of MPO on H_2O_2, have been identified as the major killer

Table 2. Effect of tuftsin-M and PMA on production of oxidants by peritoneal exudate cells of Balb/C mice in vitro

Addition	Concentration (nM)	Rate of production *		Enhancement %	
		O_2^-	H_2O_2	O_2^-	H_2O_2
None	-	20.95	37.3	-	-
Tuftsin-M	100	30.94	-	47.7	-
	200	38.40	49.5	83.3	32.7
	300	57.61	53.9	175.0	49.5
	500	33.77	-	61.2	-
	1000	24.38	-	16.4	-
PMA	160	57.87	70.8	166.7	89.8
PMA + tuftsin-M	160 + 300	65.22	78.9	211.3	111.6

* nmol/min/mg protein. The data are means of two duplicates.

agents[16]. Accordingly, the enhanced production of H_2O_2, and the substantially high level of MPO in tuftsin-M stimulated macrophages, make these cells very aggressive and potent, so that they could kill any infectious agent located intracellularly or in their vicinity. Nonetheless, the peptide could not produce a prophylactic action, as the activation of the cells was not long-lasting (fig.). The stimulation of a respiratory burst in peritoneal exudate cells appears to be a direct effect of tuftsin-M, as like PMA this peptide also instantly enhances the generation of both O_2^- and H_2O_2 (table 2). Furthermore, the enhancement pattern shown by this peptide is consistent with that of tuftsin which stimulates macrophages to release O_2^- within a range of 125–625 nM with a maximum at 350 nM[17].

Entrapment of sodium stibogluconate in tuftsin-M bearing liposomes prepared from egg lecithin and cholesterol (2:1 molar ratio) has been shown to enhance the antileishmanial activity of the drug[4]. However, the incorporation of the peptide into the bilayer of similarly-prepared liposomes caused only an insignificant (10–20%) (fig.) increase in the production of O_2^- and H_2O_2 by the peritoneal exudate cells compared with the stimulation caused by the free peptide. This suggests that the improvement in the efficacy of sodium stibogluconate brought about by liposomization may result from the respiratory burst-inducing activity of tuftsin-M, in addition to the effect of the targeted delivery of the drug to the macrophages as discussed in the report[4].

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Effect of a restricted diet on the in vitro glucose-induced insulin release of aging rats

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Abstract. To study the effect of a sudden loss of body weight on the β-cell function of aging rats, basal and glucose-induced insulin secretion was measured in pancreatic islets obtained from young (2-month-old), adult (12-month-old) and aging (24-month-old) rats, either fed ad libitum or fed a restricted diet (50% caloric restriction). Basal insulin secretion was similar in islets of young, adult and older rats. Glucose stimulated insulin release was significantly reduced in aging rats as compared to young animals. Animals fed a restricted diet showed a prolonged and higher secretory rate during first phase release when compared to animals fed ad libitum.
Key words. Aging; insulin release; pancreatic islets; dieting.

Previous reports have shown that pancreatic beta cells from collagenase-isolated and incubated or perifused islets from aging rats secrete less insulin in response to glucose or leucine stimulation than islets from younger rats[1–3]. In contrast, the total islet content of both proinsulin and insulin has been found to be increased in older animals[4] which is in accordance with the fact that average islets from older rats are bigger and contain more beta cells than average islets from young rats[5]. In the rat, the loss, with age, of insulin secretory function appears to be independent of obesity[6], and is not alleviated if obesity is prevented by long-term caloric restriction[7]. Nevertheless, a connection may exist between age-associated obesity and decreased β-cell function: female Sprague-