Journal of Peptide Science, Vol. 1, 295–302 (1995)

The Immunosuppressive Mini-domain of Human Lactoferrin

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Received 10 January 1995
Accepted 12 April 1995

Abstract: It has been found that the disulphide-bridged 231–245 pentadecapeptide loop of the lactoferrin (LF) N-lobe contains a region of immunosuppressive activity. The activity resides within a thymopentin-like sequence (Arg-Lys-Pro-Val-Asp) of the loop. Peptides related to the 575–589 loop of the LF C-lobe differ in their immunomodulatory activity from those related to the 231–245 loop. We ascribe this difference to the replacement of the Asp residue in the 231–245 loop by Thr in the 575–589 loop. Two other fragments of LF which were studied, 27–34 and 309–315, do not manifest any acitivy in the DTH test (cellular immune response), but, on testing *in vivo*, stimulate the humoral immune response. The 27–34 fragment is related to the bactericidal and immunostimulative region of LF identified by Bellamy *et al.* [1]. Our results show that the LF molecule contains, not only the known immunostimulating mini-domain, but also a region endowed with immunosuppressive activity.

Keywords: Lactoferrin; peptide immunosuppressors; thymopentin analogues

Abbreviations

DTH, delayed type hypersensitivity; GvH, graft versus host; IgG, immunoglobulin G; LF, lactoferrin; LPS, lipopolysaccharide; NK, natural killer; SRBC, sheep red blood cells.

INTRODUCTION

Lactoferrin (LF), which is released from activated neutrophils, is present in most biological fluids (see [2] for a recent review). The three-dimensional structure [3] shows two globular lobes connected by an alpha helix between residue 334 and 344. The N-lobe (residues 1–333) and the C-lobe (residues 345–691) are both subdivided into two dissimilar α/β domains of approximately 160 residues each. Each lobe contains a single iron-binding site and a single glycosylation site. Recently, Bellamy *et al.* [1] showed that the bactericidal region of human and bovine LFs is an octadecapeptide loop, closed by a disulphide bridge between Cys-19 and Cys-36. In this paper, the numbering of the amino acid residues of human LF given in [3] is used.

The C-terminus of the loop is adjacent to the sequence -Leu-Lys-Arg-Asp-Ser-; the tetrapeptide Lys-Arg-Asp-Ser, derived from human LF, was found to inhibit the platelet aggregation and release reaction [4]. According to the patent literature [5] the peptide related to bactericidal region of bovine LF potentiates the cellular immune response, promoting the release of leukotriene B4 from polymorphonuclear neutrophils and histamine from mast cells.

Each of the two lobes also contains a disulphide-bridged pentadecapeptide loop which is exposed on the surface of the molecule. The N-lobe loop comprises residues 231–245, and the C-lobe loop residues 575–589 (see Figure 1).

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CCC 1075-2617/95/050295-08

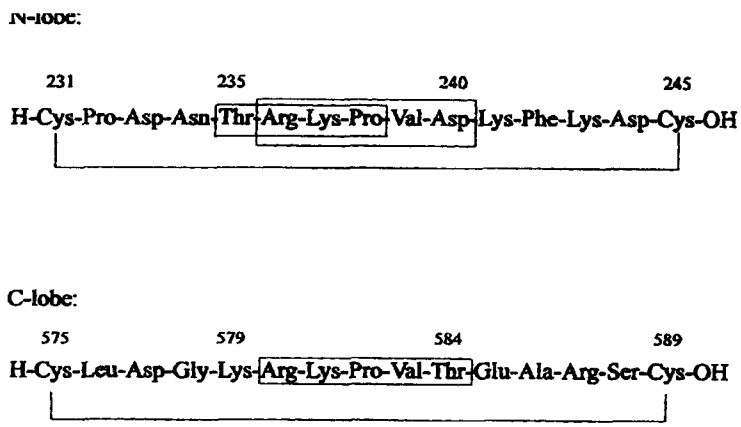

Figure 1 The sequence of the pentadecapeptide loops of the N- and C-lobes of lactoferrin. The boxes indicate the tuftsin-like and thymopentin-like fragments of the loops.

It can be seen from Figure 1 that the peptide derived from the N-lobe contains both tuftsin-like and thymopentin-like sequences, superimposed one on the other. Tuftsin, a tetrapeptide of sequence Thr-Lys-Pro-Arg, is a phagocytosis-stimulating factor, which is derived from IgG molecules. Thymopentin (Arg-Lys-Asp-Val-Tyr) is an active fragment of thymopoietin, the peptide immunostimulant produced in the thymus. The 235–238 fragment Thr-Arg-Lys-Pro of the N-lobe is, in fact, a sequential isomer of tuftsin. The corresponding fragment of the C-lobe loop is substantially changed by the replacement of Thr with Lys. However, the sequence Arg-Lys-Pro-Val, creating the main part of the thymopentin-like sequence, remains the same in both LF loops.

We recently presented data on the immunomodulatory activity of Thr-Arg-Lys-Pro(I) [6]. The peptide decreased the PFC number in the PFC *in vivo* test, but was found devoid of any activity in respect to the cellular immune response (DTH test). Unlike tuftsin, it decreased the phagocytic index of granulocytes.

The thymopentin-like fragments (Arg-Lys-Pro-Val-Asp in the N-lobe and Arg-Lys-Pro-Val-Thr in the C-lobe) of LF are very similar to Pro^3 -thymopentin (Pro^3-TP5). We found that in contrast to D-Pro^3 -TP5, which fully resembled TP5 in its biological properties, Pro^3-TP5 was inactive in the E-rosette and GvH (graft *v.* host) tests, but demonstrated some stimulatory activty on the humoral immune response [7–9].

In this paper, we present data on the immunomodulatory effects of 5 and 4 segments from the N- and C-lobe pentadecapeptide loops respectively. We also report on segments 27–34 and 309–315 of LF which are connected to the two other exposed regions of the protein. The segment 27–34 is a fragment of th 19–36 octadecapeptide loop which has been identified [5] as the location of the immunostimulating activity of LF.

MATERIALS AND METHODS

Peptides

Thymopentin (TP5) was synthesized according to Grübler *et al.* [10]. The synthesis of peptide I has previously been reported by us [6]. Peptides II–XI were obtained by a solid-phase procedure, using a chloromethylated copolymer of styrene and 1% divinylbenzene (Bio-rad), and a 9500 Peptide Synthesizer (MilliGen/Biosearch). C-Terminal amino acids were bound to the resin by the caesium salt method to a substitution level of 0.7 mmol per gram of polymer. The side chain protecting groups were the benzyl group for Thr and Asp; the nitro-group for Arg; the benzyloxycarbonyl group for Lys; and the 4-methoxybenzyl group for Cys. The couplings were performed by the DCCI/HOBt method. The cleavage of the peptides from the resin, accompanied by side chain deprotection, was accomplished by a 'high-TFMSA' procedure. The cleaved peptides were precipitated with cold diethyl ether (in the case of V, a small amount of β-mercaptoethanol was added to the ether), desalted on Sephadex G15 with 10% acetic acid, and lyophilized. In the case of V, the crude product obtained in this way was then oxidized using an iodine oxidation method. Crude products were purified by preparative reversed-phase HPLC. The proportions of the desired peptides in the crude preparations (determined by analytical HPLC) ranged from 49 to 53%, except for peptide VI, in which case

the proportion was 25%. All the peptides were transformed into acetate forms, by using ion-exchange chromatography on Sephadex SP C25 with ammonium acetate as eluant. The purity and identity of peptides were checked by RP-HPLC (purity > 99%), amino acid analysis and fast atom bombardment mass spectrometry (FAB/MS). Analytical data for all the peptides are given in Table 1.

Immunological Tests

The immunomodulatory activities of the peptides (homoral immune response) were tested by using the direct plaque-forming cell (PFC) test. The foot-pad test was used for the determination of delayed type hypersensitivity (DTH) (cellular immune response). In the determination of the PFC number, the Jerne methodology, as modified by Mishell and Dutton [12], was used. In the case of the DTH test, the methodology of Lagrange *et al.* [13] was applied. The details of all tests *in vivo* and *in vitro* have been described previously [11]. The animals used were 8–10-week old 129/Iiw and CBA/Iiw mice; the antigen was sheep red blood cells (SRBC) and the solvent was 0.9% saline solution.

Solutions of Peptides

An amount weighting 2.0–4.0 mg of each peptide was dissolved in 0.2–0.4 ml of 0.9% saline solution and the solutions were diluted to the desired concentration with 0.9% saline.

Administration of Peptides

In the experiments, 0.1–0.2 ml of peptide solution was administered intraperitoneally (i.p.) of intravenously (i.v.) (as indicated in the tables) into the animals, or added to the cell culture wells. The animals were immunized by i.p. treatment with SRBC in 0.9% saline. The peptide solution was administered twice, 3 h before and 24 h after sensitization of the animal. The results are expressed as a PFC number per 10^6 splenocytes (PFC test), and an

Table 1 Analytical Data of the Peptides II–XI

Peptide residue numbers	Sequence (one letter code)	HPLC RT[a] (min)	FAB/MS calc./found	Amino acid analysis
II 236–240	RKPVD	15.6	613.7/614.2	$Arg_{1.00}Asp_{0.98}$ $Lys_{1.00}Pro_{0.97}Val_{1.00}$
III 234–239	NTRKPV	15.4	713.8/714.2	$Arg_{0.98}Asp_{1.00}Lys_{0.99}$ $Pro_{1.05}Thr_{1.00}Val_{1.01}$
IV 233–240	DNTRKPVD	15.3	944.0/944.6	$Arg_{1.04}Asp_{3.08}Lys_{1.00}$ $Pro_{0.98}Thr_{1.00}Val_{1.01}$
V 231–245	see Fig. 1	20.6	1764.0/1765.6	$Arg_{1.00}Asp_{4.03}$ $Cys_{2.01}Lys_{2.93}Phe_{1.00}$ $Pro_{1.99}Thr_{1.01}Val_{1.00}$
VI 579–582	KRKP	13.2	527.7/528.3	$Arg_{1.00}Lys_{1.98}Pro_{1.00}$
VII 580–584	RKPVT	14.1	599.7/600.3	$Arg_{1.00}Lys_{0.98}Pro_{0.97}$ $Thr_{1.01}Val_{1.02}$
VIII 578–583	GKRKPV	14.9	683.9/684.4	$Arg_{1.04}Gly_{1.00}Lys_{2.00}$ $Pro_{1.05}Val_{1.04}$
IX 577–584	DGKRKPVT	15.6	900.1/900.8	$Arg_{1.01}Asp_{1.01}Gly_{1.00}$ $Lys_{2.00}Pro_{0.98}Thr_{0.98}$ $Val_{1.05}$
X 27–34	RKVRGPPV	30.3	908.1/908.7	$Arg_{2.02}Gly_{1.01}Lys_{1.00}$ $Pro_{2.03}Val_{2.01}$
XI 309–345	RVPPRID	20.2	852.0/852.6	$Arg_{2.05}Asp_{1.00}Ile_{1.01}$ $Pro_{2.01}Val_{1.01}$

[a]Retention time (min) in analytical RP HPLC.
Concentration 1 mg/ml. Gradient 0–100% A/60 min; A, 80% acetonitrile + 0.1% TFA in water, B, 0.1% TFA in water; column 250 × 4.6 mm RP-C18
ODS (Beckmann), flow rate 1 ml/min.

increase of foot pad thickness units (DTH test; 1 unit = 10^{-2} cm).

The results of the biological tests are summarized in Tables 2–4. For a better comparison of particular data, we have included in the tables the percentage values of immune response suppression. The values were calculated according to the equation:

$$\%\ suppression = 100\left(1 - \frac{experimental\ value}{control}\right)$$

Table 2 Direct PFC Numbers in the Mouse Spleen Cell Cultures (CBA/Iiw mice) Treated with the Peptides I–IX

Peptide	Dose μg/well	PFC/10^6	± SE[a]	*P* Student test	Suppression (%)
N-lobe					
I	Control[b]	1973	106		
	1	1896	113	NS	
	10	2258	117	NS	
II	Control	1513	55		
	1	781	101	<0.001	48
	10	317	28	<0.001	89
II + 10 μg	Control	1513	55		
TP5	1	1012	130	<0.02	33
	10	1038	93	<0.02	31
III	Control	1312	70		
	1	900	41	NS	
	10	700	23	<0.01	47
IV	Control	1312	70		
	1	860	61	<0.01	34
	10	310	22	<0.001	76
V	Control	1513	55		
	1	674	48	<0.001	55
	10	511	49	<0.001	66
C-lobe					
VI	Control	1973	106		
	1	2075	116	NS	
	10	2360	148	<0.05	
VII	Control	1973	106		
	1	2140	127	NS	
	10	1569	221	NS	
VIII	Control	1312	70		
	1	700	85	<0.01	47
	10	745	17	<0.01	43
IX	Control	1312	70		
	1	1111	232	NS	
	10	1008	10	NS	
N-lobe, peptides 27–34 and 309–315					
X	Control	1537	56		
	1	1478	97	NS	
	10	1131	102	<0.02	26
	100	984	10	<0.001	36
XI	Control	1537	56		
	1	1355	74	NS	
	10	1203	94	<0.05	22
	100	1843	40	<0.01	
TP5	Control	2740	183	NS	
	1	2817	299	NS	
	10	3201	301	<0.05	

[a]Mean ±SE (standard error) of six wells.
[b]0.9% saline solution.

Table 3 Direct PFC Numbers in Spleen Cell of CBA/Iiw Mice Treated i.p. with two doses (−3 and +24 h after SRBC Administration) of Peptides I–VII and X–XI Dissolved in 0.9% Saline

Peptide	Dose μg/well	PFC/10^6	+SE[a]	*P* Student test	Suppression (%)
N-lobe region 231–245					
I	Control[b]	2241	82		
	10	1664	156	<0.05	26
	100	1160	20	<0.001	48
II	Control	1412	183		
	10	812	118	<0.05	42
	100	470	61	<0.001	67
II + 100 μg TP5	Control	4075	437		
	10	2348	144	<0.01	42
	100	1348	202	<0.001	67
III	Control	1522	51		
	10	654	98	<0.001	57
	100	679	145	<0.001	55
IV	Control	1522	51		
	10	792	74	<0.001	48
	100	811	59	<0.01	47
V	Control	4075	437		
	10	2382	218	<0.01	42
	100	1864	132	<0.001	54
C-lobe region 577–584					
VI	Control	2241	82		
	10	2084	264	NS	
	100	1080	150	<0.001	52
VII	Control	2241	82		
	10	1559	618	NS	
	100	2446	413	NS	
VIII	Control	1482	67		
	10	1194	114	NS	
	100	1380	116	NS	
IX	Control	1482	67		
	10	1433	81	NS	
	100	1444	48	NS	
N-lobe peptides 27–34 and 309–315					
X	Control	3022	135		
	10	3976	176	<0.05	
	100	4264	227	<0.01	
XI	Control	3022	135		
	10	4630	444	<0.01	
	100	2690	382	NS	
TP5	Control	1352	375		
	10	2780	381	<0.01	
	100	2660	274	<0.01	

[a]Mean ±SE of 5 mice.
[b]0.9% saline.

These values are omitted in the tables in the cases when the experimental results were statistically non-significant (NS). They were also not calculated when the peptide showed immunoactivatory, but not immunosuppressory, activity.

Thymopentin (TP5) was used as the reference substance.

RESULTS AND DISCUSSION

The majority of the peptides belonging to the LF N-lobe 231–245 loop produce a strong immunosuppressory effect in both the humoral and the cellular immune responde. The tetrapeptide I (Thr-Arg-Lys-Pro – a tuftsin sequential isomer) is devoid of such

Table 4 The Influence of the Lactoferrin Peptides on the Inductive Phase of Delayed Type Hypersensitivity in 129/Iiw Mice

Peptide	Route of administration μg/mouse	DTH[a] Units	±SE[b]	*P* Student test	Suppression (%)
N-lobe; region 231–235					
I	Control	10.73	0.73		
i.p.	10	9.60	1.61	NS	
	100	8.00	1.53	NS	
II	Control	10.79	0.73		
i.p.	10	5.40	0.75	<0.01	50
	100	3.50	1.17	<0.001	67
II	Control	7.04	0.55		
i.v.	10	3.67	0.88	<0.01	48
	100	4.04	1.05	<0.02	43
II+100 μg TP5	Control	10.39	1.82		
	10	5.30	0.93	<0.02	49
	100	2.98	0.92	<0.001	71
III	Control	13.20	0.61		
i.p.	100	5.30	1.74	<0.01	60
IV	Control	13.20	0.61		
i.p.	100	5.60	0.78	<0.01	58
IV	Control	13.98	1.26		
i.v.	10	8.25	1.00	<0.01	41
	100	7.96	0.74	<0.01	43
V	Control	10.93	1.82		
i.p.	10	5.08	0.52	<0.01	51
	100	3.50	0.32	<0.001	66
V	Control	7.04	0.55		
i.v.	10	1.64	0.81	<0.001	77
	100	1.89	0.90	<0.001	73
C-lobe; region 577–584					
VI	Control	10.73	0.73		
i.p.	10	7.80	1.12	NS	
	100	7.50	1.36	<0.05	30
VII	Control	7.04	0.55		
i.v.	10	4.49	0.96	<0.05	36
	100	2.79	0.54	<0.001	60
VII	Control	7.04	0.55		
i.p.	10	4.46	0.89	<0.05	37
	200	1.44	0.51	<0.001	80
N-lobe; peptides 27–36 and 309–315					
VIII	Control	13.20	0.61		
i.p.	100	6.40	1.49	<0.01	52
IX	Control	13.20	0.61		
i.p.	100	11.50	0.88	NS	
X	Control	9.70	1.07		
i.p.	10	8.80	1.68	NS	
	100	6.80	1.02	NS	
XI	Control	9.70	1.07		
i.p.	10	10.07	1.26	NS	
	100	7.40	1.05	NS	
TP5	Control	5.90	0.52		
	10	5.90	0.70	NS	
	100	6.82	0.72	NS	

[a]1 unit = 10^{-2}cm.
[b]Mean ± SE of 7–10 mice.

activity as measured by PFC *in vitro* and DTH tests. However, it suppresses, to some extent, the humoral immune response *in vivo* (see Table 3). The highest suppressive activity *in vivo* and *in vitro* was observed for peptide II, a thymopentin analogue. As thymopentin is a known activator of the immune system [7,14–16], it seemed interesting to examine the effect of adminstering peptide II and thymopentin (TP5) simultaneously. The results clearly show competition between peptide II and TP5. In a dose of 10 μg/well, TP5 decreased (Table 2) the immunosuppression produced by II. In a PFC *in vivo* experiment (Table III), a high dose (100 μg/mouse) of TP5 had no influence on the effect produced by II, which may indicate that *in vivo* peptide II completely abolishes the immunostimulating influence of TP5. TP5 has no influence on the DTH reaction. In agreement with this statement, the DTH index obtained for II did not change in the presence of TP5 (see Table 4).

Removal of the C-terminal Asp residue from the peptide chain, combined with the elongation of the peptide at the N-terminus by Asn (peptide III), leads to a remarkable decrease of its immunosuppressive potency, as compared with II. Further elongation of both the N- and the C-terminus (peptide IV), results in an increase of immunosuppressive activity in the *in vitro* humoral immune response.

The whole pentadecapeptide loop (peptide V) demonstrates immunosuppressive activity comparable with that of II, at least in the DTH test.

Peptides VI, which occupies an analogous position in the C-lobe to that of peptide I in the N-lobe, and VII (a thymopentin analogue with C-terminal Thr) do not suppress the humoral immune response *in vitro*. Peptide VIII (without the C-terminal Thr but with an elongated N-terminal sequence) suppresses the humoral immune response, but its activity does not show any clear dose-dependence. In the case of peptide IX no definite immunosuppressive activity was noted.

The influence of peptides VI–IX on the humoral immune response was also tested by an *in vivo* procedure. It was found that whereas peptide VI exerted the immunosuppressive activity at a dose of 100 μg/mouse, pentapeptide VII and peptides VIII and IX were inactive in this test.

A different situation, however, was observed when the influence of those peptides on the cellular immune response was examined. Peptide VII showed definite immunosuppression after i.p., and also after i.v. administration. The activity diminished for hexapeptide VIII and tetrapeptide VI; octapeptide IX had no effect.

All these observations lead to the conclusion that the 231–245 loop of the LF N-lobe can be identified as the immunosuppressive region of the protein. The 575–589 loop of the LF C-lobe seems to be of less importance in this respect. The immunosuppressive activity seems to reside in a thymopentin-like sequence of the polypeptide chain, and is influenced by the residue occupying the C-terminal position in the thymopentin-like sequence. The presence of Asp in this position favours the immunosuppressive action, while the presence of Thr disfavours it, at least in respect to the humoral immune response.

Two other peptides related to the LF N-lobe (octapeptide X, residues 27–34, and heptapeptide XI, residues 309–315) both show practically no activity in the DTH test. They are active in the *in vivo* humoral immune response reaction, but demonstrate an immunostimulative rather than an immunosuppressive activity. In the *in vitro* experiments, both of these peptides showed a low immunosuppressive activity at a dose of 10 μg/well. However, the effect produced by peptide XI changed at a dose of 100 μg/well into an immunostimulating one. As we noted above, peptide X is related to the part of LF identified by Bellamy *et al.* [1] as the region associated with bactericidal and immunostimulatory action, and our results obtained with this peptide are consistent with their results.

CONCLUSIONS

Our experiments show that in the LF molecule there are two separate regions of very different immunomodulatory activity. The N-terminal disulphide-bridged octadecapeptide loop is the location of the immunostimulative activity of the protein. The disulphide-bridged 231–245 pentadecapeptide loop appears to be the region where immunosuppressive potency is located. The protein thus seems to be equipped with both of these immunomodulatory activities, in two different disulphide-bridged loops.

Acknowledgements

This work was supported by KBN grants Nos PB 0853/P2/93/05 and PB 0330/P2/93/05. We are also indebted to Dr J. H. Jones for the correction of the English of this text.

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