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Mycobacterium kansasii Phagocytosis Inhibition by the Oligopeptides Derived from Systemine, Cecropin A and BRCT-1 Protein Sequences*

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In this work the results of investigations of the influence of three series of peptides on the inhibition of the *Mycobacterium kansasii* phagocytosis are presented. The peptides are the fragments of systemine (the plant signaling protein), antimicrobial peptide cecropine A, and the active fragment GRGDVVNGRG of the BRCT protein. It was found that KRDVY is a weaker inhibitor of phagocytosis than its analog RKDVY. Introduction of the sequence RDG in the place of RGD (present in cecropine A) decreases the anti-phagocytic activity in comparison with the RGDVY peptide. Very interesting is a high biological activity of the palindromic peptide GRGNVVNGRG, while its parent peptide, indicated above, is inactive.

Key words: antiadhesive peptides, Mycobacteria phagocytosis, Tymopentin, Splenopentin, HLA-DQ fragments, fibronectin fragments, BRCT protein, Cecropine A, Systemin

Tuberculosis is evoked by *Mycobacterium tuberculosis*, the bacteria isolated in 1882 by German microbiologist R. Koch. At the present it is one of the most dangerous infectious diseases. Each year 3 millions people die of tuberculosis worldwide. [1]. Alcoholics, drug addicts, diabetics, and the AIDS patients are the most susceptible to fall ill with tuberculosis. Among the latter tuberculosis is 30-fold more frequent than in the rest of the population. Fighting Mycobacterium is very difficult because this bacterium can live and proliferate in leucocyte cells that normally kill the bacteria after the phagocytic act. However, the condition of the Mycobacteria phagocytosis is formation of a complex between the bacterial Ag85 antigen, located on the bacterial cell wall, and the serum protein – fibronectin [2]. Only in this form, bacteria can interact with leucocyte cell receptors of the integrin type and penetrate the host cell.

On this basis the hypothesis was put forward that the phagocytosis of Mycobacterium can be inhibited by blocking the interaction of fibronectin with the integrin receptor. The area responsible for that interaction is the sequence Gly-Arg-Gly-Asp-Ser-Pro. A

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sequence RGD is present in other adhesive proteins of extracellular matrices and blood [3]. We found that the pentapeptide fragment of the HLA – DQ protein, RGDVY, and GRGD – the fragment of fibronectin, selectively inhibit the Mycobacteria phagocytosis by 50–60%. A very good inhibitor of the phagocytosis is also the active fragment of thymic peptide hormone thymopentin, while its splenopentin analog is less active.

A similar mechanism of penetration into the host cells was found in the bacteria *Yersina pseudotuberculosis*. Yet, these bacteria do not need the presence of fibronectin to attain the cells, because they have their own protein – invasin, which makes possible the complexation with the integrin receptors of the macrophage [4]. The analysis of the interaction areas of fibronectin and invasin showed for both proteins the presence of one arginine residue and two aspartic acid residues, located at a similar distance from each other, important for the interaction [5]**.** Thus, the first Asp corresponds to the Asp residue within the RGD loop of fibronectin, whereas the second one to the Asp located in the synergy region of this protein. The distances between the C-α atoms of Arg and two Asp residues are 9.7 Å and 27.3 Å, respectively. The similarity between fibronectin and invasin is limited only to a similar structure of the interaction area, besides there is no homology between them. Based on the results of comparison of invasin and fibronectin binding regions we selected the Arg (883) and Asp (811) residues from *Y. pseudotuberculosis* Inv497 X-ray structure as a model of integrin-binding site. These residues were extracted from the protein structure and used as a template for construction of the peptides. In order to find a minimal length of poly-Gly that is able to link the Arg (883) and Asp (811) residues without changing their spatial arrangement, we connected these residues by the GG, GGG, and GGGG linkers.

In our previous publication [6] we found that among the analogs of the GRGD peptide, containing an oligo-glycine link, the inhibitory potency drastically decreases for the GRGGDV peptide and increases again after introduction of additional glycine residue. This observation was in agreement with the results of the computational analysis. These results confirmed that the distance between the C^{α} atoms of the Arg and Asp residues present in the binding site of fibronectin must be of about 9.7 Å. We also showed that even small changes in the sequence of the GRGD and RGDVY peptides cause the decrease of the inhibitory potency of the correspondent peptides. The major role in generation of the inhibitory activity is played by the Arg and Asp residues. The purpose of our further investigations was to check how a change of the order of amino acid the residues and the length of the peptide chain influence the inhibitory activity of the respectively peptides.

Also we examined three series of peptides. First one is the series of analogs of the GRGDVVNGRG peptide, which is a palindrome of the antiadhesive sequence. This is a fragment of the peptide chain of the BRCT protein [7], a regulator of the transcriptional process. Second series includes 9 peptides, the analogs of the antimicrobial peptide cecropin A, produced by the silk moth [8]. In this case we wanted to check how the change of the order of the residues in the RGD fragment affects the antipha-

gocytic activity. Last series is analogs of systemine (AVQSKPPS**KRD**PPKMQTD) [9], a plant signal peptide. The tripeptide sequence KRD is also an analog of thymopentin with the reordered Lys and Arg residues. It is known that thymopentin is a good inhibitor of the *Mycobacterium kansasii* phagocytosis [10]. We wanted to check then if a high activity of the RKD sequence is preserved in these modified structures.

EXPERIMENTAL

All the peptides were synthesized using the classical Fmoc chemistry. Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) and hydroxybenzotriazole (HOBT) were used as the coupling reagents. The β-carboxyl group of aspartic acid was protected with *t*-butyl ester (tOBu), the guanidine group of arginine was protected with the 2,2,4,6,7-pentamethyldihydrobenzenofurane (Pbf), the hydroxy group of serine with *t*-butyl, a benzyloxycarbonyl group was used for the protection of the ε-amino group of Lys. The N^{α}-Fmoc group was subsequently removed using 25% piperidine in N,N-dimethylformamide (DMF), in accordance to standard method. The protected peptides coupled to the Wang resin, (with the exception of peptides III–V coupled to the 2-chlorotrityl resin) were washed several times with DMF and MeOH and dried *in vacuo* for two hours. Then the peptides were cleaved from the resin with the K reagent (TFA/water/phenol/thioanisole). At these conditions, the side chain protecting groups were removed simultaneously. The procedure was carried out for two hours. Upon the cleavage from the resin, the products were precipitated with cold diethyl ether.

Crude peptides were then purified by a preparative HPLC on an Alltech Econsil C-18, 10 μm column (ODS 22×250 mm), flow rate 7 ml/min, determined at 223 nm. All the peptides were found to be >98% pure and were further characterized using ESI-MS and analitycal HPLC. Analytical HPLC was conducted by using a Beckman Peptide Gold System Chromatograph with a C-18, 5 μm column (ODS 4.6×250 mm) and an ultrasphere plus 4.6×4.5 mm precolumn. Solvent systems: S1: 0.1% aqueous TFA, S2: 80% acetonitrile + 0.1% TFA, linear gradient from 0–100% of S2 for 60 min, flow rate 1.0 ml/min, determined at 223 nm.

Analytical data of the peptides are summarized in Table 1.

Phagocytosis inhibition test. The values of the phagocytosis inhibition were obtained through a comparison of the phagocytic indices, determined for the human leucocytes and *Mycobacterium kansasii* incubated in the presence of the peptides and in the absence of them. The test and calculations were described by us in detail in a recent publication [10].

The samples obtained were smeared on glass microscope slides, fixed with methanol and stained using Ziehl-Nieelsen' s method. The number of bacteria internalized in 100 leucocytes per silde was scored. Visual (1500x) magnification was used to a numerate the number of bacteria that invaded the leucocyte cells. The phagocytic index was calculated as the average number of bacteria invading one cell and determin-

ed through four independent readings. The results were statistically elaborated using Student test.

Table 1. Analytical data of the peptides. Double line separates successive series of the peptides.

Peptide	HPLC (R_t) (min)[a]	ESI-MS $MW_{cal}/[MW + H]^+_{found}$
(I) KRDVY	19.04	679.4/680.2 339.7/340.3[b]
(II) GRDVY	17.87	608.3/608.6 304.2/304.2[b]
(III) KRDPP	13.98	611.3/612.6 305.7/306.7[b]
(IV) SKRDP	12.94	601.67/602.8
(V) RDGI	17.37	459.2/459.5
(VI) IRDGII	24.55	685.4/686.1 342.7/343.6[b]
(VII) NIRDGII	9.71	799.9/800.6
(VIII) RDGII	16.54	572.6/573.5
(IX)RDGV	6.54	445.5/446.3
(X) RDGIIK	17.59	700.4/701.6 350.2/351.2[b]
(XI) IRDGIIK	20.42	813.5/815.5 406.8/408.3[b]
(XII) RDGIIKA	22.22	771.5/772.1 385.7/386.4[b]
(XIII) NIRDGIIKA	23.01	998.6/1000.1 499.3/500.1[b]
(XIV) GRGDVVDGRG	11.59	987.1/987.3 493.5/494.3[b]
(XV) GRGDVVNGRG	16.75	986.1/986.4 493.1/493.5[b]
(XVI) GRGNVVNGRG	16.48	985.1/985.4 492.5/493.0[b]
(XVII) GRGNVVDGRG	17.10	493.1/493.8

[a]Retention time (min) in analytical RP-HPLC. Concentration 1 mg/ml.
Gradient 0-100% S2/60 min, S2-80% acetonitrile + 0.1% TFA in water, S1-1% aqueous TFA, column 250×4.6 mm RP-C18 ODS (Beckman), flow rate 1 ml/min.
[b]In the MS spectrum, a second doubly charged peak is present.

The values of phagocytosis indices (given in Table 2) were also recalculated into percentage values of the phagocytosis inhibition using the following equation:

$$\% \text{ inhibition} = 100 \times \left(1 - \frac{\text{phagocytic index}}{\text{control}}\right)$$

RESULTS AND DISCUSSION

The results of our investigations of peptides I–IV (systemine fragments and analogs of pentapeptide RGDVY) are summarized in Table 2. These peptides contain the reversed sequences KRD and GRD, respectively. According to the results presented

in advance by Siemion and Wieczorek [10], the RKDVY peptide shows the same inhibitory activity as the RGDVY pentapeptide. The data from Table 2 show that the rearrangement of the residues in the tripeptide sequence KRD does not evoke the complete loss of the inhibitory activity. The antiphagocytic activity of GRDVY is 50.7% at the 100 μg/m dose, like that for the RGDVY peptide [10]. However, the introduction of the reversed sequence into the RKDVY peptide causes the drop of the inhibitory activity to 27% at the 100 μg/ml dose. The presence of one or two Pro residues in the place of Val and Tyr residues (peptides III–IV) does not cause dramatic changes of the biological activity of the GRDVY peptide.

Table 2. Inhibition of *Mycobacterium kansasii* phagocytosis by human peripheral blood leucocytes with the peptides studied. Double line separates successive series of the peptides.

Preparation	Peptide dose (μg/ml)	Phagocytic index (x)	±S.E.(y)	Student test
(I) KRDVY	1	30.89/39.63	1.57/1.42	<0.05
	10	30.40/39.63	1.63/1.42	<0.05
	100	28.63/39.63	1.17/1.42	<0.05
(II) GRDVY	1	24.45/39.63	1.76/1.42	<0.01
	10	21.98/39.63	1.37/1.42	< 0.002
	100	19.56/39.63	0.81/1.42	< 0.001
(III) KRDPP	1	38.81/39.63	0.71/1.42	NS
	10	35.28/39.63	1.68/1.42	NS
	100	30.88/39.63	1.88/1.42	<0.05
(IV) SKRDP	1	37.75/39.63	1.47/1.42	NS
	10	30.40/39.63	1.60/1.42	<0.05
	100	24.64/39.63	1.58/1.42	<0.03
(V) RDGI	1	39.44/45.21	1.18/1.47	<0.05
	10	29.65/45.29	0.39/1.47	<0.01
	100	26.91/45.21	0.40/1.47	<0.01
(VI) IRDGII	1	40.71/45.21	0.94/1.47	NS
	10	32.79/45.21	0.42/1.47	<0.01
	100	28.30/45.21	0.84/1.47	<0.001
(VII) NIRDGII	1	42.08/36.14	1.06/1.59	NS
	10	30.40/36.14	1.70/1.59	NS
	100	28.93/36.14	1.78/1.59	<0.01
(VIII) RDGII	1	29.55/36.14	0.97/1.59	NS
	10	26.06/36.14	0.66/1.59	<0.02
	100	18.96/36.14	0.95/1.59	<0.001
(IX) RDGV	1	35.67/36.14	1.74/1.59	NS
	10	35.49/36.14	1.07/1.59	NS
	100	31.22/36.14	0.85/1.59	NS

Table 2 (continuation)

(X) RDGIIK	1	28.12/36.14	0.91/1.59	NS
	10	23.83/36.14	0.87/1.59	<0.02
	100	22.06/36.14	0.95/1.59	<0.001
(XI) IRDGIIK	1	40.61/45.21	1.34/1.47	NS
	10	39.20/45.21	0.83/1.47	NS
	100	35.15/45.21	1.23/1.47	<0.01
(XII) RDGIIKA	1	43.82/45.21	1.30/1.47	NS
	10	41.64/45.21	0.99/1.47	NS
	100	37.29/45.21	0.81/1.47	<0.02
(XIII) NIRDGIIKA	1	42.75/45.21	0.95/1.47	NS
	10	39.79/45.21	0.51/1.47	<0.05
	100	35.72/45.21	1.05/1.47	<0.01
(XIV) GRGDVVDGRG	1	37.91/40.23	1.90/2.14	NS
	10	37.83/40.23	1.11/2.14	NS
	100	41.67/40.23	1.83/2.14	<0.05
(XV) GRGDVVNGRG	1	37.85/40.23	0.98/2.14	NS
	10	37.34/40.23	0.88/2.14	NS
	100	38.70/40.23	0.65/2.14	NS
(XVI) GRGNVVNGRG	1	30.28/40.23	3.08/2.14	NS
	10	25.15/40.23	2.00/2.14	<0.05
	100	16.01/40.23	1.71/2.14	<0.01
(XVII) GRGNVVDGRG	1	24.91/28.94	1.65/1.94	NS
	10	24.45/28.94	1.74/1.94	NS
	100	15.72/28.94	1.21/1.94	<0.01

In control *Mycobacterium kansasii* was incubated with leucocytes for 40 min at 37°C.
In probes *Mycobacterium kansasii* was incubated with leucocytes and preparation for 40 min at 37°C.
The results are expressed as a mean ± SE of 4 probes.
(x) – phagocytic index for a particular dose/phagocytic index for a proper control.
(y) – ± SE (standard error) for a particular dose/±SE (standard error) for a proper control.

For peptides V–XIII (analogs of cecropine A), the antiphagocytic activity is generally lower in comparison with the model RGDVY peptide. The RDGI and RDGII peptides produced a 40.5% and 47.6% phagocytosis inhibition, respectively, at the highest dose applied. The antiphagocytic activity of the other peptides of this series is lower and reaches approximately 20%. The peptide of systemine series and the peptides of cecropine series, differ one to another in the ordering of R, G, and D residues. In the first case there appears a GRD or KRD sequence, whereas in the second – RDG one. The data show that a little difference in the peptide sequence leads to the quite sufficient changes in the activity. It is also visible that the activity depends also on the character of the N-terminal residue preceding RGD or KRD sequence.

The peptides belonging to the palindromic series are the analogs of natural GRGDVVNGRG sequence (peptide XV). This peptide is practically deprived of antyphagocytic activity.

The substitution of asparagine residue in this sequence (peptide XIV) by aspartic acid residue does not change the situation.

However, for the peptide XVI with two asparagines in the sequence a sufficient level (60%) of phagocytosis suppression was observed at a dose of 100 μg/ml. The inhibitory potency of peptide XVII reaches also the value of about 45% (at a dose of peptide 100 μg/ml.

Thus, the inhibitory activity of the palindromic sequence depends strongly to the localization of charged residues, bearing the negative charge. It is of interest that the complete abolishing of negative charge (peptide XVI) leads also to a very active substance. We have in the moment no clear explanation for this phenomenon.

However, as it was noted above, for the inhibitory activity of the peptide, spatial orientation of Arg 2 and Asp 4 residue is of importance. It may be that the interaction of these residues with the oppositely charged groups in the C-terminal part of the molecule destroys the spatial order in the interacting site of the peptide, what results in the loss of activity. Such the destroining can not take place in the case of XVI, a very active peptide.

The comparision of the results obtained for XV and XVII leads to the conclusion that the interaction of Asp 4 with Arg 9 is most important for the loss activity than the interaction of Arg 2 – Asp 7 type. Of course, such the ionic interaction could be realized in the folded conformations of peptides only, with the turn located in the center of the molecule.

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