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Influence of desmuramylpeptides from LK-409 series on the cytokine production in the mouse spleen cells

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Dedicated to Professor Aleš Krbavčič on the occasion of his 70th birthday

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LK-409 (7-oxooctanoyl-L-Ala-D-iGln) was found to modulate immune response. To study the impact of minimal structural changes in the LK-409 molecule on the immunomodulatory activity a series of LK-409 analogues was prepared and their activities were evaluated. After the treatment of NmRI mice at a dose of 25 μg daily for three consecutive days the isolated spleen cells were stimulated with concanavaline A and phorbol 12-myristate 13-acetate (PMA) or ionomycin and PMA, and the production of IL-2, IL-4,-IL-6, IL-10 and IFN-γ studied as a function of the different LK-409 analogues. All the compounds modulate the Th1/Th2 cytokine response, especially in the presence of ConA&PMA activation. The minimal structure modification of LK-409 strongly influences the regulation of the cytokine production.

1. Introduction

Muramyldipeptide (MDP) is the minimal constituent of the bacterial cell wall still possessing immunomodulatory activity (Adam et al. 1976). Extensive efforts have been made to discover its physiological binding site (Wolfert et al. 2002). In addition, many compounds have been synthesized in attempts to find therapeutically useful MDP analogues (Baschang 1989).

In our search for new muramyldipeptide derivatives with improved pharmacodynamic and pharmacokinetic characteristics, compared with the MDP we have synthesized several *N*-7-oxoacyl-L-alanyl-D-isoglutamines (Pečar et al. 1996). From these desmuramyldipeptides 7-oxooctanoyl-L-alanyl-D-isoglutamine (LK-409) has been selected for further structural optimization. Only the L, D combination of the configuration at the chiral centers in the peptide part of the MDP molecule gave a compound with immunostimulatory activity; other combinations either lacked activity or provoked an opposing activity (Adam et al. 1976). It is evident from an analysis of the N-acetylmuramyl part of MDP that the carbonyl group in the N-acetyl moiety could be involved in the recognition of and binding to its receptor. Based on these observations and previous reports three stereoisomers (F02, FK3, FK4), the analogue where the first amino acid was replaced with L-valine (F01) and a further analogue where the 7-oxooctanoyl residue was replaced with 5-methoxycarbonylpentanoic acid (FK8) have been synthesized in order to evaluate the influence of chirality, role of the first amino acid and position of the carbonyl group in the acyl part of LK-409 on immunomodulating activity.

The present study was designed to study the impact of minimal structural changes in the LK-409 molecule on TH1 (IL-2, IFN-γ) and TH2 (IL-4, IL-6, IL-10) cytokine production in polyclonally activated spleen cell cultures from mice treated *in vivo* with LK-409 and its analogues.

$CH_3CO(CH_2)_5CO$-L-Ala-D-iGln	LK-409
$CH_3CO(CH_2)_5CO$-D-Ala-D-iGln	FK3
$CH_3CO(CH_2)_5CO$-D-Ala-L-iGln	FK4
$CH_3OCO(CH_2)_4CO$-L-Ala-D-iGln	FK8
$CH_3CO(CH_2)_5CO$-L-Val-D-iGln	F01
$CH_3CO(CH_2)_5CO$-L-Ala-L-iGln	F02

2. Investigations and results

The influence of desmuramylpeptide LK-409 and its analogues on the synthesis of different cytokines was determined (Tables 1 and 2). Their capacities were evaluated as indexes according to the ionomycin&PMA or concanavaline A&PMA induced production. Substances with indexes below 0.8 were considered as immunosuppressive and above 1.2 as immunostimulatory.

FK8 increased the production of all cytokines tested. In the case of IL-2, IL-6 and IFN-γ stimulation is strongly dependent on the type of activator. FK8 did not increase the production of IL-6 and IFN-γ after the activation with Con A&PMA. In the alternative activation with Iono&PMA, it increased the production of IFN-γ by more than three fold. The type of activation is not important for the increased production of IL-4 and IL-10 after the treatment of mice with FK8.

LK-409 and all its analogues except FK8 decreased the levels of IL-2, independently of the type of activation.

Table 1: Effect of desmuramylpeptide analogues on Th1 cytokine (IL-2 and IFN-γ) production of mouse spleen cells after the activation with concanavalin A&PMA

Substance	IL-2 (pg/ml)	IFN-γ (pg/ml)	Sum of the scores
DMSO/saline	19155	2829	
LK-409	7755	1942	−2
I_{ss}	(0.40)	(0.69)	
Score	−1	−1	
LK-01	7110	3300	−1
I_{ss}	(0.37)	(1.17)	
Score	−1	0	
F02	4597	3300	−1
I_{ss}	(0.24)	(1.17)	
Score	−1	0	
FK3	3380	2139	−2
I_{ss}	(0.18)	(0.76)	
Score	−2	−1	
FK4	3863	3300	−1
I_{ss}	(0.20)	(1.17)	
Score	−1	0	
FK8	24770	3300	−1
I_{ss}	(1.29)	(1.17)	
Score	+1	0	

In parentheses, the stimulation or suppression indexes (I_{ss}) are given according to ConA&PMA production. The quantutative evaluation of indexes is presented in scores: −2 ($I_{ss} \leq 0.1$), −1 ($0.1 < I_{ss} < 0.8$), 0 ($0.8 \leq I_{ss} \leq 1.2$), +1 ($1.2 < I_{ss} < 2$), +2 ($2 \leq I_{ss}$)

F01, FK-4 and, particularly, FK3 strongly decrease its production. Only FK8 and F02 induced production of IFN-γ, while LK-409 down-regulated the synthesis of this cytokine very strongly. LK-409 decreased the amount of IFN-γ with both activators, while F02 and FK8 increased its concentration, especially after activation with Iono&PMA.

Furthermore, F01, F02 and FK3 have shown the most significant decrease in IL-4 production in contrast to LK-409, FK4 and FK8 which increased the production of IL-4 fourfold when the cells were stimulated with ConA&PMA. A higher concentration of IL-4 was observed after stimulation with ConA&PMA, while FK8 elevated it to a greater extent in combination with Iono&PMA.

Table 2: Effect of desmuramylpeptide analogues on Th2 cytokine (IL-4, IL-6, IL-10) production of mouse spleen cells after the activation with concanavalin A&PMA

SUBSTANCE	IL-4 (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	Sum of the scores
DMSO/saline	7.8	66.5	27.6	
LK-409	32.4	74.3	53.7	+3
I_{ss}	(4.20)	(1.12)	(1.95)	
Score	+2	0	+1	
LK-01	1	85.6	39.9	+1
I_{ss}	(0.13)	(1.30)	(1.44)	
Score	−1	+1	+1	
F02	1	61.3	47.6	0
I_{ss}	(0.13)	(0.92)	(1.72)	
Score	−1	0	+1	
FK3	1	31.3	26.1	−3
I_{ss}	(0.13)	(0.47)	(0.95)	
Score	−1	−1	−1	
FK4	33.2	10.8	32.2	+1
I_{ss}	(4.3)	(0.16)	(1.17)	
Score	+2	−1	0	
FK8	17.2	69.5	76.8	+4
I_{ss}	(2.20)	(1.04)	(2.78)	
Score	+2	0	+2	

In parentheses, the stimulation or suppression indexes (I_{ss}) are given according to ConA&PMA production. The quantutative evaluation of indexes is presented in scores: −2 ($I_{ss} \leq 0.1$), −1 ($0.1 < I_{ss} < 0.8$), 0 ($0.8 \leq I_{ss} \leq 1.2$), +1 ($1.2 < I_{ss} < 2$), +2 ($2 \leq I_{ss}$)

FK3 suppressed the production of IL-6 independently of activators, in contrast to FK4 which decreased it only after activation with ConA&PMA. F02 and LK-409 had no effect on the level of this cytokine, while F01 slightly increases it if the cells were activated with ConA&PMA.

FK8 elevated the concentration of IL-10 in combination with both types of activators, while FK3 increased it only with Iono&PMA. FK4 down-regulated the synthesis of IL-10 after activation with Iono&PMA while it had no effect on the production of this cytokine after treatment with ConA&PMA. The opposite effect was observed with LK-409 and F02. These two compounds augmented the production of IL-10 when cells were activated with ConA&PMA, but strongly decreased its level with Iono&PMA.

3. Discussion

Our results show that LK-409 and its analogues modulate the production of cytokines and therefore influence the immune response, probably through the differentiation of Th0 cells into active Th1 or Th2 cells. The balance between Th1 and Th2 plays an important role in many infectious diseases, specifically *via* production of different cytokines. Th1 cells produce IL-2 and IFN-γ and favor cell-mediated response and macrophage activation while Th2 cells generate increased levels of IL-4, IL-5, IL-6 and IL-10. In this way, Th1 and Th2 stimulate humoral immunity and, on the other hand, allergic response (Trinchieri 1997). The results show that the compounds investigated are able to initiate the differentiation of mature Th cells to Th1 or Th2 cells.

The activity of LK-409 analogues is extremely dependent on their structure; even the configuration at the chiral centers influences the production of cytokines.

LK-409, which has the L and D configuration at the two chiral centers, strongly enhanced the production of IL-4 and IL-10 after stimulation with ConA&PMA. The L,L-analogue (F02) decreased the production of IL-4 and elevated the concentration of IL-10 and specially IFN-γ, in contrast to the D,L-analogue (FK4) which elevated the production of IL-4. The D,D-analogue (FK3) had the most pronounced suppressive effect on the production of all cytokines measured.

The replacement of L-alanine in LK-409 by L-valine influenced the production of IL-4. In contrast to the valine derivative F01 strongly decreased IL-4 production. Replacement of the 7-oxooctanoyl moiety of LK-409 with 5-methoxycarbonyl-pentanoic acid improved the immunological profile, increasing the production of all cytokine measured.

This suggests that LK8 and FK3 should be candidates for further immunological investigation and structural optimization.

4. Experimental

4.1. Test compounds and animals

LK-409, F01, F02, FK3 and FK4 and FK5 were synthesized as described previously (Sollner et al. 1993; Sollner 1993). All other substances were of pharmaceutical grade.

Female (4–5 weeks old) HAN-NmRI mice (outbreed), without signs of any infection and weighing 20–25 g received LK-409 and its analogues intraperitoneally. The substances were dissolved in pyrogen-free DMSO (Sigma, USA) and diluted with apyrogenic sterile saline to a final concentration of 0.14 mM. 0.1% (v/v) DMSO in saline was used in vehicle treated control animals. The compounds were injected intraperitoneally at a dose 0.07 μmol for three consecutive days. On the fourth day mice were sacrificed by cervical dislocation.

4.2. Cytokine production

Spleens from mice from treated and control animals were homogenized in RPMI 1640 lacking L-glutamine. Spleen cells were washed twice with RPMI 1640. After the second centrifugation they were resuspended in RPMI 1640 containing 5% heat-inactivated FCS (Sigma, USA), antibiotics (100 U of penicillin and 100 μg of streptomycin/ml of medium) and L-glutamine. Mononuclear cells were counted and adjusted to a concentration of 2×10^6/ml. Cell suspensions (1 ml) were plated on 24-well flat bottom microculture plates (T grade, Nunc, Denmark) together with a polyclonal activators-combination either of cocanavalin A (ConA, Pharmacia, Sweeden) and phorbol myristate acetate (PMA, Sigma, USA) (ConA&PMA) or ionoycin (Iono, Sigma, USA) and PMA (Iono&PMA). The activators were used at a final a concentration of 0.33 ng/ml PMA, 5.33 μg/ml ConA and 500 nM Iono. Cells were incubated for 24 h at 37 °C in 5% CO_2, 95% humidity atmosphere. Cell-free supernatants collected and stored at −70 °C before being evaluated for various cytokines. The concentrations of cytokines were measured by commercially available mouse ELISA kits (Endogen, USA).

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