3. L. V. Devoino and E. L. Al'perina, Fiziol. Zh. SSSR, 70, No. 2, 239 (1984).
4. V. I. Ratnikov, N. E. Ryabinina, and R. U. Ostrovskaya, Byull. Éksp. Biol. Med., No. 10, 56 (1982).
5. E. B. Sopotsinskaya, I. A. Lisnyak, and V. A. Yakimenko, Ukr. Biokhim. Zh., 52, No. 4, 434 (1980).
6. L. A. Frangulyan, R. A. Manukyan, N. P. Babayan, et al., Neurohumoral Regulation of Immune Homeostasis [in Russian], Leningrad (1986), pp. 122-123.
7. J. Descotes, R. Tedone, and J. C. Evreux, Immunol. Lett., 5, 41 (1982).
8. J. Descotes, R. Tedone, and J. C. Evreux, J. Neuroimmunol., 9, 81 (1985).
9. M. Didier, M. F. Belin, M. Aguera, et al., Neurochem. Int., 7, 481 (1985).
10. W. Haefely, Agents Actions, 7, 353 (1977).
11. T. Nishikawa and B. Scatton, Brain Res., 331, 81 (1985).
12. W. Sieghart, J. Neural Transm., 63, 191 (1985).
13. B. L. Waszczak, N. Eng, and J. R. Walters, Brain Res., 188, 185 (1980).
14. P. L. Wood, P. Etienne, S. Lal, and N. P. V. Nair, Prog. Neuro-Psychopharmacol., 6, 471 (1982).

EFFECT OF DALARGIN, A SYNTHETIC ENDOGENOUS OPIOID ANALOG, ON NATURAL CYTOTOXICITY OF HUMAN LYMPHOCYTES

E. N. Aleksandrova, E. L. Nasonov, V. A. Vinogradov, and M. I. Titov

UDC 612.112.94.071.4.014.46:615.31:[547.95:547.943

KEY WORDS: dalargin; human lymphocytes; natural cytotoxicity.

Interest in the study of the role of opioid peptides in regulation of the immune system has increased considerably in recent years [2, 15]. Data have been obtained on the influence of opioid peptides on the proliferative response of mitogen-stimulated lymphocytes [5], on the suppressor activity of lymphocytes [11], antibody synthesis [6], the phagocytic function of neutrophils and macrophages [12, 14], and cellular cytotoxic reactions [7, 9, 10]. The writers showed previously that dalargin, a synthetic analog of Leucine-enkephalin with the structural formula Tyr-D-Ala-Gly-Phe-Ley-Arg possesses marked immunomodulating activity in vitro, and among other properties it stimulates the ability of T lymphocytes to form rosettes with sheep's red blood cells, it influences the proliferative response of lymphocytes stimulated by concanavalin A and by pokeweed mitogen, and it stimulates the phagocytic activity of leukocytes [1, 2].

The aim of this investigation was to study the action of dalargin on natural cytotoxicity (NCT) of human peripheral blood lymphocytes.

EXPERIMENTAL METHODS

Mononuclear cells were isolated by the method in [3]. Adherent cells were removed by incubation on plastic Petri dishes for 1 h at 37°C [8]. Nonadherent cells were resuspended in complete medium RPMI 1640 (flow Laboratories, England) with the addition of 10% embryonic calf serum (ECS; Flow Laboratories), 2 mM L-glutamine (Merck, West Germany), 10 mM HEPES (Flow Laboratories), and 50 μg/ml of gentamicin, the cell concentration being adjusted to $2 \cdot 10^6$/ml. The effector cells were preincubated with dalargin in a final concentration of 10^{-6}-10^{-14} M or with the control culture medium for 1 h at 37°C, washed twice, and studied in the cytotoxic test.

Human erythroleukemia cells line K-562, cultured in complete RPMI 1640 medium with 10% ECS were used as the targets.

All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow.
(Presented by Academician of the Academy of Medical Sciences of the USSR I. K. Shkhvatsabaya.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, vol. 105, No. 4, pp. 442-445, April, 1988. Original article submitted July 17, 1987.

TABLE 1. Effect of Dalargin on Number of Effector–Target Conjugates (in %)

Group of subjects	Concentration of dalargin, M					
	0	10^{-14}	10^{-12}	10^{-10}	10^{-8}	10^{-6}
Donors						
№ 1	9,6	18,0	18,5	13,8	16,2	12,3
№ 2	12,3	21,6	14,8	21,7	13,0	13,9
№ 3	9,8	18,3	16,4	16,3	18,6	17,7
№ 4	22,5	21,3	27,1	26,8	29,5	18,4
Patient with SLE	15,4	12,9	15,1	20,1	21,8	14,2
Total . . .	13,9±2,5	18,4±1,7	18,4±2,4	19,7±2,5	19,8±3,2	15,3±1,2

TABLE 2. Effect of Dalargin on Number of Conjugates with Killed Targets (in %)

Group of subjects	Concentration of dalargin, M					
	0	10^{-14}	10^{-12}	10^{-10}	10^{-8}	10^{-6}
Donors						
№ 1	9,1	15,4	12,0	11,5	18,2	7,7
№ 2	5,6	6,4	7,6	5,6	9,6	5,8
№ 3	10,0	14,4	24,6	15,4	10,9	8,8
№ 4	7,3	20,7	9,1	30,8	15,9	20,4
Patient with SLE	4,7	11,6	18,0	9,7	6,2	10,5
Total . . .	7,3±1,0	13,7±2,7	14,3±3,3	14,6±4,8	12,2±2,3	10,6±2,8

TABLE 3. Effect of Dalargin on Number of "Active" NK Cells (in %)

Group of subjects	Concentration of dalargin, M					
	0	10^{-14}	10^{-12}	10^{-10}	10^{-8}	10^{-6}
Donors						
№ 1	0,87	2,77	2,22	1,59	2,94	0,94
№ 2	0,69	1,38	1,12	1,22	1,25	0,81
№ 3	0,98	2,63	4,0	2,51	2,02	1,56
№ 4	1,64	4,41	2,47	8,25	4,66	3,75
Patient with SLE	0,72	1,50	2,72	1,94	1,35	1,49
Total . . .	0,98±0,18	2,54±0,58*	2,50±0,58	3,10±1,35	2,44±0,65	1,71±0,56

Legend. *$p < 0.05$.

The NCT of the lymphocytes was determined by estimating the cytotoxicity of single cells in agarose [13]. Effector cells and target cells, $2 \cdot 10^5$ of each, were mixed in test tubes (12 × 75 mm) in a total volume of 0.2 ml of complete RPMI 1640 medium with 15% ECS. The mixture of cells was centrifuged for 5 min at 250 g and incubated for 10 min at 37°C. The residue was carefully resuspended and added to 0.5 ml of a 0.5% solution of agarose (Agarose A, Pharmacia, Sweden) in RPMI 1640 medium with 10 mM HEPES (before addition of the cells the agarose was cooled at room temperature to 39°C). The mixture of cells with agarose was quickly transferred into Petri dishes 60 mm in diameter (Falcon Plastics, USA), covered beforehand with 0.5 ml of agarose. The layer of agarose with cells, solidified at room temperature, was covered with 6 ml of complete RPMI 1640 medium with 15% ECS, after which the dishes were incubated for 3 h at 37°C in a humid atmosphere with 5% CO_2. At the end of incubation the preparations were stained with 0.1% trypan blue solution, washed, and fixed in 0.5% glutaraldehyde solution. Control samples contained target cells and effector cells separately in agarose. All tests were carried out in two parallel determinations. The preparations were examined under the microscope with a magnification of 400. The percentage of effector–target conjugates, the percentage of conjugates with killed targets, and the percentage of "active" natural killer (NK) cells [13] were calculated.

EXPERIMENTAL RESULTS

Preincubation of lymphocytes with dalargin in a concentration of 10^{-14}-10^{-8} M increased the number of effector–target conjugates on average by 40.1 ± 17.8 to 50.0 ± 16.8%

TABLE 4. Effect of Naloxone on Level of NCT, Stimulated by Dalargin

Preparation	No. of effector–target conjugates	No. of conjugates with killed targets	No. of "active" NK cells
Control	12,3	5,6	0,69
Dalargin (10^{-8} M)	13,0	9,6	1,25
Naloxone (10^{-6} M)	16,7	8,1	1,25
Dalargin + Naloxone	15,4	10,8	1,66

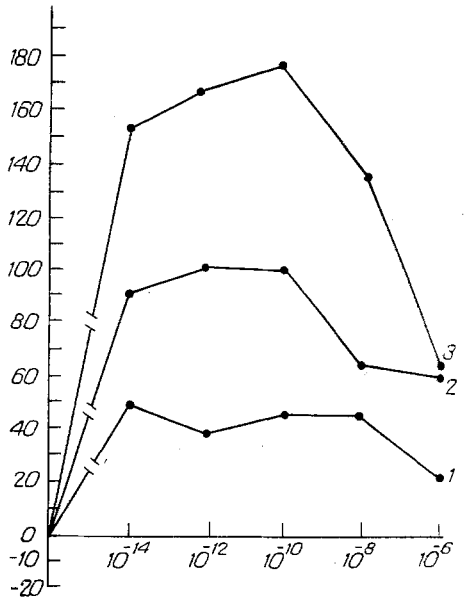

Fig. 1. Effect of dalargin on NCT of lymphocytes. Abscissa, concentration of dalargin (in M); ordinate, change in number (in % of control) of effector–target conjugates (1), of conjugates with killed targets (2), and of "active" NK cells (3). Mean data of five experiments are shown.

(Table 1, Fig. 1). The most marked stimulating effect of dalargin on the ability of NK cells to form conjugates with targets was observed in donor No. 1 (in a concentration of 10^{-14}-10^{-12} M) and No. 3 (in a concentration of 10^{-14}-10^{-8} M), with an initially smaller number of effector–target conjugates compared with the other donors. In a patient with systemic lupus erythematosus (SLE) dalargin increased the percentage of conjugates moderately (by 30.5-51.6%, in a concentration of 10^{-10}-10^{-8} M). In some cases dependence of the stimulating activity of dalargin on its dose was bimodal in character (in donors Nos. 1, 2, and 3).

Data on the effect of dalargin on the stage of lysis of the targets by NK cells are given in Fig. 1 and Table 2. Dalargin in a concentration of 10^{-14}-10^{-8} M caused a dose-

dependent increases in the cytolytic activity of the NK cells, increasing the number of conjugates with killed targets by 66.1 ± 20.8 to 104.3 ± 49.5%. The potentiating action of dalargin on the cytotoxic function of the NK cells was observed in all subjects, and in some cases it reached 300% (donor No. 4, patient with SLE). Under these circumstances, in three of the four donors and in the patient with SLE two peaks of stimulating activity of dalargin were found: in concentrations of 10^{-14}-10^{-12} and 10^{-10}-10^{-6} M.

Between concentrations of 10^{-14} and 10^{-6} M dalargin induced a dose-dependent increase in the number of "active" NK cells by 64.0 ± 23.1 to 177.6 ± 62.5% (Table 3, Fig. 1). Preincubation of the lymphocytes with dalargin in a concentration of 10^{-14} M led to an increase in the number of "active" NK cells by 152.8 ± 22.7% relative to the control (from 0.98 ± 0.18 to 2.54 ± 0.58%; $p < 0.05$). A marked increase in the percentage of "active" NK cells under the influence of dalargin was observed in all donors and in the patient with SLE; in three of the five subjects tested, the potentiating action of dalargin was bimodal, with peaks of activity at concentrations of 10^{-14}-10^{-12} and 10^{-10}-10^{-6} M.

A potentiating effect of dalargin was thus observed both at the stage of binding of NK cells with targets and at the stage of lysis. In all cases the increase of NCT by dalargin could be observed in physiological concentrations, similar to the level of endogenous opioid peptides in the blood. According to data obtained by other workers, β-endorphin (10^{-14}-10^{-8} M) and Met-enkephalin (10^{-9} M) significantly enhance the functional activity of NK cells in vitro [7, 9, 10]. Under these circumstances the potentiating action of the opioid peptides was blocked by naloxone, i.e., it was mediated through specific opiate receptors. We showed that naloxone can act, not as an antagonist, but as an agonist of opioid receptors, intensifying the formation of effector–target conjugates and the cytotoxic power of the NK cells (Table 4). In some cases a bimodal kinetics of the stimulating effect of dalargin on NCT was observed with two peaks of activity of the peptide in the region of concentrations of 10^{-14}-10^{-12} and 10^{-10}-10^{-6} M. It can be postulated that dalargin modifies the function of NK cells by binding with several types of receptors. Similar patterns were obtained in a study of the effect of β-endorphin and Met-enkephalin on the production of T-cell chemotaxic factor [4].

The potentiating action of opioid peptides on NCT is similar to that of interferon, and it may be partly mediated by interferon-dependent mechanisms [7, 9]. According to our preliminary data, dalargin stimulates the function of NK cells more effectively than interferon. Within the range of concentrations from 10^{-14} to 10^{-8} M dalargin stimulated the formation of effector–target conjugates by 16.9-31.1%, of conjugates with killed targets by 24.7-321.9%, and of active NK cells by 50.6-403.0%, whereas recombinant human α-interferon (reoferon), in a concentration of 10-1000 IU, increased the values of the same parameters by 0-28.0, 27.4-72.6, and 63.4-67.7%, respectively.

The data on the stimulating effect of dalargin on NCT of lymphocytes are evidence that the preparation may be used in clinical practice for the correction of immunologic disturbances in pathological states characterized by weakening of the function of NK cells.

LITERATURE CITED

1. V. A. Vinogradov, E. V. Vasil'eva, E. L. Nasonov, et al., Ter. Arkh.. No. 11, 114 (1984).
2. E. L. Nasonov and V. A. Vinogradov, Byull. Vses. Kardiol. Nauch. Tsent., No. 1, 3 (1987).
3. A. Boyum, Scand. J. Clin. Lab. Invest., 21, Suppl., 97 (1968).
4. S. L. Brown and D. E. Van Epps, J. Immunol., 134, 3384 (1985).
5. S. C. Gilman, J. M. Schwarts, R. J. Milner, et al., Proc. Natl. Acad. Sci. USA, 79, 4226 (1982).
6. H. M. Johnson, E. M. Smith, B. A. Torres, and J. E. Blalock, Proc. Natl. Acad. Sci. USA, 79, 4171 (1982).
7. N. Kay, J. Allen, and J. E. Morley, Life Sci., 35, 53 (1984).
8. K. Kumagai, K. Itoh, and S. Hihuma, J. Immunol. Meth., 29, 17 (1979).
9. R. N. Mandler, W. E. Biddison, R. Mandler, and S. A. Serrate, J. Immunol., 136, 934 (1986).
10. P. M. Mathew, C. J. Froelich, W. L. Sibbit, et al., J. Immunol., 130, 1658 (1983).
11. M. W. McCain, I. B. Lamster, and J. Billotta, Int. J. Immunopharm., 8, 443 (1986).
12. B. M. Sharp, W. F. Keane, H. J. Suh, et al., Endocrinology, 117, 793 (1985).
13. M. Ulberg and M. Jondal, J. Exp. Med., 153, 615 (1981).
14. D. E. Van Epps and L. Salant, J. Immunol., 132, 3046 (1984).
15. J. Wybran, Fed. Proc. Fed. Am. Soc. Exp. Biol., 44, 92 (1985).