BRAIN, BEHAVIOR, AND IMMUNITY 6, 223–233 (1992)

Naloxone Is an Inappropriate Antagonist of Met-Enkephalin-Modulated Superoxide Anion Release

TANJA MAROTTI AND HELENA HABERSTOK

Institute R. Boskovic, Department of Experimental Biology and Medicine, Bijenicka 54, 41000 Zagreb, Croatia

Met-enkephalin (MENK) increased superoxide anion release by human polymorphonuclear cells (PMNs) at a physiological (10^{-10} *M*) concentration and decreased release at a relatively high (10^{-8} *M*) concentration. Surprisingly, naloxone (NAL), used as a specific antagonist in these experiments, displayed immunomodulatory actions of its own, increasing superoxide anion release at 10^{-10} and 10^{-8} *M* concentrations. Although both were effective, the dose–response curves were different for NAL and MENK. NAL and MENK also had a combined influence on O_2^- release. The stimulative effect of 10^{-10} *M* MENK could be abolished by 10^{-8} *M* NAL in seven of eight cases. Unexpectedly, the stimulatory effect of 10^{-8} and 10^{-10} *M* NAL could be abrogated by MENK in five of eight cases as well. The fact that NAL and MENK mutually interfere with one another in their effect upon O_2^- release by human PMNs discredits NAL as an appropriate antagonist in this system.

INTRODUCTION

Opioid peptides regulate the immune response and alter cellular functions related to microbicidal and cytotoxic activity of phagocytic cells *in vivo* and *in vitro*. For example, Met-enkephalin (MENK) simulated chemotaxis of human monocytes (Vann Epps & Saland, 1984) and enhanced lysis of antibody-coated target cells by rat peritoneal macrophages (Foris, Medgyesi, & Hauck, 1986), but suppressed phagocytosis of IgG2a-coated ^{51}Cr-labeled sheep red blood cells (Foris, Medgyesi, Gyimesi, & Hauck, 1984). There are studies indicating that naloxone (NAL), as a specific antagonist, can reverse some of the effects of the opioid peptides (Foris et al., 1986; Hucklebridge, Hudspith, Muhamed, Lydyard, & Brostoff, 1989). However, numerous other effects not reversible by NAL have been reported (Plotnikoff, Murgo, Miller, Corder, & Faith, 1985; Murgo, Plotnikoff, & Faith, 1985).

Our previous results (Marotti, Sverko, & Hrsak, 1990) demonstrated that MENK modulates superoxide anion (O_2^-) release from unstimulated human polymorphonuclear cells (PMNs). Thus, the present study was undertaken to define: (a) whether NAL exhibits immunomodulatory actions of its own and (b) whether the modulation of superoxide anion release by MENK can be abrogated by NAL.

MATERIAL AND METHODS

Isolation of Human Neutrophils

Polymorphonuclear leukocytes from adult healthy subjects were isolated from heparinized blood (10 units/ml of preservative-free heparin) by dextran sedimentation (3% dextran-T-500, Pharmacia, Uppsala, Sweden; 1 ml dextran/ml blood, 45–60 min at room temperature) and centrifugation over Ficoll–Hypaque (Boyum, 1968). Contaminating red blood cells were removed by hypotonic lysis with deionized water. PMNs were suspended in phenol-free Hanks' balanced salt solution

0889-1591/92 $5.00

(HBSS). This procedure typically yielded preparations containing more than 95% PMNs.

Experimental Design

Neutrophils were treated with MENK (Sigma, St. Louis, MO) or/and NAL (Sigma) in concentrations ranging from 10^{-14} to 10^{-7} *M* and 10^{-12} to 10^{-6} *M*, respectively. A 100 *M* excess of NAL was added to PMNs 10 min before MENK. Cells were incubated with MENK, NAL, or both for 10 min at room temperature. The agents were removed by centrifugation, and reactivity of the cells was tested by the addition of cytochrome *c* in phenol-free HBSS.

Assay for Superoxide Anion (O_2^-) Release

Superoxide release was measured as superoxide dismutase (SOD) (Sigma) inhibitive reduction of ferricytochrome *c* by using a modification of the method of Johnson, Godzik, and Cohn (1978). The samples contained 1 ml of cytochrome *c* (1 mg/ml, Type III; Sigma) in phenol-free HBSS and 1×10^6 cells in 100 μl of medium. Specificity of the reaction was tested by the addition of 60 IU SOD/ml reaction mixture. After incubation at 37°C in 5% CO_2 for 30 min, the reaction mixture was centrifuged for 5 min at 2000 rpm. Optical density of the supernatant was determined spectrophotometrically at 550 nm. Concentration of reduced cytochrome *c* was calculated by using the formula $E_{550\ nm} = 2.1 \times 10^4\ M^{-1}cm^{-1}$. Protein content of each tube was determined by the method of Lowry et al. (1951) following lysis of the cells by the addition of 0.1 *N* NaOH overnight. The tubes contained 100 to 200 μg of protein. The results were expressed as nmol O_2^-/mg protein/30 min.

Statistics

The Kruskal–Wallis nonparametric analysis of variance was used to assess variability between the groups (controls, suppression, stimulation) vs variability within the groups. Differences between the groups were evaluated by the Mann–Whitney *U* test and by linear regression (Spiegel, 1980). The level of significance was set at 5%.

RESULTS

The Effect of MENK upon O_2^- Release by Human PMNs

Polymorphonuclear cells of 17 healthy donors were incubated with 10^{-7} to 10^{-14} *M* MENK for 10 min at room temperature (Fig. 1). Suppression or stimulation of O_2^- release was observed (Fig. 1) at each MENK concentration examined. We therefore divided our results for each MENK concentration into three groups: no effect or less or more O_2^- release than the respective control value and tested by the Kruskal–Wallis analysis of variance whether the variability among the three groups was greater than the variability within the groups, i.e., whether it was possible to further compare the groups by the Mann–Whitney *U* test. Since only the 10^{-8} and 10^{-10} *M* MENK concentrations induced stimulation or suppression relative to control values ($\chi^2 = 6.76$, $p = 0.034$, and $\chi^2 = 8.15$, $p = 0.016$, respectively) they were further examined with the Mann–Whitney *U* test. Of 17 donors tested, MENK at 10^{-8} *M* concentration significantly ($p = 0.04$) decreased O_2^- release from PMNs of 8 donors. At 10^{-10} *M* MENK concentration in

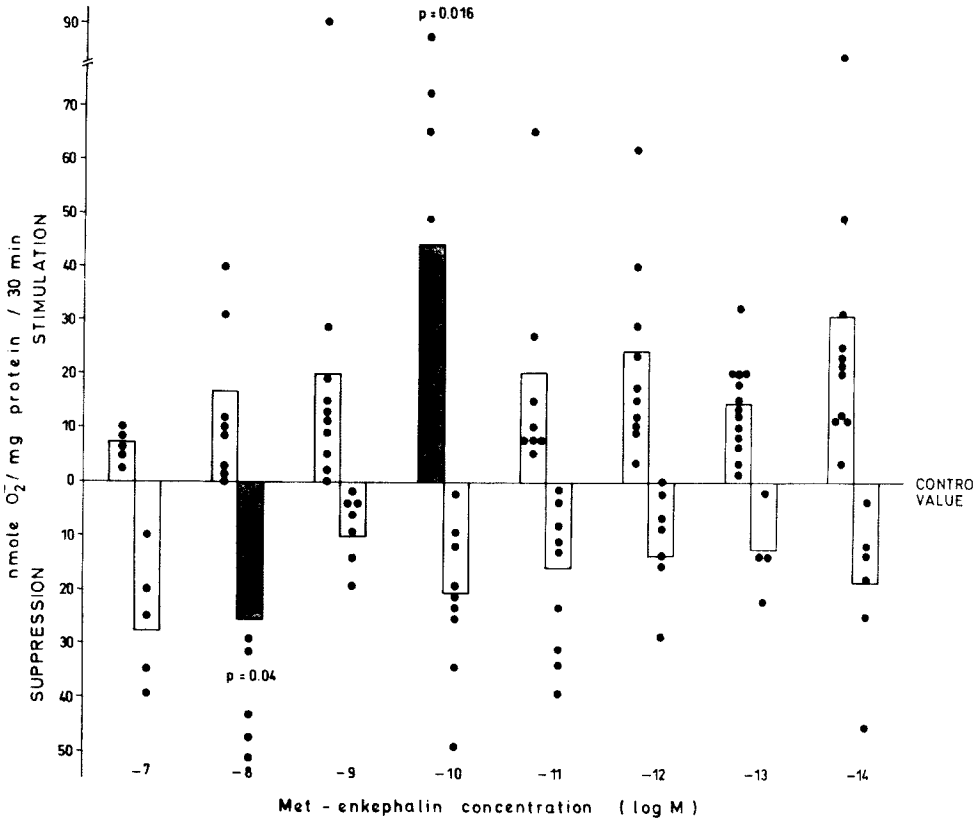

FIG. 1. Effect of Met-enkephalin on O_2^- release from human polymorphonuclear cells. The value of O_2^- release from control (untreated) PMNs has been taken as 0. Thus, each point represents magnitude of change above (stimulation) or below (suppression) the donor's baseline value. Each dot represents one donor. The results are expressed as nmol O_2^- release/mg protein/30 min. Shaded areas represent significant stimulation (10^{-10} *M* MENK) or suppression (10^{-8} *M* MENK) of O_2^- release relative to control values.

8 of 17 donors O_2^- release was significantly increased ($p = 0.016$). Other MENK concentrations induced donor-dependent variations of O_2^- release without statistical significance.

The results for any given donor, whose PMNs are stimulated at 10^{-10} *M* MENK, are considerably consistent across other MENK concentrations. That is, of 100% of donors that show increase at 10^{-10} *M* MENK, 60% of the same donors are stimulated at 10^{-8} and 10^{-9} *M* MENK, 88% at 10^{-11} *M* MENK, and 65% at 10^{-12} *M* MENK. The results were less consistent for any given donor whose PMNs were suppressed at 10^{-8} *M* MENK. That is, only at the 10^{-10} *M* MENK concentration did 78% of the same donors show a decrease in O_2^- release, while at other MENK concentrations only about 35% of the donors showed suppression of superoxide anion release.

The Effect of NAL upon O_2^- Release by Human PMNs

Polymorphonuclear cells of 17 healthy donors were incubated with 10^{-6} to 10^{-12} *M* NAL for 10 min at room temperature (Fig. 2). Suppression or stimulation

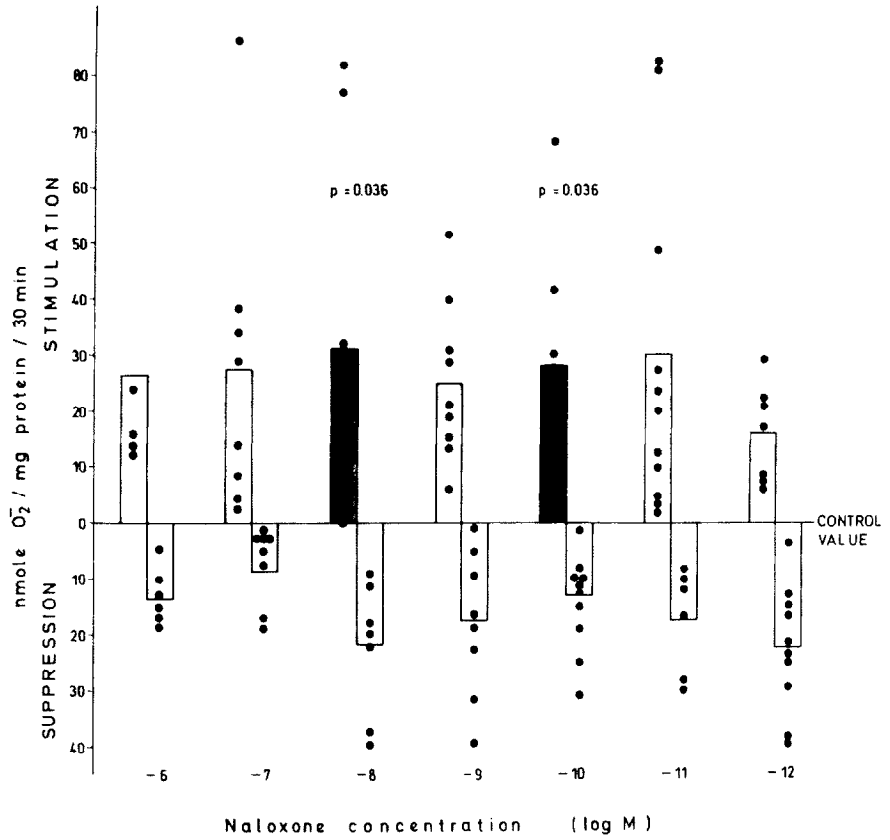

FIG. 2. Effect of naloxone on O_2^- release from human polymorphonuclear cells. The value of O_2^- release from (control) untreated human PMNs has been taken as 0. Thus, each point represents the magnitude of change above (stimulation) or below (suppression) the donor's baseline value. Each dot represents one donor. Results are expressed as nmol O_2^- release/mg protein/30 min. Shaded areas represent significant stimulation (10^{-10} and 10^{-8} *M* NAL) of O_2^- release relative to control values.

of O_2^- release (Fig. 2) was observed for each NAL concentration examined. The Kruskal–Wallis analysis of variance revealed that at the 10^{-8}, 10^{-9}, and 10^{-10} *M* NAL concentrations, donors with suppressed and stimulated O_2^- release were significantly different from the corresponding control groups ($\chi^2 = 7.6$, $p = 0.02$; $\chi^2 = 8.1$, $p = 0.017$; $\chi^2 = 9.3$, p = 0.009, respectively). However, significant stimulation of O_2^- release was obtained from PMNs of 10 of 17 donors at 10^{-8} *M* NAL and from PMNs of 7 of 17 donors at 10^{-10} *M* NAL ($p = 0.036$ for both concentrations). None of the NAL concentrations examined suppressed O_2^- release to a statistically significant degree.

The results for any given donor whose PMNs were stimulated at 10^{-8} and 10^{-10} *M* NAL were consistent across other NAL concentrations, the stimulatory effect varying between 83 and 100%.

Comparison of MENK and NAL Effect upon O_2^- Release

Our results have shown that, although NAL and MENK are both effective at 10^{-8} and 10^{-10} *M* concentrations, they did not exhibit the same pharmacological profile (Figs. 3A and 3B). None of the NAL concentrations studied suppressed

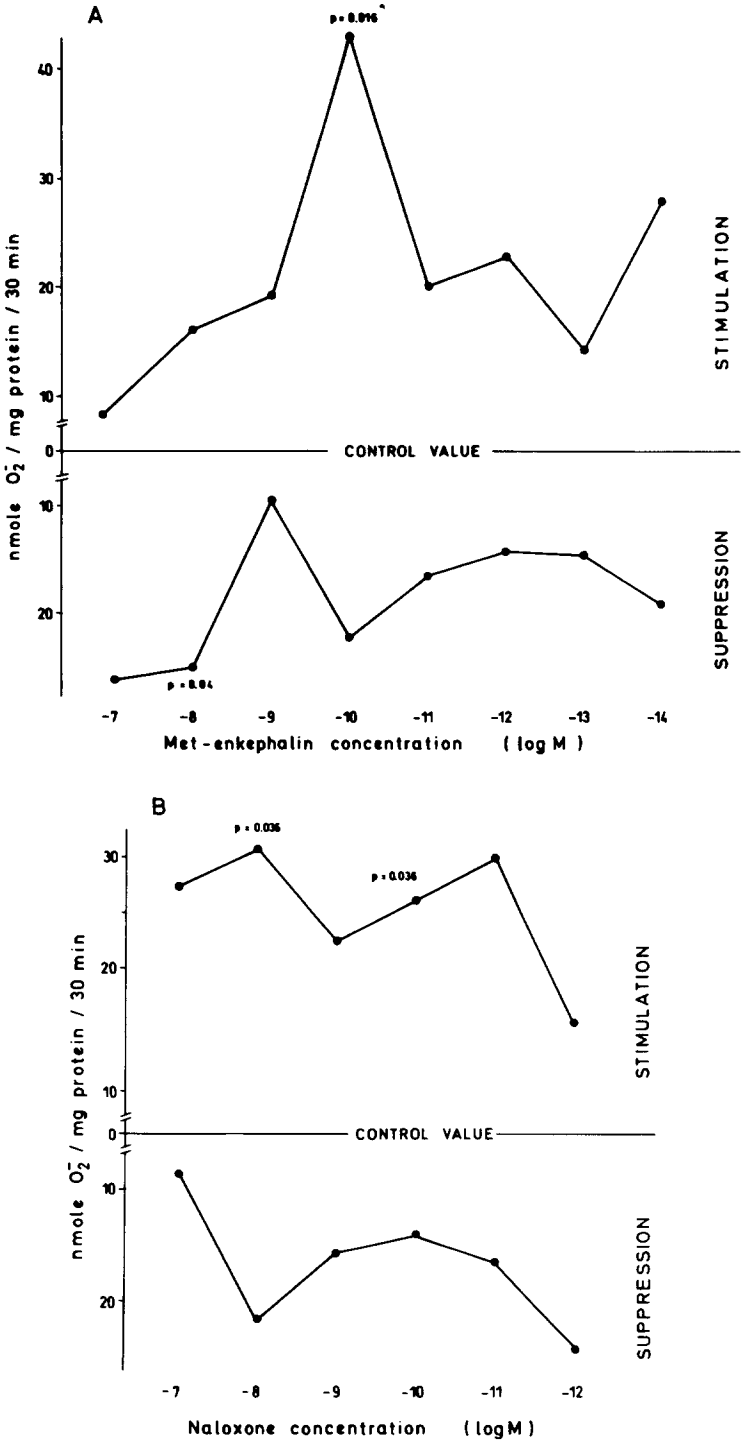

FIG. 3. Comparison of concentration-dependent stimulative or suppressive effect of Met-enkephalin or naloxone. The value of (control) untreated human polymorphonuclear cells has been taken as 0. Each dot represents mean of the magnitude of increased (stimulation) or decreased (suppression) O_2^- release from PMNs of 17 donors at each concentration of either MENK (A) or NAL (B). Results are expressed as nmol O_2^-/mg protein/30 min.

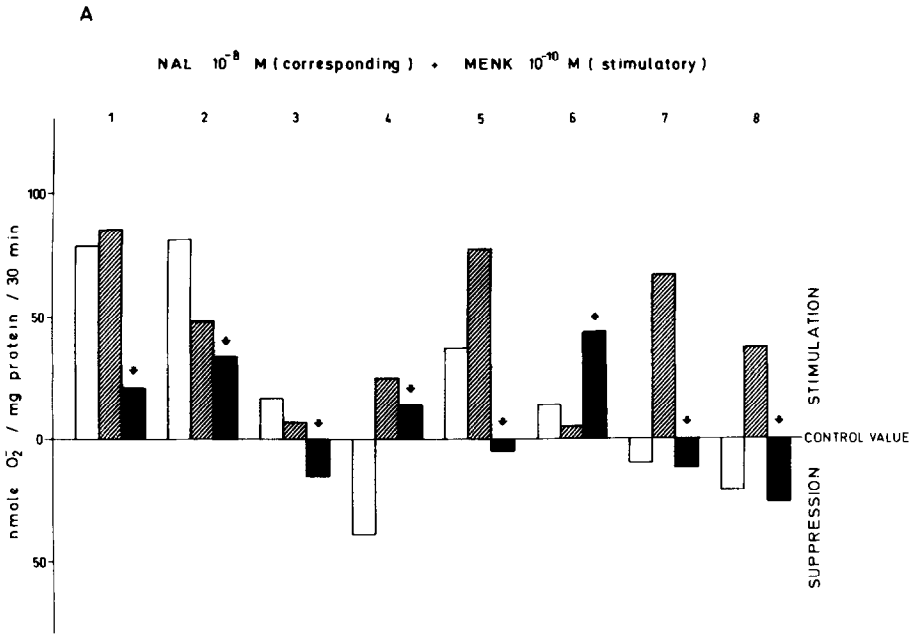

FIG. 4. The effect of combined use of naloxone and Met-enkephalin on O_2^- release from human polymorphonuclear cells. Each bar represents the magnitude of change in O_2^- release above (stimulation) or below (suppression) the donors' own baseline value of control, untreated PMNs. Naloxone treated (□), Met-enkephalin treated (▨), 100 *M* excess of naloxone prior to Met-enkephalin (■). Arrows indicate the direction of change in O_2^- release: combined additive effect (↑) or combined opposing effect (↓), either to Met-enkephalin (A) or to naloxone (B,C). Each number represents one donor.

O_2^- release. There was no dose–response in NAL-induced O_2^- release ($r = 0.53$ for 10^{-7} to 10^{-12} *M* NAL concentrations) and no evident stimulation peak since the amount of O_2^- release at 10^{-8} and 10^{-10} *M* NAL was not statistically different (Fig. 3B). On the contrary, the concentration curve for MENK was bell-shaped, showing a bidirectional effect with one suppressive peak at 10^{-8} *M* and one stimulative peak at 10^{-10} *M* MENK concentration (Fig. 3A).

The Effect of Combined Use of NAL and MENK upon O_2^- Release

In order to investigate whether the combined use of NAL and MENK results in opposing effects in relation to NAL or MENK treatment alone, PMNs were treated with a 100 *M* excess of NAL 10 min prior to MENK treatment. Results are shown in Figs. 4A–4C. In 8 of 17 donors whose PMNs incubated with 10^{-10} *M* MENK released increased amounts of O_2^- (control versus MENK, $p = 0.01$) naloxone alone at 10^{-8} *M* concentration was not effective (control versus NAL, $p = 0.26$). However, in the same 8 people, combined treatment of cells with 10^{-8} *M* NAL and 10^{-10} *M* MENK abrogated the stimulative effect of MENK in 7 of 8 of them (control versus NAL + MENK, $p = 0.6$). Only donor 6 released more O_2^- after combined treatment than with MENK or NAL alone (Fig. 4A). In 8 of 17 donors whose PMNs incubated with 10^{-8} *M* NAL released increased amounts of O_2^- (control versus NAL, $p = 0.036$) treatment with 10^{-10} *M* MENK was not effective (control versus MENK, $p = 0.5$). However, combined treatment re-

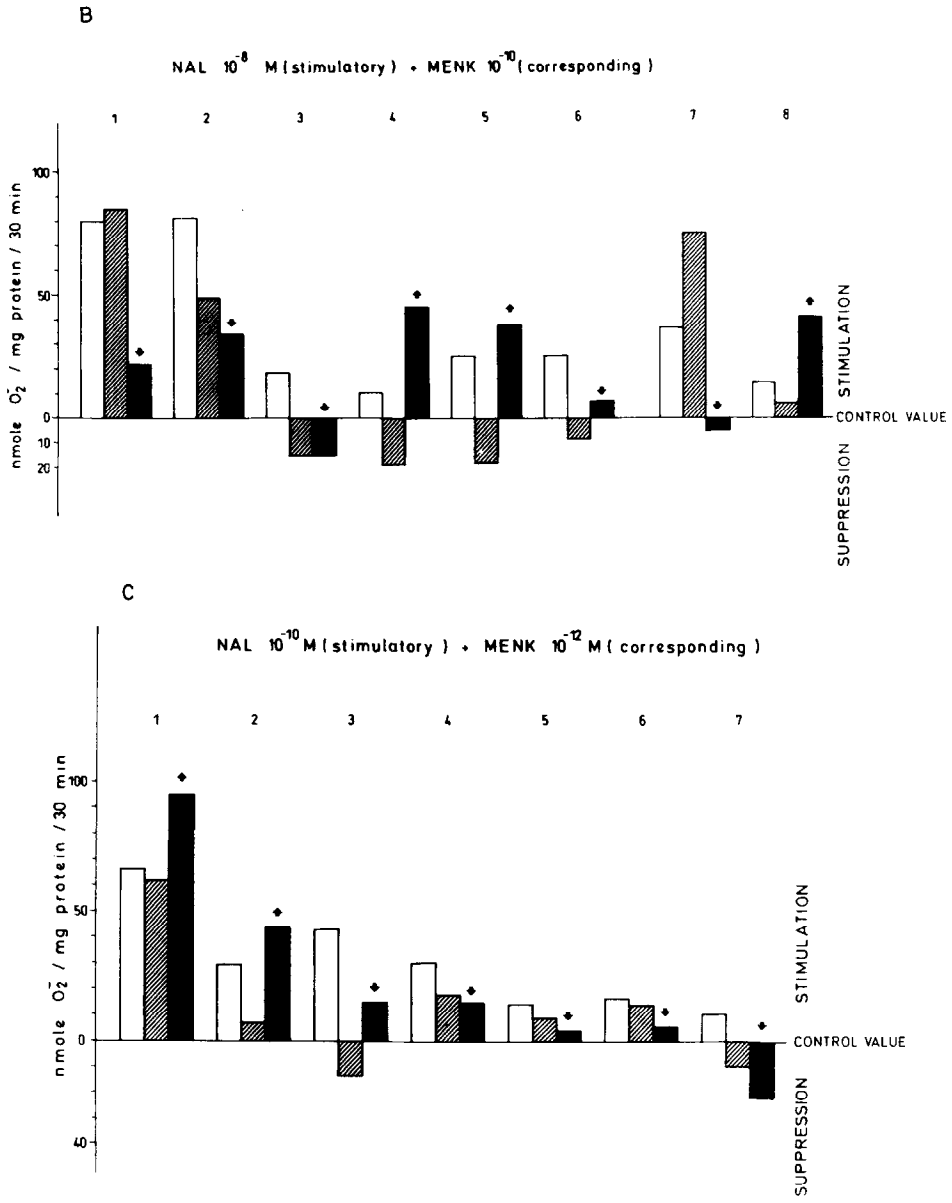

FIG. 4.—*Continued*

duced the stimulatory effect of NAL in 5 donors and additionally stimulated O_2^- release in 3 donors (Fig. 4B). There was no statistical difference between the values of O_2^- release from control nontreated PMNs and values of O_2^- released from PMNs treated with 10^{-8} *M* NAL and 10^{-10} *M* MENK (control versus NAL + MENK, $p = 0.4$). This was also the case in PMNs of 7 of 17 donors whose O_2^- release was stimulated by 10^{-10} *M* NAL (control versus NAL, $p = 0.036$) in which 10^{-12} *M* MENK had no effect (control versus MENK, $p = 0.8$) (Fig. 4C). That is, combined treatment of cells with 10^{-10} *M* NAL and 10^{-12} *M* MENK abolished the stimulative effect of NAL in 5 donors and further stimulated O_2^- release in 2 donors.

DISCUSSION

The present work confirms the results of our previous study (Marotti et al., 1990) of MENK-modulated superoxide anion release by human PMNs. Namely, in 8 of 17 donors tested, O_2^- release was decreased at 10^{-8} *M* and increased at 10^{-10} *M* MENK concentration. Those results substantiate previous investigations indicating the "donor-dependent effect" mediated by opioid peptides (Oleson & Johnson, 1988; Brummitt, Sharp, Gekker, Keane, & Peterson, 1988).

However, significant stimulation of O_2^- was obtained with 10^{-8} and 10^{-10} *M* NAL alone (in 10 of 17 or 7 of 17 donors, respectively). These data conform to other literature indicating that NAL displays an immunomodulatory action of its own. For example, immunoregulation by NAL and a δ-specific antagonist, ICI 174 864, was noted in natural killer (NK) lysis (Oleson & Johnson, 1988) and in the generation of cytotoxic T cells (Carr & Klimpel, 1986). Rowland et al. (1987) demonstrated that NAL, alone, added to cultures of mouse splenocytes primed with sheep erythrocytes was able to increase the plague-forming cells (PFC) response. NAL at 10^{-5} to 10^{-7} *M* stimulates nervous tissue growth in culture and further enhances the responses to Leu-enkephalin (Ilyinsky et al., 1987). Heijnen, Bevers, Kaavelaars, and Balieux (1986) showed that NAL added to cultures of human lymphocytes had an agonistic effect on the PFC response. Our results (Marotti & Batinic, 1990) are in agreement with those mentioned, since NAL alone at 10^{-4} to 10^{-6} *M* almost completely blocked the antibody response of human lymphocytes *in vitro*. Jankovic and Maric (1988) pointed out that NAL and ICI 174 864 when used alone suppressed humoral immune reactions in sheep erythrocyte-primed mice.

NAL might act at the opiate receptor level or through a nonopioid receptor mechanism. In human polymorphonuclear cells, specific binding of opiate antagonist [^{3}H]-naloxone was demonstrated (Falke, Fischer, & Martin, 1985). Madden, Donahoe, Zwemer-Collins, Shafer, and Falek (1987) showed significant individual variations in NAL binding to human T lymphocytes, the number of molecules of NAL bound per lymphocyte being 9560 ± 5230. This might at least partly explain individual variations of NAL effects in the population examined in our study.

Opposing the opioid receptor-bound effect of NAL, Simpkins, Ives, Tate, and Johnson (1985) suggested that NAL does not act at an opiate receptor since the active as well as the inactive stereoisomer of 10^{-5} to 10^{-7} *M* NAL inhibited O_2^- release from human neutrophils. The authors claim that 10^{-8} *M* NAL showed no effect on O_2^- release but did not present the data. The nonopioid receptor-mediated effect of NAL might be the result of alteration of membrane characteristics. Curtis and Lefer (1980) showed that NAL decreased enzyme release from liver large-granule fractions as a result of stabilization of lysosomal membranes. Moreover, Carratu and Mitolo-Chieppa (1982) reported that NAL inhibited Na^+ and K^+ currents.

The possibility of NAL acting through a nonopioid receptor mechanism seems to emerge from our study too, since, although both are effective, NAL and MENK exhibit different pharmacological profiles. MENK showed a characteristic bell-shaped dose–response curve, suggesting the presence of multiple receptors which mediate opposing biological effect; in contrast, no dose–response relationship could be demonstrated with NAL.

Since many studies have used antagonism by naloxone as a criterion of an

opioid receptor-mediated effect, we have examined the effect of the combined use of NAL and MENK on superoxide anion release by human PMNs. According to the literature, NAL seems to block the modulating effect of opioid peptides on some, but not all, immune reactions. Thus, NAL abrogated the MENK-stimulated proliferation of human lymphocytes *in vitro* (Hucklebridge et al., 1989). MENK-enhanced lysis of antibody-coated target cells by macrophages (Foris et al., 1986), and MENK-stimulated chemotaxis of human peripheral blood cells (VanEpps & Saland 1984). β-Endorphin-stimulated production of O_2^- in human polymorphonuclear cells was markedly reduced by naloxone (Sharp et al., 1985). In contrast, NAL, at the concentration of 10^{-7} *M*, failed to block the effect of MENK on the PFC response of mouse spleen cells primed with sheep erythrocytes (Rowland et al., 1987). NAL was unable to eliminate a significant neural tissue growth-promoting effect of MENK (Ilyinsky et al., 1987). Some other effects, not reversible by naloxone, have been reported by Plotnikoff, Murgo, Corder, and Faith (1985) and Murgo, Plotnikoff, and Faith (1985). Our study revealed some interesting results obtained by the combined use of NAL and MENK. In 8 of 17 donors whose PMNs incubated with 10^{-10} *M* MENK released increased amounts of O_2^- and 10^{-8} *M* NAL alone was not effective, combined treatment blocked the stimulative effect of MENK in 7 of these donors. Unexpectedly, in 8 of 17 donors whose PMNs incubated with (10^{-8} or 10^{-10} *M*) NAL released increased amounts of O_2^- and (10^{-10} or 10^{-12} *M*, respectively) MENK was not effective, combined treatment blocked the stimulative effect in 5 donors and additionally stimulated O_2^- release in 3 donors.

It seems, therefore, that besides being effective alone, NAL and MENK, combined, can either block each other or, to a lesser degree, invigorate each others' stimulatory effect. NAL seems to be able to block the effect of MENK and vice versa because, although low concentrations of NAL show very strong affinity for the μ-receptor, higher concentrations will also block the δ-receptor. However, its selectivity for δ as opposed to μ binding sites is approximately 10-fold lower (Holaday, 1983). MENK, however, possesses highest affinity for the δ-receptor, but, according to Peterson, Robson, and Kosterlitz (1983), also possesses a considerable cross-reactivity to the μ-receptor. Madden et al. (1987) demonstrated that NAL specifically bound to human T lymphocytes was partially displaceable by various opiate agonists including morphine (56%), β-endorphin (61%), and Met- and Leu-enkephalin (40%). NAL also inhibited 65–78% of ^{3}H-SUPERFIT (selective δ-class opioid receptor ligand) binding to human peripheral blood mononuclear cells (Carr et al., 1988). Investigation of μ- and δ-class opioid receptors indicates that their binding sites may be functionally and physically associated (Schoffelmeer, Rice, & Heijnam, 1988), probably in the membrane-bound form (Carr et al., 1990). Thus, δ binding sites (preferential for enkephalins) and μ binding sites (preferential for naloxone) seem to be in close enough proximity to modulate the ligand binding capabilities of one another. NAL may replace receptor-bound endogenous opioids; alternatively, it may upregulate the binding sites and thus increase sensitivity to opioid peptides because, as shown recently by Vindrola, Padros, Sterin-Prync, Finkielman, and Nahmod (1990), human polymorphonuclear cells contain and release proenkephalin-derived peptides.

Thus, our study clearly demonstrated NAL agonistic and antagonistic effects upon superoxide anion release by human polymorphonuclear cells. Whether the effect is opioid receptor-mediated or is a nonopioid phenomenon needs further

examination, especially since it is known that a ligand selective for a particular type of receptor in a binding assay does not necessarily work the same way in a pharmacologic assay and that pharmacological selectivity of an agonist can differ in various tissues.

REFERENCES

Boyum, A. (1968). Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* **21**(Suppl. 97), 77–89.

Brummit, C. F., Sharp, B. M., Gekker, G., Keane, W. F., & Peterson, P. K. (1988). Modulatory effects of beta endorphin on interferon-gamma production by cultured peripheral blood mononuclear cells. *Brain Behav. Immun.* **2**, 187–197.

Carr, D. J., DeCosta, B. R., Kim, C. H., Jacobson, A. E., Bost, K. L., Rice, K. C., & Blalock, J. E. (1990). Antiopioid receptor antibody recognition of a binding site on brain and leucocyte opioid receptors. *Neuroendocrinology* **51**, 552–560.

Carr, D. J., Kim, C. H., De Costa, B., Jacobson, A. E., Rice, K. C., & Blalock, J. E. (1988). Evidence for δ class opioid receptor on cells of the immune system. *Cell. Immun.* **116**, 44–51.

Carr, D. J., & Klimpel, G. R. (1986). Enhancement of the generation of cytotoxic T cells by endogenous opioids. *J. Neuroimmunol.* **12**, 75–83.

Carratu, M. R., & Mitolo-Chieppa, D. (1982). Inhibition of ionic currents in frog node of ranvier treated with naloxone. *Brit. J. Pharmacol.* **77**, 115–119.

Curtis, M. T., & Lefer, A. M. (1980). Protective action of naloxone in haemorrhagic shock. *Am. J. Physiol. H* **239**, 416–421.

Flake, N. E., Fischer, E. G., & Martin, R. (1985). Stereospecific opiate binding in living human polymorphonuclear leucocytes. *Cell. Biol. Int. Rep.* **9**, 1041–1047.

Foris, G., Medgyesi, G. A., Gyimesi, E., & Hauck, M. (1984). Met-enkephalin induced alteration of macrophage functions. *Mol. Immunol.* **21**, 747–750.

Foris, G., Medgyesi, G. A., & Hauck, M. (1986). Bidirectional effect of Met-enkephalin on macrophage effector functions. *Mol. Cell. Biochem.* **69**, 127–137.

Heijnen, C. J., Bevers, C., Kaavelaars, A., & Balieux, R. (1986). Effect of alpha-endorphin on the antigen-induced primary antibody response of human blood B cells in vitro. *J. Immunol.* **136**, 213–216.

Holaday, J. W. (1983). Cardiovascular effects of endogenous opiate system. *Annu. Rev. Pharmacol. Toxicol.* **23**, 541–546.

Hucklebridge, F. H., Hudspith, B. N., Muhamed, J., Lydyard, P. M., & Brostoff I. (1989). Methionine-enkephalin stimulates in vitro proliferation of human peripheral lymphocytes via opioid receptors. *Brain Behav. Immun.* **3**, 183–189.

Ilinsky, O. B., Kozlova, M. V., Kodrikova, E. S., Kalentchuk, V. U., Titov, M. I., & Bespalova, ZD. D. (1987). Effect of opioid peptides and naloxone on nervous tissue in culture. *Neuroscience* **22**, 719–735.

Jankovic, B. D., & Maric, D. (1988). Enkephalins modulate in vivo immune reactions through delta and mu-opioid receptors. *Ann. N.Y. Acad. Sci.* **540**, 691–693.

Johnson, R. B., Godzik, C. A., & Cohn, Z. A. (1978). Increased superoxide anion production by immunologically activated and chemically elicited macrophages. *J. Exp. Med.* **148**, 115–127.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.

Madden, J. J., Donahoe, R. M., Zwemer Collins, J., Shafer, D. A., & Falek, A. (1987). Binding of naloxone to human T lymphocytes. *Biochem. Pharmacol.* **36**, 4103–4109.

Marotti, T., & Batinic, D. (1990). Enkephalins regulate in vitro antibody response of human lymphocytes. *Period. Biol.* **92**, 197–202.

Marotti, T., Sverko, V., & Hrsak, I. (1990). Modulation of superoxide anion release from human polymorphonuclear cells by Met- and Leu-enkephalin. *Brain Behav. Immun.* **4**, 13–22.

Murgo, A. J., Plotnikoff, N. P., & Faith, R. E. (1985). Effects of methionine enkephalins plus ZnCl2 on active T rosettes. *Neuropeptides* **5**, 367–375.

Oleson, D. R., & Johnson, D. R. (1988). Regulation of human natural cytotoxicity by enkephalins and selective opiate agonists. *Brain Behav. Immun.* **2**, 171–186.

Peterson, S. J., Robson, L. E., & Kosterlitz, H. W. (1983). Classification of opioid receptors. *Brit. Med. Bull.* **39**, 31–36.

Plotnikoff, N. P., Murgo, A. J., Miller, G. C., Corder, C. N., & Faith, R. E. (1985). Enkephalins: Immunomodulators. *Fed. Proc.* **44,** 118–127.

Rowland, R. R. R., Chukwuocha, R., & Tokuda, S. (1987). Modulation of the in vitro murine immune response by Met-enkephalin. *Brain Behav. Immun.* **1,** 342–348.

Schoffelmeer, A. N. M., Rice, K. C., & Heijnam, H. (1988). Fentanylisocyanat reveals the existence of physically associated mu and delta-class opioid receptors mediating inhibition of adenylate cyclase in rat neostriatum. *Eur. J. Pharmacol.* **149,** 179–182.

Sharp, B. M., Keane, W. F., Suh, H. J., Gekker, G., Tsukayama, D., & Peterson, P. K. (1985). Opioid peptides rapidly stimulate superoxide production by human polymorphonuclear leucocytes and macrophages. *Endocrinology* **117,** 793–795.

Simpkins, O. C., Ives, N., Tate, E., & Johnson, M. (1985). Naloxone inhibits superoxide release from human neutrophils. *Life Sci.* **37,** 1381–1386.

Speigel, M. R. (1980). *Theory and problems of probability and statistics,* pp. 263–264, 218–219. McGraw–Hill: New York.

Vann Epps, D. E., & Saland, L. (1984). Beta-endorphin and Met-enkephalin stimulate human peripheral blood mononuclear cell chemotaxis. *J. Immunol.* **132,** 3046–3052.

Vindrola, O., Padros, M. R., Sterin-Prync, A., Ase, A., Finkielman, S., & Nahmod, V. (1990). Proenkephalin system in human polymorphonuclear cells. *J. Clin. Invest.* **86,** 531–537.

Received March 6, 1991