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Inhibition of Pulmonary Metastases and Enhancement of Natural Killer Cell Activity by Methionine-Enkephalin

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Studies were performed to investigate the effects of the endogenous opioid peptide methionine enkephalin on experimental metastasis of the murine B-16 melanoma and on murine splenic natural killer cell activity. Methionine enkephalin was shown to significantly inhibit tumor metastasis and significantly enhance splenic natural killer cell activity. These results indicate that the endogenous opioids can modulate the immune response and tumor defense and that methionine-enkephalin may prove to be a beneficial adjunct to the therapy of neoplastic disease.

INTRODUCTION

The development of new and more effective means of treatment of cancer has been an area of intensive study for a number of years. Attention has been focused on a number of therapeutic measures, including chemotherapy and immunotherapy. Recently there has been increasing interest in using immunoenhancing endogenous factors as therapeutic agents, or adjuncts to therapy, in cases of neoplastic disease. Interferons, interleukin-2, and thymic hormones have all received considerable attention, and have shown some promise, in this area. Somewhat more recently, several studies have shown that the endogenous opioid peptides, endorphins and enkephalins, have immunomodulatory properties.

Following the initial report by Wybran, Appleboom, Famaey, and Govaerts (1979) that T-lymphocytes possess receptors for methionine enkephalin a number of studies have been performed investigating the effects of the enkephalins (methionine-enkephalin and leucine-enkephalin) on various parameters of immune function. Enkephalins have been shown to enhance active T-cell rosette formation in peripheral blood lymphocytes from both normal volunteers and lymphoma patients following *in vitro* treatment, stimulate the PHA induced proliferative response of murine splenic lymphocytes treated *in vitro,* cause increased thymic weights in mice following *in vivo* treatment, prolong survival time of mice innoculated with L1210 tumor cells, inhibit the local growth of B16 melanoma in challenged mice, and increase natural killer (NK) cell activity in peripheral blood lymphocytes from both normal human volunteers and cancer patients (Faith,

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Liang, Plotnikoff, Murgo, & Nimeh, 1987; Murgo, 1985; Murgo, Faith, & Plotnikoff, 1986; Zagon & McLaughlin, 1986).

It is now evident that the enkephalins can act as modifiers of a number of immune functions. Since a number of observations indicate enhancement of T-cell functions and natural killer cell activity following *in vitro* treatment of responding cells, the studies reported here were undertaken to investigate the effects of methionine enkephalin treatment on metastasis of the B16 melanoma and NK cell activity in mice.

METHODS

Animals. Four-week-old male C57BL/6 mice were purchased from either Jackson Laboratories (Bar Harbor, ME) or Harlan Sprague–Dawley, Inc. (Indianapolis, IN). Upon arrival at the research facility the animals were allowed 1 week to recover from shipping stress before being placed on study. Animals from Jackson Laboratories were utilized in studies of metastasis while animals from Harlan Sprague–Dawley, Inc. were used in the natural killer cell studies.

Test chemicals. Methionine-enkephalin used in the tumor metastasis studies was purchased from Peninsula Labs (Belmont, CA) while the methionine-enkephalin used in the NK activity studies was purchased from Sigma Chemical Co. (St. Louis, MO). Naloxone was purchased from Sigma Chemical Co. Chemicals used in the tumor metastasis studies were diluted in phosphate-buffered saline (PBS) on the day of the experiment. The methionine-enkephalin used in the NK activity studies was diluted in Hanks' Balanced Salt Solution (HBSS) and stored at −70°C until used. Mice were injected sc with test chemicals or with diluent in the case of control animals.

Tumor challenge. B16-BL6 melanoma cells were prepared from monolayer tissue culture by brief exposure to trypsin EDTA, washed, and suspended in Mg^{2+} and Ca^{2+} free PBS. Each mouse was challenged with 5×10^4 or 10^5 tumor cells iv on Day 0. The mice were sacrificed on Days 12–14, the lungs placed in formalin, and the number of metastases counted with the use of a dissecting microscope.

Natural killer cell assays. All NK cell assays were performed using freshly isolated splenic lymphocytes as effector cells. Five-week-old male C57Bl/6 mice were injected with varying doses of methionine-enkephalin and 18 h later the mice were sacrificed by cervical dislocation. Their spleens were removed aseptically and pooled in groups of two. Single cell suspensions were prepared from these spleens and used as effector cells in NK assays. Both the YAC-1 and B16 melanoma tumor cell lines were used as target cells for these assays.

The NK cell assays were performed as previously described (de Landazuri, Lopez-Botet, Timonen, Ortaldo, & Herberman, 1981). Aliquots containing 2×10^6 target cells/ml were labeled with 150 μCi of ^{51}CR solution/ml of cells (Amersham, Arlington Heights, IL) by incubation at 37°C in 5% CO_2 in air for 45 min. After washing the cells three times, 5×10^3 viable cells in 0.1 ml of RPMI 1640 medium were pipetted into 96-well Linbro plates (Linbro Scientific, Hamden, CT). Various effector cell concentrations in 0.1 ml of RPMI 1640 medium were added to triplicate wells to give effector:target ratios of 200:1, 100:1, and 50:1. After incubation at 37°C in 5% CO_2 in air for 4 h, 100 μl of supernatant from each

well was collected and counted for 2 min in a Packard autogamma counter. The percentage of isotope released was used as a measure of cytotoxicity and was calculated by the formula:

$$\% \text{ specific release} = \frac{\text{cpm experimental release} - \text{cpm from medium control}}{\text{cpm maximum release} - \text{cpm from medium control}} \times 100,$$

where cpm experiment release = counts released after incubation of target cells with effector cells, cpm from medium control = counts spontaneously released by target cells incubated in medium alone, and cpm maximum release = counts released by lysis of target cells with 1% Triton X-100.

Statistical analyses. Several statistical tests were employed to analyze the data generated in these studies. The χ^2 test was used to determine differences in the incidence of metastases between treatment groups, the Mann–Whitney test was used to determine differences in number of metastases between treatment groups, and the Student *t* test was used to determine differences in NK activity between treatment groups.

RESULTS

Effect of Methionine-Enkephalin on Tumor Metastasis

The effect of various doses of methionine-enkephalin on the development of experimental pulmonary metastases with B16 melanoma is shown in Table 1. The number of metastatic lesions was significantly reduced in mice treated with 0.5 mg (30 mg/kg) of enkephalin. Table 2 illustrates results obtained when mice were treated with naloxone and methionine-enkephalin or naloxone alone. Naloxone suppresses the inhibitory effect of methionine-enkephalin on tumor metastasis but when given alone also inhibits tumor metastasis. Naloxone is an antagonist of many opioid effects, but as seen here, it is not uncommon for naloxone to mimic opioid effects when given alone (Murgo et al., 1986).

TABLE 1
Effect of Methionine-Enkephalin on Pulmonary Metastasis

Dose (mg/animal)[a]	Incidence (%)	*p*	Number of metastases median (range)	*p*
0	16/20 (80)		3.0 (0–16)	
0.05 (3.0)	8/10 (80)	NS	4.0 (0–10)	NS
0.2 (12.0)	9/10 (90)	NS	3.0 (0–18)	NS
0.5 (30.0)	11/20 (55)	<.1	1.0 (0–6)	<.005

Note. Methionine-enkephalin was administered to 5-week-old male C57BL/6 mice sc on Day −1. B16-BL6 melanoma cells were prepared from monolayer tissue culture by brief exposure to trypsin–EDTA, washed, and suspended in Mg^{2+} and Ca^{2+} free PBS. Each mouse was challenged with 5×10^4 tumor cells iv on Day 0. The mice were sacrificed on Day 14; the lungs were placed in formalin and the number of metastases counted with the use of a dissecting microscope. The *p* values indicate differences from control. Differences in incidence were determined by χ^2 while differences in number of metastases were determined using the Mann–Whitney test. NS = not significant.

[a] Numbers in parentheses indicate approximate dosage in milligrams per kilogram body weight.

TABLE 2
Effect of Methionine-Enkephalin and Naloxone on Pulmonary Metastasis

Group	Incidence (%)	p	Number of metastases median (range)	p
PBS (control)	8/10 (80)		4.5 (0–24)	
(Met)Enk alone	3/10 (30)	<.05	0 (0–1)	<.007
Naloxone + (Met)Enk	7/10 (70)	NS	1.5 (0–10)	NS
Naloxone alone	4/10 (40)	<.1	0 (0–8)	<.049

Note. Naloxone (1.0 mg/animal or approximately 60.0 mg/kg body wt) and methionine-enkephalin, (Met)Enk, (0.5 mg/animal or approximately 30 mg/kg body wt) were administered to 5-week-old male C57Bl/6 mice on Day −1. Control mice received injections of diluent (PBS). B16-BL6 melanoma cells (10^5) were injected iv on Day 0. Half of the mice from each group were sacrificed on Days 12 and 14 and the number of lung metastasis was determined. The p values indicate differences from control. Differences in incidence were determined by χ^2 while differences in number of metastases were determined using the Mann–Whitney test. NS = not significant.

Effect of Methionine-Enkephalin on Natural Kill Cell Activity

To investigate the effects of methionine-enkephalin on NK activity, C57Bl/6 mice were injected with varying doses of enkephalin and 18 h later their spleens were removed and splenic NK activity was determined. Two target cells were used in these studies. These were the YAC-1 tumor cell line and B-16 melanoma cells. Several dosage levels of methionine-enkephalin resulted in significant enhancement of splenic NK activity at three different effector:target cell ratios (200:1, 100:1, and 50:1) against both target cell lines (Fig. 1). Enhancement of NK activity against the B-16 melanoma cells tended to occur at higher dosage levels of enkephalin than that for YAC-1 cells.

DISCUSSION

The results of these studies are similar to other studies which have shown other endogenous factors to inhibit tumor metastasis. The thymic factor, thymostimulin, has been shown to inhibit growth and pulmonary metastasis of Lewis lung carcinoma (Yagi, Tamashita, & Tsubura, 1985); recombinant human interferon has been shown to inhibit melanoma metastasis in mice (Yokoyama, Yoshie, Aso, Ebina, and Ishida, 1986); and recombinant Interleukin-2 has been shown to inhibit B-16 melanoma metastasis in mice (Saijo, Ozaki, Nakano, Sahurai, Takahashi, Sasaki, & Hoshi, 1986). The model system utilized in these studies may appear to be somewhat overly sensitive to some. While the percentage of animals developing metastases was reasonable in control animals, the number of metastases per animal (0–24) was somewhat lower than desirable. This may have been a result of the tumor line used (B16-B6). Recent reports indicate a range of 13–40 metastases per animal when a similar number of B16-F10 melanoma cells was injected (2.5×10^4 as compared to 5.0×10^4 B16-B6 cells) into C57BL/6 mice (Hanna & Burton, 1981). We plan to repeat these studies using the B16-F10 cell line in an attempt to increase the number of metastases in control animals. Additionally, some may argue that the age of the mice when placed in the study (5 weeks old) may increase the sensitivity of the system because of the correlation between age and NK cell

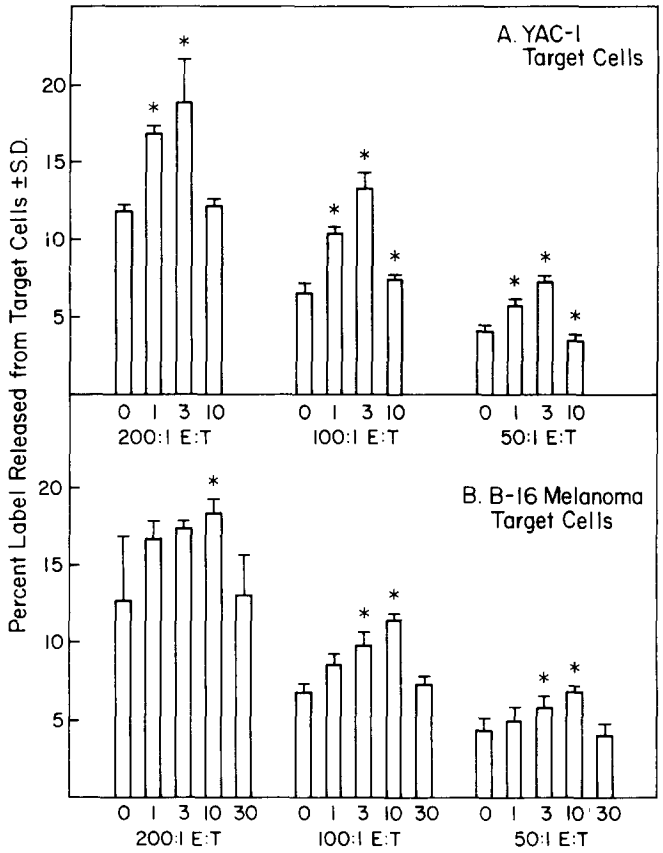

FIG. 1. Effect of methionine-enkephalin on splenic NK cell activity in C57Bl/6 mice. Five-week-old male C57Bl/6 mice were injected with varying doses of methionine-enkephalin (0, 1, 3, 10, or 30 mg methionine-enkephalin/kg body wt). Eighteen hours later the mice were sacrificed by cervical dislocation. Their spleens were removed aseptically and pooled in groups of two. Single cell lymphocyte suspensions were prepared from these spleens and used as effector cells in NK assays. Both the YAC-1 and B-16 melanoma tumor cell lines were used as target cells for these assays. Target cells were labeled with ^{51}Cr by incubation in Na $^{51}CrO_4$ solution. NK activity of effector cells was determined by a 4-h cytotoxicity assay performed in round-bottomed microtiter plates. Assays were performed at effector:target cell ratios of 200:1, 100:1, and 50:1. The results are reported as the mean percentages of specific label released from target cells for each of the treatment groups. The data shown represents pooled data from two experiments wherein there were six animals in each dosage group. The bars indicate the mean values obtained for each group and the vertical lines represent the SD. The dose of methionine-enkephalin for the group is represented by the numbers below the bars. *Significant difference ($p < .05$) from the group receiving no methionine-enkephalin. These differences were determined using Student's t test.

activity in mice. NK cell activity in murine splenic cells shows definite age relationships and the mice used in the NK portions of these studies were of the proper age to exhibit maximal splenic NK activity. It has been reported that NK activity in peripheral blood lymphocytes of mice matures around 4 weeks of age and is maintained in circulation throughout a major portion of adult life (Lanza & Djeu,

1982), indicating that the age of the mice used for the tumor metastases studies should not be a factor in the sensitivity of these studies.

The population of lymphocytes known as NK cells are capable of spontaneous cytotoxicity against a variety of cell targets *in vitro*. While it is not entirely certain what the *in vivo* function of these cells are, there are indications that their functions include anti-viral and tumor surveillance activities (Hanna & Burton, 1981; Herberman & Holden, 1978; Herberman & Ortaldo, 1981). In addition to these observations, there has been considerable interest recently in the use of lymphokine activated killer cells (LAK) as a method of cancer immunotherapy (Ettinghausen & Rosenberg, 1986; Lafreniere & Rosenberg, 1985; Yang, Mule & Rosenberg, 1986). These cells appear to offer considerable promise for immunotherapy of certain types of neoplasms. Recent evidence indicates that LAK cells are not a unique cell type, but rather a functional phenomenon, and that most of the peripheral blood, splenic, and bone marrow LAK activity is mediated by interleukin-2 activated NK cells (Lotzova & Herberman, 1987). This implies that NK cells may be of special interest to the studies reported here. *In vitro* treatment of human peripheral blood lymphocytes with the enkephalins has been shown to enhance NK cell activity in both normal volunteers and cancer patients (Faith, Liang, Murgo & Plotnikoff, 1984; Faith et al., 1987) and now *in vivo* treatment with metenkephalin has been shown to enhance murine splenic NK cell activity. The inhibition of tumor growth following enkephalin treatment reported earlier (Murgo, 1985) and the inhibition of tumor metastasis reported here may be due to enkephalin-induced enhancement of NK cell activity or to some other, as yet undetermined, effect of enkephalin treatment. Further studies are required to determine the mechanism(s) involved. In the observations reported here, the optimal dose of Met-enkephalin for inducing splenic NK cell activity was different from that which inhibited tumor metastasis. There are several possible explanations for this observation: (1) peripheral blood NK cells would presumably be more important in inhibiting the metastases, and optimal doses of enkephalin for enhancing peripheral NK activity may be different than for enhancing splenic NK activity; (2) the mice used for NK cell activity and tumor metastasis were purchased from different sources and conceivably could exhibit different dose responses to Met-enkephalin; and/or (3) the source of Met-enkephalin used for NK cell activity and tumor metastasis studies was different, and while very unlikely (since both formulations are greater than 99.9% pure) might result in differences in dose response. Studies are planned to resolve these issues and to determine whether NK cells are of prime importance in inhibiting tumor metastases in this model system.

For the studies reported here, it was decided to assess NK cell activity early after enkephalin dosing. This corresponds to the NK cell activity state that tumor challenged animals were in at the time of iv tumor challenge. This presumably is the time when NK cell activity would be most critical in inhibiting tumor metastasis. It is felt that perhaps the multiple enkephalin dosing, both prior to and following tumor challenge, might result in a greater inhibition of tumor metastasis. Studies are planned to investigate this possibility and to assess NK cell activity at various times following multiple enkephalin treatments.

Evidence now indicates that while NK activity is normal in some patients with solid tumors it is depressed in other patients, especially those with lymphoid tumors and also in individuals at high risk of developing cancer (Lipinski, Dokhelar & Tursz, 1982; Lotzova, Savary, Keating, & Hester, 1985; Platsoucas et al., 1980; Pross, Rubin, & Baines, 1982). This leads to the belief that agents which enhance NK activity may be beneficial in the treatment of neoplastic disease. While the data is still preliminary, the authors believe that there is sufficient evidence to continue to investigate the potential usefulness of the enkephalins as adjuncts in the therapy of neoplastic disease. The authors believe that the enkephalins may prove to be useful adjuncts in the therapy of neoplastic disease. This belief is based on the fact that the enkephalins have been shown to enhance a number of cellular immune functions (Faith et al., 1987; Murgo, 1985; Murgo et al., 1986) and inhibit local tumor growth (Murgo, 1985) and spontaneous metastases (Scholar, Violi, & Hexum, 1987). Furthermore, the studies reported here demonstrate that experimental pulmonary metastases can be reduced and splenic NK cell activity enhanced by pretreatment with methionine-enkephalin. The fact that the enkephalins enhance NK cell activity is of interest in the inhibition of tumor metastasis as NK cells have been shown to have the ability to inhibit metastasis of some tumors (Barlozzari, Leonhardt, Wiltrout, Herberman, & Reynolds, 1985; Hanna & Burton, 1981). In addition, helper T-cells have recently been shown to produce methionine-enkephalin (Zurawski, Benedik, Kamb, Abrams, Zurawski, & Lee, 1986) and enkephalin has been shown to induce interleukin-2 receptors on T-cells and possibily induce release of interleukin-2 (Plotnikoff, Wybran, Nimeh, & Miller, 1986).

Finally, a comment regarding the observation of the effect of naloxone seems necessary. As shown in Table 2, naloxone is an antagonist of Met-enkephalin but exhibits agonistic effects when given alone. This is not an uncommon observation. Naloxone has shown agonistic activity similar to β-endorphin and Met-enkephalin in inhibiting tumor growth (Murgo, 1985), in inhibiting T-lymphocyte chemotactic factor (Brown and Van Epps, 1985), and in enhancing the generation of cytotoxic T cells (Caar and Klimpel, 1986). Both naloxone and Met-enkephalin bind the opioid receptor, but it is believed that naloxone may bind to a different region on the receptor than Met-enkephalin binds (Thorpe, 1984). It may be that the binding of one or the other compound results in conformational changes in the receptor that induces agonistic activity while binding of the receptor by both compounds results in an antagonistic outcome.

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