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METHIONINE ENKEPHALIN: IMMUNOMODULATOR IN NORMAL VOLUNTEERS, CANCER AND AIDS PATIENTS

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Clinical studies of the immunological effects of methionine enkephalin in normal volunteers, cancer, and AIDS patients are summarized. The major immunology changes seen were increases in T cell subsets, natural killer cell activity, as well as mitogen blastogenesis. Clinically, the cancer and ARC patients did not develop infections.

Recent reviews on the subject of neuropeptides and psychoneuroimmunology have emphasized the need to extrapolate basic science data to the clinical level (Plotnikoff et al., 1986a, b). Thus the enkephalins-endorphins have been shown to stimulate mitogen-induced lymphocyte proliferation and NK cell activity *in vitro*, (Murgo et al., 1986). In addition, more recently Wybran & Schandene (1986) have shown, *in vitro*, that methionine enkephalin increases numbers of T cell subsets including OKT 3, OKT 4, OKT 8, OKT 9, OKT 10, and OKT 11. Finally a number of studies appeared showing that chemotaxis was also enhanced by various neuropeptides (Pert et al., 1986). Enkephalin-endorphin studies on antibody formation indicated biphasic effects, augmentation at low doses and suppression at high doses (Johnson et al., 1982; Jankovic & Maric, 1986).

MATERIALS AND METHODS

Methionine Enkephalin – Methionine enkephalin (Sigma-Aldrich Chem. Co., Sr. Louis, MO) was dissolved in sterile saline and filter sterilized by the hospital pharmacy. It was dissolved in 60 ml of saline at a concentration sufficient to infuse at 2 ml minute for 30 minutes to give a total delivered dose of 10 to 25 micrograms per kilogram per body weight.

Cell preparations – Heparinized peripheral blood was collected, incubated with 25 microliters of a 1:100 dilution of latex bead preparation/10^6 cells for 30 minutes at 37C, diluted with phosphate buffered saline (PBS), layered over a Ficoll-Paque (Pharmacia) gradient and centrifuged for 30 minutes at 400 x g. The mononuclear cell band was collected, washed twice with PBS. resuspended in RPMI and quantified.

Active T cells – An equal volume of 1 x 10^6 lymphocytes and heat inactivated fetal calf serum was incubated at 37 C for 60 minutes. The fetal calf serum had been absorbed against both sheep and human AB erythrocytes. The lymphocytes were then gently mixed with an equal volume of sheep red blood cell (SRBC) at 4 x 10^7 /ml suspended in saline, centrifuged at 200 x g at 4 C for 5 minutes, gently resuspended and rosettes quantified using a hemacytometer. An active rosette forming cell is defined as a lymphocyte which has bound three or more SRBC.

T lymphocytes and T subsets – 10^6 cells in 50 microliters PBS with 0.1% sodium azide was incubated for 30 minutes at 4 C with monoclonal antibodies [MoAb-S (10 micrograms/ml)]. The cells were washed twice, incubated with FITC labeled goat anti-more serum at 4 C for 30 minutes, washed twice again with PBS with sodium azide and gently resuspend in 1 drop of PBS with the soduim azide and quantified under a Nikon epi-illumination fluoresent microscope. Three hundred cells were counted: the monoclonal antibodies utilized were OKT 3, OKT 4, OKT 8, and OKT 11 (Ortho Pharmaceu., N.J.).

Lymphocyte proliferation assay – Lymphocyte proliferation, stimulated by Phytohemagglutinin (PHA), pokeweed mitogen (PWM). concanavalin A (con A) and Staphylococcal Protein A was performed in triplicate. The mitogens, in varying concentrations, were plated with 1 x 10^5 cells per well in 96 microculture plates and were incubated for 96 hours. Sixteen hours before harvesting, 1 microCurie of ^{3}H thymidine (2 Ci/mmole; Amersham, Arlingotn Heights, Bethseda, MD) was placed in a suction-filter apparatus and the activity of tritium bound to acid insoluble material was quantified in a liquid scintillation spectometer.

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Natural Killer (NK) cell assay – K562 target cells, adjusted to 2 x 10^6 cells in 0.2 ml RPM 1640, were labeled with $Na^{51}CrO_4$ (1 mCi) for 45 minutes at 37 C in 5% CO_2. The cells were washed twice, resuspended in 2 ml RPMI 1640 and adjusted to 5 x 10^6 cells per ml. Peripheral blood lymphocytes were collected on a Ficoll-Paque gradient, washed twice and adjusted to 5 x 10 cells/ml and 3 serial 3 fold dilutions made. An equal volume of effector cells and target cells were added to wells on microtiter plates. These cell mixtures were incubated at 37 C in 5% CO_2 for 4 hours. A 0.1 ml aliquot was collected from each of the wells and quantified. The killing capabilities of the NK cells was calculated as follows:

$$\text{NK ratio} = 1 - \frac{\text{Experimental counts} - \text{SR} \times 100}{\text{Total counts} - \text{SR}}$$

where SR = spontaneous release of ^{51}Cr.

Interleukin 2 receptor – Lymphocytes collected from a Ficoll-Paque gradient were incubated with Phytohemagglutinin for 96 hours, the cell harvested, washed twice and incubated with anti-human IL-2 receptor (Becton-Dickinson, Mtn. View, Calif.) for 30 minutes at 4 C. The cells were washed twice with PBS, with sodium azide, incubated with FITC goat anti-mouse antibody, washed twice with PBS, resuspended in 1 drop of PBS with sodium azide and examined under the fluroescent microscope. Three hundred cells were counted.

RESULTS

Our group initiated *in vivo* studies of methionine enkephalin in normal volunteers in 1983 (Plotnikoff et al., 1986a,b). We studied the immunobiological effects of methionine enkephalin, at doses of 1, 10, 50, 100, 150, 200 and 250 micrograms/kg in a 30 minute saline infusion, on T cell subsets and NK cell activity as well as classical pharmacological parameters including behavioral effects.

No significant effects were seen in terms of blood pressure, heart rate, EKG, respiration, body temperature, or neurological reflexes. This finding is illustrated in Table I showing the evaluation of a dose of 150 micrograms/kg of methionine enkephalin. However, it is interesting to see that a certain degree of mood elevation was observed with the infusion of methionine enkephalin (Table II).

TABLE I

Normal Volunteer (150 micrograms/kg)/Clinical Pharmacology

	HR S/E	BP	BP	Temp	Resp
3/11/84					
8:00 a.m.	62/76	120/80	130/80	97.4	14
8:55 a.m.	76/80	120/80	130/80	98.2	13
9:00 a.m.	Methionine Enkephalin Infusion 150 micrograms/kg				
9:10 a.m.	90	120/80		98	13
9:20 a.m.	81	120/80		98	12
9:30 a.m.	73	120/80		98	12
10:00 a.m.	74/102	120/80	130/80	98	14
11:00 a.m.	87/100	120/80	130/80	98	12
1:00 p.m.	74/80	120/80	130/80	98	13
4:00 p.m.	76/80	120/80	130/80	98	12
3/12/84					
8:55 a.m.	72/80	120/80	130/80	98	13

The most significant changes seen were in the immunological parameters studied. The actual data is shown in Table III. Significant increases were seen in the numbers of lymphocytes, T cell rosettes, OKT 3, OKT 4, OKT 8, and OKT 11, as well as, mitogen (PHA, Con A, Pokeweed) induced proliferation. Similar effects of methionine enkephalin were seen at all doses treated.

Cancer patients – In a series of *in vitro* studies, our group found that methionine enkephalin could increase numbers of T cells forming SRBC rosettes in lymphoma patients and augment NK cell activity in a large variety of cancers (Table IV).

Similar effects were also seen *in vivo* in a number of cancer patients including melanoma,

hypernephroma, and lung cancer. An example of the findings are shown in Table V. As can be seen there are significant increases in numbers of lymphocytes and T cell subsets following methionine enkephalin infusion.

Arc-AIDS Kaposis sarcoma patients – These studies were based on our earlier findings that methionine enkephalin increased the number of OKT 4 cells in normal voluteers. Since the OKT 4 helper T cell is the major target of the HIV (AIDS virus), it seemed appropriate to test the effects of methionine enkephalin in AIDS patients. Our initial studies were in Kaposis Sarcoma patients whom we followed over a time

TABLE II

Normal Volunteer/Mood Scale – 100 mm Line Test/ Methionine Enkephalin – 150 micrograms/kg

Time	Millimeters
8:00 a.m.	22
8:30 a.m.	50
8:55 a.m.	50
10:00 a.m.	45
11:00 a.m.	32
1:00 p.m.	17
4:00 p.m.	46
8:00 a.m.	22

TABLE III

Normal Volunteers (150 micrograms/kg) Immunological Parameters

	0hr	2hr	24hr
Lymphocytes			
Percent	32%	45%	38%
Absolute	1760/mm^3	2430/mm^3	2660/mm^3
B Lymphocytes (smIg)			
Percent	16%	15%	15%
Absolute	282/mm^3	365/mm^3	399/mm^3
T Lymphocytes (E rosettes)			
Percent	55%	69%	52%
Absolute	968/mm^3	1677/mm^3	1383/mm^3
T Lymphocytes (OKT 11)			
Percent	81%	82%	73%
Absolute	1426/mm^3	1993/mm^3	1942/mm^3
T Helper Lymphocytes (OKT 4)			
Percent	47%	44%	45%
Absolute	827/mm^3	1069/mm^3	1197/mm^3
T Suppressor Lymphocytes (OKT 8)			
Percent	30%	19%	28%
Absolute	528/mm^3	462/mm^3	745/mm^3
T Helper/T Suppressor Ratio	1.566	2.134	1.607
NK Cells (Leu-7)			
Percent	22%	23%	20%
Absolute	387/mm3	559/mm^3	532/mm^3
Blastogenesis			
Phytohemagglutinin	151X	152X	176X
Concanavalin A	215X	292X	179X
Pokeweed	23X	33X	19X
Staphylococcus protein A	4.4X	8.0X	1.9X

The absolute increase of lymphocytes at the 2 hr time period was due to a proportional increase in the T and B lymphocytes plus NK cells. The increase of T lymphocytes was due to an increase of T helper cells. The T suppressor cells did not increase until the 24 hr specimen. This observation is evident in the T helper to T suppressor ratio at the 2 and 24 hr periods. The function of the lymphocytes was demonstrated by mitogen stimulated blastogenesis. PHA, a T helper cell mitogen, was identical at the 2 hr period and a slight increase at the 24 hr period. Con A, a T helper and T suppressor cell mitogen was increased at the 2 hr period while being slightly decreased at the 24 hr period. Pokeweed, a T dependent B cell mitogen, and Staphylococcus aureus Cowan strain I, a B cell mitogen, mimicked the Con A results. The suppressor cells increased at the 24 hr period and obviously expressed their influence on the latter three mitogen's blastogenesis. The relative level of NK cells remained consistent throughout the 24 hr period but the absolute number was increased.

TABLE IV

Types of Cancer Patients Treated with Methionine Enkephalin

Lung Carcinoma	Non-Hodgkin's Lymphoma
Breast Cancer	Hodgkin's Lymphoma
Ovarian Cancer	Large Cell Lymphoma
Gastric Carcinoma	Lymphocytic Lymphoma
Thyroid Carcinoma	Lymphoma
Acute Myelocytic Leukemia	Diffuse Histiocytic Lymphoma
Chronic Myelogenous Leukemia	Nodular Histiocytic Lymphoma
B-Cell Lymphoma	

TABLE V

Melanoma Patient Recieving Methionine Enkephalin (40 micrograms/kg)

	1/24	2/19
WBC	11,100	8,000
Lymphocytes		
Percent	13%	15%
Absolute	1443/mm^3	1200/mm^3
T Lymphocytes and Subsets		
T Lymphocytes (OKT 3)		
Percent	60%	84%
Absolute	865/mm^3	1008/mm^3
T Lymphocytes (OKT 11)		
Percent	78%	87%
Absolute	1126/mm^3	1044/mm^3
T Helper Cells (OKT 4)		
Percent	26%	52%
Absolute	375/mm^3	624/mm^3
T Suppressor (OKT 8)		
Percent	47%	42%
Absolute	678/mm^3	504/mm^3
T helper/T suppressor ratio	0.6	1.2

TABLE VI

AIDS/Kaposis Sarcoma Patient Recieving Methionine Enkephalin (10 micrograms/kg)

	1/27	2/26
WBC	2500/mm^3	3300/mm^3
Lymphocyte	725/mm^3	726/mm^3
T Lymphocytes and Subsets		
T Lymphocyte (OKT 3)		
Percent	73%	63%
Absolute	529/mm^3	457/mm^3
T Lymphocyte (OKT 11)		
Percent	78%	81%
Absolute	566/mm^3	588/mm^3
T Helper (OKT 4)		
Percent	9%	12%
Absolute	65/mm^3	87/mm^3
T Suppressor (OKT8)		
Percent	63%	57%
Absolute	457/mm^3	414/mm^3
T helper/T Suppressor Ratio	0.1	0.2

period of several months. The patients had a perceptible elevation of mood, gained weight, and were found to have a healing (crusting) of suppurating Kaposis Sarcoma nodules. Although the nodules healed and the dark coloration and the size of the nodules diminished, methionine enkephalin did not prevent the appearance of new nodules. The immunological parameters measured all increased in number and shown in Table VI. Most striking, in this regard, were the increases, seen in T cell subsets as well as mitogen-induced proliferation.

AIDS related complex (ARC) patients experiencing fevers, diarrhea, weight loss, lymphadenopathy, and immunological deficiencies showed the greatest response to methionine enkephalin treatment. All of these patients showed several of the ARC symptons. All of the patients gained weight and were able to resume full time employment. Currently they are being maintained on infusions of methionine enkephalin once a week (Wybran et al., 1986). The greatest increases seen were in PHA-induced proliferation as well as NK cell activity shown in Figs. 1 and 2. T cell subsets were also seen to increase.

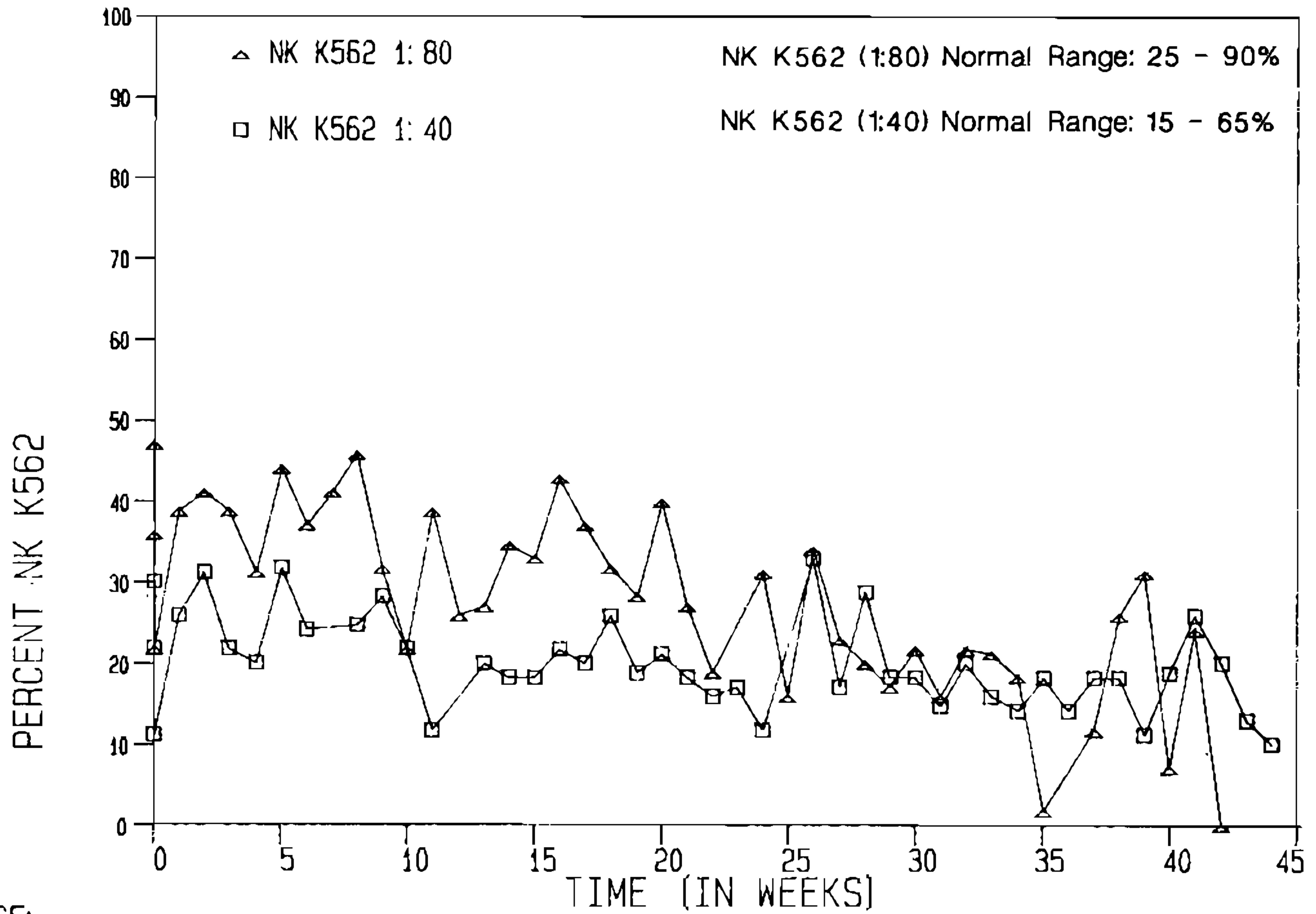

Fig. 1: Natural killer (NK) activity of peripheral blood lymphocytes from ARC patients as a function of treatment with methionine-enkephalin (MEK). The cell line K562 was used as target for measuring NK activity.

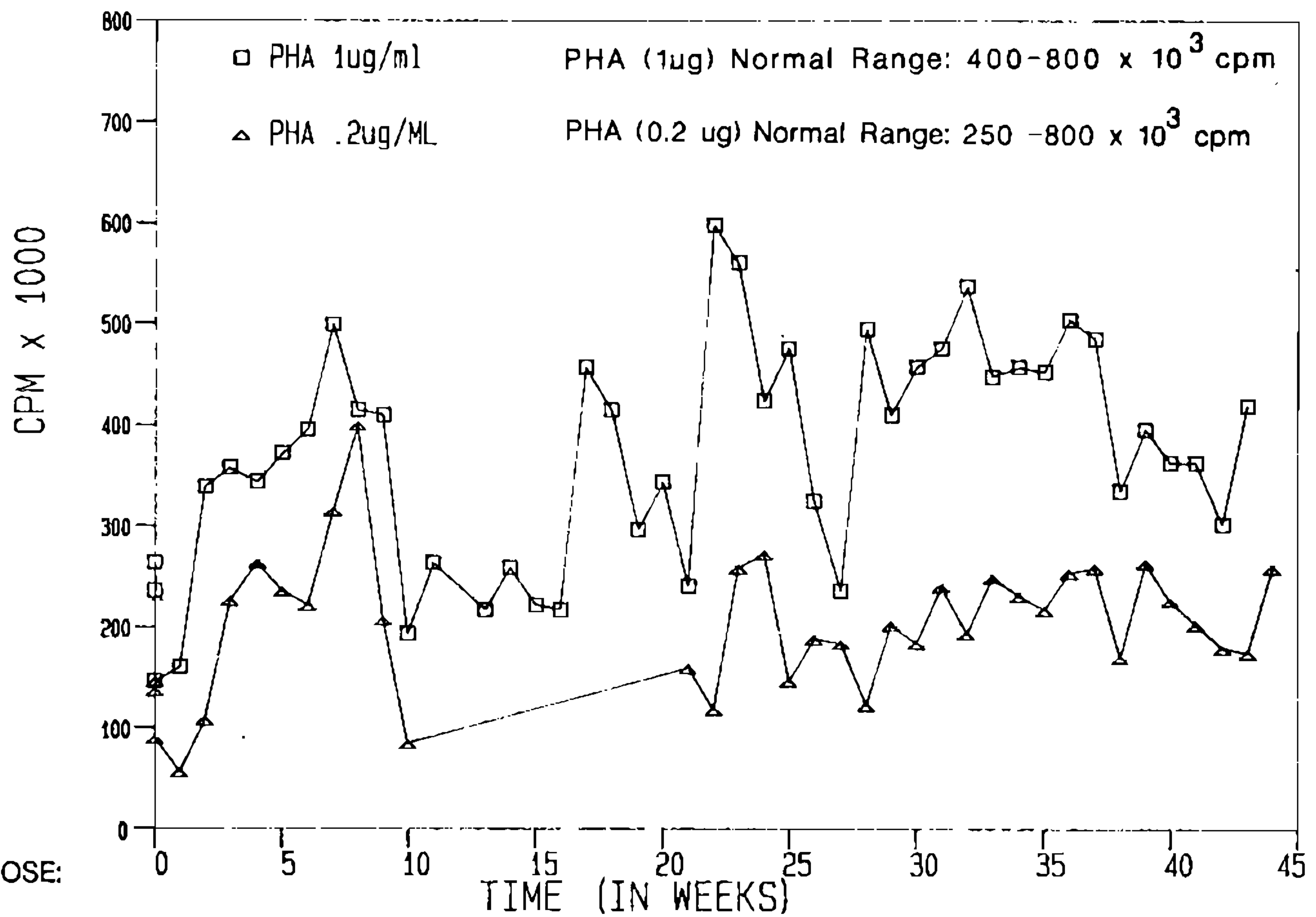

Fig. 2: Phytohemagglutinin (PHA) mitogenic response of lymphocytes from ARC patients, alongy with treatment with methionine enkephalin (MET).

DISCUSSION

Our studies in normal volunteers, cancer patients, as well as AIDS patients are confirmatory of one another in that methionine enkephalin augmented the number of lymphocytes and in particular, the T cell subsets (OKT3, OKT4, OKT8, OKT11) and blastogenesis to the mitogens PHA, ConA, and pokeweed. In addition, methionine enkephalin treatment enhanced NK cell activity as measured by K562 cells or by Leu 7 marker. The mechanism(s) of action are complex since lymphokine production (IL-I, IL-II, and gamma-interferon) are increased by methionine enkephalin treatment (Brown & Van Epps, 1985; Plotnikoff et al., 1985; Youkilis et al., 1985). In addition, Zurawski et al. (1986) recently demonstrated that T helper cells express pre proenkephalin A (in the presence of a mitogen) and release methionine enkephalin into the media. Thus, methionine enkephalin itself can now be classified a lymphokine being released from cells of the immune system. Similar findings have also been documented for beta-endorphin and ACTH by Smith & Blalock (1986). The enkephalins have also been found to modulate cycle nucleotides, calcium, and prostaglandins as well as catecholamines (Foris et al., 1984).

Our clinical studies with methionine enkephalin raise the possibility that this immunomodulator may be useful in the treatment of immunodeficiences associated with disease (AIDS), cancer treatments (chemotherapy) or resistant infections (antibiotics). If disease states and/or stress can alter processing of the prohormones (POMC, proenkephalin A, and prodynorphin), then the immune system as well as the central nervous system may require replacement therapy (Bloom, 1983; Lewis & Stern, 1983; Akil et al., 1984). Our clinical studies suggest that methionine enkephalin may serve this role in the treatment of cancer and AIDS patients (Evans et al., 1986; Udenfried & Kirkpatrick, 1984; Hollt, 1986; Weber & Pert, 1984).

We invite you to join us in this new approach to immunotherapy employing prohormone fragments (Brown et al., 1986; Ruff & Pert, 1986).

CONCLUSIONS

Our clinical studies of methionine enkephalin in normal volunteers included in a wide dosage range of 1 to 250 micrograms/kg.

No significant effects were seen in terms of blood pressure, heart rate, EKG, respiration, body temperature, or neurological reflexes. However, there were significant increases in T cell subsets, NK cell activity, as well as, mitogen -induced proliferation. Similar effects were in a variety of cancer patients as well as ARC (AIDS) patients. In ARC patients, constitutional symptoms (fevers, weight loss, and lymphadenopathy) were eliminated and immunological competence improved. It is our hope that methionine enkephalin may be found useful in the treatment of various immunodeficient states.

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