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ALTERATION OF T AND NULL LYMPHOCYTE FREQUENCIES IN THE PERIPHERAL BLOOD OF HUMAN OPIATE ADDICTS: *IN VIVO* EVIDENCE FOR OPIATE RECEPTOR SITES ON T LYMPHOCYTES[1]

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Street opiate addiction produces a significant depression in the absolute number of total T lymphocytes in peripheral blood as measured by the ability of the lymphocytes to rosette sheep red blood cells (SRBC). Associated with the decrease in T cells, there is an increase in the absolute number of null lymphocytes but no significant changes in B lymphocytes or total white blood cell count. The T cell values for 2 different populations of addicts (n = 12 and 32) are 31.8% and 23.1%, whereas the null cell values are 51.1% and 57.6%, respectively. The values for comparable control populations (n = 18 and 10) are: T% = 70.7% and 67.4%, and null % = 9.2% and 14.5%. Self-reported use of marihuana does not significantly alter the distribution of cell populations. A 1- to 3-hr incubation of addict-derived lymphocytes with 10^{-6} to 10^{-7} M Naloxone reverses both T cell depression and null cell increase by allowing the null cells to express SRBC receptors. Cyclic AMP and dibutyryl cyclic AMP can also convert the null cells to T cells. The conversion of null to T lymphocytes has additionally been measured by monitoring the increase in PHA-stimulated growth in 72-hr cultures as determined by tritiated thymidine incorporation into DNA. These results support the hypothesis that opiates can alter T lymphocyte number and function *in vivo*, and that this alteration may produce a significant degeneration in the immune competence of street opiate addicts.

Lymphocytes from street opiate addicts have been shown to exhibit a variety of dysfunctions, including a severe reduction in their ability to respond to mitogenic stimulation by PHA, pokeweed mitogen, or Con A (1). Since these mitogens, particularly PHA and Con A, primarily affect T lymphocytes, the conclusion was drawn that T lymphocyte function was altered in street opiate addicts. Recently, Wybran *et al.* (2) showed that morphine and met-enkephalin reduced the frequency of low-avidity (active) human T lymphocytes *in vitro*. This effect was reversible by incubation with (−)-naloxone, which suggested that some of the T lymphocytes might have opiate receptor sites analogous to those demonstrated to exist on neuronal cells. Lopker *et al.* (3), using radioisotopically labeled dihydromorphine, levorphanol, and dextrorphan, showed that phagocytic leukocytes had stereospecific opiate receptor sites. β-Endorphin, on the other hand, bound to cultured lymphocytes, but the binding was not antagonized by naloxone, morphine, or a variety of enkephalin analogs (4). These results confirmed the hypothesis that leukocytes can be a prime binding site for exogenous opiates or endogenous opioids in the peripheral circulating system.

We would now like to report a significant decrease in total T lymphocytes *in vivo* in street opiate addicts, a concomitant increase in null lymphocytes, and the ability of (−)-naloxone to reverse both of these effects *in vitro*.

MATERIALS AND METHODS

Sources of blood. Two 10-ml tubes of heparinized peripheral blood were drawn from controls and from opiate addicts beginning drug therapy in Georgia, Illinois, and Massachusetts. Each control and patient completed a medical history form, including drug usage information, and signed an informed consent form. The blood samples and forms were coded by number to guarantee the objectivity of the study and privacy of the subjects and controls. Blood from local Atlanta addicts was obtained from the Central Intake Unit, Georgia Drug Abuse Program, and was assayed within 24 hr of drawing. Blood from addicts was also obtained from the Illinois Dangerous Drugs Rehabilitative Systems, Chicago Central Intake Unit, and the Alcohol and Drug Abuse Research Center, McLean Hospital, Belmont, MA; flown to Atlanta by United States Postal Express or Delta Dasher; and assayed usually within 24 hr of drawing. Control bloods were obtained from staff members at the Georgia Mental Health Institute and the Illinois Dangerous Drugs Rehabilitative Systems.

Separation of lymphocytes and quantitation of T, B, and null lymphocytes. Heparinized blood was diluted 1:2 in ABG buffer.[3] The diluted blood was layered over Ficoll-Paque (Phar-

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[3] Abbreviations used in this paper: cAMP, adenosine-3′5′-cyclic monophosphate; dBcAMP, N^6,$O^{2'}$-dibutyryladenosine-3′5′-cyclic monophosphate; ^{3}H-dT, tritiated thymidine; POPOP, *p*-bis[2-(5-phenyloxazolyl)]benzene; PPO, 2,5-diphenyloxazole; far UV, ultraviolet light (200 to 300 nm); ABG buffer, buffer containing 50 μM $CaCl_2$, 980 μM $MgCl_2$, 5.4 mM KCl, 0.145 M NaCl, 0.1% glucose, 14.5 mM Tris, pH 7.6;

TABLE I

White blood cell number and lymphocyte percentages in opiate addicts and controls

	Cell No. or % (± SD)					
	Atlanta		Chicago		F[a]	p ≤
	Controls	Addicts	Controls	Addicts		
Population	18	12	10	32		
WBC	7755	7208	6665	7365		
	(862)	(1674)	(1620)	(1873)	0.03	0.85
B cells	17.7	16.1	17.8	16.4		
	(3.0)	(3.9)	(3.0)	(3.1)	3.33	0.07
T cells	70.7	31.8	67.4	23.1		
	(5.1)	(8.3)	(3.8)	(9.3)	429.7	0.0001
Null-cells	9.2	51.1	14.5	57.6		
	(4.0)	(9.5)	(3.7)	(9.0)	467.1	0.0001

[a] Two way analysis of variance (7).

macia Fine Chemical) and centrifuged at 400 × g for 30 min at room temperature. Lymphocytes were collected from the interface region and washed twice in large volumes of either Hanks' balanced salt solution (HBSS) or ABG. The final cell concentration was adjusted to 10^7 cells/ml in RPMI 1640 with 50% FCS. All FCS used in these determinations was heat inactivated and preabsorbed with SRBC to remove nonspecific agglutinating activity for SRBC. In the E-rosette analysis for T lymphocytes (5), 10^6 cells were added to duplicate tubes containing 0.1 ml of FCS and 0.1 ml of 2.5% aged (2 wk) SRBC in RPMI 1640 and 50% FCS. These tubes were centrifuged gently for 5 min before incubating overnight at 4°C. The tubes were again gently pelleted by centrifugation and chilled in an ice bath. Immediately before microscopic examination, the pellets were gently resuspended and stained with 1% methylene blue. Two hundred cells from each tube were counted. An E-rosette-positive cell was described as a mononuclear cell with 3 or more adhering SRBC.

Surface immunoglobulin-positive cells (B lymphocytes) were quantitated in 2 ways: by adherence of Bio-Rad immunobead (6) and by binding of fluorescein-labeled rabbit antibody specific for human IgA, IgG, and IgM (Bio-Rad) (5). In the immunobead assay, mononuclear cells were incubated with 0.25 ml of the immunobead reagent for 30 min at room temeprature, and B lymphocyte rosettes and monocytes containing ingested beads were quantitated microscopically (430 ×). In the fluorescein-labeled antibody assay, 10^6 cells were first incubated with 0.1 ml of a 1% latex suspension (1.1 μ diameter, Dow Chemical Co.) to measure phagocytic ingestion by monocytes. The cell and latex suspension was incubated at 37°C for 30 min and layered over 1.0 ml of undiluted FCS. This preparation was centrifuged at 100 × g for 5 min at room temperature. The cellular pellet was washed in HBSS with 0.2% sodium azide, and the latex-treated cells were reacted with 0.1 ml of fluorescein-conjugated antiserum along with 0.2% sodium azide in 5% FCS. Quantitation of B cells was accomplished by alternately viewing fields with tungsten and ultraviolet light at 630 ×. The results were expressed as the number of latex bead-negative, fluorescent-positive cells divided by the total number of latex bead-negative cells detected by tungsten illumination. The number of null cells was estimated by subtracting the total of E-rosette-positive lymphocytes (T lymphocytes) and surface immunoglobulin-positive cells (B lymphocytes) from the total number of lymphocytes. The percentage of latex-positive, phagocytic monocytes was generally <1%, and therefore this term was usually ignored in our calculations.

PHA stimulation of T lymphocytes. Lymphocytes (10^8 cells/ml RPMI-20% FCS) were treated for 0 to 3 hr with 10^{-9} to 10^{-2} M naloxone. The lymphocytes were then tested for B, T, and null cell percentages and the ability of PHA to stimulate tritiated thymidine (^{3}H-dT) incorporation into acid precipitable material. In the latter experiment, 10^7 cells were cultured for 72 hr in Chromosome 4 media (GIBCO) containing PHA, supplemented with 10 μCi ^{3}H-dT (6 Ci/mmol). The cells were harvested at 72 hr by centrifugation (400 × g for 10 min), and the excess radioactivity was removed with 3 washes of 5 ml of HBSS. The DNA was precipitated with 25% TCA at 0°C for 30 min, transferred onto a Whatman glass fiber filter GF/C, and washed 2 times with 5% ice-cold TCA and 2 times with ice-cold 95% ethanol. The filters were dried and counted in 10-ml scintillation solution (0.1 gm POPOP, 6 gm PPO (see Abbreviations), 1 liter toluene) in a Beckman LS-7000 scintillation counter.

RESULTS

Effects of street opiates on T lymphocyte percentages. The white blood count and percentages of B, total T, and null lymphocytes were determined for 44 street opiate addicts and 28 nondrug controls derived from either the local Atlanta or Chicago population (Table I). Shipping did not significantly alter the values for either the addict group or controls, and street drug addiction did not change white blood cell count or B lymphocyte percentage. On the other hand, a significant reduction was found in total T lymphocyte percentage between the addict and nondrug control populations ($F = 429.7$, $p<0.0001$) (7). At the same time, there was a concomitant and parallel increase in null lymphocyte percentage ($F = 467.1$, $p<0.0001$). All of the control individuals had significantly higher T lymphocyte percentages than any of the addicts. As seen in Table II, the self-reported use of marihuana did not alter the T cell depression seen in the opiate addicts. Thus, "street opiate" abuse clearly depresses both the percentage and the absolute number of T lymphocytes, and the reduction is mirrored in the increase in null lymphocytes.

The effects of the street opiates are long lived. Five patients, who entered a long-term drug therapy program at McLean Hospital, were followed for T, B, and null lymphocyte percentages longitudinally. After 3 wk (19 days) of drugfree detoxification, the % T lymphocytes = 28.6 ± 4.6, % B lymphocytes = 18.4 ± 2.7, and % null lymphocytes = 54.2 ± 6.5. This suggests that lymphocytes, like neuroblastoma (8), have a long-term memory for prior narcotic treatments.

Reversal of street opiate effects with (−)-naloxone. (−)-Naloxone, an antagonist of the binding of opiates by neuronal opiate receptors, reversed the T cell depression seen in opiate addicts, but it had no effect on T lymphocyte numbers from control subjects (Table III). The increase in T cell percentage and concomitant decrease in null cell percentage produced by naloxone were concentration dependent, with a maximum effective concentration between 10^{-7} to 10^{-6} M (Fig. 1). The

TABLE II

T cell frequency as a function of marihuana use in opiate addicts

Marihuana Use	Atlanta		Chicago	
	No. of subjects	Mean T cell % ± SD	No. of subjects	Mean T cell % ± SD
Yes	14	21.8 ± 8.2	9	30.9 ± 8.7
No	18	24.1 ± 10.3	3	34.7 ± 8.5

FCS, fetal calf serum; PHA, phytohemagglutinin; ConA, concanavalin A; TCA, trichloroacetic acid.

TABLE III

Lymphocyte response to naloxone and cyclic nucleotides

Opiate use	Marihuana use	T-, B-, and Null-cell Frequencies (%)																	
		Pretreatment			Naloxone 10^{-6} M			0.1 μM dBcAMP			1.0 μM dBcAMP			0.1 μM cAMP			1.0 μM cAMP		
		T	B	Null	T	B	Null	T	B	Null	T	B	Null	T	B	Null	T	B	Null
Control	−	70	17	13	69	16	15				63	12	25						
Control	−	71	22	7	70	20	10	71	20	9	68	20	12	68	18	14	70	19	11
Heroin	+	47	18	35	64	20	16	61	20	19				59	17	24	62	17	21
Heroin	−	49	16	35	74	18	8	69	17	14	71	20	9	64	20	16	70	18	12
Methadone (8 yr)	−	58	21	21	62	15	23				63	14	23						
Methadone (3 yr)	+	69	18	13	70	16	14				71	18	11						
Methadone (1 yr)	+	70	16	14	72	14	14				74	18	18						

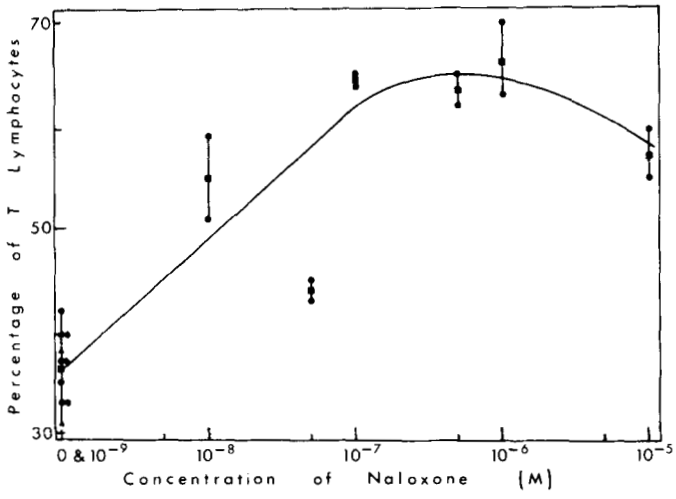

Figure 1. Effect of varying (−)-naloxone concentration of T lymphocyte percentage. Lymphocytes from a street heroin addict were treated for 1 hr at 37°C with naloxone; 0 M (●) and 10^{-9} (▲) naloxone produced identical results and were plotted together.

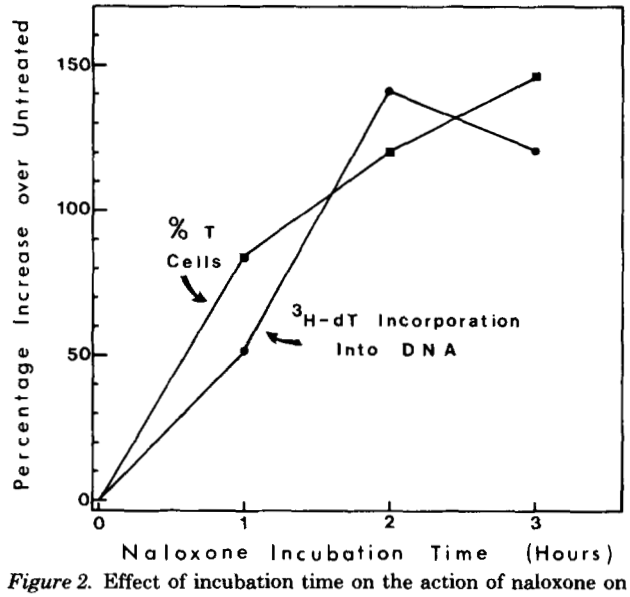

Figure 2. Effect of incubation time on the action of naloxone on T lymphocyte percentage. Lymphocytes from a street heroin addict were treated with 10^{-6} M naloxone at 37°C for varying periods of time. B-, T-, and null-lymphocyte percentages were then determined.

increase was time dependent, with an optimum time of incubation of the lymphocytes with 10^{-6} M naloxone of between 1 and 3 hr at 37°C (Fig. 2).

Along with an increase in T cell percentage, the naloxone treatment also improved the appearance of the E-rosettes from the opiate addict blood. Before treatment, the rosettes from the opiate addicts tended to be ragged, with only a few (3 to 6) SRBC surrounding the T lymphocytes, whereas rosettes from the control subjects had many (>10) SRBC completely surrounding the lymphocyte. After treatment, the E rosettes from the lymphocytes of the opiate addicts were tightly formed and resembled those from the controls.

During this treatment protocol (up to 3 hr incubation at 37°C in RPMI-10% FCS), the lymphocytes of both controls and addicts became sticky and difficult to resuspend after harvesting by centrifugation at 400 × g for 10 min. We have recently found that this latter effect can be minimized by lowering the incubation temperature to 22 to 24°C (room temperature) and adding a 5-fold excess of RPMI-50% FCS at the end of the incubation period. The cells were then centrifuged at 200 × g for 15 min. The lymphocytes produced by this milder treatment were easier to resuspend, although the results they gave were not different from those found for the sticky lymphocytes.

dBcAMP and cAMP reversal of street opiate effects. Sharma *et al.* (8, 9) have demonstrated that opiates modulate the action of adenylate cyclase in neuronal cells. dBcAMP can reverse the effects of opiate binding to neuronal cells, whereas cAMP is not absorbed by these cells and is not effective in reversing opiate action. In lymphocytes, we have found that both dBcAMP and cAMP can reverse the effects of opiate binding and increase the percentage of T lymphocytes to the same extent as naloxone (Table III).

PHA stimulation of (−)-naloxone-treated cells. The T cell increase among addicts produced by (−)-naloxone is paralleled by an increase in the ability of PHA to stimulate cellular incorporation of ^{3}H-dT into TCA-precipitable material (a measure of cell division and DNA synthesis). In Figure 3, the increase in T cell number and the increase in PHA-stimulated ^{3}H-dT incorporation were plotted as a function of time of incubation with naloxone. Although there were parallel increases in E-rosette-positive cells and ^{3}H-dT incorporation in the opiate addict, there were no comparable increases for the control subject. Thus, the increase in T cell number produced by naloxone parallels an increase in functionality of the lymphocyte population.

DISCUSSION

Opiate receptor sites on neuronal cells have been extensively

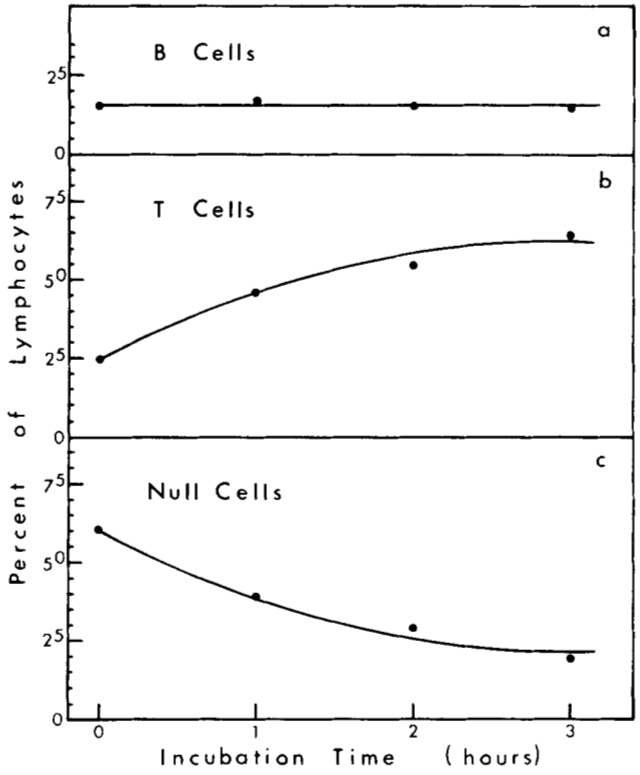

Figure 3. Effect of incubation time on the action of naloxone on T lymphocyte percentage and incorporation of ^{3}H-dT into DNA. Lymphocytes from a street heroin addict were treated with 10^{-6} M naloxone at 37°C for 0, 1, 2, and 3 hr; 0 hr represents untreated lymphocytes. T lymphocyte percentages were determined immediately after naloxone incubation (■). ^{3}H-dT incorporation was measured as acid-precipitable material after the naloxone-treated lymphocytes had been cultured for 72 hr with ^{3}H-dT (●).

characterized as to function, specificity, and mechanism of action (10). Recent reports have suggested that other tissues, including rat kidney (11), rat adrenal glands (11), rat pancreas (12), and human leukocytes (2–4) might also be sites of specific opioid action. Such important physiologic processes as endocrine control of the circulating glucose level (12) and cell-mediated immunologic response (2) have thus been tied to central nervous system function through the mediation of normal circulating factors, the endogenous opioids.

The evidence for an opioid link to the immune system rests on the demonstrations of the alteration of lymphocyte character by opiates and the ability of lymphocytes to bind opiates stereospecifically. Wybran *et al.* (2) showed that after 1 hr of incubation, morphine, met-enkaphalin, and dextromoramide, but not levomoramide, reduced the number of active T lymphocytes, i.e., those that form rosettes rapidly with SRBC. The total number of T lymphocytes assayed after an overnight incubation with SRBC was not affected. (−)-Naloxone reversed the decrease in active E rosettes caused by the opiates. Lopker *et al.* (3) demonstrated stereospecific binding of opiates to both granulocytes and monocytes and concluded that there were opiate receptors on phagocytic leukocytes. It was not clear from these *in vitro* results whether these opiate receptors had physiologic significance in the control of leukocyte metabolism and the immune system.

Our results show that *in vivo* administration of opiates, albeit illicit ones, produces a reduction in T lymphocyte number and function, and a concomitant increase in null cells. This T cell reduction and null cell increase can be reversed by incubating the cells with (−)-naloxone, dBcAMP, or cAMP (see Abbreviations). It must be reemphasized that only nonimmune SRBC adherence and lymphocyte proliferation were measured. This opiate binding to T lymphocytes seems to block expression of their SRBC receptors and to convert them into apparent null cells. Naloxone removes the block by either competitive inhibition with the opiate or by promoting an allosteric change in the membrane conformation, allowing the SRBC receptor to be actively expressed.

In neuronal cells, both naloxone and dBcAMP will reverse the effects of opiate binding, but not cAMP, because cAMP is not taken up by these cells. Lymphocytes, on the other hand, have been shown to incorporate cAMP directly (13), and this would explain its ability to affect opiate action in the T lymphocytes but not in neuronal cells. The concentrations of naloxone, dBcAMP, and cAMP found to be effective in lymphocytes are similar to those used to reverse opiate effects in neuronal cells, indicating that the effects in lymphocytes are as specific as those found for neuronal opiate receptors. The results of Wybran *et al.* (2) and Lopker *et al.* (3) further confirm the stereospecificity and avidity of the opiate binding sites in leukocytes. Our lymphocyte results with dBcAMP and cAMP also suggest that the opiate action may be mediated by neucleotide cyclases in a manner similar to that described for neuroblastoma (7, 8). Indeed, both Hadden *et al.* (14) and Parker (15) have implicated cyclic nucleotide concentration in the control of lymphocyte function by such diverse endogenous agents as prostaglandins, histamine, acetylcholine, catecholamines, and β-adrenergic antagonists, among others.

Since most street heroin addiction involves polydrug use, including chronic use of marihuana, barbiturates, hallucinogens, and other licit and illicit substances, the hypothesis can be proposed that the depression in T lymphocyte percentage was caused by another drug or combination of drugs, or by the effect of drug use on the addict's general physical health and nutrition, i.e., the addict milieu. There have been reports that chronic marihuana use depresses T lymphocyte number and function (16), but other workers have not been able to confirm these findings (17). As seen in Tables II and III, self-reported use of marihuana did not affect the lymphocyte alterations reported in this study, including the depression of T lymphocyte percentage in opiate addicts and its reversal by naloxone, cAMP, or dBcAMP. Regardless of whether the subject reported marihuana use or not, the result was the same: a reduction in T lymphocyte percentage, which could be reversed by (−)-naloxone. Thus, it apparently was the use of opiates that caused the depression in T lymphocyte number, because opiate abuse was the only element common to all of the addicts. The ability of (−)-naloxone to produce a time- and concentration-dependent increase in T lymphocyte percentage among the lymphocytes from opiate addicts argues strongly for a direct effect of the opiate on the T lymphocyte.

The binding of opiates to the T lymphocytes might also be manifest in a variety of other ways with severe physiologic consequences. Madden *et al.* (18) have reported that lymphocytes from opiate addicts have a significantly reduced capacity for repair of far UV damage to DNA. In analogous genetic diseases such as xeroderma pigmentosum, the low DNA repair level leads to an increased rate of mutation and carcinogenesis (19). In fact, Sadeghi *et al.* (20) have shown that among over 12,000 opium addicts in Iran, the rate of bladder cancer goes up as much as 19-fold in addicts who smoke tobacco, whereas either tobacco or opium alone only raises the rate 2-fold over that for nonusers. The opium appeared to potentiate the car-

cinogenic effects of the known mutagen, i.e., the tobacco smoke, although the increased cancer rate might also be explained by a reduction in immune system activity of the T cells toward the cancer cells.

Whether the binding of the opiates to human cells is limited to the leukocytic, neuronal, adrenal, and pancreatic tissue or whether it is a more generalized phenomenon involving other tissue has yet to be determined. Geller and Stimmel (21) have discussed pathologic changes induced by opiates in lymphatic, hepatic, subpyloric, and peripancreatic tissue indicative of opiate action in/on these cell types. The possible presence of opiate-binding sites in these tissues has profound health implications for not only opiate addicts but also those patients who are receiving opiates for relief of chronic, severe pain, particularly since the phenomenon was not reversed even after 3 wk of detoxification. In summary, the results described above support the hypothesis that there are opiate receptor sites on T lymphocytes that can affect the function of these cells through the moderation of cyclic nucleotides in an analogous manner to the opiate receptors found in neuronal tissue. Whether the lymphocyte receptor sites are identical to the neuronal receptors remains to be determined.

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