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BINDING OF NALOXONE TO HUMAN T LYMPHOCYTES

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Abstract—Purified T lymphocytes have a specific binding site for naloxone, the opiate antagonist. The K_D for the site was 50.6 ± 2.4 nM, while the Hill coefficient (n) was 1.67 ± 0.16, indicating a degree of positive cooperativity of ligand binding. The bound naloxone was partially displaceable by various opiate agonists including morphine (56%), β-endorphin (61%), met^5- and leu^5-enkephalin (40% each), [D-ala^2, D-leu^5]-enkephalin (78%) and [D-ala^2, D-leu^5]-enkephalinamide (66%). Virtually all of the binding capacity was recovered in the particulate membrane fraction after sonic lysis of the cells. There was great interindividual variability in B_{max} between samples, suggesting a possible mechanistic basis for the variability in drug action seen between different individuals.

The first evidence that opiates alter immunological functioning was reported by Archard *et al.* [1] in 1909. Interest in this topic has intensified over the past two decades with the realization that opiate abusers are prone to a variety of opportunistic infectious [2] and oncogenic [3–5] diseases that are often the result of immunodeficiency. In 1974, Brown *et al.* [6] elaborated on the immunological changes accompanying heroin addition when they showed that lymphocytes from addicts have depressed mitogenic responsiveness and that addicts have elevated circulating levels of immunoglobulins [6]. Our group and others have since found that lymphocytes from heroin addicts exhibit a depressed capacity for both total [7] and active [8, 9] T-cell E-rosette formation. Also, T-helper and T-suppressor lymphocyte ratios (Th/Ts) for heroin addicts are frequently severely depressed [10, 11]. It is important to note that the depression of E-rosetting and Th/Ts ratios occurs in the absence of the involvement of human immunodeficiency virus [11, 12] and that these immunological parameters are known indicators of compromised immunological capacity [13, 14]. Furthermore, various *in vivo* animal models, and *in vitro* human studies, have indicated that opiates directly influence the immunological functioning of T cells [15–18], NK cells† [19], macrophages [20] and polymorphonucleocytes [21].

The observations on the immunocompromising properties of opiates have been supplemented with studies to determine whether cells of the immune system have relevant opiate receptors. Abood and co-workers showed that erythrocytes [22], monocytes [23] and granulocytes [23, 24] possess pharmacologically specific, opiate binding-sites. Hazum *et al.* [25] describe a β-endorphin binding site on cultured lymphoblastoid cells from which the β-endorphin is not displaceable by other opiates. Other, more limited, studies also suggest that lymphocytes have specific opiate binding sites [26]. Moreover, both *in vivo* [27] and *in vitro* [15] studies have shown that some of the effects of opiate on T-cell functions are reversible by naloxone, which also has encouraged the speculation that T cells have opiate receptors.

Because T-cell function is of such critical importance to host immune defenses, and because many of the immunological effects of opiates are mediated through T cells, the existence of opiate-binding sites on these cells remains an important but unresolved issue. The major problem with previous studies aimed at clarifying this issue [26, 28] has been that those studies were conducted with T-cell preparations that were actually mixtures of hematological elements that separately have the capacity to bind and/or trap opiates. Thus, the present study was undertaken to define the ability of essentially pure, freshly isolated T cells to bind the opiate antagonist naloxone with appropriate pharmacological specificity.

METHODS

Isolation of mononuclear leukocytes on Ficoll. Mononuclear leukocytes were isolated from plateletpheresis residues obtained from the Atlanta Chapter of the American Red Cross. The procedures

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† Abbreviations: NK cell, natural killer cell; DADLE, [D-ala^2, D-leu^5]-enkephalin; DADL-enkephalinamide, [D-ala^2, D-leu^5]-enkephalinamide; Th lymphocyte, T-helper/inducer lymphocyte; Ts lymphocyte, T-suppressor/cytotoxic lymphocyte; HBSS, Hanks' Balanced Salt Solution; FBS, fetal bovine serum; PL, Ficoll/Percoll purified lymphocytes; NAD^+, nicotinamide-adenine dinucleotide; PI, pelletable cell sonicate; DTT, dithiothreitol; and AIDS, acquired immune deficiency syndrome.

of Segel *et al.* [29] were used for isolation of the mononuclear cell fraction as described in several of our previous reports [10, 11].

Purification on Percoll. The cells were diluted to 5×10^7 cells/ml and 5 ml was layered on top of a 35-ml preformed Percoll gradient along with colored density beads. The gradients were spun at 400 *g* for 5 min, and the top 4 ml containing mainly platelets was removed. The gradients were respun at 800 *g* for 20 min, and the cell layer between the red and blue density beads was selected. This lymphocyte layer was washed (400 *g*, 15 min) three times with Hanks' Balanced Salt Solution lacking calcium and magnesium (HBSS, GIBCO), and the final pellet was resuspended to 2×10^7 cells/ml in RPMI 1640 (GIBCO) plus 5% heat-inactivated fetal bovine serum (FBS, GIBCO).

Plastic adhesion. A 30-ml sample of the cell suspension was aspirated into a 150 cm^2 tissue culture flask (Corning) and incubated for 1 hr at 37°. Non-adherent cells (which are mainly lymphocytes) were dislodged from adherent monocytes by gentle agitation and poured into fresh flasks, and the incubation was repeated. The non-adherent cells were centrifuged (400 *g*, 15 min) and resuspended in 5 ml RPMI 1640 plus 5% FBS.

Nylon wool column. The method of Julius *et al.* [30] was used for removal of B cells and macrophages. The cells from four flasks used for absorption of macrophages, as above, were combined, placed into a nylon wool column, and covered with parafilm. The column had been prepared by shredding 2.5 g nylon wool (Cellular Products Inc., Buffalo, NY) into a 50-ml syringe, autoclaving, and finally rinsing with medium just before use. The cells and column were incubated for 45 min at 37° and then eluted with 50 ml HBSS plus 5% FBS, and stored overnight at 4°. This procedure produced from 10^8 to 10^9 purified lymphocytes (PL) composed of 96–99% sheep erythrocyte (E) rosette positive lymphocytes, 1–4% B and null lymphocytes, <1% monocytes and <1 platelet/5 lymphocytes along with an occasional erythrocyte.

Cell sonication. The naloxone binding capacity of the lymphocytes was assayed with either whole cells or cell sonicates. Whole cells were washed once with ice-cold HBSS (200 *g*, 15 min), diluted to 5×10^7 cells/ml and held on wet ice until assayed. If the cells were disrupted by sonication (Artex Sonic Dismemberer, Farmingdale, NY), they were first washed as above and then diluted to 2×10^8 cells/ml (4× whole cell concentration) with ice-cold HBSS. The cell suspension was sonicated at 0° for 1–4 min, depending upon solution volume, until <5% whole cells remained as measured by the Coulter Counter F (Hialeah, FL). Samples were counted approximately every 30–40 sec. The sonicated suspension was either diluted 4-fold (to make the membrane content equivalent to the whole cell assays) and assayed, or centrifuged immediately at 4° at 25,000 rpm for 10 min in a Beckman L5–50 ultracentrifuge with a 50.1 rotor. The pellet was resuspended in a volume of cold HBSS, equivalent to the discarded supernatant fraction, and homogenized with a cold ground glass homogenizer. The suspension was washed twice by centrifugation. The final pellet (P1) was diluted with a volume of cold HBSS equal to four times the original whole cell-buffer volume and held on ice for assay. Protein concentration of these samples was determined by the method of Lowry *et al.* [31].

Binding assay. Specific binding of [^{3}H]naloxone to whole lymphocytes and sonicated lymphocyte membranes was measured by the method of Simon *et al.* [32] as the difference between total binding (the amount of [^{3}H]naloxone which binds to the preparation in the absence of competing ligand) and non-specific binding (the amount of [^{3}H]naloxone which binds in the presence of a 1000-fold excess of unlabeled naloxone). Triplicate assay tubes (Sarstadt, 125 mm × 10 mm) were prepared; they contained 0.1 ml of twice the desired concentration of [^{3}H]naloxone (New England Nuclear, Boston, MA; 44.1 Ci/mmol). A duplicate set containing the same volume and concentration of [^{3}H]naloxone and a 1000-fold excess of non-radioactive competing ligand (naloxone unless otherwise specified) was also prepared. To these, 0.1 ml whole lymphocytes (5×10^7 cells/ml) or an equivalent concentration of sonicate was added, and the mixture was incubated on wet ice for 1 hr, except where noted. The mixture was then rapidly filtered, in sets of 3, on Whatman GF/C glass fiber filters (2.4 cm) with a Hoefer multiwell filtering apparatus maintained at 100 mm Hg. The filters were immediately washed twice with 4 ml of ice-cold HBSS, removed, and placed in a scintillation vial with 0.5 ml of 0.1 M KOH for 30 min. Beckman Ready-Solv HP/b scintillation solution (10 ml) was added, and the samples were held overnight before counting in a Beckman LS-5810 liquid scintillation counter using an automatic quench correction program.

RESULTS

Competition among lymphocytes, other cells, and platelets for naloxone. Many blood components can bind opiates [22–24], so that it is essential to purify the T lymphocytes to the point where binding to these other elements becomes truly negligible. Previous workers reported opiate binding [26], or lack of it [28], to lymphocyte preparations that had significant monocyte (>5%) and/or platelet concentrations (>5 platelets/lymphocyte) as well as erythrocyte and other hematologic contaminants. When we studied the effects such contaminants had on opiate binding, we found that contamination at these levels prevented us from obtaining reproducible results. For example, when platelet concentration was high (>10 platelets/lymphocyte), a high level of non-specific binding was found (Fig. 1, curve A), whereas a lymphocyte preparation with a lower platelet contamination (curve B) had a significantly lower level of non-specific binding. Erratic platelet concentrations in the final preparation could be eliminated by including Percoll centrifugation in the protocol. Curves C and D (Fig. 1) represent the degree of non-specific binding seen for lymphocytes purified by the complete scheme described in Methods. That platelets were the component responsible for the erratic non-specific binding can be seen from Table 1. The Ficoll–Percoll purified lympho-

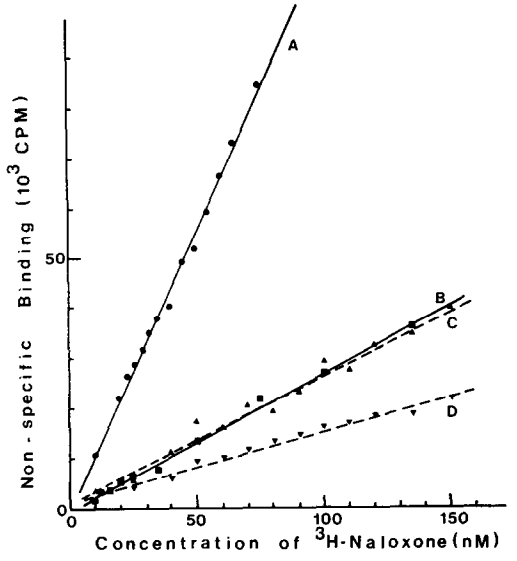

Fig. 1. Non-specific binding by lymphocyte preparations from four individuals. Curve A: high platelet concentration (>10 platelets/lymphocyte). Curve B: low platelet concentration (<4 platelets/lymphocyte). Both A and B were purified by Ficoll and absorption but not Percoll. Curves C and D: purified by complete protocol including Percoll centrifugation. Both C and D had low platelet contaminants (<1 platelet/lymphocyte).

cytes (PLs) demonstrate 2.5-fold greater specific binding compared to non-specific binding. Platelets alone, in a concentration similar to that found in preparations not purified on Percoll (10^8 platelets/ml), had an extraordinarily high level of non-specific binding which masked the specific binding of the lymphocytes when they were combined and assayed. Also, variability between repeated assays increased dramatically when platelet contamination was present, perhaps due to fragility of the platelets.

Monocytes also evidence a significantly high level of non-specific binding commensurate with their large cell membrane area compared to the size of the average lymphocyte, as noted previously [26]. When naloxone binding was compared for equal numbers of lymphocytes and monocytes, the monocytes clearly had a disproportionately high level of non-specific to specific binding (Table 1).

Media effects. It was possible to measure significant specific binding of naloxone to T lymphocytes in a maintenance medium such as HBSS, but specific binding was not detected with an enriched medium such as RPMI-1640 (Table 2). In investigating which compound in RPMI-1640 was responsible for the loss in specific binding, it was determined that the sulphydryl reagent, glutathione, at a concentration equal to that found in RPMI-1640, significantly depressed specific binding (Table 2). Interestingly, glutathione did not affect non-specific binding, but RPMI-1640 decreased non-specific binding by half. Several other compounds contained in RPMI-1640 were tested (NAD^+ and nicotinamide) but had no effect on either specific or non-specific binding.

Table 1. Effects of platelet and monocyte contamination on [^{3}H]naloxone binding by T lymphocytes

	Cell type	Total binding (cpm)	Non-specific binding (cpm)	Specific binding (cpm)
(A)*	Lymphocytes alone	37,800	11,500	26,200
	Platelets alone	62,800	58,700	4,100
	Lymphocytes plus platelets	75,200	61,800	13,400
(B)†	Lymphocytes alone		1,000–2,000 (N = 6)	2,000–4,000 (N = 6)
	Monocytes alone		10,000–16,000 (N = 3)	1,000–4,000 (N = 3)

* Ability of platelets and lymphocytes alone and in combination to bind to 50 nM [^{3}H]naloxone.

† Comparative ability of monocytes and lymphocytes to bind 10 nM [^{3}H]naloxone.

Time course. At 0°, specific binding reached saturation at about 45 min and remained stable for another 35–45 min (Fig. 2). At higher temperatures (23° and 37°), the rate of binding was much faster than at 0°, but the maximal amount of specific binding was less.

Binding of [^{3}H]naloxone to T lymphocytes. Using the lymphocyte preparation and binding assay described in Methods, it was possible to obtain a saturable, specific binding curve (Fig. 3). The non-specific binding curve was a straight line which did not reach a plateau with increasing concentrations of ligand. The competitive binding reached a plateau at approximately 100–110 nM, yielding a K_D of approximately 50–55 nM. Specific binding was 2 to 2.5 times greater than the non-specific binding; nevertheless, non-specific binding, probably attributable to uptake, was a significant aspect of the total binding of naloxone by the T lymphocytes.

When lymphocytes were obtained from several individuals and tested separately for binding capacity

Table 2. Effect of the medium on the binding of 20 nM [^{3}H]naloxone to lymphocytes

HBSS	(100)	(100)
RPMI-1640	6	49
HBSS + 1.6 μM glutathione	48	99
HBSS + 0.75 μM nicotinamide	91	98

Results are normalized to the values obtained in HBSS alone. Counts per minute equivalent to 100% specific binding were 8000, while for the non-specific binding 100% was 8300.

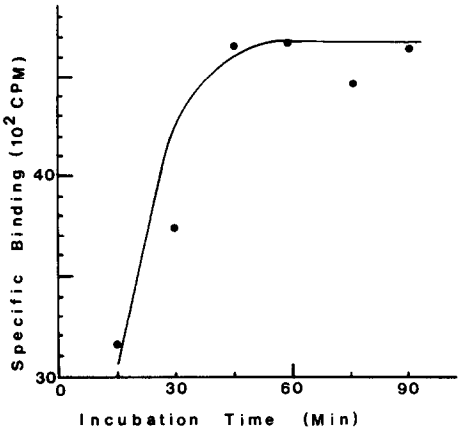

Fig. 2. Time versus specific binding curve of 25 nM [^{3}H]naloxone to highly purified lymphocytes at 0°.

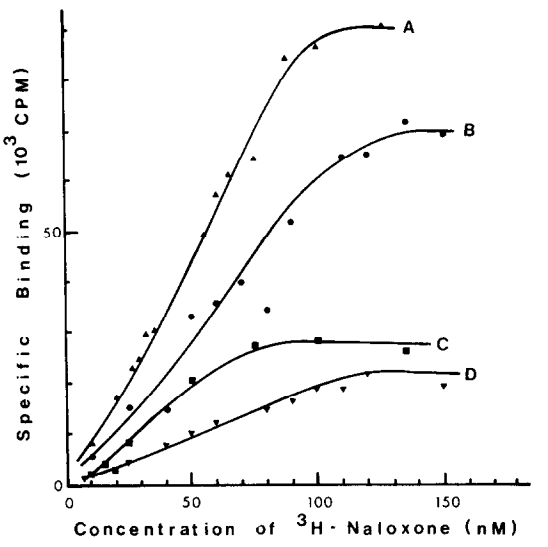

Fig. 4. Specific binding curves for purified lymphocyte preparations from four different individuals.

(Fig. 4), the approximate K_D values for three of four individuals (A, B and D) were similar (48.3, 50.6 and 53.0 nM respectively) while one subject (C) had a lower K_D (approximately 34.8 nM) as determined by Hill plots (Fig. 5). The Hill coefficients (n) derived from these Hill plots were 1.55, 1.61 and 1.85 for A, B and D and 2.45 for C, suggesting some cooperativity in ligand binding. Of greater importance, the height of the curves at saturation (B_{max}) varied greatly among subjects (Fig. 4) so that an almost 5-fold difference existed between the lowest and the highest capacity. From these maximal values at saturation, the number of molecules of naloxone bound per lymphocyte was 15,290, 12,620, 6000 and 4340 for A through D, respectively, the mean being 9,560 ± 5,230.

Ability of sonicates to bind naloxone. To eliminate some of the problems associated with uptake of the labeled compound by the whole cell, the cells were lysed by sonication for progressively longer periods of time until <1% remained intact. Aliquots with various percentages of whole cells were tested for their capacities to bind 20 nM [^{3}H]naloxone (Fig. 6). Both specific and non-specific binding occurred even after essentially complete cell lysis. For example, at 50% cell lysis, >90% specific binding remained, while at 100% cell lysis, >30% remained. The optimum cell concentration for sonication proved to be 2×10^8 cells/ml, a concentration which was four

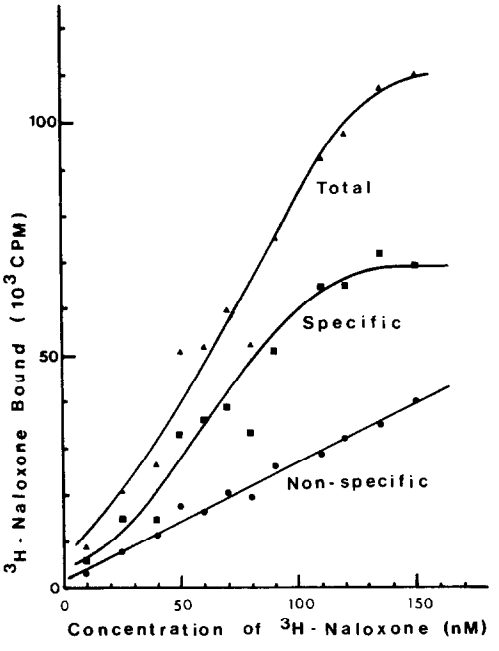

Fig. 3. Typical binding curves for intact, purified lymphocytes from a single individual.

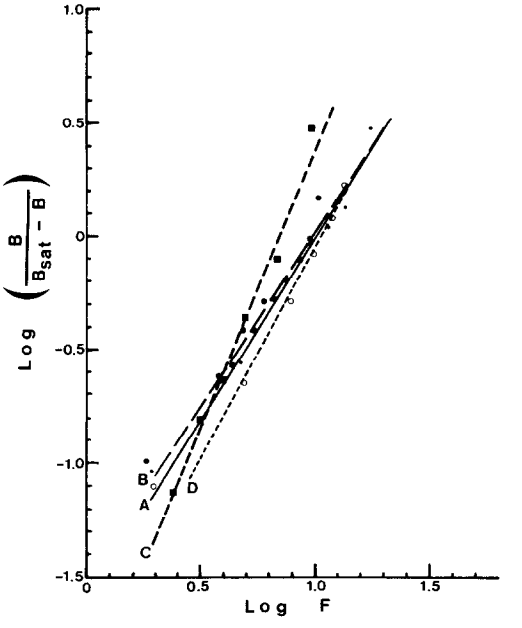

Fig. 5. Hill plot [33] of the specific binding data presented in Fig. 4 for the four individuals.

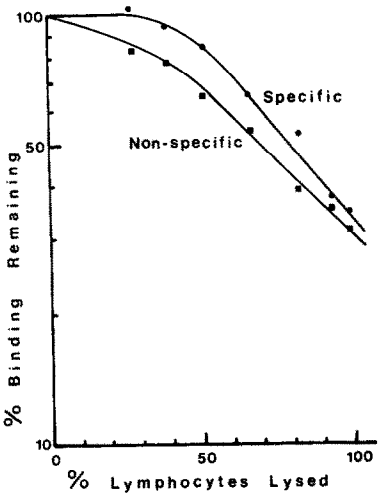

Fig. 6. Effect of sonication on specific and non-specific binding by lymphocytes. Both the supernatant and precipitable fractions were present in the sonicate.

Table 4. Displacement of [^{3}H]naloxone binding to whole lymphocytes and lymphocyte sonicates (PI) by opiate agonists

Drug	Displacement by ligand (%)	
	Whole cell	PI
Naloxone	(100)	(100)
Morphine	56	61
β-Endorphin	61	69
met^5-Enk	40	45
leu^5-Enk	40	37
DADLE	78	71
DADL-enkephalinamide	66	

The ability of opiate agonists to displace [^{3}H]naloxone from T lymphocytes is compared to the displacement ability of naloxone itself. The results for each displacement ligand are expressed as the percent of the counts displaced by naloxone, i.e. (cpm displaced by ligand/cpm displaced by naloxone) $\times$ 100 = %. The [^{3}H]naloxone was present at a concentration of 20 nM, while that of the unlabeled ligand was 20 μM.

times greater than the optimum concentration for the whole-cell binding assay.

When the sonicate was separated by ultracentrifugation, virtually all of the binding activity was found in the pellet (P1, Table 3). When the supernatant fraction was added back to the pellet, the level of binding dropped to that seen in the non-centrifuged sonicate.

Naloxone displacement by opiate agonists. Previous reports [25] of β-endorphin binding to dividing lymphoblasts indicate that the β-endorphin is not displaceable by opiate agonists and antagonists. In this study, we found that naloxone could be displaced from T-lymphocyte whole cells and from T-lymphocyte membrane sonicates by a variety of mu and delta opiate receptor agonists (Table 4). The relative displacements abilities for each agonist were similar for both preparations, suggesting that the naloxone binding sites were the same for each.

DISCUSSION

Highly purified T lymphocytes can bind naloxone in a saturable, competitive manner. The binding site has some physiologically important characteristics including displaceability by opiate agonists. The K_D, however, is higher than that found in neuronal tissue for naloxone (50 nM versus 1–5 nM respectively). Several of the reports describing the alteration of leukocyte functions by various concentrations of opiates generally predict a lower K_D for the physiologically relevant phenomena. Thus, while the binding of ligand to this low affinity site occurs within the concentration range that produces some changes in lymphocyte function and/or antigenicity [15, 34] other studies [15, 34] suggest that certain immunological effects of opiates are mediated through higher affinity sites. Nevertheless, the low affinity, 50 nM, site reported here represents an important starting point to address mechanisms by which opiates interact with lymphocytes.

In studying naloxone binding sites on T lymphocytes, it is important to consider the non-specific component that accounts for one-fourth to one-third of the total binding. This non-specific component probably arises from the uptake and trapping of naloxone by the lymphocyte [35, 36]. Whether this occurs by penetration, endocytosis or receptor-mediated endocytosis is not clear, although the use of low temperatures (4°) in these assays should elim-

Table 3. Binding of [^{3}H]naloxone to whole lymphocyte cell sonicate and sonicate fractions

Fraction	Total binding (cpm)	Non-specific binding (cpm)	Specific binding (cpm)	Specific binding compared to whole cell (%)
Whole cell	9900	3900	6000	(100)
Sonicate	6000	3900	2100	35
Supernatant	1700	700	1000	17
P1	7800	3400	4400	74

Whole lymphocytes were sonicated until <1% remained. Half of the sonicate was centrifuged, and the supernatant and precipitate (P1) were obtained. Each fraction (whole cell, sonicate, supernatant and P1) was assayed for specific binding of 30 nM [^{3}H]naloxone.

inate energy-requiring processes. The fate of the trapped naloxone is also not known, although the lymphocyte is not usually thought to be a site of naloxone detoxification through conjugation. Even the lymphocyte sonicates had a remarkably high non-specific binding component. This may have been due to folding of the sonically disrupted membranes into vesicles capable of trapping naloxone. Thus, there are at least two, not necessarily mutually exclusive, mechanisms (receptor mediation and uptake) by which opiates may affect lymphocyte physiology.

The possibility of multiple pathways for the interaction of naloxone and T lymphocytes may be further complicated by different media requirements for each pathway and by interindividual variability. It is significant that specific binding of naloxone to lymphocytes was measurable only in a minimal maintenance medium and not in a highly enriched one generally favourable for lymphocyte activity and function. Further, reduced glutathione inhibited specific, but not non-specific, naloxone binding by lymphocytes because in the neuronal system reducing agents (DTT) enhance specific binding [37]. The presence of a critical reduced sulfhydryl group in the neuronal receptor can be inferred even more strongly from the inhibition of opiate agonist binding by oxidizing agents (Cu^{2+}) which is reversible by reduced thiol reagents [37]. Whether these differences are tissue and/or species specific or reflect a difference between ligands used (agonists vs antagonist) can only be better determined when the biochemical nature of lymphocyte opiate receptors is better characterized.

One of the principal results of these experiments arose from the significant interindividual variation in naloxone binding capacity seen with different batches of cells. Figure 4 clearly demonstrates major differences in B_{max} among four different individuals. For this reason, absolute results for each experiment shown should be compared only within that experiment (and batch of cells) and not between experiments. It was evident (data not shown) that each batch of cells had roughly the same constituent percentages of lymphocyte subtypes. Thus, simple cell type variation does not seem to explain the inter-experimental variation in naloxone binding. While the exact cause of this variation has not been established, our current data suggest that such variation represents a significant physiological difference in drug binding between individuals. Because drug addition leads to both a compromised immune system [6, 7] and opportunistic infections (AIDS, hepatitis) in some individuals and apparently not others, this interindividual variation in naloxone binding capacity may provide at least a partial explanation for these medical observations.

Further experimentation is needed to resolve the agonist-ligand specificity of the binding sites occupied by naloxone. Displacement of naloxone by various mu and delta ligands suggests that naloxone may occupy mu-like and delta-like sites on the lymphocyte that are comparable in specificity to neuronal sites. The ability of naloxone to antagonize the effects of both morphine and met-enkephalin on T-cell E-rosette formation, as reported by Wybran *et al.* [15], is further evidence for the involvement of mu and delta sites in the interaction of opiates with T cells. On the other hand, Grevel *et al.* [38] reported recently a "weakly saturable" naloxone binding-site (lambda) on neuronal tissue with a K_D of approximately 50 nM. Since naloxone *per se* produces specific effects on antigenic marker expression of T lymphocytes in the absence of other opiates [39,*], naloxone may act on its own functionally distinctive T-cell binding site. Thus, elucidation of the pharmacological type of naloxone binding site present on the T lymphocyte and its similarity to analogous sites in the central nervous system will be the direction of future experimentation. The methodology developed in this report provides the means to pursue such experiments.

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