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# Studies of Cyclic AMP Effect on Release of Unsaturated Vitamin $B_{12}$ Binding Capacity from Human Blood Cells[1] (37885)

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Previous work in our laboratory has suggested that a substantial amount of human plasma unsaturated vitamin $B_{12}$ binding capacity (UBBC) is released from granulocyte granules (1). It was further shown that NaF inhibited, and lithium salts ($Li^+$) stimulated, the release of UBBC from granulocyte-containing blood samples (8). Fluoride is able to stimulate the activity of adenylate cyclase, resulting in the production of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) (2). $Li^+$ is capable of inhibiting adenylate cyclase in tissues (2, 3). Changes in intracellular cyclic AMP levels may control release of other leucocyte granule enzymes (4–6). The current studies were executed to determine whether UBBC release is mediated by the inhibition of adenylate cyclase.

*Materials and Methods.* Venous blood was collected directly into 10-ml Vacutainer test tubes (Becton-Dickinson, Rutherford, NJ) containing crystalline EDTA ($K_3$), (3200 XF40), or, for studies with NaF, containing crystalline EDTA/NaF (3200 XF92). Dibutyryl cyclic AMP, cyclic AMP, norepinephrine, epinephrine, isoproterenol, and lithium chloride were dissolved or diluted in saline. Prostaglandin $E_1$ was initially dissolved in 95% ethanol and then diluted in saline. Each reagent, in 25–250 μl volume, was dispensed into four new 10-ml test tubes. Lithium chloride, to yield 50 mEq/liter final concentration, was added to 2 of the 4 tubes containing reagent, as well as to 2 of the 4 control tubes. Two-and-one-half milliliters of whole blood was then added to each tube. After mixing, two sets of tubes, one set containing $Li^+$ and one set without $Li^+$, were centrifuged immediately ($t_0$); the supernatants were removed and stored at −20°. The other two sets of tubes, one set containing $Li^+$ and one set without $Li^+$, were incubated at 23° for 24 hr ($t_{24}$), after which the samples were centrifuged and the supernatants removed and stored at −20°.

Assay for human plasma UBBC was performed by the method of Gottlieb *et al.* (7) with the following modifications: 0.1 ml plasma was added to 1.0 ml saline containing 500 pg $^{57}Co$-$B_{12}$ and the mixture incubated in a water bath for 30 min at 37° with constant agitation. At the conclusion of incubation, 1.0 ml of a 1:20 mixture of hemoglobin-coated charcoal was added to each tube. The tubes were mixed on a vortex mixer, centrifuged, and the supernatants decanted. Radioactivity of the supernatants was determined in a Picker Autowell II counter and the UBBC calculated by comparison with a standard solution of $^{57}Co$-$B_{12}$.

*Results.* The data are shown in Table I. At 24 hr, in the presence of lithium, the control and all samples, except those incubated with NaF, registered a sharp increase in the UBBC. In the absence of lithium,

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TABLE I. Effect of Cyclic AMP and Agents that may Increase Cyclic AMP Levels on Release of UBBC from Human Blood Cells.

| Test substance | Concentration ($M$) | UBBC (pg/ml) Without $Li^+$ $t_0$ | Without $Li^+$ $t_{24}$ | With $Li^{+,b}$ $t_0$ | With $Li^{+,b}$ $t_{24}$ |
|---|---|---|---|---|---|
| Control[a] | — | 902 ± 36 | 1008 ± 73 | — | 4408 ± 20 |
| Cyclic AMP | $10^{-4}$ | 736 ± 27 | 947 ± 16 | 706 ± 10 | 3904 ± 375 |
| Dibutyryl cyclic AMP | $10^{-4}$ | 829 ± 13 | 981 ± 38 | 835 ± 17 | 4654 ± 18 |
| Prostaglandin $E_1$ | $10^{-6}$ | 994 ± 38 | 1092 ± 23 | 935 ± 5 | 4856 ± 33 |
| Norepinephrine | $10^{-4}$ | 797 ± 17 | 975 ± 11 | 871 ± 6 | 3944 ± 670 |
| Isoproterenol | $10^{-4}$ | 805 ± 2 | 978 ± 1 | 834 ± 2 | 4269 ± 12 |
| Epinephrine | $10^{-4}$ | 949 ± 56 | 1027 ± 63 | 832 ± 6 | 4256 ± 45 |
| NaF | $4.7 \times 10^{-2}$ | 847 ± 23 | 793 ± 12 | 723 ± 13 | 756 ± 25 |

[a] Samples, including control, collected in 4 m$M$ EDTA.

[b] $Li^+$ added as LiCl to yield 50 mEq/liter blood concentration.

there was a slight increase in the UBBC in the control and samples not incubated with NaF.

After 24 hr, the samples incubated with NaF showed a slight decrease in UBBC whether or not lithium was present.

*Discussion.* Scott *et al.* have demonstrated *in vitro* variability in human serum or plasma UBBC levels, depending upon time, temperature, and choice of anticoagulant (8). This variation is due to *in vitro* release of Transcobalamin III (TC III) from cells. (Transcobalamins I and III are vitamin $B_{12}$ binding proteins that appear to be derived from granulocytes.) Collection of blood in EDTA at 4° or in EDTA/NaF appears to prevent TC III release and will yield plasma containing about 10% total TC I and TC III. Lithium ion, when added *in vitro,* will produce a profound release of TC III, increasing the UBBC (8).

Fluoride is a potent stimulator of adenylate cyclase in many tissue preparations, and lithium is reported to antagonize adenylate cyclase and the action of fluoride on that enzyme (2). Catecholamines, prostaglandins, and methylxanthines are capable of producing increased levels of cyclic AMP, either by direct action on adenylate cyclase or by inhibition of the cyclic AMP degradative enzyme, phosphodiesterase. The release of leucocyte granule contents, such as histamine (4, 5) and β-glucuronidase (6), has been shown to be inhibited by exogenous cyclic AMP and dibutyryl cyclic AMP and by catecholamines and theophylline. In another study, however, the authors report they were unable to correlate changes in cyclic AMP levels within rat mast cells with histamine release (9).

In this experiment, there was not a significant change from control values in the amount of UBBC measured at 24 hr when agents capable of increasing endogenous cyclic AMP—prostaglandin $E_1$, norepinephrine, isoproterenol, or epinephrine—or cyclic AMP or dibutyryl cyclic AMP were incubated with human blood cells.

In contrast, the addition of NaF markedly inhibited the release of UBBC; the $t_{24}$ values in this experiment, either with or without lithium, were actually slightly lower than the $t_0$ value.

These results suggest that UBBC release is not mediated primarily through changes in intracellular cyclic AMP levels. This would be in agreement with the observation that fluoride action on adenylate cyclase apparently occurs only in broken cell preparations and not in intact cells (2). Fluoride is also an inhibitor of several other enzyme systems (10). It inhibits the action of at least two glycolytic enzymes, enolase and diphosphofructose phosphatase, and may exert its effect on UBBC release in that way. How lithium works to promote UBBC release is not known. It may alter membrane permeability, possibly by par-

tial substitution for $Na^+$ or $K^+$, or by interfering with solute or water flow (11), thereby allowing release of stored UBBC.

Failure to find substantial cyclic AMP effect on UBBC release does not appear related to sequestration of ionic calcium by EDTA. Although ionic calcium is required for some cyclic AMP-mediated responses, it apparently is not required for catecholamine stimulation of adenylate cyclase (2). Greater UBBC increment occurs with NaF-heparin than with NaF-EDTA (8). This is opposite to the expected effect if ionic calcium depletion (brought about by EDTA) were inhibiting adenylate cyclase.

*Summary.* Cyclic AMP and other agents capable of elevating cyclic AMP levels were incubated with human blood cells to test ability to inhibit unsaturated vitamin $B_{12}$ binding protein release. Except for NaF, none of the agents tested showed a significant effect on UBBC increment at 24 hr compared to controls. As shown previously, NaF fully prevented UBBC release, even in the presence of added $Li^+$. From these data, it is concluded that release of unsaturated vitamin $B_{12}$ binding protein from human blood cells is not mediated primarily through changes in intracellular cyclic AMP levels.

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