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In Vitro Observations on the Binding of Vitamin B_{12} by Serum Proteins and the Effect of Cells on the Process (38121)

RALPH CARMEL AND KAREN BJORNDAL
(Introduced by Ananda S. Prasad)
Division of Hematology, Department of Medicine, Wayne State University School of Medicine, and the Special Hematology Laboratory, The Grace Hospital, Detroit, Michigan 48201

The intravascular kinetics of vitamin B_{12} have been the subject of many studies (1). The roles of transcobalamin (TC) I as a circulating storage compartment which binds vitamin B_{12} tightly and TC II as a transient serum binder which facilitates transport of B_{12} into cells are generally accepted. The function, if any, of serum third binder is not known, but it appears to take some part in B_{12} transport under physiological conditions (2).

This report presents serial observations on *in vitro* binding of trace amounts of vitamin B_{12} by the 3 serum binders and the effect of the presence of blood cells on this process.

Methods and Materials. From each of 3 healthy volunteers with normal blood counts, serum unsaturated vitamin B_{12}-binding capacity (UBBC), and binder fractions, were collected clotted blood and blood anticoagulated with EDTA. Serum was extracted from the former, and cells from the latter were washed 3 times with 0.9% NaCl–10 m*M* $CaCl_2$.

In each case, 15 ml of serum were put into each of 4 siliconized flasks, and approximately 12 ml of washed cells were divided equally between 2 of the flasks. To one of the flasks with cells and one without were also added 1500 pg $^{57}CoB_{12}$ in 0.15 ml, specific activity 10 μCi/48 μg (Amersham-Searle, Arlington Heights, Ill.). This amount of $^{57}CoB_{12}$ saturated less than 10% of the binding capacity of the serum.

Incubation was done at 37°, and aliquots were removed from each of the 4 flasks at 0.5, 4, 24 and 52 hr intervals. In the cell-containing samples, after hematocrit and white blood cell count were determined each time, the cells and serum were separated by centrifugation. The serum B_{12}-binding proteins of the samples incubated with trace amount of $^{57}CoB_{12}$ were fractionated in duplicate determinations on 0.5 ml aliquots by batch separation with DEAE cellulose (3) and by precipitation with 2 *M* ammonium sulfate.[1] TC I remains uneluted from the DEAE-cellulose pellet by 0.06 *M* phosphate buffer, pH 6.3, (TC II and third binder being eluted), while TC II is precipitated in 0.4 *M* K_2HPO_4 by 2*M* ammonium sulfate (leaving TC I and third binder in the supernate).[1] Thus TC I and TC II can be quantitated separately, the remainder of the UBBC, after subtraction of the 2 values, being third binder.[1] In some instances fractionation was further checked by Sephadex G-75 gel chromatography using 0.1 *M* Tris—1 *M* NaCl, pH 8.6. The cells from the flask containing trace amounts of $^{57}CoB_{12}$ were washed twice with 0.9% NaCl, and $^{57}CoB_{12}$ adsorbed by cells was determined by counting the radioactivity of the lysate.

Duplicate samples of serum from the flasks incubated without $^{57}CoB_{12}$ were saturated with excess $^{57}CoB_{12}$, specific activity 10 μCi/667 μg. After the unbound $^{57}CoB_{12}$ was removed with a pellet of coated charcoal, fractionation of the B_{12}-binding proteins was carried out as above and results expressed as pg UBBC/0.5 ml serum aliquot.

Results. Results in the 3 subjects were identical (a single exception is noted below), and therefore all the data are represented by the mean values.

Cell counts and cell uptake (Table I). Hematocrit and white cell counts remained con-

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[1] Carmel, R., Amer. J. Clin. Path., in press.

TABLE I. Cell Counts and Cell Uptake of $^{57}CoB_{12}$ at Various Incubation Intervals.[a]

	0.5 hr	4 hr	24 hr	52 hr
Hematocrit (%)	24	24	25	19
WBC/μl	4630	5170	4270	3830
Cell uptake of $^{57}CoB_{12}$ (pg/ml cells[b])	66	64	72	42

[a] The values represent the mean of the results in the 3 normal subjects.

[b] It is unknown whether uptake is confined to any single cell type in our system. Therefore cell uptake refers to that by 1 ml of the total cell population in the flask.

stant over 24 hr but fell by 52 hr incubation. A similar pattern was seen with cell uptake of $^{57}CoB_{12}$.

UBBC. On cell-free incubation, UBBC remained constant at 0.5, 4 and 24 hr, but fell by 52 hr (508, 519, 495, and 453 pg/0.5 ml respectively). As shown in Fig. 1 A, there was no significant change in any of the 3 component binders over 24 hr except for a minimal decrease in TC II. This decrease in TC II over 24 hr was not seen consistently among the 3 subjects, however. The fall in UBBC at 52 hr was entirely due to decreased TC II. In contrast, serum UBBC continually rose (447, 526, 650 and 862 pg/0.5 ml respectively) when incubated with cells. Figure 1 B demonstrates that the rise was due entirely to a striking increase of TC I and third binder, while TC II remained constant throughout. Interestingly, the level of TC II was lower than when serum was incubated without cells (until 52 hr when the two levels were identical). Levels of TC I and third binder were initially identical in both incubation situations.

Trace $^{57}CoB_{12}$. Fairly constant amounts of a trace dose of $^{57}CoB_{12}$ were recoverable from serum whether incubated with or without cells. Figure 1 C and D show the change with time of the binder distribution of these trace amounts of $^{57}CoB_{12}$. When cells were not present (Fig. 1 C) the distribution of $^{57}CoB_{12}$ remained constant except for a slight increase in that bound to TC I and a lesser decrease in that bound to TC II. By 52 hr, however, binding by TC II fell noticeably. In the presence of cells (Fig. 1 D) $^{57}CoB_{12}$ carried by TC II fell more immediately and more strikingly. The $^{57}CoB_{12}$ carried by TC I increased slightly with a more striking increase in that carried by third binder. As was the case with the UBBC, the amount of the trace dose of $^{57}CoB_{12}$ bound initially (i.e., at 0.5 hr) to TC II was greater in the cell-free incubation than when incubated with cells.

Discussion. In vivo kinetic studies have shown that the bulk of a trace dose of vitamin B_{12} is bound by TC II, the binder that transports B_{12} to cells (4). The half-life of the TC II-B_{12} complex is 15–18 hr (5). As time passes, relatively more of the B_{12} is carried by TC I which holds the vitamin tightly and has a half-life of many days (4, 5). Whether TC II transfers B_{12} directly to TC I or indirectly by first transferring B_{12} to a cell is not clear and it has been suggested that such transfer may be inconsequential (6). The role

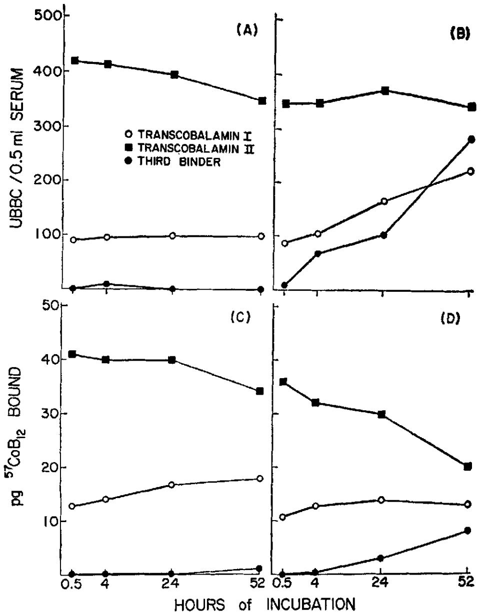

FIG. 1. Binding by the 3 serum vitamin B_{12}-binding proteins incubated with and without blood cells. (A) UBBC changes with time; (B) As in A, but with incubation in the presence of blood cells; (C) Changes with time in distribution of a trace dose of $^{57}CoB_{12}$ among the 3 serum binders; (D) As in C, but with incubation in the presence of blood cells.

of third binder has not been determined. TC I and third binder appear to be released by white blood cells (7, 8) while the source of TC II is unknown.

The *in vitro* studies presented here support the above facts and provide a few insights into the serum transport of vitamin B_{12}. TC I is shown to be a fairly stable protein, being apparently unchanged even after 52 hr incubation at 37°, by when TC II begins to disappear or at least to lose its B_{12}-binding capacity (Fig. 1 A). Levels of TC I and third binder increase strikingly in the presence of cells, illustrating the role of the blood cells in their release. Third binder increase particularly exceeds that of TC I once cell lysis occurs, confirming the origin of third binder in blood cells, probably predominantly granulocytes (9). We have earlier postulated that serum third binder and TC I are related though different (10). The different patterns of increase of the two binders further suggest that they are not identical and are released differently by cells or are released by different cells.

The bulk of a trace dose of $^{57}CoB_{12}$ preferentially binds to TC II (80% vs. the 74% TC II fraction of serum UBBC). TC II loses its $^{57}CoB_{12}$ quickly in the presence of cells but does not in the absence of cells, which appears to reflect progressive attachment to cells and to binders released by cells rather than protein denaturation since TC II UBBC remains unchanged. Transfer to cells is supported by the fact that TC II binding is lower initially in the presence of cells than in the absence of cells. That this finding is as true of TC II UBBC as it is of TC II carrying $^{57}CoB_{12}$ suggests that TC II attaches to blood cells whether it is carrying vitamin B_{12} or not.

TC I binding of $^{57}CoB_{12}$ appears to increase slightly with time. The role of third binder is difficult to evaluate from the present data since, as is usual in normal serum,[1] it was present in barely detectable amounts in the serum. When present, third binder appears to be more avid than TC I for $^{57}CoB_{12}$ since its binding of trace $^{57}CoB_{12}$ (Fig. 1 D), unlike TC I, parallels its increasing presence in serum (Fig. 1 B). While it is not possible to say whether transfer from TC II to TC I and third binder is direct or indirect, binding of $^{57}CoB_{12}$ by cells need not be an intermediate between release by TC II and binding by TC I since TC I binding of $^{57}CoB_{12}$ over 24 hr rose equally irrespective of absence or presence of cells, and by 52 hr was actually greater in the absence of cells. Since TC II UBBC remains fairly constant, transfer of $^{57}CoB_{12}$ was probably direct from TC II to TC I and third binder rather than a result of $^{57}CoB_{12}$ release by denatured TC II. Transfer to TC I appeared in our study to be very gradual and small in amount. Particularly in the early phases, transfer to it and to third binder, when present, was quantitatively overshadowed by $^{57}CoB_{12}$ transfer to cells. Average uptake by the 6 ml of cells present was 350–400 pg of the original 1500 pg $^{57}CoB_{12}$ in each flask.

Summary. In vitro studies of the binding of vitamin B_{12} in the presence and absence of cells were done. Sera from 3 healthy volunteers were incubated with a trace amount of $^{57}CoB_{12}$ and aliquots were incubated with and without autologous peripheral blood cells. At the same time, the sera were incubated in this fashion but without added $^{57}CoB_{12}$ to determine the effect of the incubation conditions on the UBBC.

The results showed the following: (1) UBBC and its 3 component binding proteins remained constant over 24 hr, indicating that related observations were probably not due to denaturation of proteins. Fall in UBBC after 24 hr was due to decrease, possibly denaturation, of TC II while TC I remained constant; this may reflect the short half-life of TC II and the long half-life of TC I *in vivo*. (2) In the presence of cells, however, serum UBBC rose with time as a result of increasing TC I and third binder. Both binders rose equally over 24 hr while the cells remained intact, third binder becoming particularly increased thereafter as cell destruction became noticeable. These findings demonstrate that, unlike TC II, TC I and third binder are derived from blood cells. The differing patterns of their increase suggest that the latter 2 proteins are not identical. (3) Trace $^{57}CoB_{12}$ binds preferentially to TC II and is initially transferred predominantly to cells. Later, transfer occurs to third binder, if present, and to a lesser extent to TC I. It appears, but is not certain, that transfer to TC I and third binder is direct, since it takes place even in the absence of cells. (4) TC II appears to attach to cells whether it is carrying $^{57}CoB_{12}$ or not.

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