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Competition Between Apo and Holo Transcobalamin II (TC II) For the TC II-Mediated Uptake Process[1] (40172)

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Approximately equal cellular, receptor or tissue uptake of either holo TC II (TC II which is carrying Cbl) or apo TC II (the carrier protein only, free of Cbl) has been reported (1, 2). This seemed to be an unlikely behavior for TC II since its function is to deliver Cbl. If one accepts that about 90% of serum TC II is apo TC II (3), and some would put the figure even higher, then equal competition between apo and holo TC II would create a 9:1 disadvantage for an essential transport function. Looking at analogous transport systems, the promotion of iron uptake by transferrin for example, the holo form is much better taken up than the apo (4).

The models of the uptake process for the present study were HeLa cells and cultured lymphocytes. The HeLa cell is an established model for testing the function of TC II and although the peripheral lymphocyte is known to be responsive to TC II-Cbl when stimulated (5), the use of a cultured, established human cell line constitutes a new model.

The cellular uptake of Cbl can be divided into several phases and through preliminary work we defined the events encompassed by the conditions for 3 hr uptake at 37° used in the main part of the study. (a) Uptake of TC II-Cbl reaches a plateau and TC II-Cbl is at a maximum within the cell. (b) The incorporated Cbl has the potentiality for conversion to coenzymes but the process is just beginning. (c) No free Cbl has been released by the HeLa cell and only small amounts have begun to leave the lymphocyte.

This work has been published in abstract form (6).

Materials and methods. Materials. The lymphocyte line, RPMI 6410, and the culture media, RPMI 1640, were purchased from Associated Biomedic Systems Inc. Other materials for cell culture and the HeLa cell were as described (7). The semipurified TC II was prepared from human Cohn fraction III (generously supplied by Dr. J. Fenton of the Division of Laboratories, New York State Dept. of Health) as before (7). The CN [^{57}Co] Cbl (7) was reduced to a specific activity of about 45 mCi/mg.

Cultures. The lymphocytes were maintained in 75 ml Falcon Culture flasks in RPMI 1640 medium pH 7.0 containing 20% fetal calf serum, 100 units/ml of penicillin and 50 μg/ml of streptomycin. The cultures were adjusted to 3.0–5.0 × 10^5 viable cells after feeding and allowed to grow to 1.0–1.3 × 10^6 viable cells/ml. The viability of both cells was determined by the Trypan blue exclusion method.

Preparation of the holo and apo TC II. The starting material was always from a single lot of Cohn fraction III. Workable batches were made by batch absorption using CM-Sephadex (7). Each batch was checked for Cbl content and for unsaturated binding capacity for Cbl (3, 8) giving the total amount of TC II as well as the apo and holo fractions. Aliquots of a size suitable for a day's work were frozen and thawed as needed. Both holo and apo TC II were prepared from the same batches. To obtain a preparation of only holo TC II, the binding sites of an aliquot were fully saturated with either radioactive or nonradioactive CN Cbl, depending on the needs of the experiment. To do so, CN Cbl was added to give a concentration 15% greater than the known binding capacity, the mixture incubated for 20 min at 37° and the free CN Cbl removed by overnight dialysis against 100 vol of medium M-199. Preparations of apo TC II were dialyzed in the same way and then the binding capacity for Cbl checked. Although binding sites for Cbl remained intact, it was necessary to check the biological integrity of the apo TC II by converting it to holo TC II and testing it in the lymphocyte system. Since all preparation of apo TC II

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contained some holo TC II (the holo TC II derived from the serum from which the Cohn fraction III was obtained), the following quantitative adjustments were made. The mixture of holo and apo TC II in the competition studies contained a fixed amount of CN [^{57}Co]Cbl-TC II and increasing amounts of either nonradioactive holo TC II or nonradioactive apo TC II. The amount of TC II carrying nonradioactive Cbl naturally was included in the value for holo TC II with the dilution effect serving as a reduction in the specific activity of the radioactive holo TC II. Thus the final values for holo and apo TC II were as stated for each individual experiment.

Uptake studies. Medium M-199 was used in all of the uptake studies since the RPMI 1640 maintenance medium for the lymphocytes contained Cbl. First the concentration of holo TC II-Cbl needed to saturate the uptake process of a fixed number of each cell line was determined. This ratio of number of cells to amount of TC II-Cbl was a crucial part of the experiments since the end point was a competition for a limited capacity for uptake. There were 3×10^6 lymphocytes per tube containing 3 ml of medium to which was added 2.0 ng of CN [^{57}Co]Cbl. Approximately 1×10^6 HeLa cells were plated as a monolayer in 5 ml of medium per flask; the amount of CN [^{57}Co]Cbl was the same, 2.0 ng.

The principles of the studies were the same for both cell lines. First it was shown that the addition of unlabeled holo TC II in increasing amounts over the range of 1.0–10.0 ng depressed the amount of CN [^{57}Co]Cbl-TC II taken up as there was competition for the available but limited uptake process. Then, in parallel sets of studies potential competition by equal amounts of apo TC II was tested.

For the main studies the uptake by both cell lines was conducted at 37° for 3 hr. In order to limit uptake to the primary phase in a few studies, the uptake was measured after 15′ at 4°. All other conditions were the same as for the main studies. The system for recovery of the HeLa cells and the determination of uptake has been described (7). The lymphocyte cultures were centrifuged at 250 g for 5 min at 4° and the supernatant discarded. The cells were washed 3 times in this fashion with 5 ml of cold saline "A" (7) before counting.

Results. The relationship between a fixed number of cells and the amount of TC II-Cbl taken up is shown in Fig. 1. The saturation point of the process for 1×10^6 lymphocytes was reached at a concentration of 0.22 ng per ml corresponding to 2.0 ng per culture tube of 3×10^6 cells in 3 ml. Saturation of 1×10^6 HeLa cells was at 0.40 ng of TC II-Cbl per ml, corresponding to 2.0 ng per culture flask of 1×10^6 cells in 5 ml. For convenience, during the remainder of the studies, identical amounts of TC II-Cbl were added per culture container, the volume of media and numbers of cells being adjusted to correct for the difference between the capacities of HeLa cells and lymphocytes.

Although the promotion of uptake of Cbl by TC II has been well established for HeLa cells (9) and for stimulated human peripheral lymphocytes (5), the effect of TC II on established lines of lymphocytes had not. Figure 1 shows that TC II does promote the uptake of Cbl by RPMI 6410 cells.

The studies of the upper part of Table I show that the experiments were conducted at approximate saturation of the cell uptake and that nonradioactive holo TC II competed with radioactive holo TC II for the process. With increasing amounts of added nonradio-

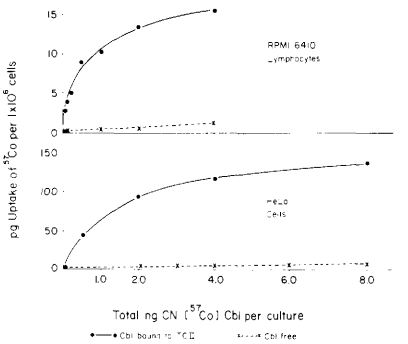

FIG. 1. The amount of holo TC II necessary to saturate the TC II-Cbl uptake of lymphocytes (upper) and HeLa cells (lower). The lymphocyte cultures contained 3×10^6 cells and HeLa cell cultures 1×10^6 cells. Fewer points were used in the HeLa cell studies because the level of TC II-Cbl required for saturation had been well studied earlier. Note that the uptake of free Cbl at different concentrations is also expressed for each cell type showing that binding to TC II enhanced the uptake of Cbl.

TABLE I. COMPETITION BETWEEN HOLO AND APO TC II FOR THE UPTAKE OF TC II-Cbl 3 HR AT 37°.

Form and amount (in ng) of TC II per culture			Uptake of ^{57}Co label as pg per 1 × 10^6 cells			
			By RPMI 6410 lymphocytes		By HeLa cells	
[^{57}Co] Cbl-TC II	Cbl-TC II	TC II	Mean	±1 SD[a]	Mean[b]	Range
2.0	—	—	17.36	0.55	136.26	(131.62–142.33)
2.0	1.0	—	12.53	0.63	92.78	(90.15–95.50)
2.0	3.0	—	8.23	0.47	54.72	(51.02–58.88)
2.0	5.0	—	6.25	0.49	40.09	(38.10–42.77)
2.0	10.0	—	3.95	0.51	26.59	(23.23–28.98)
2.0	—	—	17.36	0.55	136.80	(127.84–154.76)
2.0	—	1.0	16.28	1.49	127.47	(121.69–131.94)
2.0	—	3.0	15.65	1.77	116.14	(103.86–121.65)
2.0	—	5.0	15.94	1.49	115.91	(104.30–125.57)
2.0	—	10.0	15.11	1.36	111.19	(98.58–123.92)

[a] Mean and SD calculated from ten individual sets of data.
[b] Mean calculated from four individual sets of data.

active holo TC II the uptake of a fixed amount of CN [^{57}Co] Cbl-TC II declined to the expected degree. When expressed as Scatchard plot, the competition was linear.

Identical studies were performed substituting nonradioactive apo TC II for the nonradioactive holo TC II but keeping the CN [^{57}Co] Cbl-TC II the same, Table I. Inhibition at its maximum was about one-fourth that of holo TC II. Figure 2 gives the same information in graphic form. The same sets of experiments were repeated with HeLa cells instead of lymphocytes, Table I, with similar results.

Although we were more interested in the complete uptake process than its component parts, we did carry out a few studies for 15′ at 4° to impair uptake beyond the primary phase, Table II. Under these conditions there was much greater competition by apo TC II for the primary phase in both cell lines. The degree varied with the cell line, but was not as great as provided by holo TC II.

Discussion. There was some competition by apo TC II for the uptake process for holo TC II under the physiologic conditions of 37°, but by no means enough to prevent entry of TC II-Cbl into the cell. Whereas the HeLa cell was the more established of the two cell models used here, the lymphocyte may be the more physiologic because this cell type is known to need TC II for efficient Cbl uptake in man *in vivo* (10). The present observations are supported by observations from two laboratories showing the consequences of *in vivo* conversion of apo TC II to holo TC II (11, 12). Both holo and apo TC II were maintained at a constant level but with conversion

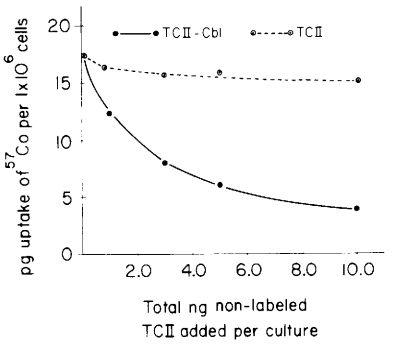

FIG. 2. A graphic expression of the competition for uptake by RMPI 6410 cells between radioactive and nonradioactive holo TC II and the lack of competition by apo TC II. Each point is the mean of ten experiments. The number of lymphocytes was 3 × 10^6 cells in each experiment and the amount of CN [^{57}Co] Cbl was 2.0 ng.

of the predominant apo TC II to all holo TC II, there was movement of the holo TC II out of circulation. The apo TC II was then restored to its prior level.

The observations of the present study did not agree with those of Schneider et al (1) who injected rabbit apo TC II labeled with ^{131}I and rabbit holo TC II labeled with ^{125}I injected into the rabbit. The apo TC II label was cleared from the circulation somewhat more rapidly than the holo TC II label and tissue uptake was similar for both forms. Differences in species, possible alterations of TC II through iodination and the fact that one model was *in vivo* and the other *in vitro* might form the basis for the difference in observations between their study and the present one. Also the present study was addressed only to the question of competition

TABLE II. COMPETITION BETWEEN HOLO AND APO TC II FOR THE UPTAKE OF TC II-Cbl 15 MIN AT 4°.

Form and amount (in ng) of TC II per culture			Uptake of ^{57}Co label as pg per 1 × 10^6 cells[a]			
			By RPMI 6410 lymphocytes		By HeLa cells	
[^{57}Co] Cbl-TC II	Cbl-TC II	TC II	Mean	Range	Mean	Range
2.0	—	—	11.15	(10.33–12.20)	12.22	(11.25–13.18)
2.0	1.0	—	7.29	(6.73–7.85)	9.68	(8.69–10.90)
2.0	3.0	—	4.67	(4.13–5.21)	7.64	(6.63–8.52)
2.0	5.0	—	3.46	(2.95–3.97)	5.93	(5.64–6.17)
2.0	10.0	—	1.96	(1.72–2.26)	4.30	(4.13–4.46)
2.0	—	—	11.15	(10.33–12.20)	11.17	(9.03–12.83)
2.0	—	1.0	8.71	(8.46–9.21)	9.44	(9.01–10.01)
2.0	—	3.0	6.69	(6.05–7.42)	8.06	(6.83–8.98)
2.0	—	5.0	5.85	(5.49–6.29)	7.33	(5.59–8.21)
2.0	—	10.0	3.94	(3.57–4.35)	5.31	(4.62–6.34)

[a] Mean calculated from four individual sets of data.

between apo and holo TC II for the same process. Should apo TC II enter cells by a mechanism different from that of holo TC II, uptake would be detected by the model of Schneider et al but not in the present study. Our observations at 4° were very close to those of Friedman *et al.* (2) made from the model of a preparation of cell membranes from human placenta containing receptors for TC II-Cbl. Apparently there is substantial, although not complete, competition for the primary phase of TC II-Cbl uptake by apo TC II but not for the complete, biologically active penetration of TC II-Cbl into the cell. Whether the placental cells handle apo TC II in any special way, as has been postulated (13) is unknown.

Summary. The uptake process of transcobalamin II-cobalamin (TC II-Cbl) of HeLa cells and RPMI 6410 lymphocytes was evaluated for its capacity to respond to apo TC II, that not carrying Cbl. At 4° there was partial competition by apo TC II for holo TC II in the primary phase of uptake. However, at 37° for 3 hr there was only slight competition for the process by which TC II-Cbl penetrates the cell.

While the present manuscript was in the process of review and revision, a relevant article, "Binding and Uptake of Transcobalamin II by Human Fibroblast" by Youngdahl-Turner P., Rosenberg LE., and Allen RH appeared in the Journal of Clinical Investigation **61,** 133–141, January 1978. The article reports a comprehensive study covering many phases of the topic, and included were studies of competition by apo TC II for uptake of holo TC II at 4°. Competition under those circumstances was similar to that observed at 4° in the present study. Competition was not tested at 37°.

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