"SPECIFIC" BINDING OF RADIOIODINATED TRANSFERRIN TO POLYPROPYLENE CULTURE TUBES

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Summary. Radioiodinated human serum transferrin has been found to interact with polypropylene culture tubes in a manner that mimics the specific binding of a protein to a cellular receptor. The magnitude of this transferrin-culture tube interaction is such that any true binding of the protein to cells present is masked. Characteristics of this non-specific "binding" process as well as kinetic and thermodynamic parameters are compared with previously reported values for the interaction of transferrin with both reticulocytes and chinese hamster fibroblasts.

Introduction. In order to study the interaction of various biologically active macromolecules with their supposed receptors on the surface of target cells, it has been necessary to employ a radioactively-labeled macromolecule. To this end, numerous studies have been performed in which the binding of radioiodinated polypeptides to their "specific" cellular receptors has been investigated (see refs. 1 and 2 for review). These studies have provided great insight into both the nature of hormone-receptor interactions and the mechanisms of action of polypeptide hormones. Recently, however, Cuatrecasas and Hollenberg (3) demonstrated that the binding of radioiodinated insulin to inert materials, such as Millipore filters and talc particles, can mimic the binding of insulin to cellular receptors. Indeed, the binding of radioiodinated insulin to inert materials in the absence of tissue demonstrated saturability, reversibility, high affinity, and some specificity.

In an attempt to demonstrate specific receptors on human lymphocytes for serum transferrin, we have employed radioiodinated transferrin for binding studies. In this paper, we describe "specific"

binding of the labeled transferrin to polypropylene culture tubes. The kinetics and thermodynamics of this interaction resemble closely data obtained previously in studies with serum transferrin and both reticulocytes (4) and chinese hamster fibroblasts (5).

Methods. Iodination. Human serum transferrin (Behring Diagnostics) was iodinated as the iron comples (2 atoms Fe^{3+} per molecule transferrin) by the method of Katz (6) employing I_2, [^{125}I]NaI (carrier free, New England Nuclear) and cold NaI. The amount of iodine was calculated to produce an average of 1 residue of monoiodotyrosine per molecule of transferrin (7). Iron was removed as described previously (7) and the radioiodinated protein then was dialyzed and freeze-dried.
Lymphocyte isolation and culture technique. Techniques for the isolation and culture of human peripheral blood lymphocytes have been described (8). Cells were incubated in 12x75 mm polypropylene culture tubes (Falcon Plastics).
Binding assay. Interest in this laboratory is in the role of transferrin in zinc metabolism. Consequently, sufficient 0.02 M zinc acetate was added to a solution of iodinated transferrin in Hank's balanced salt solution to form the 2Zn:1 transferrin complex, denoted ^{125}I-ZnTf. To assess binding, an amount of ^{125}I-ZnTf (10-200µg in 25µl) was added to 1.5 x 10^6 lymphocytes in 1 ml serum free RPMI-1640. Cells were incubated for 30 minutes at 37° for routine binding assays or for other time periods as indicated in the text. To terminate the incubation, 2 ml ice-cold Hank's balanced salt solution were added and the cells centrifuged at 1000 rpm for 10 minutes. The pellet was resuspended in 2 ml ice-cold Hank's solution and centrifuged again. This procedure was repeated one additional time. After decanting the final supernatant, the radioactivity associated with the cell pellet was counted directly (i.e., the washed cell pellet still in the original culture tube) in a Nuclear Chicago gamma counter. Control experiments were performed in which no cells were present. To correct for "non-specific" binding, a 50-100 fold excess of unlabeled ZnTf was added to some cultures prior to the addition of ^{125}I-ZnTf. The radioactivity counted after the washing procedure was subtracted from the radioactivity associated with the corresponding cultures containing ^{125}I-ZnTf and no cold protein. This final figure (Hot only - (cold + hot)) represents "specific binding" of the labeled transferrin.

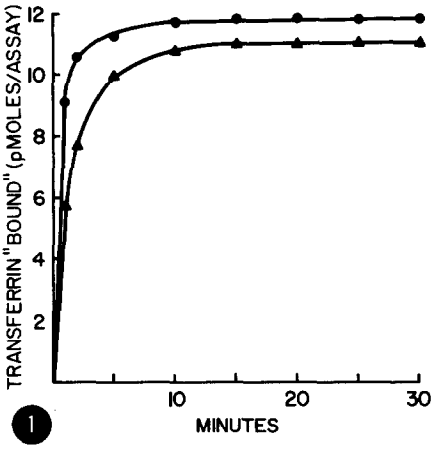
Results. Iodination of transferrin. Human serum transferrin was iodinated to a specific activity of 10-100mCi/µmole. Although this specific activity is considerably lower than that obtained for various hormones, such as insulin (9,10), the quantities of transferrin used in our binding studies (5-200µg ^{125}I-ZnTf/ml) while physiological, are much greater than those of the various hormones whose binding properties have been investigated. Activity of the protein was found to be unchanged by two criteria. First, the ability of the protein to bind both Zn^{2+} and Fe^{3+} was not altered, as judged by UV difference spectroscopy (11). Also, the visible absorption spectrum of the iron-transferrin complex was not changed. Second, the

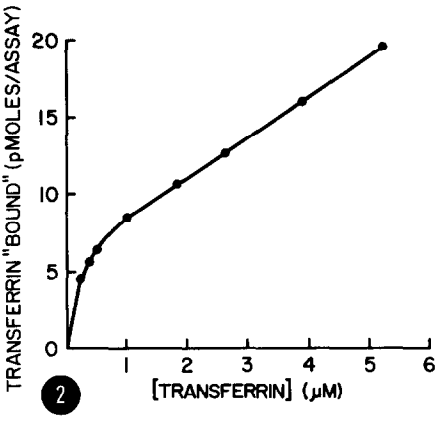
ability of the ^{125}I-ZnTf to enhance nucleic acid synthesis in phytohemagglutinin-stimulated human lymphocytes was the same as control ZnTf (8).

"Specific" binding of ^{125}I-ZnTf. Fig. 1 presents the "specific" binding of ^{125}I-ZnTf to human lymphocytes as a function of time. Curves are presented for binding at both 37° and 4°. Identical data was obtained in the presence or absence of lymphocytes. From a graph of log [free transferrin] vs time (not shown) a pseudo-first order rate constant for association was determined. The value at 37° was 0.56×10^{-4} sec^{-1}. Additionally, from an Arrhenius plot showing the temperature dependence of the rate constant (graph not shown), an energy of activation for the association reaction of +2.6 kcal/mole was determined.

The association of ^{125}I-ZnTf with the polypropylene culture tubes was essentially irreversible. After incubation of ^{125}I-ZnTf in culture tubes with no cells present, followed by normal washing procedures and then reincubation at 37° in fresh medium, no radioactivity dissociated from the tubes for periods or reincubation up to 24 hours.

Fig. 2 shows the concentration dependence of transferrin "binding" at 37°. Identical data were obtained in the presence or absence of 1.5×10^6 lymphocytes. The curve obtained appears to be characteristic of a system demonstrating two distinct classes of binding sites. Consequently, the data were analyzed by Scatchard analysis (12), as seen in Fig. 3. It should be noted that Scatchard analysis of these data is not valid, since the system does not demonstrate reversibility. A Scatchard plot was made, however, simply for comparison of our values for number of binding sites and association constant with previously obtained values for the transferrin-reticulocyte interaction (4) and the transferrin-chinese hamster fibroblast interaction (5). Accordingly, the Scatchard plot of Fig. 3 indicates two classes of binding sites, differing in both number and association constant. Extrapolation of the more steeply sloping line to the X-axis reveals that there are approximately 1.53×10^{12} molecules of ^{125}I-ZnTf bound per culture





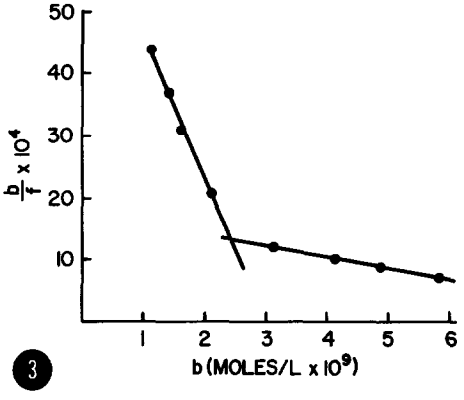


Fig. 1. "Binding" of ^{125}I-ZnTf to polypropylene culture tubes as a function of time. Data is presented for "binding" at 37° (O) and at 4° (Δ). Identical was obtained in the presence or absence of cells. Incubations were performed as described under Methods. Each experiment was run in duplicate and replicated three times. Each data point represents "specific binding", which was calculated by subtracting the radioactivity counted in the presence of cold ZnTf (50-100 fold excess over labeled protein) from the total radioactivity counted when only ^{125}I-ZnTf was present.

Fig. 2. "Binding" of transferrin as a function of ^{125}I-ZnTf concentration. Each experiment was run in duplicate and replicated three times. Incubations were performed as described under Methods with an incubation time of 30 min. Each data point represents "specific binding", as described for Fig. 1. Identical data was obtained in the presence or absence of lymphocytes.

Fig. 3. Scatchard plot for the binding of transferrin to polypropylene culture tubes. Values for bound (b) and free (f) transferrin were calculated from the data of Fig. 4.

tube. If, however, the interaction of ^{125}I-ZnTf to the culture tubes had gone unrecognized and the "binding" assumed, therefore, to be specific binding of the protein to lymphocytes, this would yield a value of approx-

imately 8.5 x 10^5 - 1 x 10^6 receptors for transferrin per lymphocyte. From the slope of the more steeply sloping line, an association constant of 2.25 x 10^6 M^{-1} at 37° can be calculated. The "lower-affinity site" has an association constant at 37° of about 1.8 x 10^5 M^{-1}.

From the data presented, certain thermodynamic papameters for the transferrin-culture tube interaction can be estimated. The standard free energy change, ΔG^o, for the association reaction can be estimated from the association constant. The standard enthalpy change, ΔH^o, can be estimated from the van't Hoff equation. Finally, the standard entropy change, ΔS^o, can be estimated from the values of ΔG^o and ΔH^o. These data are presented in Table I. Additionally, Table I compares the "binding" parameters of this study with values reported for the interaction of serum transferrin with both rabbit reticulocytes (4) and chinese hamster fibroblasts (5).

Effect of trypsin on labeled-transferrin"bound" to culture tubes. Culture tubes with "bound" transferrin were incubated for 10 min at 37° with 3ml 0.5% trypsin in Hanks' salt solution. The liquid was decanted, and both the liquid and culture tube counted for radioactivity. It was found that trypsin quantitatively released the labeled transferrin associated with the tube.

Discussion. These studies demonstrate the apparently "specific" binding of radioiodinated transferrin to a supposedly inert component of the assay system, the polypropylene culture tube. This "binding" is of such a magnitude that any truly specific binding of transferrin to lymphocytes is masked. Our initial binding assay, as described in this report, was chosen for its convenience, since incubations, washings, and gamma counting could be done in a single tube. Caution must obviously be exercised if this procedure is followed in other studies. We have since modified our binding assay to allow a more facile and accurate assessment of specific transferrin binding to human lymphocytes. This will be reported elsewhere (J.L. Phillips, manuscript in preparation).

TABLE I. Kinetic and Thermodynamic Parameters for Transferrin-Culture Tube, Transferrin-Reticulocyte, and Transferrin-Fibroblast Interactions.

Parameter	System: Culture Tube	Reticulocyte (Ref.4)	Fibroblast (Ref.5)
Time for maximum binding to occur	10-15 min	10-15 min	10-15 min
Rate constant for Association Reaction (37^{o})	0.56×10^{-4} sec^{-1}	0.565×10^{-4} sec^{-1}	–
Activation Energy for Association Reaction	2.6 kcal/mole	10.8 kcal/mole	–
Number of "Binding Sites" per cell (37^{o})	$8.5 \times 10^{5} - 1.0 \times 10^{6}$[a]	$2.0 \times 10 - 5.6 \times 10^{5}$	9.3×10^{5}
Association Constant (37^{o})	$2.25 \times 10^{6} M^{-1}$ ("high affinity") $1.8 \times 10^{5} M^{-1}$ ("low affinity")	$2.07 \times 10^{5} M^{-1}$	$2.3 \times 10^{6} M^{-1}$
ΔG^{o} (37^{o})	-8.9kcal/mole	-8.19kcal/mole	–
ΔH^{o} (37^{o})	+1.2kcal/mole	+1.5kcal/mole	–
ΔS^{o} (37^{o})	+32.7cal/mole·deg	+31cal/mole·deg	–

[a] There are approximately 1.53×10^{12} molecules of transferrin "bound" per culture tube. If these moleclues were erroneously assumed to be bound to $1.5 - 1.8 \times 10^{6}$ lymphocytes per culture tube, as in our standard binding assay, these values would result.

The similarity of our binding data to that reported by Baker and Morgan (4) and by Messmer (5) for the binding of transferrin to rabbit reticulocytes and chinese hamster fibroblasts, respectively, is striking. Baker and Morgan(4) used a binding assay in which the radioactivity associated with their incubation tubes, rather than the reticulocytes, may have been measured. No cell-free controls were reported. Messmer (5) incubated radioiodinated transferrin with fibroblasts attached to tissue culture dishes (glass or plastic was not specified). She released the cells from the dishes using trypsin and then counted the whole solution of cells plus trypsin. Based on data presented here, any labeled transferrin associated with the tissue culture plates would be removed by the trypsin and then erroneously interpreted as protein bound to the cells. It is suggested that the binding of transferrin to reticulocytes and to fibroblasts be reexamined to assure that the "binding" reported was indeed to the cells, and not to a non-specific component of the system.

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