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LIGAND INTERACTION

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ABSTRACT Immunohistological studies have indicated that membrane sites binding transferrin are present upon activated human peripheral blood lymphocytes. In this study, we have investigated transferrin uptake in human lymphocytes exposed to phytohemagglutinin (PHA), by quantitative radiobinding and immunofluorescence in parallel. In stimulated lymphocytes, binding was maximal after a 30-min incubation, being greatest at 37°C, and greater at 22°C than at 4°C. Although some shedding and endocytosis of transferrin occurred at 22° and 37°C, these factors, and resulting synthesis of new sites, did not affect measurement of binding which was found to be saturable, reversible, and specific for transferrin (K_a 0.5–2.5 × 10^8 M^{-1}). Binding was greater after a 48-h exposure to PHA than after 24 h, and was maximal at 66 h. Sequential Scatchard analysis revealed no significant elevation in affinity of interaction. However, although the total number of receptors increased, the proportion of cells in which binding of ligand was detected immunohistologically increased in parallel, and after appropriate correction, the cellular density of receptors remained relatively constant throughout (60,000–80,000 sites/cell). Increments in binding during the culture period were thus due predominantly to expansion of a population of cells bearing receptors. Similar differences in binding were apparent upon comparison of cells cultured in different doses of PHA, and in unstimulated cells binding was negligible. Transferrin receptors appear, therefore, to be readily detectable only upon lymphocytes that have been activated.

INTRODUCTION

The existence of specific receptor sites with high affinity for radiolabeled transferrin upon immature erythrocytes and reticulocytes is a well-recognized phenomenon (1–3). The full range of possible biological functions of these receptor sites has not been conclusively established. However, they appear to play a role in the fixation of transferrin at the cell surface as a prelude to internalization and utilization of complexed metallic cations, particularly iron, although whether endocytosis of transferrin per se is required remains controversial (4). A number of studies have been performed in a search for similar membrane structures on other cells that might be anticipated to have high metabolic requirements for cations such as iron or zinc. Transformed cell lines such as those of lymphoblastoid or malignant origin, which have a large growth fraction, possess transferrin binding sites with similar characteristics which may be demonstrated using either radiolabeled transferrin (5, 6) or immunofluorescence techniques (7). We have also recently described evidence indicating the ability of a fetal tissue, human syncytiotrophoblast (8, 9), and of normal human peripheral blood lymphocytes activated by mitogenic lectins in vitro (10) to bind human transferrin. However, although the immunohistological procedures employed (8–10) allow assessment of the proportion of cells participating in the transferrin binding reaction and also permit studies of the topography and mobility of binding sites within the membrane (7, 11), they are not suitable for the accurate measurement of the saturability and affinity of inter-

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action (11). Similarly, quantitative measurements of the effects upon binding of transferrin after complex formation with iron or other cations, an important consideration in terms of receptor function (3), are not currently practical using immunohistological methods. These factors provide a strong stimulus for composite studies making use of both radiobinding and immunofluorescence techniques.

Such a combined approach may also assist in resolving certain discrepancies that have arisen concerning the presence of transferrin receptor sites on peripheral blood lymphocytes. We observed transferrin binding by immunofluorescence only upon activated lymphocytes; never >5% of resting or quiescent cells bound transferrin and in most cases such unstimulated cells were completely negative, regardless of whether they had been cultured in the absence of mitogenic stimulus or had been freshly obtained from peripheral blood (10). However, in the one previous study of the cellular binding of transferrin by normal human lymphocytes which was performed using radioiodinated ligand (12), evidence of transferrin binding was reported in nonactivated lymphocytes, and in addition membrane uptake did not appear to increase significantly subsequent to stimulation with mitogen, although the same lectin, phytohemagglutinin (PHA),[1] was employed. In the present study, we have measured binding of transferrin to human peripheral blood lymphocytes by both radioisotopic and immunohistological binding assays in parallel. The results obtained demonstrate that cellular activation is a prerequisite for the appearance of significant transferrin binding, and that the latter phenomenon represents a specific interaction between this ligand and membrane receptors of high affinity.

METHODS

Human transferrin. Human transferrin containing <0.05% ferric iron (Sigma Chemical Co., St. Louis, Mo.) gave a single protein peak upon Sephadex G-100 gel chromatography in 0.15 M phosphate-buffered saline (PBS) pH 7.4, and a single precipitin line upon double radial immunodiffusion against antiserum to whole human serum (Miles Laboratories, Inc., Elkhart, Ind.). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to Laemmli (13). After staining with Coomassie Blue, gels were subjected to optical density analysis in an Ortec gel scanner (Ortec Inc., Oak Ridge, Tenn.), which confirmed that >95% of protein occupied a peak with relative mobility (R_f) corresponding to a molecular weight of ~78,000.

Radiolabeling of transferrin. Human transferrin was conjugated with ^{125}I by means of lactoperoxidase either in liquid (14) or solid phase (15). The initial step involved saturation of transferrin with iron, to prevent iodination of iron binding sites and to reduce the risk of radiation-induced damage (1). Saturation was achieved by addition of 2:1 chelates of iron with nitrilotriacetic acid, or ferric ammonium citrate, to transferrin solutions at physiological concentrations (2–4 mg/ml in PBS, pH 7.4) to achieve a ratio of iron: protein of 1.5 μg/mg, and after reaction at 22°C for 60 min, unbound iron and chelator were removed by dialysis against PBS. The amounts of iron added were sufficient to promote >98% saturation, and the latter was monitored by spectrophotometric analysis (1) and urea electrophoresis as described by Leibman and Aisen (16).

Lactoperoxidase-catalyzed radioiodination was carried out using various amounts (50–500 μg) of transferrin and various quantities (500–2,000 μCi) of carrier-free ^{125}I as NaI (sp act 17 Ci/mg, New England Nuclear, Boston, Mass.). For solid-phase labeling, enzymobeads conjugated with lactoperoxidase and glucose oxidase (Bio-Rad Laboratories, Richmond, Calif.) were employed together with beta glucose (15), whereas for the liquid-phase procedure, 1 μg of lactoperoxidase (Sigma Chemical Co.) was used with 0.003% hydrogen peroxide and potassium iodide (KI) as described by Marchalonis (14). Unbound ^{125}I was then removed by desalting chromatography on Sephadex G-25, and aliquots of the final preparations of radioiodinated transferrin (^{125}I-Trf) were checked to ascertain that >95% of counts were protein-bound as judged by 20% trichloroacetic acid (TCA) precipitation. The efficiency of incorporation of radiolabel was ~30–45% for solid-phase iodination and 70–80% for the liquid-phase procedure and the specific activity was in both cases 0.5–5.0 μCi/μg. The structure and functional integrity of ^{125}I-Trf were shown to be unaltered as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13), thin-layer analytical polyacrylamide gel isoelectric focusing with print immunofixation (17–19), assays of iron-binding capacity (16), immunohistological studies of binding to trophoblast and transformed cells (7–10), and immunochemical studies of antigenicity including double radial immunodiffusion and solid-phase affinity chromatography (20).

For comparative studies of the binding of iron-saturated transferrin and apoprotein, ^{125}I-Trf was depleted of iron by addition of 0.3 M acetate buffer pH 5.5 containing 0.01 M EDTA. After reaction for 1 h, the resulting apoprotein was then dialyzed to equilibrium against 0.15 M PBS, pH 7.4, to remove Fe-EDTA chelates. Portions were retained as apoprotein, whereas other aliquots were then reacted with Fe: nitrilotriacetic acid or ferric ammonium citrate as described above to achieve the desired level of iron saturation.

Radioiodinated transferrin binding assays. The cells examined included freshly obtained mononuclear cells, and similar cell preparations which had been cultured in the presence or absence of optimum amounts of PHA as previously described in detail (10). The binding reactions were performed in 12 × 75-mm glass tubes (Fisher Scientific Co., Pittsburgh, Pa.) in the presence of 1% human serum albumin (Miles Laboratories, Inc.). Unbound ligand was removed by passage of cells through a 0.5-ml density cushion of 30% sucrose (P = 1.125) in a 1.5-ml conical-bottom polypropylene tube (Fisher Scientific Co.). The reaction mixture was centrifuged at 15,000 rpm (12,800 g) for 5 min in an Eppendorf centrifuge, and the supernate and majority of the sucrose cushion were aspirated. The tips of the vials containing the cell pellet were then severed with a scalpel, transferred to Biovials (Beckman Instruments, Inc., Fullerton, Calif.), and examined in a gamma counter. The sucrose cushions employed resulted in the passage of >99% mononuclear cells into the tube tip, whereas the proportion of unbound ^{125}I-Trf in the pellet was negligible (<0.1%). This procedure was used for all subsequent experiments, except for immunofluorescence procedures and studies of the reversibility of

[1] *Abbreviations used in this paper*: PBS, phosphate-buffered saline; PHA, phytohemagglutinin; ^{125}I-Trf, radioiodinated transferrin (^{125}I).

binding which required viable cells throughout; washing in these experiments was accomplished by three cycles of addition of PBS and centrifugation at 400 *g* for 10 min. In each experiment, an additional assay was run containing a 1,000-fold excess (usually 1 mg) of unlabeled transferrin to allow correction for binding of ^{125}I-Trf unrelated to ligand:receptor interactions, a phenomenon usually termed nonsaturable or "nonspecific" binding of ligand (21–23). The magnitude of nonsaturable binding was in every case <5% of the total counts, and was generally <1%.

Several substances were tested for their ability to block uptake of ^{125}I-Trf including apotransferrin preparations purified from human, bovine, rabbit, and equine serum (Miles Laboratories, Inc.) which were shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be >90% pure, and certain other purified serum proteins, including bovine serum albumin, human serum albumin and immunoglobulin (Ig)G (Miles Laboratories, Inc.). In addition alpha$_1$-acid glycoprotein (orosomucoid), alpha$_1$-antitrypsin, and Gc component were purified from normal human serum using a previously described protocol (24, 25) which included sequential depletion of albumin by means of affinity chromatography upon Blue-Sepharose 6B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.), and preparative column isoelectric focusing (model 8100-1, LKB Instruments, Inc., Rockville, Md.) performed according to Vesterberg (26). After unpacking the focusing column, protein fractions (55 × 2 ml) were analyzed by means of fused rocket immunoelectrophoresis as described by Svendsen (27), using defined antisera to Gc globulin, alpha$_1$-antitrypsin, and alpha$_1$-acid glycoprotein (Meloy Laboratories, Inc., Springfield, Va.), to transferrin (Dakopatts, Accurate Chemical & Scientific Corp., Hicksville, N. Y., U. S. Distributor), and whole human serum. Fractions of ascending pH, enriched respectively, in alpha$_1$-acid glycoprotein, alpha$_1$-antitrypsin, and Gc globulin were then pooled and dialyzed against 0.15 M PBS, pH 7.4, to remove sucrose and ampholytes. To remove trace amounts of transferrin, fractions were then subjected to chromatography using AH Sepharose 4B to which was coupled antiserum to transferrin (20). The adsorption and removal of transferrin was monitored by addition of a trace amount of ^{125}I-Trf (60,000 cpm/column run). For alpha$_1$-antitrypsin and Gc globulin, an additional step involving ion-exchange chromatography on DEAE-Sephacel (Pharmacia Fine Chemicals) with elution by increasing molarity (0.00–0.25 M NaCl) was performed to improve the purity of the final preparation. All protein preparations to be used in studies of specificity of the transferrin-binding reaction were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13) and isoelectric focusing with print immunofixation (17, 18). All these preparations were found to be >90% pure, did not contain detectable transferrin; and none were appreciably altered, as judged by molecular weight, the isoelectric points of the isomers and in the case of alpha$_1$-antitrypsin by retention of tryptic inhibitory capacity (28).

Immunohistological transferrin binding assays. To perform direct comparative studies of the radiobinding assay described in this investigation and the immunofluorescence procedure that has been used extensively in our previous studies (7–11), binding of transferrin was assessed in certain experiments by radioisotopic and immunohistological means in parallel. The latter methods have been previously described, together with the controls and specificity checks employed (9, 10). The protocol involved serial reaction of the cell population under investigation with a source of human transferrin, rabbit antiserum to human transferrin (Dakopatts), and a fluorescein isothiocyanate labeled sheep antiserum to rabbit immunoglobulin with three washes in PBS between each layer. In the present studies, cells were reacted with 1 μg unlabeled or ^{125}I-conjugated transferrin at 22°C. After the two subsequent reactions with antisera at 4°C, one aliquot of each cell preparation was washed by centrifugation, mounted in 50% glycerol in PBS, pH 7.4, and examined by fluorescence microscopy (10), and the other aliquot was centrifuged through a 30% sucrose cushion and submitted to gamma counting.

RESULTS

Derivation of optimum conditions for radiolabeled ligand binding assays. Assays carried out using a wide variety of cell concentrations and reaction volumes revealed that most reproducible results were obtained at cell concentrations of 1–2.5 × 10^7/ml, and for the experiments reported in this study, each assay contained 100 μl of cell suspension (1–2.5 × 10^6 cells) together with appropriate quantities of ^{125}I-Trf and other reaction components in 0.15 M PBS pH 7.4 to a final volume of 500 μl. The optimum time of incubation of cells with radiolabeled ligand was examined by reaction of cells with a constant amount of ^{125}I-Trf (200 ng) for increasing time intervals. Binding attained a maximum within 30 min, and the profile was similar at 4°, 22°, and 37°C; therefore a 30-min incubation time was used for all subsequent experiments, with the exception of studies of the reversibility of binding (see below). Investigation of the temperature dependence of ^{125}I-Trf binding was carried out by comparison of isotherms constructed at 4°, 22°, and 37°C, the time of incubation with ^{125}I-Trf being 30 min throughout. These studies showed significantly higher binding values at 22° and 37° than at 4°C (Fig. 1 and Table I), but it was noted that the figures obtained when ^{125}I-Trf was added in large amounts (e.g., 1,000 ng) at 22° or 37°C were reduced in comparison with the quantities bound when smaller amounts of ligand (400 or 600 ng) were employed. This observation led to the examination of several factors that might have influenced the results obtained.

Firstly, degradation of ligand during interactions with cells at 22° and 37°C has been shown with certain hormones such as insulin (21, 22) to effect substantial inaccuracies in the estimation of ligand binding. Nevertheless, results obtained in the present study by concurrent treatment of reaction supernates with 20% TCA together with 3% bovine serum albumin as a coprecipitating agent did not reveal any increases in nonprecipitable counts (Table I). Secondly, we have recently demonstrated that lateral mobility of binding sites may occur at 22° and 37°C, culminating in some endocytosis and shedding of bound transferrin (29). However, although pretreatment of cells with sodium azide, cytochalasin B, or colchicine, confirmed the suppression of capping reactions and endocytosis described previously (29), this was accompanied by

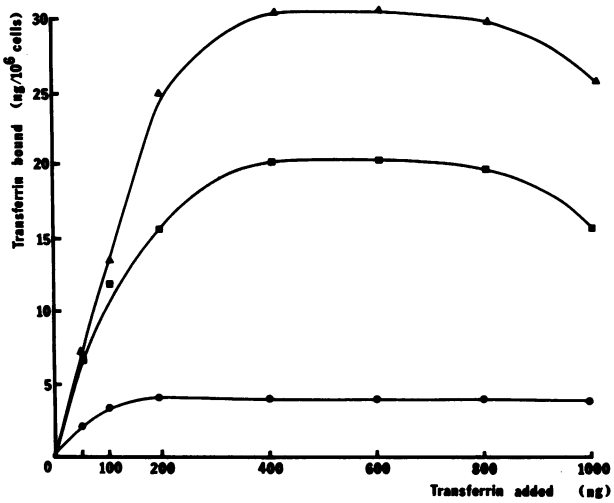

FIGURE 1 Temperature dependence of ^{125}I-transferrin binding. Peripheral blood lymphocytes were stimulated with 10 μl PHA for 66 h, and aliquots of 2.0 × 10^6 cells were then incubated at 4°, 22°, or 37°C with increasing amounts of ^{125}I-transferrin for 30 min. Unbound ligand was removed by centrifugation of cells through a 0.5-ml density cushion of 30% sucrose. After subtraction of nonsaturable binding, the amount of transferrin bound (ng/10^6 cells) was uniformly greater at 22°C (■) than at 4°C (●), and was maximal at 37°C (▲).

no significant changes in quantitative binding of ^{125}I-Trf. Thirdly, the possible reappearance of membrane binding sites secondary to endocytosis (29) was examined. In other experiments, we have demonstrated that this phenomenon required active protein synthesis and could be blocked by cycloheximide.[2]

[2] Galbraith, R. M., and G. M. P. Galbraith. The role of protein, RNA and DNA synthesis in the development and expression of transferrin binding sites on mitogen-activated peripheral blood lymphocytes. Manuscript submitted for publication.

In the present study, when cells were preincubated with cycloheximide (25 μg/ml) for 30 min at 22°C and washed twice in PBS, no significant changes in the amount of transferrin bound could be discerned either at 4°, or at 22° or 37°C. These findings indicated that under the experimental conditions used, neither ligand degradation, endocytosis and shedding, nor receptor reappearance caused significant inaccuracies in the estimation of binding at either 22° or 37°C. Therefore subsequent experiments were performed at 22°C except where indicated.

Characteristics of the transferrin binding reaction. The binding isotherms obtained (Fig. 1 and Table I) demonstrated that the binding reaction was saturable, although the maximum amount of ^{125}I-Trf bound in any given experiment varied from 1 to 30 ng/10^6 cells depending not only upon such factors as the time and temperature of interaction (see above), but also upon the degree of cellular activation and to a minor extent upon iron saturation of transferrin (see below). The affinity of binding was examined by means of competitive binding experiments, the results being expressed in the form of Scatchard plots (23). A representative example derived with cells exposed to PHA for 66 h is shown in Fig. 2. No evidence indicating the existence of subsets of receptors with differing affinities (21, 22) was obtained, and in cells from five normal subjects affinities ranged from 0.5 to 2.5 × 10^8 M^{-1}. The numbers of transferrin binding sites per cell were estimated from the intercept of the abscissa to be 60,000–80,000 sites/cell.

The specificity of the interaction between transferrin and cell membranes was investigated by binding experiments in which incubation with ^{125}I-Trf (200 ng) was preceded by reaction with increasing amounts of the unlabeled substance under test. As illustrated in Fig. 3, the greatest inhibition of binding of ^{125}I-Trf

TABLE I
*Investigation of Ligand Degradation**

		Amount of transferrin added, ng					
	Temperature	50	100	200	400	800	1,000
^{125}I-Transferrin bound, ng	4°C	2.2	3.2	4.1	3.9	3.8	3.8
TCA-precipitable cpm, %		86.7	87.9	89.0	92.1	93.3	93.9
^{125}I-Transferrin bound, ng	22°C	6.4	11.6	15.5	20.4	19.8	15.4
TCA-precipitable cpm, %		84.8	86.0	89.9	91.8	92.9	93.5
^{125}I-Transferrin bound, ng	37°C	7.1	13.6	24.8	30.8	29.8	25.3
TCA-precipitable cpm, %		81.4	83.7	88.0	91.1	91.7	92.7

* Cells cultured in the presence of an optimum dose of PHA (10 μl) were harvested at 66 h and aliquots of 10^6 cells reacted with increasing amounts of ^{125}I-transferrin as indicated at 4°, 22°, or 37°C. Bound ligand was separated from free transferrin by centrifugation of cells through 30% sucrose cushions, and the quantity of ligand bound in each case was calculated after subtraction of nonsaturable binding. The TCA precipitability of the counts in the reaction supernates were measured by addition to a 10-μl sample of 700 μl 20% TCA together with 290 μl 3% bovine serum albumin as co-precipitant, and are expressed as a percentage.

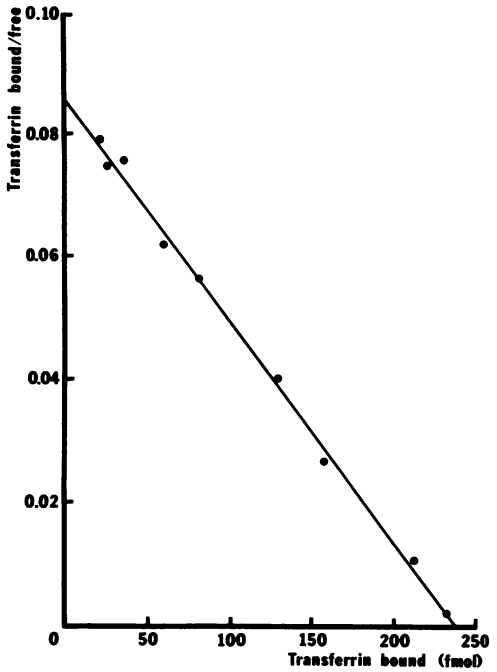

FIGURE 2 Scatchard plot of bound transferrin against the ratio of bound to free ligand. Cells were cultured as for Fig. 1 for 66 h, and reacted (2.0×10^6/assay) in competitive binding assays with a constant amount of ^{125}I-transferrin (25 ng) and increasing amounts of cold ligand (1 pg–10 ug). Separation of bound and unbound ligand was accomplished by passage of cells through a 30% sucrose cushion. After correction for nonsaturable binding the results were plotted as the mean of duplicate determinations. The correlation coefficient (r^2) as determined by least-squares analysis was 0.97. The affinity of association (K_a) as calculated from the slope was -1.8×10^8 M^{-1}, and the average number of receptor sites as estimated from the x intercept, Ro, after correction for the proportion of cells positive for transferrin binding by immunofluorescence (82%), was ~80,000 receptor sites/cell.

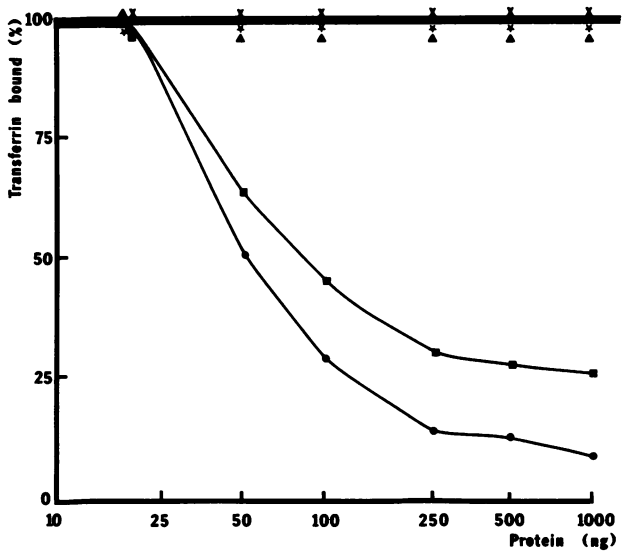

FIGURE 3 Specificity of binding. Cells exposed to PHA for 66 h were reacted in aliquots of 2.5×10^6 with varying quantities of preparations of human (●), rabbit (■), and bovine (*) transferrin, IgG (▲), and human serum albumin (×), for 30 min at 22°C, washed three times with PBS (400 g for 10 min), and then incubated in the normal fashion with a constant quantity (200 ng) of ^{125}I-transferrin for a further 30 min at 22°C. Bound and unbound ligand were separated by centrifugation of cells through 30% sucrose cushions (12,800 g for 5 min), and after correction for nonsaturable binding, results were expressed as the percentage of transferrin bound in relation to figures obtained with cells undergoing initial reaction with PBS. Equine transferrin, alpha$_1$-acid glycoprotein, alpha$_1$-antitrypsin, and Gc globulin also failed to induce significant reduction of transferrin binding, and for clarity have not been included in this figure.

was effected by unlabeled human transferrin. Purified rabbit transferrin also caused significant inhibition of binding, although of lesser degree dose-for-dose, but prior reaction with comparable amounts of bovine transferrin whether saturated with iron or as apoprotein did not result in any detectable reduction in binding of ^{125}I-Trf (Fig. 3). These results are compatible with previous immunohistological findings indicating that transferrin present in the fetal calf serum used for PHA cultures did not bind detectably to human peripheral blood lymphocytes (10). Similar results (data not shown) were obtained with transferrin of equine origin. Moreover, no interference with binding was apparently produced by human serum albumin or IgG (Fig. 3), or by alpha$_1$-antitrypsin, alpha$_1$-acid glycoprotein, Gc globulin, or bovine serum albumin (results not shown). The reversibility of transferrin binding was investigated after incubation of activated cells at 22°C for 30 min with 200 ng ^{125}I-Trf by washing with PBS, addition of increasing quantities of unlabeled transferrin, and further incubation for various periods of time. Such incubation with a large excess of cold ligand (1 mg) demonstrated that cell-bound counts were reduced by >50% within 30 min of secondary incubation, and when this incubation time was kept constant, reversibility was observed with as little as 100 ng unlabeled transferrin (Fig. 4). As anticipated, displacement of ^{125}I-Trf by cold ligand occurred more rapidly when secondary incubation was performed at 37° than at 22°C.

Relationship between transferrin binding and cellular activation. This was investigated by comparison of binding isotherms in cells exposed to different doses of PHA for 66 h, or in cultures stimulated with a constant amount of lectin but for various time periods. In dose-response studies, negligible binding was observed in the absence of PHA or at suboptimal concentrations (1 μg/culture), but at higher doses of lectin cellular uptake increased progressively, being maximal in cultures containing 10 and 25 μl PHA. Moreover, a close relationship was found to exist between binding of ^{125}I-Trf, the amount of PHA in culture, and extent of activation as judged by uptake

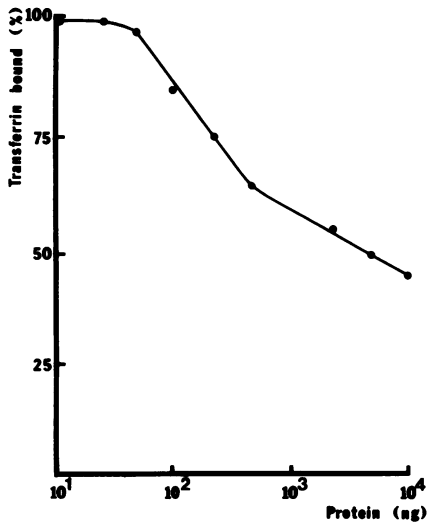

FIGURE 4 Reversibility of binding. Cells stimulated with PHA for 66 h were reacted in aliquots of 2.2×10^6 with a constant quantity of ^{125}I-transferrin (200 ng) for 30 min at 22°C, washed three times in PBS with centrifugation at 400 *g* for 10 min, and then incubated for a further 30 min at 22°C with varying amounts of cold ligand. Displaced ^{125}I-transferrin was removed by passage of cells through a 30% sucrose cushion and reversibility was expressed as the percentage transferrin remaining bound in comparison with control cells exposed to PBS during the secondary reaction.

of [^{3}H]thymidine (Table II). Negligible amounts of ^{125}I-Trf were taken up by freshly obtained noncultured cells, <0.25 ng being bound per 10^6 cells even after incubation in the presence of 2 μg ^{125}I-ligand. In time-course experiments, a typical isotherm profile was obtained at 24 h of culture with further progressive increases in binding of ^{125}I-Trf at 48 and 66 h for each amount of radiolabeled ligand employed. As with the dose-response studies, these time-course binding curves again revealed a close association between [^{3}H]thymidine uptake as a parameter of cellular activation and quantitative transferrin binding (data not shown).

Sequential changes in receptors during exposure to PHA. The progressive augmentation in transferrin binding demonstrated as a function of the amount of PHA employed, or the length of exposure to this lectin, was investigated by immunohistological examination of cells to determine sequential changes in the proportion binding ^{125}I-Trf, and by parallel Scatchard plots to assess temporal alterations in affinity and average number of receptor sites per cell. Upon immunofluorescent examination of cells cultured for 24, 48, and 66 h, the proportion positive for transferrin increased (Fig. 5) in a manner comparable to the degree of augmentation of ^{125}I-Trf binding over the same range of concentrations (100–1,000 ng). Scatchard plots derived from these experiments, however, demonstrated no significant increase in K_a and in some cases, the values obtained at 48 or 66 h were slightly lower than at 24 h (Table III). Moreover, although the intercept on the abscissa indicated a progressive increase in the average number of receptor sites per cell, this conclusion fails to take into account the progressively larger proportions of cells demonstrated immunohistologically to be binding transferrin at 48 and 66 h. Thus, after appropriate correction, the average number of receptors per cell actually remained relatively constant throughout the culture period (Table III).

Effects of iron saturation upon transferrin binding. Binding curves were performed with ^{125}I-apoprotein and ^{125}I-iron saturated transferrin using cells stimulated with PHA for 66 h. Upon comparison of the isotherms obtained, binding was found to be slightly but consistently higher for iron-saturated ligand than for apoprotein (Fig. 6).

TABLE II

*Relationship between Uptake of [^{3}H]Thymidine, Binding of ^{125}I-Transferrin, and Proportion of Cells Positive for Transferrin Fluorescence as a Function of Amount of PHA**

	Amount of PHA added, μl				
	0	1	5	10	25
[^{3}H]Thymidine uptake, *cpm*	460	4,532	30,532	61,892	52,413
^{125}I-Transferrin binding, *cpm*	790	930	6,080	11,300	9,700
Proportion binding transferrin, %	0	8	58	85	81

* Cells were cultured in the presence of different amounts of PHA for 66 h, and pulsed with [^{3}H]-thymidine for the terminal 18 h of culture. ^{125}I-Transferrin binding assays were performed as in Table I, except that cells were incubated in each case with 200 ng ^{125}I-transferrin at 22°C, and immunohistological examination of cells for transferrin binding was undertaken by sequential reaction of cells with ^{125}I-transferrin, rabbit anti-transferrin, and fluorescein isothiocyanate-labeled sheep anti-rabbit Ig.

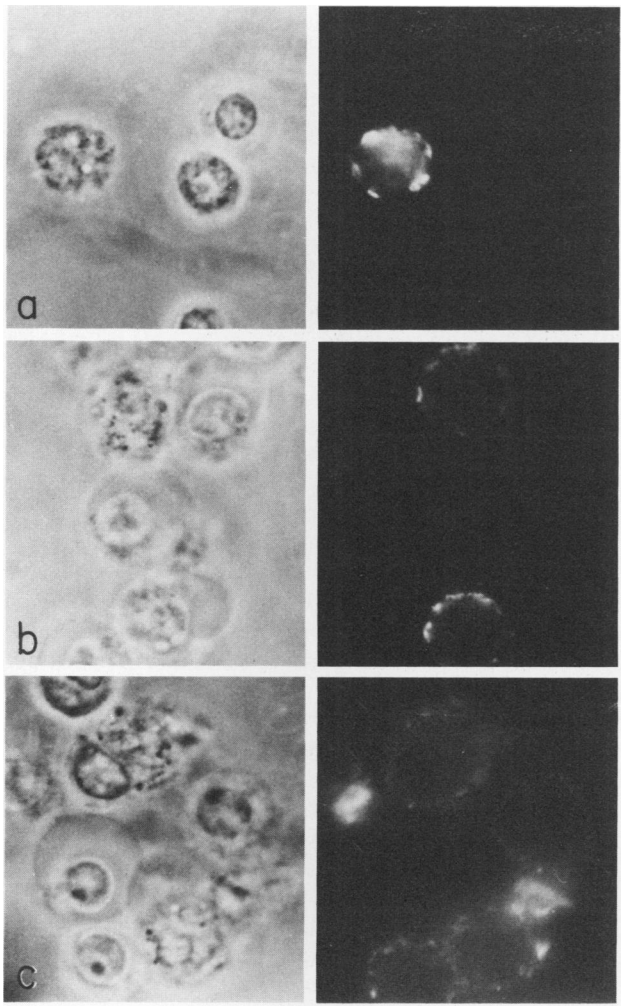

FIGURE 5 Sequential change in the proportion of cells positive for transferrin binding by immunofluorescence. Peripheral blood mononuclear cells were exposed to PHA for 24, 48, and 66 h, harvested and serially reacted with ^{125}I-transferrin (1,000 ng), rabbit anti-human transferrin, and fluorescein isothiocyanate sheep anti-rabbit Ig with intervening washes. The progressive increase in the percentage positive for transferrin fluorescence from 5% at 24 h (*a*) to 36% at 48 h (*b*), and to 90% at 66 h (*c*) is clearly seen upon comparison of the appearances obtained upon white light microscopy (left) and fluorescein isothiocyanate fluorescence excitation (right). Note correspondence between fluorescence and blastoid appearances. ×500.

DISCUSSION

This study demonstrated that interactions between transferrin and membrane binding sites on activated human peripheral blood lymphocytes are of high affinity and specificity, and display saturability and reversibility. These findings therefore satisfy the major criteria required for designation of ligand-binding structures as specific receptors (30, 31). Transferrin binding as assessed immunohistologically is also characteristic of immature erythrocytes and reticulocytes (4), human trophoblast (8, 9), and lymphoblastoid and other transformed cells (7). That such binding does indeed involve high affinity receptors for transferrin has been demonstrated by investigations using radiobinding assays in these tissues (1–6, 32, 33), and it is notable that the affinity constant, K_a, obtained in activated normal human lymphocytes in the present study was very similar to those reported in the majority of those other tissues for which information is currently available (2, 5, 33). The final criterion frequently applied in the definition of hormonal receptors, namely tissue specificity in relation to physiological role, was not addressed in the present investigation. Binding of transferrin is likely to be a first step in the provision of iron and possibly other complexed metallic cations to cells in metabolic need (1–4). Indeed the addition of human transferrin to in vitro cultures of stimulated lymphocytes has been found to be associated with increased DNA synthesis (34, 35), whereas transferrin of bovine origin, which does not bind in detectable amounts (10), does not stimulate comparable metabolic effects (35). However, the precise consequences of cellular interactions with transferrin have not yet been fully explored, and it remains to be determined if specific receptors upon activated lymphocytes are necessarily consistent with tissue specificity in relation to biological activities.

The binding of radiolabeled transferrin to normal human peripheral blood lymphocytes has previously been examined by Phillips (12), although data was only presented for unstimulated cells. Direct comparison of these findings with our own shows both obvious similarities and important differences. For example, the K_a recorded by Phillips (12) is similar to the affinity of transferrin receptors in the present study. However, it was indicated that under optimal conditions (37°C for 30 min), a maximum of approximately 9.4 ng zinc-transferrin was bound per 10^6 cells (12), whereas maximal binding of iron-transferrin in the present study under similar experimental conditions was much higher (25–30 ng/10^6 cells) even upon exposure to amounts of ^{125}I-ligand almost three orders of magnitude lower (nanograms as compared to micrograms). It is difficult to ascribe these discrepancies to methodological differences such as in the method of radioiodination, since the specific activity of ^{125}I-transferrin (0.13–1.3 μCi/ug) was comparable to that obtained in the present study, and no evidence of substantial physicochemical alteration was apparently observed (12). Similarly, in both studies, allowance was made for nonsaturable binding of transferrin and care was taken to exclude counts bound to the assay

TABLE III
*Sequential Changes in Transferrin Receptors on Lymphocytes during Exposure to PHA**

	Exposure to PHA, *h*		
	24	48	66
Positive by immunofluorescence, %	5.0	36	90
^{125}I-Transferrin bound, *ng*	1.2	7.0	9.6
Association constant, K_a, $M^{-1} \times 10^8$	4.3	2.6	1.5
Number of receptors per cell	4,108‡	25,534‡	64,990‡
	82,154§	70,928§	72,208§

* Cells cultured as in Table I and harvested at 24, 48, or 66 h. The table shows the proportion of cells at each point positive for transferrin fluorescence determined as in Table II. The amount of ^{125}I-Trf bound (nanograms) upon reaction with 400 ng at 22°C, and studies of affinity and receptor density as calculated by Scatchard analysis (23), were determined as outlined in Table I.

‡ Uncorrected.

§ Corrected for proportion of cells positive for transferrin binding.

tubes. It is possible that the use of zinc-transferrin (12) rather than iron-saturated ligand may in part explain the large differences in binding obtained, but, comparison of ^{125}I-apoprotein binding shows even greater divergence between the two studies. In fact binding of apoprotein in Phillips' study more closely approximates to that observed in the present investigation in unstimulated lymphocytes. Thus, although Phillips stated that treatment of lymphocytes with PHA did not alter binding of zinc transferrin (12), these observations raise the possibility that the much lower figures obtained might represent binding to the very small proportion of cells bearing receptors (5%), which we have sometimes observed before activation (10).

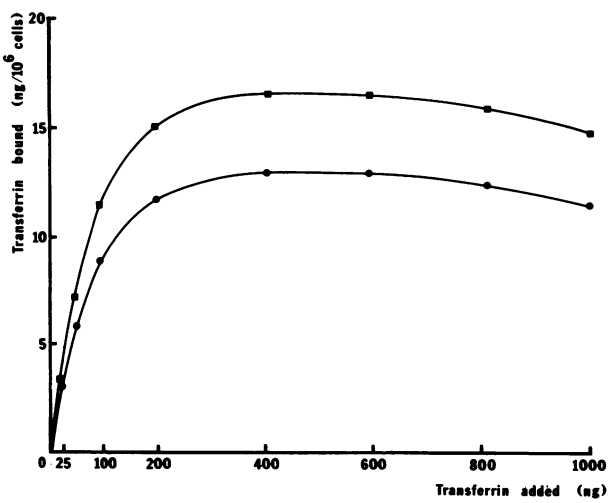

FIGURE 6 Comparative binding of ^{125}I-apotransferrin and ^{125}I-iron-saturated ligand. Cells optimally stimulated with PHA were harvested at 66 h and reacted (2.1×10^6 cells/assay) with increasing amounts of ^{125}I-ligand, either as apoprotein (●) or iron-saturated (■). After removal of unbound transferrin by centrifugation of cells through 30% sucrose cushions (12,800 *g* × 5 min), allowance was again made for nonsaturable binding using an excess of cold apotransferrin and cold iron-saturated ligand, respectively.

The use of radiobinding and immunohistological assays in parallel offers certain advantages. For example, with immunofluorescence procedures, it is possible to check rapidly that ^{125}I-Trf remains in native form and can still bind comparably to receptor sites after radioiodination. Secondly, in the case of the decrease in binding of ^{125}I-Trf observed in certain instances at the upper end of binding isotherms performed at 22°or 37°C (e.g. Fig. 1), this phenomenon was not only found to be unrelated to ligand degradation, but was also shown by parallel immunohistological studies to be independent of the lateral mobility, shedding, endocytosis and receptor reappearance which characterize transferrin receptors upon occupation by ligand at 22° or 37°C (29). Thirdly, the specificity and reversibility of transferrin:receptor interactions can be investigated in parallel by immunofluorescence (9, 10). Finally, knowledge of the actual proportion of cells binding transferrin may be an important adjunct to competitive binding studies involving Scatchard analysis (23) because the raw data obtained for number of receptors/cell can be corrected to yield a more accurate figure. This point was evident in studies of transferrin binding in relation to length of exposure to PHA (Table III) in which progressive increases in binding of transferrin appeared to be due in large part to the continued appearance of cells bearing a relatively constant number of transferrin receptors. Thus, such immunohistological experiments indicate that not all cells necessarily bound ligand, and also provide a simple method for applying an appropriate correction.

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REFERENCES

1. Jandl, J. H., and J. H. Katz. 1963. The plasma-to-cell cycle of transferrin. *J. Clin. Invest.* **42:** 314–326.
2. Baker, E., and E. H. Morgan. 1969. The kinetics of the interaction between rabbit transferrin and reticulocytes. *Biochemistry.* **8:** 1133–1141.
3. Baker, E., and E. H. Morgan. 1969. The role of iron in the reaction between rabbit transferrin and reticulocytes. *Biochemistry.* **8:** 2954–2958.
4. Van der Heul, C., M. J. Kroos, and H. G. Van Eijk. 1978. Binding sites of iron transferrin on rat reticulocytes. Inhibition by specific antibodies. *Biochim. Biophys. Acta.* **511:** 430–441.
5. Hamilton, T. A., H. G. Wada, and H. H. Sussman. 1979. Identification of transferrin receptors on the surface of human cultured cells. *Proc. Natl. Acad. Sci. U. S. A.* **76:** 6406–6410.
6. Larrick, J. W., and P. Cresswell. 1979. Transferrin receptors on human B and T lymphoblastoid cell lines. *Biochim. Biophys. Acta.* **583:** 483–490.
7. Galbraith, G. M. P., R. M. Galbraith, and W. P. Faulk. 1980. Transferrin binding by human lymphoblastoid cell lines and other transformed cells. *Cell. Immunol.* **49:** 215–222.
8. Galbraith, G. M. P., R. M. Galbraith, A. Temple, and W. P. Faulk. 1980. Demonstration of transferrin receptors on human placental trophoblast. *Blood.* **55:** 240–242.
9. Galbraith, G. M. P., R. M. Galbraith, and W. P. Faulk. 1980. Immunological studies of transferrin and transferrin receptors of human placental trophoblast. *Placenta.* **1:** 33–46.
10. Galbraith, G. M. P., J. M. Goust, S. M. Mercurio, and R. M. Galbraith. 1980. Transferrin binding by mitogen-activated human peripheral blood lymphocytes. *Clin. Immunol. Immunopathol.* **16:** 387–395.
11. Galbraith, R. M., and G. M. P. Galbraith. 1980. Recent progress in immunohistology. *Ann. Clin. Lab. Sci.* **10:** 1–8.
12. Phillips, J. L. 1976. Specific binding of zinc transferrin to human lymphocytes. *Biochem. Biophys. Res. Commun.* **72:** 634–639.
13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* **272:** 680–685.
14. Marchalonis, J. J. 1969. An enzymic method for the trace iodination of immunoglobulins and other proteins. *Biochem. J.* **113:** 299–305.
15. Karonen, S. L., P. Mörsky, M. Siren, and U. Seuderling. 1975. An enzymatic solid-phase method for trace iodination of proteins and peptides with ^{125}Iodine. *Anal. Biochem.* **67:** 1–10.
16. Leibman, A., and P. Aisen. 1979. Distribution of iron between the binding sites of transferrin in serum: methods and results in normal human subjects. *Blood.* **53:** 1058–1065.
17. Arnaud, P., R. M. Galbraith, G. M. P. Galbraith, R. C. Allen, and H. H. Fudenberg. 1978. A new allele of human alpha$_1$-antitrypsin: Pi^{NHampton}. *Am. J. Hum. Genet.* **30:** 653–659.
18. Arnaud, P., G. B. Wilson, J. Koistinen, and H. H. Fudenberg. 1977. Immunofixation after electrofocusing: Improved method for specific detection of serum proteins with determination of isoelectric points. I. Immunofixation print technique for detection of alpha-1-protease inhibitor. *J. Immunol. Methods.* **16:** 221–231.
19. Saravis, C. A., and M. Zamcheck. 1979. Isoelectric focusing in agarose. *J. Immunol. Methods.* **29:** 91–96.
20. Cambiaso, C. L., A. Goffinet, J-P. Vaerman, and J. F. Heremans. 1975. Glutaraldehyde-activated aminohexyl-derivative of Sepharose 4B as a new versatile immunoabsorbent. *Immunochemistry.* **12:** 273–278.
21. Galbraith, R. A., C. Wise, and M. G. Buse. 1980. Insulin binding and response in erythroblastic leukemic cells. *Diabetes.* **29:** 571–578.
22. De Meyts, P. 1976. Insulin and growth hormone receptors in human cultured lymphocytes and peripheral blood monocytes. *Methods Mol. Biol.* **9:** 301–383.
23. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51:** 660–675.
24. Arnaud, P., E. Gianazza, P. G. Righetti, and H. H. Fudenberg. 1980. The role of sialic acids in the microheterogeneity of alpha$_1$-acid glycoprotein: study by isoelectric focusing and titration curves. *In* Electrophoresis. J. Radola, Editor Springer-Verlag, Berlin. 151–163.
25. Arnaud, P., R. M. Galbraith, C. Chapuis-Cellier, G. M. P. Galbraith, and H. H. Fudenberg. 1979. Purification of proteins by recycling subtraction affinity chromatography. *Protides Biol. Fluids Proc. Colloq.* **26:** 649–652.
26. Vesterberg. O. 1971. Isoelectric focusing of proteins. *Methods Enzymol.* **22:** 389–412.
27. Svendsen, P. J. 1973. Fused rocket immunoelectrophoresis. *Scand. J. Immunol. Suppl. 1* **2:** 69–70.
28. Metais, P., H. Schirardin, and J. Warter. 1966. Miesure de la capacité d'inhibition tryptique du serum humain. *Clin. Chim. Acta.* **13:** 602–610.
29. Galbraith, G. M. P., and R. M. Galbraith. 1980. Metabolic and cytoskeletal modulation of transferrin receptor mobility in mitogen-activated human lymphocytes. *Clin. Exp. Immunol.* In press.
30. Cuatrecasas, P., M. D. Hollenberg, K. J. Chang, and V. Bennett. 1975. Receptor complexes and their modulation of membrane function. *Recent Prog. Horm. Res.* **31:** 37–84.
31. Roth, J., C. R. Kahn, M. A. Lesniak, P. Gorden, P. De Meyts, K. Megyesi, D. M. Neville, J. R. Gavin, A. H. Soll, P. Freychet, I. D. Goldfine, R. S. Bar, and J. A. Archer. 1975. Receptors for insulin, NSILA-s, and growth hormone: applications to disease states in man. *Recent Prog. Horm. Res.* **31:** 95–126.
32. Seligman, P. A., R. B. Schleicher, and R. H. Allen. 1979. Isolation and characterization of the transferrin receptor from human placenta. *J. Biol. Chem.* **254:** 9943–9946.
33. Wada, H. G., P. E. Hass, and H. H. Sussman. 1980. Transferrin receptor in human placental brush border membrane: Studies on the binding of transferrin to placental membrane vesicles and the identification of a placental brush border glycoprotein with high affinity for transferrin. *J. Biol. Chem.* **254:** 12629–12635.
34. Tormey, D. C., and G. C. Mueller. 1972. Biological effects of transferrin on human lymphocytes *in vitro*. *Exp. Cell. Res.* **74:** 220–226.
35. Phillips, J. L., and P. Azari. 1975. Effect of iron transferrin on nucleic acid synthesis in phytohemagglutinin-stimulated human lymphocytes. *Cell. Immunol.* **15:** 94–99.