Combined radiolabel-binding and immunocytochemical evaluation of receptor–ligand interactions

Studies of transferrin receptors on activated lymphocytes

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A protocol that involved both immunohistological and radiolabel-binding procedures was devised for the study of transferrin–receptor interactions. This composite approach yielded considerably more information than did either technique used alone, and also provided a simple means for exclusion of several common potential sources of error.

For many biologically active compounds the first step in the sequence of events that culminates in an appropriate cellular response involves an interaction with a specific plasma-membrane receptor site. Previous studies (Cuatrecasas *et al.*, 1975) have defined the criteria by which such ligand-binding sites may be designated as receptors: thus the binding reaction should show specificity, saturability, reversibility, high affinity of binding and specificity of cellular response. All techniques used to date for this purpose have drawbacks. Thus in the traditional radiolabel-binding assay membrane ligand binding is quantified with a biologically compatible radioisotopic label; this permits a mathematical determination of the first four criteria listed above, and also the number of receptor sites, but does not readily yield information at a morphological level. On the other hand, techniques that permit the direct examination of membrane events involved in ligand binding, e.g. immunocytochemical and ‘rosetting’ methods (Dickler, 1976), do not readily lend themselves to accurate quantification, and their sensitivity is considered to be low. We therefore devised a complementary approach making use of radiolabel-binding and immunohistological protocols in tandem for our investigations of the transferrin receptors that appear on phytohaemagglutinin-activated human peripheral-blood lymphocytes (R. M. Galbraith *et al.*, 1980). In the present study we further investigate the potential value of this combined approach, using activated lymphocytes as a model system.

Experimental

Cells

Viable suspensions of normal human peripheral-blood lymphocytes were examined after 66 h in macroculture with and without phytohaemagglutinin (G. M. P. Galbraith *et al.*, 1980b; R. M. Galbraith *et al.*, 1980). Cells were obtained with the informed consent of donors and with approval of the local Institutional Review Board.

Radiolabel-binding and immunofluorescence assays

Purified apotransferrin (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used in solution in phosphate-buffered saline (0.14 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.4) at various concentrations. The purity of this preparation was confirmed by both physicochemical and immunochemical means, and samples were saturated with iron as required (R. M. Galbraith *et al.*, 1980). Competitive radiolabel-binding assays were performed with ^{125}I -labelled transferrin as previously described (R. M. Galbraith *et al.*, 1980). Cells (10^6) were incubated at 4, 22 and 37°C with constant amounts of ^{125}I -labelled transferrin (25 ng) concurrently with increasing concentrations of unlabelled ligand (0–5000 ng) in a final volume of 500 μl . All assays included an additional tube containing a 1000-fold excess of unlabelled transferrin to allow estimation of, and correction for, non-saturable binding. The results were expressed as binding isotherms and also subjected to Scatchard

analysis (Scatchard, 1949). In immunofluorescence studies, incubation with transferrin was followed by serial reaction with rabbit antiserum to human transferrin (prepared locally) and a fluorescein isothiocyanate-labelled sheep antiserum to rabbit immunoglobulin (Wellcome Laboratories, Research Triangle Park, NC, U.S.A.), with intervening washes. The characterization of antisera, and controls performed for reaction specificity, are detailed elsewhere (G. M. P. Galbraith *et al.*, 1980a,b).

Mixed-population studies

Transferrin binding was examined in lymphocyte populations prepared by deliberate mixing of known positive and negative cells. Thus lymphocytes were cultured for 66 h in the presence of phytohaemagglutinin (PHA⁺, 60–80% of cells activated) and in its absence (PHA⁻, 0–5% activated). These populations were mixed in the following proportions by cell number, 100:0, 75:25, 50:50 and 0:100, and examined for transferrin binding.

Sensitivity of immunofluorescence binding assay

Competitive radiolabel-binding assays were also used to determine the sensitivity of detection of transferrin binding by immunofluorescence methods. Samples of 10⁶ cells were taken after reaction with transferrin at 22°C, cooled to 4°C and serially treated with antiserum to transferrin and fluorescein isothiocyanate conjugate. The percentage of positive cells at each point was then superimposed on the binding isotherms constructed. Finally, the washing supernatants from each assay were examined for trichloroacetic acid-insoluble γ -radioactivity to determine if bound transferrin was removed from its receptor either by antibody fixation or by the cooling step required for immunohistological examination.

Results and discussion

In cells maintained in culture for 66 h without mitogen, binding of transferrin was detected immunohistologically in <5%, whereas 60–80% of cells stimulated with phytohaemagglutinin for the

same time period showed positive membrane fluorescence. Parallel competitive radiolabel-binding assays demonstrated quantitatively that transferrin was bound by activated but not by unstimulated lymphocytes. These results confirm a relationship between transferrin binding and cellular activation suggested by previous studies employing both radiolabel-binding and immunofluorescence methods (G. M. P. Galbraith *et al.*, 1980b; R. M. Galbraith *et al.*, 1980). In the latter study, transferrin binding increased concomitantly with length of exposure to mitogen, and Scatchard analysis suggested that, although the equilibrium association constant (K_a) did not change, there was a dramatic increase in the total number of receptors. When these data were corrected for the increased percentage of cells binding transferrin in each preparation, the calculated numbers of receptors per cell actually remained constant throughout the culture period. If substantiated, this finding would indicate that increased transferrin binding reflects continued expansion of a population of cells bearing a relatively constant number of receptors rather than an increase in the number of receptors on each individual cell.

In the present investigation, mixed-population studies were therefore performed, in which the proportion of receptor-bearing cells was varied artificially by mixing populations containing receptor-positive (PHA⁺) and receptor-negative (PHA⁻) lymphocytes. The results of one representative experiment are shown in Table 1. The radioisotopic data for the populations under study appeared to yield widely divergent results for receptor number (from 17000 to 28000/cell). However, since it was known that cells negative for fluorescence did not bind significant amounts of ligand, the data were corrected for the percentage of cells in each population detectably binding transferrin by immunofluorescence. The number per individual receptor-bearing cell was then very similar in the three populations containing activated cells (47000 to 49000), a further indication that fluorescence-negative cells bound negligible amounts of ligand.

Table 1. *Transferrin binding by mixed populations of lymphocytes*
For experimental details see the text.

Lymphocytes	...	PHA ⁻	PHA ⁺	PHA ⁻	PHA ⁺	PHA ⁻	PHA ⁺	PHA ⁻	PHA ⁺
Ratio	...	100	0	0	100	25	75	50	50
Receptors/cell (uncorrected)*		—		28 218		24 284		17 204	
Fluorescence-positive cells (%)		0		60		49		35	
Receptors/cell (corrected)		—		47 030		49 559		49 154	

* Calculated from Scatchard analysis of competitive binding studies with ¹²⁵I-labelled transferrin.

† Cells were serially treated with purified human transferrin (100 µg/ml), rabbit anti-transferrin serum and fluorescein isothiocyanate-labelled sheep antiserum to rabbit immunoglobulin, and the percentage of fluorescing cells was calculated.

This observation clearly holds important implications for the accurate calculation of receptor number per cell, and the sensitivity of the immunohistological binding assay was therefore investigated directly by performing immunofluorescence assays at each point of competitive radiolabel-binding procedures. It is important to stress that less than 5% of cell-bound trichloroacetic acid-insoluble radioactivity was removed as a result of the joint effects of cooling to 4°C for immunofluorescence and subsequent reactions with the two antibody layers. When superimposed on binding isotherms constructed at 4, 22 and 37°C (Figs. 1a–1c), the immunofluorescence data demonstrated that at saturation, achieved at all temperatures on the addition of 275 ng of transferrin, binding was detected in 38, 70 and 62% of cells respectively, and these percentages remained unchanged when larger amounts of ligand were added. However, when such cells were treated with progressively smaller, non-saturating, amounts of ligand (<275 ng), a decrease in the percentage positive was detected in each case, but not until the amounts added were decreased below 125 ng at 4°C (Fig. 1a) and 75 ng at 22°C and 37°C (Figs. 1b and 1c). At these 'sensitivity points', the binding curve at 4°C showed a total of 0.48 ng of transferrin bound/10⁶ cells, or, with correction for the percentage of positive cells, 1.26 fg/cell. Similar amounts of transferrin were bound at 22°C (1.30 fg/cell) and at 37°C (1.58 fg/cell). The sensitivity of the immunofluorescence assay, expressed as the lowest amount of ligand detectable on each of every ligand-binding cell in a

given population, was therefore between 1.26 and 1.58 fg of transferrin/receptor-bearing lymphocyte in the illustrated experiment, and in repeated assays was always between 1.0 and 2.5 fg/cell. In addition, these values were not significantly altered on saturation of transferrin with iron.

Given this definition of sensitivity, the use of different incubation temperatures had little effect. However, the binding isotherms clearly show that, whereas saturation was achieved by addition of the same amount of ligand at each temperature, the amounts of ligand bound at saturation were consistently greater at the higher temperatures (Fig. 1), as noted previously (R. M. Galbraith *et al.*, 1980). In practice, therefore, although the sensitivity of immunofluorescence at 4°C was near to the saturation point as defined radioisotopically, it occurred considerably below saturation at higher temperatures.

It is also possible to express sensitivity in terms of the number of transferrin molecules bound per cell at each assay point. For example, in the illustrated experiment, at the 'sensitivity point' at 22°C incubation of 10⁶ cells with 75 ng of transferrin resulted in binding of a total of 0.91 ng of protein (Fig. 1). Given that the molecular weight of transferrin is 78 000, and Avogadro's number (6.022×10^{23}), then a total of 7.029×10^9 molecules of transferrin was bound. Since at this assay point 70% of cells were shown by immunofluorescence to be positive for bound transferrin, it can be calculated that each receptor-bearing cell bound an average of 10040 molecules of transferrin. Similar calculations

Fig. 1. Composite binding isotherms showing cellular uptake of transferrin expressed both as the absolute amounts bound radioisotopically (—) and as the percentage of cells positive immunohistologically (---)

Results are included from experiments performed at 4°C (a), 22°C (b) and 37°C (c). In each case, saturation, as derived from Scatchard analysis, is shown by an arrow on the ¹²⁵I-labelled transferrin curve, and the 'sensitivity point' below which the percentage of positive cells decreased, is marked by an arrow on the immunofluorescence curve. For experimental details see the text.

revealed that, at the 'sensitivity points' at 4°C and 37°C, each receptor-bearing cell bound an average of 9750 and 12200 molecules of transferrin respectively. Thus the minimum number of bound transferrin molecules recognized by immunofluorescence methods in activated lymphocytes averaged 10000/cell, representing a fractional receptor occupancy of approx. 20%. These data therefore provide further evidence that cells negative by fluorescence are unlikely to bind significant quantities of ligands.

These results illustrate certain points that may be of general relevance to the study of membrane receptor-ligand interactions. The immunohistological detection and enumeration of ligand-binding cells is clearly a useful adjunct to quantitative assays that employ radiolabelled ligand. In addition to the ability to monitor phenomena such as endocytosis and shedding (Galbraith & Galbraith, 1980), which may then be investigated to determine their possible influence on measurement of ligand binding to the membrane (R. M. Galbraith *et al.*, 1980), a combined approach permits more accurate determination of the cellular density of receptors. This is important, since previous radiolabel-binding studies that have derived values for receptor density expressed as the number per cell have of necessity assumed binding of equal amounts of ligand by every cell in the population under study. Finally, the sensitivity of immunofluorescence, although generally assumed to be low, was shown in the present study to be adequate for recognition of receptor expression down to a fractional occupancy of 20% or less. Sensitivity, as quantitatively defined above, clearly varies with such factors as the ligand

employed, and the need for direct labelling of ligand, for example in model systems where cooling or subsequent addition of antibody significantly enhances ligand stripping from the membrane. However, it is stressed that the present results were obtained with standard microscopic equipment, and it is likely that the use of image intensification will increase sensitivity still further.

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