THE USE OF CYTOSTAR-T™ SCINTILLATING MICROTITRE PLATES FOR MONITORING RECEPTOR BINDING USING LIVE CELLS.

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Introduction

The binding of a ligand to a receptor often represents the first step in a complex cascade of biochemical events associated with a signal transduction pathway. Consequently the receptor provides an attractive accesible target for intervention by therapeutic agents.

Receptor binding assays have been extensively used to characterise and classify receptors and to identify receptor agonists and antagonists as potential drugs. Classically these assays have utilised radiolabelled ligands and membrane preparations derived by disruption of cells or tissues containing the receptor of interest[1]. As this approach generally results in loss of cellular integrity, certain aspects of the regulation of receptor expression cannot be studied. Furthermore, cells which normally grow in a well defined orientation often lose phenotypic characteristics when dissociated.

The Cytostar-T plate enables the study of cell cultures which normally grow as an adherent monolayer on culture treated plastic or on a layer of extracellular matrix proteins. This system is therefore potentially suitable for carrying out ligand binding assays in an homogeneous format without disturbing the equilibrium between bound and free ligand, and whilst maintaining the cells in a more physiological environment.

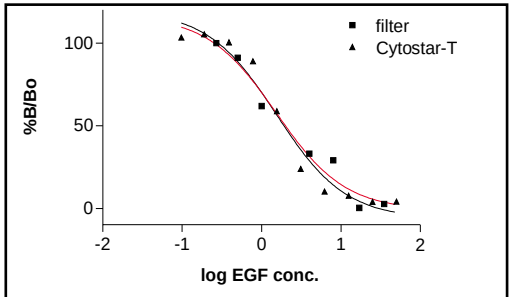

Figure 1. *Competition binding of [^{125}I]EGF to EGF-Rs expressed on A431 cells measured by Cytostar-T plate and filtration. For Cytostar-T assay 80% confluent A431 cell monolayers (Cytostar-T) were incubated at 37°C for 2 hours in assay buffer (20mM HEPES, 2mM $CaCl_2$, 0.1% BSA) containing [^{125}I]EGF (300pM final concentration) and unlabelled EGF at a concentration range of 0.1-50nM. Plates were counted on a Wallac 1450 MicroBeta™ scintillation counter. For filter assay 9µg/214µl aliquots of A431 cell fractions were incubated at 37°C for 2 hours in assay buffer containing [^{125}I]EGF (300pM final concentration) and unlabelled EGF at a concentration range of 0.1-50nM. Reactions were loaded onto Whatman GF/C filters, washed through with assay buffer and counted in liquid scintillant on an LKB Rackbeta 1209 counter. Results are means (n=3).*

Experimental

The interaction of [^{125}I]EGF (AmershamTM, IM196, >750Ci/mmol) with EGF-R expressed by the A431 human cell line was studied as a model system to investigate the performance of Cytostar-T plates in the measurement of kinetic events in comparison with SPA and filtration techniques.

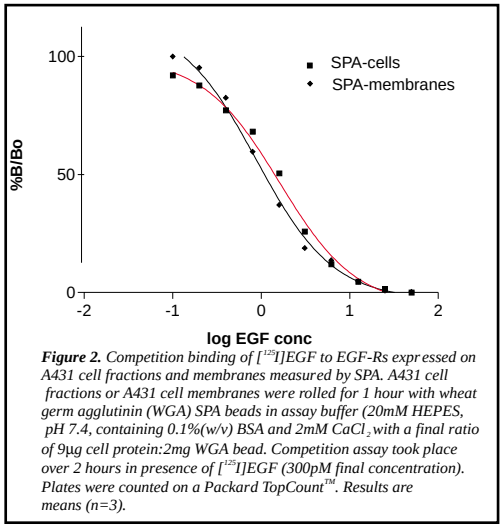

Figure 2. *Competition binding of [^{125}I]EGF to EGF-Rs expressed on A431 cell fractions and membranes measured by SPA. A431 cell fractions or A431 cell membranes were rolled for 1 hour with wheat germ agglutinin (WGA) SPA beads in assay buffer (20mM HEPES, pH 7.4, containing 0.1%(w/v) BSA and 2mM $CaCl_2$ with a final ratio of 9µg cell protein:2mg WGA bead. Competition assay took place over 2 hours in presence of [^{125}I]EGF (300pM final concentration). Plates were counted on a Packard TopCount™. Results are means (n=3).*

A comparison of competition assay data was carried out with varying confluencies of A431 cells. This is an analogous step to SPA membrane protein optimization. A cell confluency of 80% gave the highest signal:noise and the most reproducible results. It is likely that the expression of growth factor receptors is highest in actively dividing sub-confluent cells[2].

A feature of the Cytostar-T assay is that intact living cells are used as a receptor source. Thus post-binding events, including receptor dimerisation, internalisation and ligand degradation are coupled to the receptor binding assay. These events are known to be temperature dependent. It is thought that post binding events are minimised at 4°C[3] and that there is a block at the endosome internalisation stage at 18°C[4]. Experiments showed 37°C to give optimum binding results.

Competition studies were performed and the K_i calculated from the experimentally determined IC_{50} value for EGF-R Cytostar-T assays in comparison with SPA (whole cells and membranes) and filtration techniques. Typical plots of competition data obtained by the four assay methods are shown in Figures 1 and 2 and are summarised in Table 1. IC_{50} and K_i values for SPA and filtration methods were in good agreement with those obtained for Cytostar-T.

The association and dissociation constants of [^{125}I]EGF binding to EGF-R expressed by A431 cells were determined for each assay system. Dissociation curves were achieved by the addition of an excess of unlabelled EGF. The Cytostar-T plate and SPA assay techniques allowed a single set of assay wells to be monitored continuously over time. Figure 3 shows typical data obtained by the Cytostar-T plate technique and kinetic parameters are summarised in Table 2.

Equilibrium saturation binding experiments were performed using SPA and Cytostar-T. The K_D and B_{max} values are summarized in Table 1.

	IC_{50}	K_i	K_D nM	B_{max} 10^{-10} moles/litre
Cytostar-T plate	1.55 1.56	1.19 1.20	1.84 0.99	1.66 3.00
SPA (whole cells)	1.06 1.70	0.80 0.23	0.60 0.18	0.95 1.40
Filtration	1.53 1.50	0.47 0.23	0.11 0.16	1.1 1.9

Table 1. Summary of IC_{50} and affinity constants determined by [^{125}I]EGF competition studies in Cytostar-T plate, SPA and filter methods.
Table shows results from 2 separate experiments.
Kinetic constants calculated using the computer program PRISM™.

	k_{ob} $x10^{-2}$ min^{-1}	k_1 $x10^{-7}$ M^{-1} min^{-1}	k_{-1} $x10^{-2}$
Cytostar-T	3.3 4.1	0.89 4.76	2.63 2.91
SPA	4.3 2.4	5.60 0.78	1.51 1.80

Table 2. Summary of kinetic constants calculated for [^{125}I]EGF association/dissociation on Cytostar-T and SPA.
Table shows results from 2 separate experiments.
Kinetic constants calculated using the computer program PRISM™.

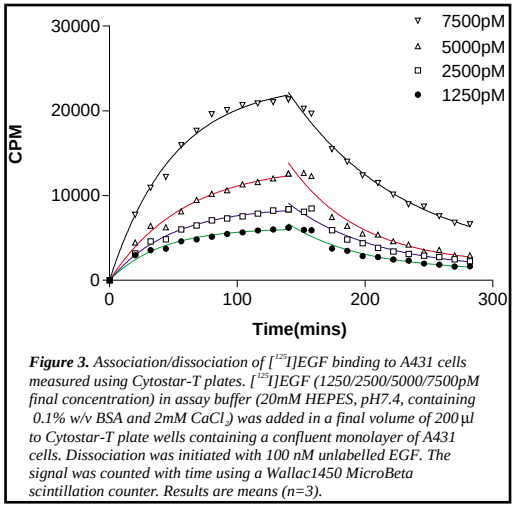

Figure 3. *Association/dissociation of [^{125}I]EGF binding to A431 cells measured using Cytostar-T plates. [^{125}I]EGF (1250/2500/5000/7500pM final concentration) in assay buffer (20mM HEPES, pH7.4, containing 0.1% w/v BSA and 2mM $CaCl_2$) was added in a final volume of 200µl to Cytostar-T plate wells containing a confluent monolayer of A431 cells. Dissociation was initiated with 100 nM unlabelled EGF. The signal was counted with time using a Wallac1450 MicroBeta scintillation counter. Results are means (n=3).*

Studies have commenced on a second model system of a receptor-ligand binding assay on Cytostar-T plates. Initial results in figure 4 show the association of [^{125}I] testosterone with the receptor-expressing cell line MCF-7 compared with the non-expressing control cell line MDA/MB/231. This illustrates the potential use of Cytostar-T plates for live cell receptor assays in other cell lines.

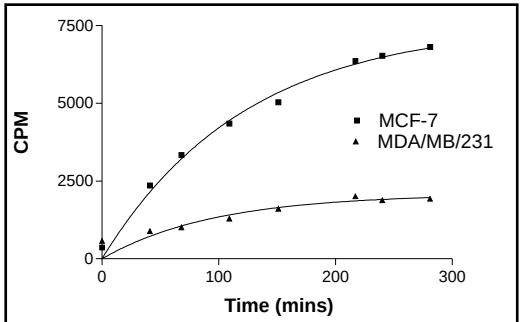

Figure 4. Association of [^{125}I] testosterone binding to MCF-7 cells measured using Cytostar-T plates. [^{125}I] Testosterone (1nM final concentration) in RPMI 1640 media minus phenol red was added in a final volume of 200µl to Cytostar-T plate wells containing a confluent monolayer of MCF-7 cells. The signal was counted with time using a Wallac 1450 MicroBeta scintillation counter. Results are means (n=3).

Conclusions

The results of this study show that the kinetic parameters obtained for the EGF-R on A431 cells by Cytostar-T plate are compatible with those obtained by SPA and filtration methods. Total cpm measured are lower on Cytostar-T compared to SPA due to differences in counting efficiency but competition curves and IC_{50} values were virtually identical across the assay methodologies. Association/dissociation rates and saturation binding curves were also similar. The slightly higher K_D and K_i values obtained with Cytostar-T compared with SPA and filtration were probably due to the influence of post-binding events that occur in the viable cell[5,6,7].

It can be concluded from this study that both Cytostar-T plates and SPA beads have the sensitivity required to measure kinetic events without causing interference with the receptor/ligand interaction. The advantages of Cytostar-T plates and SPA beads over traditional methods are significant in that the technology is able to measure real-time binding events, allowing the researcher to make considerable savings in both labour and reagents. The Cytostar-T scintillating microplates have the additional advantage in that cells can be assayed in a more physiological environment, allowing the continuous monitoring of biochemical and morphological events over short or extended time periods without any disruption of the cells.

References

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