Optimization of Cellular ELISA for Assay of Surface Antigens on Human Synoviocytes

ABSTRACT

The cellular enzyme-linked immunosorbent assay (CELISA) permits assay of cell surface antigens on intact fixed cells. Using a monolayer of cells as the solid phase, the CELISA offers an inexpensive alternative to flow cytometry. In addition, this protocol has the decided advantages of miniaturization (small numbers of cells) and ease of replication (the 96-well cluster plate format). In efforts to optimize CELISA for detecting surface antigens on human fibroblast-like synoviocytes, the authors found that cell number, serum proteins, choice of culture plate, pipetting technique and fixatives may all impact the results of the CELISA.

UNITRODUCION

It is a small step from the development of the enzyme-linked immunosorbent assay (ELISA) (8) to adaptation of the technology for detection of cell surface and/or cytoplasmic antigens on or in intact cells (19). In a cellular ELISA (CELISA), monolayers of cells adhering to the bottom of wells of a 96-well polystyrene cluster plate comprise the solid phase of a more typical ELISA, in which an antigen is adsorbed to the bottom of each well.

Advantages of the CELISA include: (i) utilization of a relatively intact, adherent, fixed cell as the target of the assay in lieu of an insolubilized protein; (ii) the utilization of the 96-well format, which facilitates measurements in replicate and requires a relatively small number of cells per well; (iii) conservation of costly reagents, given the small assay volume; and (iv) a relatively inexpensive assay using a plate reader rather than a flow cytometer.

Despite these obvious advantages, we have found that the CELISA is an underutilized protocol, with most references pertaining to its use being restricted to immunological journals (2,3,7,14,20) with a notable exception in BioTechniques (4). Although several papers are available pertaining to optimization of ELISA (1,11,13), few papers address optimization of CELISA (18,20). This report characterizes our techniques and modifications of CELISA relevant to our principal applications. By using similar techniques, the CELISA can be adapted to numerous other cell types.

MATERIALS AND METHODS

Human Synoviocyte (HSC) Cultures

Surgical specimens of synovial tissue were obtained with approval of the University of Kansas Medical Center Human Subjects Committee from patients undergoing arthroscopy. Synovial lining cells were cultured as described previously (5,15).

Mononuclear Cell/T-Cell Preparation

Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized venous blood obtained from normal, healthy, adult volunteers by centrifugation using Histopaque 1077 (Sigma Chemical, St. Louis, MO, USA). To remove adherent cells, PBMCs were suspended in medium containing 10% fetal calf serum (FCS) and plated twice for 45 min at 37°C on plates coated with 1% FCS. In some cases, T-cell-enriched populations were prepared by passage through nylon wool (Polysciences, Warrington, PA, USA) (16).

Typical CELISA Protocol

This protocol was modified from that of Rothlein et al. (17) using biotinylated anti-IgG antibodies and avidin coupled to horseradish peroxidase (HRP) (15). Cells were cultured at subconfluent numbers (10^4/well) for 24–72 h in a 96-well tissue culture plate and then exposed to various experimental treatments. After, typically, 24–48 h, cell layers were washed with warm
Dulbecco's modified Eagle medium (DMEM), fixed with 1% paraformaldehyde for 30 min at room temperature (RT), or 12 min at 37°C, and washed 2x with assay buffer [0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (pH 7.2–7.4) with 0.01% thimerosal]. Nonspecific binding was blocked for 10 min at 37°C or 1 h at RT with 1% BSA or nonfat dry milk dissolved in PBS, containing 0.02% sodium azide. After washing again with the assay buffer, the wells were exposed to the primary antibody for 1 h at 37°C, washed 3x and exposed to a biotinylated anti-mouse IgG conjugate diluted 1:2500 (Organon Teknika/Biotechnology Research Institute [OT/BRI], Rockville, MD, USA) at 37°C for 15 min. Then, extensive washing (3–5x) was followed by the addition of TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA).

Spectrophotometric readings were made at 650 nm using a Vmax™ Microplate Reader (Molecular Devices, Menlo Park, CA, USA). Cell monolayers were quantified by crystal violet staining, solubilizing the dye with 1% sodium lauryl sulfate and measuring absorbance at 590 nm (6). Data were reduced using Molecular Devices’ Softmax™ software and imported to a spreadsheet (Excel®; Microsoft, Redmond, WA, USA) for analysis. Isotype-matched irrelevant antibody or pre-immune serum controls were used in each assay.

**Antibodies**

We have used numerous monoclonal antibodies (MAbs) as the primary antibody directed towards cell surface determinants in our applications. They include anti-HLA Class I (W6/32 or MB40.5), anti-HLA Class II (L243), anti-intercellular adhesion molecule-1 (ICAM-1) (RR1/1 [supplied by Dr. Robert Rothlein, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT, USA] or 84H10), numerous antibodies directed towards mononuclear cell membrane markers (MO1, OKT3, OKT4 and OKT8), a panel of anti-dermal fibroblast MAb (supplied by Dr. Joseph Korn, Boston University School of Medicine, Boston, MA, USA) and two irrelevant isotype control antibodies, MSIgG1 and IgG2a (P1.17). In addition we have used polyclonal antibodies produced in sheep or rabbits and directed at the cellular secretory proteins collagenase and stromelysin (data not shown). Secondary antibodies have included the following biotinylated commercial products: anti-mouse IgG produced in rabbit (Vector Laboratories) and anti-sheep or anti-rabbit IgG produced in goat (OT/BRI).

**Flow Cytometry (FC)**

To further characterize HSC membrane markers, flow cytometric analysis was done on a FACStar® (Becton Dickinson, San Jose, CA, USA) using single-color staining as described previously (15).

**RESULTS AND DISCUSSION**

**Adherent Cells**

Success of the CELISA depends initially upon the culture conditions chosen for the particular cell type to be assayed. Detachment of cells for transfer into a 96-well plate may lead to transient modulation of surface antigens. Culturing these cells for at least 48 h before the initiation of the experiment usually allows return to a steady state. The majority of our protocols have been carried out on primary cultures of HSC used from passages 2 through 8.

We have also used the CELISA on two lines of human lung fibroblasts (HFL 1, CCL 153; and IMR-90, CCL 186; ATCC, Rockville, MD, USA), a line of human embryonic kidney cells (CRL 1573; ATCC), primary cultures of human kidney epithelial cells and primary cultures of human umbilical vein endothelial cells.
When tested with HSC, the CELISA performed most efficiently with subconfluent monolayers (Figure 1). Ten thousand cells per well were optimal for this application (ca. 4 x 10^4 HSC/well approached confluence). This number of cells generates a strong signal (Figure 1, inset) while not wasting cells or reagents because of reduced activity with increased cell number. Each cell type should be tested with a range of cell numbers, as cells may differ in size and reactivity.

Nonadherent Cells

In addition to detecting surface antigens on adherent cells, we have been able to perform the CELISA on normally nonadherent cells, including PBMCs and purified T cells. These cells can be bound to the bottom of a cluster plate well by a capturing agent such as poly-L-lysine (9), by use of a commercial tissue adhesive (Cel-Tak; Collaborative Research, Bedford, MA, USA) or even by use of a fixative such as glutaraldehyde (19).

We have also performed CELISA directed at surface determinants present on normally nonadherent cells after adherence to a monolayer of attached cells (Figure 2, Panel B). The optimal number of nonadherent cells initially utilized in this assay was determined as previously described (10).

Effects of Culture Medium and Serum

The selection of culture medium with associated proteins, sera and growth factors resides ultimately upon a compromise between the optimal conditions for growth and/or maintenance of the adherent cell type, and the optimal conditions for presentation of the surface antigen targeted by the CELISA. With HSCs, the presence and concentration of serum added to the culture medium can make a significant difference in the reactivity of the surface determinant of interest (Figure 3). Reasons for this variability may include the presence of inhibitors, of stimulants such as cytokines or, inadvertently, of endotoxin.

To avoid undesirable serum effects, we use a serum-free medium (SFM) containing 0.2% lactalbumin hydrolysate (LAH; Sigma Chemical) after two washes with Hanks’ balanced salt solution (HBSS; Life Technologies, Gaithersburg, MD, USA) 24 h before the initiation of the experiment. Supplementation of DMEM with LAH supports most of the antigen expression seen in the presence of serum (Figure 3).

Surface Charge

We have tried several brands of polystyrene cluster dishes, and by using crystal violet to monitor the monolayers, we have found as much as 34% reduced staining with some brands (data not shown). Of those tested, Plastek® M negatively charged plates (MatTek Corporation, Ashland, MA, USA) promoted maximal attachment of HSC. However, some MAb bound nonspecifically to the negatively charged surface of these plates, resulting in high backgrounds. Regardless of the type of plate selected, a blocking step (as described in Materials and Methods) before the CELISA is needed to suppress nonspecific binding.

Uniformity

It is also necessary to confirm the uniformity of the colorimetric readings resulting from any particular brand of plate and to check for variability produced by any particular protocol. This is tested by treating each well identically and then checking for uniform color development. Variation should not exceed 10%. As in most ELISA protocols, peripheral wells most often show the highest degree of variability.

Several possible causes could explain this, including a defective plate or lid, effects of ambient light upon a colorimetric substrate, unequal heating across the plate and loss of enzyme activity due to drying (1). We minimize these sources of error by comparing brands of plates for uniformity, incubating plates in a covered water bath and by restricting the period of time...
Research Reports

during which a well is devoid of fluid to less than 30 s. Also, during lengthy protocols (tissue culture incubations ≥72 h), we fill the outer 36 wells with sterile buffer or water to serve as a barrier against peripheral well evaporation, thus limiting assay wells to the inner 60 wells.

Pipetting Technique

As with any ELISA, some common sources of error are caused by inadequate mixing of reagents and by pipetting mistakes. The former is particularly noticeable with a failure to adequately mix the two-solution substrate (tetramethylbenzidine; TMB) or to resuspend cells immediately before seeding a culture plate. Proper use of a manifold pipet includes careful and gentle placement of tips at the edge of the wells to avoid laceration of the monolayer.

To monitor the loss of a cell sheet during the multiple washes, we have performed several experiments comparing the use of a multichannel pipet with (i) flicking the contents and subsequently draining the wells on paper towels, (ii) a semiautomatic washer (e.g., Nunc-Immunowash 12; Nunc, Roskilde, Denmark) or (iii) aspiration of individual wells by means of a vacuum pump. In our hands, washing with a multichannel pipet is the most efficient (data not shown), as seen for ELISA (1).

Fixatives

Although we have tried to perform CELISA in the absence of fixation, there is often a significant loss of the monolayer due to washes. We have tested a number of fixatives in an effort to maximize persistence of an intact cell sheet and still preserve antigenicity of surface molecules. We have tested acetone, formaldehyde, paraformaldehyde, and glutaraldehyde. Undiluted acetone was unsatisfactory because it etched the surface of the polystyrene wells, and formaldehyde at three dilutions produced variable results (data not shown). Paraformaldehyde or glutaraldehyde produced the best results. We have a preference for paraformaldehyde, as it is also the fixative of choice for ELISA (1).

Figure 3. Modulation of IFNγ-stimulated (100 U/mL) HLA class I or class II expression by culturing cells in the presence of DMEM alone, DMEM plus 0.2% lactalbumin hydrolysate (D/LAH) or DMEM plus 1%, 5% or 10% FCS (D/F1, D/F5 or D/F10). Class I expression on cells cultured in LAH or serum was significantly different (P <0.01) from cells cultured in DMEM alone, but not significantly different from each other. HLA class II expression on cells cultured in D/LAH or D/F10 was significantly lower (P <0.01) than expression on cells cultured in D/F1 or D/F5. Expression was significantly higher in all four groups cultured in DMEM supplemented with LAH or serum than in cells cultured in DMEM alone (P <0.01). Data are corrected for nonspecific binding, bars represent the mean of triplicate wells with SEM and results are representative of 10 experiments.

Figure 4. Modulation of ICAM-1 at 24 h by IL-1β (10 U/mL), IL-6 (30 U/mL) or platelet-derived growth factor (PDGF; 10 U/mL) as assayed by CELISA (left ordinate) and FC (right ordinate). Comparison is similar to more than 10 experiments run to correlate results of CELISA with those of FC.
commonly used for FC analyses, but glutaraldehyde may work as well in many applications. If 0.05% glutaraldehyde is used, reactive aldehyde groups must be blocked with 0.2 M glycine in PBS for 20 min at RT to avoid covalent binding of proteins added at subsequent steps. The shortest fixation time that maintains the integrity of the monolayer is the desired goal.

**Titration of Primary and Secondary Antibodies**

The titration of primary and secondary antibodies is identical to that performed for a typical ELISA and is probably best achieved using a criss-cross, serial-dilution protocol described in numerous reports (1,12,13). The primary antibody should be used at saturation.

**CELISA vs. Flow Cytometry**

We consider FC to be a principal method of standardization and validation of any CELISA protocol. Figures 2 and 4 show results of similar protocols assayed by these two complementary methods. Both assays have advantages and disadvantages. FC analyses characterize cells individually, whereas the CELISA is restricted to analysis of cell populations. If cells are fixed at all in a FC protocol, they are fixed after staining; whereas with CELISA, cells are fixed before addition of the MAb, possibly resulting in some loss of antigenicity. In contrast, the detachment of adherent cells required for FC, especially if an enzymatic release agent is used, may also result in some loss of antigenicity.

The 96-well format of the CELISA is also advantageous in performing replicate determinations with far fewer numbers of cells/determination. Using HSC, the CELISA requires $1 \times 10^4$ cells/well, whereas the FC protocol requires staining approximately $3-5 \times 10^5$ cells/determination. Finally, a microplate reader is considerably less expensive to acquire and operate than a flow cytometer.

In summary, we have found the CELISA to be a useful and efficient tool for analyses of cell surface antigens on relatively intact cells. Many factors impact the results of any particular protocol. For best results, it is necessary to run a number of preliminary assays to optimize protocol variables, not only for the cells and antibodies used, but also for the methods of culture and for the reagents used. Confirmation of results with FC analyses is an appropriate means of validating the protocol.

**ACKNOWLEDGMENTS**

This work was supported, in part, by a grant from Pfizer, Inc. (H.B.L.); and by the Carey and Evans Arthritis Funds, Kansas University Endowment Association.

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Received 11 March 1996; accepted 2 October 1996.

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Vol. 22, No. 5 (1997)