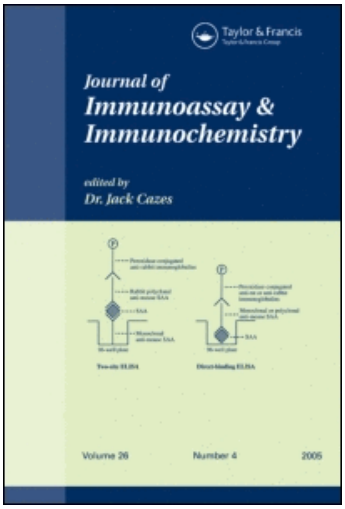

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AN IMMUNOENZYME PROCEDURE FOR ENUMERATING FIBRONECTIN-SECRETING CELLS

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ABSTRACT

A recently described solid phase immunoenzyme procedure (ELISPOT) has been adapted for the detection of individual cells secreting fibronectin. Simple and sensitive, this technique should find useful application for studying fibronectin production at the cellular level.
(KEY WORDS: Fibronectin, ELISPOT, single cells)

INTRODUCTION

In the past decade, a large number of biological functions has been ascribed to fibronectin, a high molecular weight glycoprotein found in body fluids, in vertebrate basement membranes and in loose connective tissue. Fibronectin interacts with native and denatured collagens, and a variety of other macromolecules including fibrin, heparin, hyaluronic acid and certain gangliosides (reviewed by Ruoslahti et al. (1)). There is compelling evidence that fibronectin, through its multiple ligand binding interactions, plays an important role in cellular adhesion, malignant transformation,

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reticuloendothelial clearance function and tissue morphogenesis; for a recent review see Yamada (2). A striking characteristic of fibronectin is its existence as an insoluble connective tissue matrix protein as well as a soluble protein in plasma and other body fluids. Although both forms are very similar biochemically and antigenically, their functional relationship still has to be resolved (3, 4, 5, 6).

Various bioassay systems, pulse-chase isotopic labelling methods and immunological procedures have been used to detect and/or to quantify cellular fibronectin. These methods generally involve cumbersome and time-consuming procedures involving preculture of the cells with subsequent extraction of the secreted fibronectin from the culture supernatants (reviewed in Yamada and Olden (7)). Moreover, these methods do not enable an appreciation of the frequency of fibronectin-secreting cells within a given cell population. The latter information should be particularly valuable for studying fibronectin metabolism at the single cell level in physiological situations as well as in certain diseases.

The recently described enzyme-linked immunospot assay (ELISPOT), originally described for the detection of antibody-secreting cells (8) and subsequently applied to the detection of antigen-secreting cells (9), toxin- producing bacteria (10), and rheumatoid factor-secreting cells (11), has in the present report been adapted for the detection of fibronectin secreted by individual cells.

MATERIALS AND METHODS

Cell Lines

Human embryonic fibroblasts and Epstein Barr virus transformed human lymphoblastoid cells (P3HR-1) (12) were grown for approximately one week at 37°C in Eagle's minimal essential medium (MEM) (Gibco Europe, Scotland) supplemented with antibiotics and 10 % foetal bovine serum (Gibco). Outgrown cells were harvested and trypsinised in order to remove membrane-associated fibronectin (13). After three washings with cold serum-free MEM, the cells were resuspended, counted and adjusted to the appropriate density in serum-free medium. All cell suspensions were kept on ice prior to being assayed (within thirty minutes).

Serological Reactants

Horseradish peroxidase (HRP) conjugated rabbit anti-human fibronectin, rabbit anti-human fibrinogen and swine anti-rabbit immunoglobulins (Dakopatts, Copenhagen, Denmark) were diluted 1:250 in phosphate-buffered saline (0.015 M, pH 7.4 (PBS) containing 0.05% Tween 20 and 1% BSA before use. Preliminary checkerboard titration experiments established that this dilution was optimal for the present experimental conditions. The immunoglobulin fractions of rabbit anti-human fibronectin and rabbit anti-human fibrinogen (Dakopatts) were diluted in serum-free MEM before use. The immunoglobulin fraction of a normal rabbit serum was obtained after caprylic acid precipitation according to Steinbuch and Audran (14).

Enzyme-Substrate

Para-phenylene-diamine (PPD) (Sigma Chemical Company, St Louis, Mo) was dissolved in methanol in a concentration of 5 mg/ml, mixed with $H_2O_2$0 (final concentration 0.3%) and immediately added to 20 volumes of a 1% solution of Noble agar (Difco Lab., Detroit, Mi) in PBS previously molten and then cooled to 48°C. The agar-substrate solution was used within 10 minutes.

Enzyme-Linked Immunospot Assay (ELISPOT) for Enumeration of Fibronectin-Secreting Cells

Petri dishes (5 cm diam, NUNCLON, Roskilde, Denmark) were filled with 3 ml of PBS containing 0.02% NaN_3 and either gelatin (Difco), in a final concentration of 0.05% (w/v), or 1% BSA (Sigma). After overnight incubation at room temperature, the plates were rinsed twice with PBS and exposed to 3 ml of PBS containing 1% BSA for one hour at 37°C in order to saturate remaining binding sites on the plastic surface. The coated dishes were then decanted and filled with 1.5 ml of serum-free MEM containing various numbers of cells (fibroblasts or lymphoblastoid cells). Incubation was carried out at 37°C for four hours in an atmosphere containing 10% CO_2 and 100% humidity. The dishes were rinsed twice with PBS and incubated for 10 minutes with cold PBS containing 0.05 M EDTA. After three additional washings with PBS, the dishes were exposed for three hours at room temperature to 3 ml of HRP-conjugated rabbit anti-human fibronectin. Following four washes with PBS the plates were either developed with agar-substrate solution or further exposed to 3 ml of HRP-labelled

swine anti-rabbit immunoglobulin conjugate for two hours at room temperature and then washed with PBS as above. Colour development by the boundenzyme was performed by adding agar-PPD substrate solution onto the dishes. The plates were immediately decanted in order to leave a thin layer of gel. After a few minutes, the dishes were examined for appearance of dark-brown spots which were enumerated with the naked eye or under low magnification (x 16).

RESULTS

Detection of Fibronectin-Secreting Cells by the ELISPOT Test:

When plated for a few hours into gelatin-coated dishes, human embryonic fibroblasts produced fibronectin that bound to the solid-phase within the close vicinity of the secreting cells. Zones of gelatin-bound fibronectin could be readily visualized upon stepwise addition of enzyme-labelled anti-human fibronectin antibodies and agar-substrate solution. Dark-brown foci or "spots" appeared within minutes at the location of the previously plated fibronectin-secreting cells and could be enumerated directly with the naked eye or under low magnification (figure 1). Occasional artifacts due to cell debris and/or aggregates were easily distinguished from the distinct appearance of positive reactions, i.e. dark, circular and homogeneously granulated foci.

As shown in figure 2, as few as 10^3 fibroblasts could generate a significant number of spots when plated for four hours in gelatin-coated dishes. The number of spots was found to be pro-

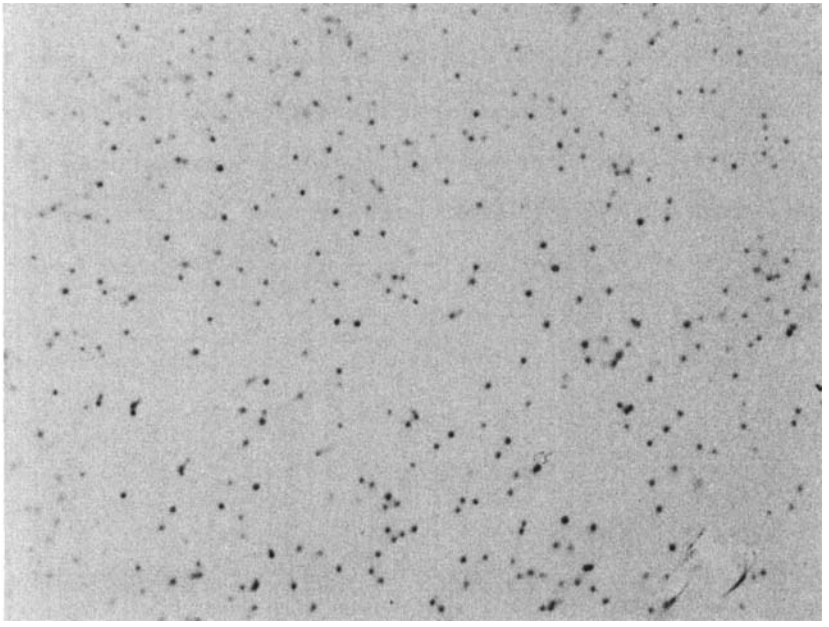

FIGURE 1:
Typical appearance of fibronectin-mediated spots as visualised by the *ELISPOT* test.
One thousand embryonic fibroblasts were plated for four hours in gelatin-coated dishes and zones of gelatin-bound fibronectin were visualised after development with peroxidase-labelled anti-fibronectin antibodies and agar-substrate (x 16).

portional to the number of cells plated, accounting for almost 10% of the number of cells assayed. In addition, the sensitivity of the immunoenzyme procedure could be increased by adding a second layer of HRP-labelled anti-immunoglobulin antibodies, i.e. swine anti-rabbit immunoglobulin conjugate. Both the intensity of the reactions and their numbers were increased when using this artifice of amplification (data not illustrated).

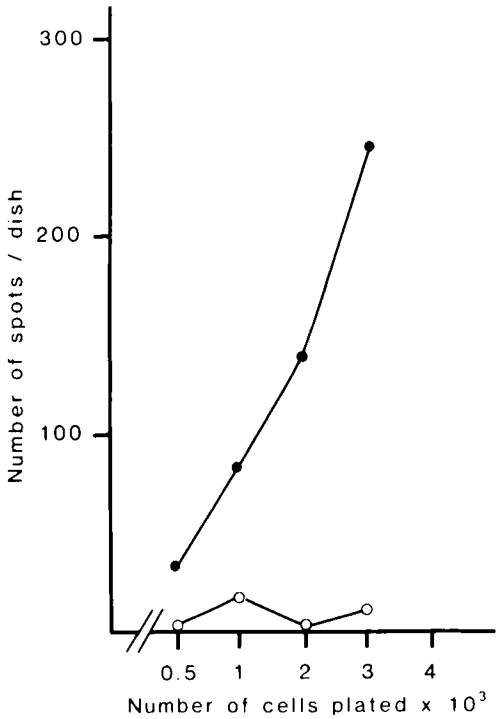

FIGURE 2:
Sensitivity of the ELISPOT test for detection of fibronectin--secreting cells.
Various numbers of fibroblasts were incubated in gelatin-coated dishes (closed symbols) or in BSA-coated dishes (open symbols) for four hours and assayed for numbers of fibronectin-secreting cells by means of the ELISPOT test.

Specificity

Although spots were observed when human fibroblasts had been plated in uncoated dishes, the number was generally ten to fifty times lower than the number observed in gelatin-treated dishes. However, exposure of uncoated petri dishes to BSA prior to cell plating prevented the direct binding of secreted fibronectin to the

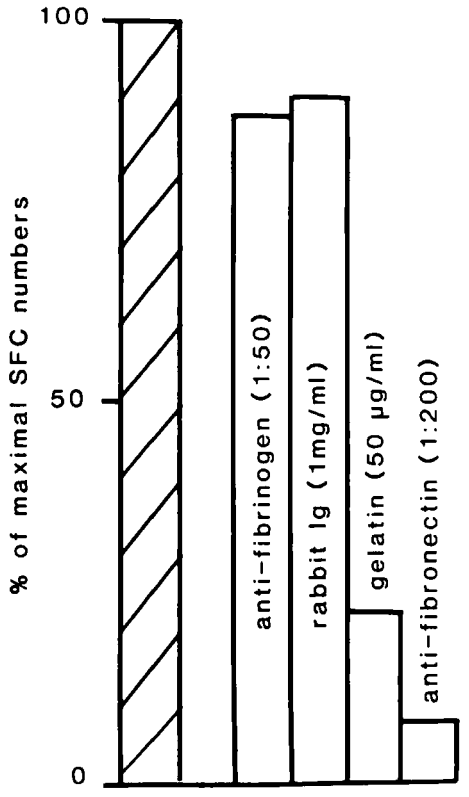

FIGURE 3:
Specificity of the ELISPOT assay for detection of fibronectin--secreting cells.
One thousand fibroblasts were plated into gelatin-coated dishes in the presence of various additives tested for their ability to inhibit fibronectin-mediated spot formation. Results are expressed as percentage of maximum spot-forming cell (SFC) numbers observed without (hatched bars) and with (open bars) additives.

plastic surface as shown by the absence of reactions observed in BSA-coated dishes.

Unlike fibroblasts, Epstein Barr virus transformed lymphoblastoid cells failed to generate any detectable reactions in gelatin-coated dishes.

Addition of rabbit anti-human fibronectin antibodies or gelatin during cell plating inhibited the formation of spots whereas incorporation of either rabbit anti-human fibrinogen antibodies or normal rabbit immunoglobulins had no detectable effect (figure 3).

Thus, the generation of spots by human fibroblasts appeared to be due to the specific binding of fibronectin molecules secreted by individual cells.

DISCUSSION

The assay system described here provides a simple and sensitive method whereby the secretion of fibronectin by individual cells may be analysed in short term cultures.

When evaluated for the detection of fibronectin-secreting human embryonic fibroblasts, the procedure showed adequate specificity. Thus, reactions could only be observed in dishes that had been coated with an appropriate ligand, i.e. gelatin. Although spot formation could be observed in uncoated dishes, the latter observation is not surprising in view of the known affinity of fibronectin for plastic substrates (15). This property is thought to mediate the anchorage of numerous cell types to plastic culture vessels. However, exposure of uncoated plates to BSA prior to cell plating prevented the direct binding of secreted fibronectin to the plastic surface.

When compared with most bioassay systems and immunological methods used to detect cellular fibronectin secreted and accumulated

in culture supernates, the procedure described here offers several practical advantages. It does not require any special equipment; registration of the reactions is achieved with the naked eye; it avoids laborious and time-consuming steps for production and extraction of secreted fibronectin; it makes use of relatively short culture incubation periods, thus minimising the risks of extensive proteolytic degradation of secreted fibronectin.

More importantly, the ELISPOT test enables an estimation to be made of the frequency of fibronectin-secreting cells rather than of the absolute amount of secreted product. However, the latter information can easily be obtained by simply substituting a liquid substrate for the agar-substrate as described elsewhere (8). Thus, the assay system described here might provide additional information concerning the average rate of fibronectin secretion for a given population of cells.

We have successfully applied this method to study fibronectin secretion by a variety of cells including peritoneal macrophages and liver granuloma cells during the course of experimental schistosomiasis in mice (in preparation). The ELISPOT assay can thus be adapted to various types of receptor-ligand interactions, e.g. gelatin-fibronectin, providing a versatile methodological principle for studying the metabolism of virtually any immunoreactive substance at the single cell level.

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Ms Lena Carlsson and Ms Krystyna Pawlowska are greatfully acknolwledged for their excellent technical assistance.

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REFERENCES

1. Ruoslahti E., Engvall E. and Hayman, E.G. Fibronectin: Current Concepts of its Structure and Functions. Coll. Res. 1981; 1:95-128.

2. Yamada K.M. Cell Surface Interactions with Extracellular Materials. Ann. Rev. Biochem. 1983;52: 761-799.

3. Yamada K.M. and Kennedy D.W. Fibroblast Cellular and Plasma Fibronectins are Similar but not Identical. J. Cell. Biol. 1979; 80: 492-498.

4. Fukuda M., Levery S.B. and Hakomori S. Carbohydrate Structure of Hamster Plasma Fibronectin. J. Biol. Chem. 1982;257: 6856-6860.

5. Hayashi M. and Yamada K.M. Differences in Domain Structures between Plasma and Cellular Fibronectins. J. Biol. Chem. 1981;256: 11292-11300.

6. Atherton B.T. and Hynes R.O. A Difference between Plasma and Cellular Fibronectins Located with Monoclonal Antibodies. Cell 1981;25: 133-141.

7. Yamada K.M. and Olden K. Fibronectins - Adhesive Glycoproteins of cell Surface and Blood. Nature 1978;275: 179-184.

8. Czerkinsky C.C., Nilsson L.-Å., Nygren H., Ouchterlony Ö. and Tarkowski A. A solid-Phase Enzyme-Linked Immunospot (ELISPOT) Assay for Enumeration of Specific Antibody-Secreting Cells. J. Immunol. Methods 1983;65: 109-121.

9. Czerkinsky C.C., Tarkowski A., Nilsson L.-Å., Ouchterlony Ö., Nygren H. and Gretzer C. Reverse Enzyme-Linked Immunospot Assay (RELISPOT) for the Detection of Cells Secreting Immunoreactive Substances. J. Immunol. Methods 1984; 72:489-496.

10. Czerkinsy C.C. and Svennerholm A.-M. Ganglioside GM1 Enzyme-Linked Immunospot Assay for Simple Identification of Heat-Labile Enterotoxin-Producing Escherichia coli. J. Clin. Microbiol. 1983;17: 965-969.

11. Tarkowski A., Czerkinsky C.C., Nilsson L.-Å., Nygren, H. and Ouchterlony Ö. Solid-Phase Enzyme-Linked Immunospot (ELISPOT) Assay for Enumeration of IgG Rheumatoid Factor-Secreting Cells. J.Immunol. Methods 1984; in press.

12. Hinuma Y., Konn M., Yamaguchi J., Wudarski D.J., Blakeslee J.R.Jr. and Grace J.T.Jr. Immunofluorescence and Herpes Type Virus Particles in the P3HR-1 Burkitt Lymphoma Cell Line.) J. Virol. 1967;1: 1045-1051.

13. Hynes R.O. Alteration of Cell-Surface Proteins by Viral Transformation and by Proteolysis. Proc. Natl. Acad. Sci. (USA). 1973;70: 3170:3174.

14. Steinbuch M. and Audran R. The Isolation of IgG from Mammalian Sera with the Aid of Caprylic Acid. Arch. Biochem. Biophys. 1969;134: 279-284.

15. Grinnell F. Cellular Adhesiveness and Extracellular Substrata. Int. Rev. Cytol. 1978;53: 65-144.