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Enzyme-linked Lectin Assay (ELLA)

II. *Detection of Carbohydrate Groups on the Surface of Unfixed Cells*

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An enzyme-linked lectin assay (ELLA) has been developed to detect specific carbohydrate units on the surface of unfixed cells. The assay may be read in standard ELISA plate readers, since the cell-bound enzyme–lectin conjugate is specifically eluted from the cells prior to development of the conjugate. ELLA, when read in an enzyme-linked immunosorbent assay (ELISA) plate reader, allows better detection and relative quantitation of specific surface carbohydrate units than is possible by standard immunofluorescence with fluorescein-conjugated lectins.

Enzyme–lectin conjugates have been used as specific probes for carbohydrate moieties on cells and tissue sections for well over a decade [1, 2, 3]. To date, however, these probes have been used exclusively as cytochemical stains, being developed with insoluble substrates and evaluated with either light or electron microscopy. Recently, we described the use of an enzyme–lectin conjugate for the semi-automated and quantitative detection of specific carbohydrate end groups on immobilized glycoproteins [4]. This assay, which we have termed ELLA (enzyme-linked lectin assay), is performed on microtiter plates in a manner analogous to the common ELISA (enzyme-linked immunosorbent assay) technique in which enzyme-linked reagents are used to detect specific antigen–antibody reactions. In the current study, we extend the use of ELLA methodology to the detection of specific carbohydrate moieties on the surface of viable cells in suspension using soluble substrates in order that the amount of lectin binding may be determined quantitatively using automated ELISA plate readers.

The lectins used in this study are well-characterized isolectins derived from *Griffonia simplicifolia* seeds, as previously described [5]. These two isolectins, GS I-A_4 and GS I-B_4, are tetramers comprised of homogeneous subunits. The GS I-A_4 exhibits a primary specificity for α-D-GalNAc*p* (*N*-acetyl-α-D-galactosaminyl groups) but also reacts with α-D-Gal*p* (α-D-galactopyranasyl groups) units, whereas GS I-B_4 shows a marked specificity for α-D-Gal*p* end groups [5]. Both lectins were conjugated to alkaline phosphatase for use in this study, but the

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majority of the experiments were performed with alkaline phosphatase-conjugated GS I-B_4 because of its stricter specificity.

MATERIALS AND METHODS

Cells

Human erythrocytes (types A, B and O) and several transformed or tumorigenic cell lines of murine origin were used in this study. The human erythrocytes were provided by W. John Judd, Department of Pathology, University of Michigan, and were obtained from normal blood donors. The parent Swiss 3T3 cells and a variant line of 3T3 cells deficient in terminal α-D-galactopyranosyl groups (3T3 B_4^r) were obtained from Dr Barry Peters, Department of Pharmacology, University of Michigan, and have been described in a previous report [6]. Murine L-929 cells, and a variant of the L-929 cells deficient in surface α-D-galactopyranosyl end groups (L-929 Abn^r) were obtained from Dr E. H. Y. Chu, Department of Human Genetics, University of Michigan, and have previously been described [7]. A second α-D-Galp-negative variant line of L-929 cells was selected in our laboratory using anti-human blood group type B sera and was designated L-929/anti-B^r. The murine tumor lines were all derived from three 3-methylcholanthrene-induced fibrosarcomas. Two of the lines were uncloned parental lines (1.1 and 1.2) from two of the tumors and one line was cloned from a lung metastasis of the third tumor (1.0/L1). The remaining three lines (1.0/anti-B^r, 1.1/anti-B^r, and 1.2/anti-B^r) were selected from the parent tumors by cytotoxicity with anti-sera to the human blood group B antigen. The selection and characterization of these lines have been detailed in several of our recent publications [8, 9, 10].

ELLA

Alkaline phosphatase-conjugated *Griffonia simplicifolia* I-B_4 isolectin was prepared using a one-step glutaraldehyde method, as described previously [4]. Enzyme–lectin conjugates were tested for their ability to react specifically with glycoproteins containing α-D-galactopyranosyl end groups using the ELLA technique previously described [4]. Single-cell suspensions of either cells harvested from tissue culture or human blood were washed three times in isotonic phosphate-buffered saline (PBS), pH 7.2. The cells were counted with the use of a hemocytometer and a predetermined number of cells were added to sterile plastic test tubes. The cells were pelleted by centrifugation (250 g for 5 min) and the supernatant gently removed. AP-GS I-B_4 (250 µl) at a concentration of 5 µg lectin/ml were added to each test tube. The cells were then resuspended by gentle pipetting, and the tubes were capped and allowed to incubate for 30 min at room temperature with frequent shaking. Following this incubation period the cells were pelleted by centrifugation (250 g for 5 min) and the supernatant containing unbound lectin removed. The cells were washed once with 1 ml PBS, pH 7.2 then transferred to fresh plastic test tubes. The cells were then washed twice more with PBS, pH 7.2, 1 ml per wash. After the final wash, 250 µl of 50 mM methyl α-D-galactopyranoside (Me α-D-Gal*p*) in 0.1 M carbonate buffer, pH 9.6 containing 0.15 M NaCl were added to each test tube to elute the enzyme–lectin conjugate from the cells. As controls, elution procedure were followed using either 0.1 M carbonate buffer, pH 9.6 containing only 0.15 M NaCl or containing both 50 mM methyl α-D-glucopyranoside and 0.15 M NaCl. Additional controls consisted of initially incubating the cells in isotonic PBS without AP-GS I-B_4, followed by standard elution protocols. This controlled for release of endogenous phosphatase. After addition of the haptenic sugar solution to the cell pellets, the cells were gently resuspended by pipetting and the suspensions of cells were incubated for 30 min at room temperature. After incubation the cells were pelleted by centrifugation and triplicate 50-µl aliquots of the supernatant withdrawn and added to wells of a plastic microtiter plate (Falcon cat. no. 3070). To each well containing 50 µl of the supernatant an additional 50 µl of *p*-nitrophenyl phosphate (2 mg/ml in 0.1 M carbonate buffer, pH 9.6) were added. The supernatant containing any eluted enzyme–lectin conjugate and the phosphatase substrate solution were allowed to incubate in the wells at room temperature for various lengths of times, then the A_{405} of each well was measured using a Titertek Multiskan ELISA-plate reader (Flow Laboratories, Inc., McLean, Va). In separate experiments, *Griffonia simplicifolia* I-A_4 isolectin, which has a much higher affinity for *N*-acetyl α-D-galactosaminyl (α-D-GalNAc*p*) end groups than for α-D-Gal*p* end groups, was conjugated to alkaline phosphatase using the methodology described above. Five mM GalNAc was used to protect the binding sites of the GS I-A_4

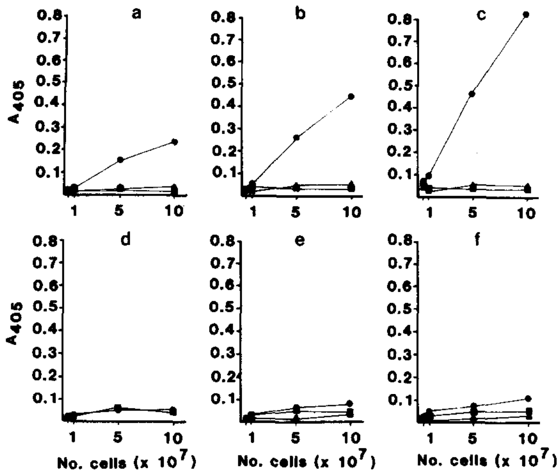


Fig. 1. Reactivity of human erythrocytes with AP-GS I-B_4. Erythrocytes assayed were: ●, Type B; ■, type 0; ▲, type A. Reactivity after (*a, d*) 30 min; (*b, e*) 1 h; (*c, f*) 2 h incubation of the substrate. (*a–c*) The ELLA reactivity represents elution of the AP-GS I-B_4 from cells with 50 mM methyl α-D-gal*p*; (*d–f*) reactivity after elution with 50 mM methyl α-D-glucopyranoside.

isolectin during the conjugation rather than 5 mM Me α-D-Gal*p*. ELLA assays were then performed using AP-GS I-A_4 (at a final concentration of 10 μg lectin/ml) and human erythrocytes. Elution of the AP-GS I-A_4 was accomplished using 20 mM GalNAc.

Immunofluorescence

Fluorescein isothiocyanate-labeled GS I-B_4 was available in our laboratory from a previous study and was used to stain the cell lines grown in vitro using modifications of our previously described method [11].

RESULTS

After conjugation of the enzyme to the lectin each new batch of AP-GS I-B_4 was routinely screened for its specific reactivity against α-D-gal*p* end groups on immobilized glycoproteins, such as laminin. By testing various dilutions of AP-GS I-B_4 in this system, the concentration of conjugate which reacted rapidly with α-D-Gal*p* end groups, and gave a low background, was determined. Subsequently it was this concentration of conjugate which was used to detect α-D-Gal*p* end groups on unfixed cells. With most preparations of AP-GS I-B_4, a 1 : 100 dilution (5 μg/ml final concentration of lectin) was used.

Using the methodology described above the AP-GS I-B_4 was used as a probe for terminal α-D-Gal*p* units on unfixed cells. The first cells studied were human erythrocytes. These were chosen because type B erythrocytes contain α-D-Gal*p* end groups and bind GS I-B_4 [5, 12], while type A and type O erythrocytes do not contain this carbohydrate end unit [12] and consequently do not bind this isolectin. Additionally these erythrocytes represent relatively homogeneous cell populations. Fig. 1 illustrates the reactivity obtained in the ELLA against several types of human erythrocytes. As can be seen type B erythrocytes were strongly reactive in this assay, whereas type A and type O erythrocytes were not. The

intensity of the ELLA reaction was directly proportional to the number of type B erythrocytes present and to the length of time the substrate was allowed to react.

Six well characterized murine tumor lines were then screened by ELLA for the presence of terminal α-D-Gal*p* groups. Previous studies have shown that three of these lines, 1.0/L1, 1.1 and 1.2, contain α-D-Gal*p* end groups and bind GS I-B_4, while the remaining three, 1.0/anti-B^r, 1.1/anti-B^r and 1.2/anti-B^r, do not contain terminal α-D-Gal*p* groups and do not bind the lectin [8, 10]. The results of the ELLAs on these cells are shown in fig. 2. The immunofluorescent staining of these cells with FITC-conjugated GS I-B_4 is shown in fig. 3. As can be seen 1.0/L1, 1.1, and 1.2 were all highly fluorescent when stained with the FITC-GS I-B_4, whereas the 1.0/anti-B^r, 1.1/anti-B^r and 1.2/anti-B^r were not. Similarly, 1.0/L1, 1.1, and 1.2 were all reactive in ELLA, while 1.0/anti-B^r, 1.1/anti-B^r and 1.2/anti-B^r were not (fig. 2). As with type B erythrocytes, the intensity of the ELLA reaction was directly proportional to the number of 1.0/L1, 1.1 or 1.2 cells added to the assay.

ELLA assays were also conducted using AP-GS I-B_4 to screen murine 3T3 and L-929 cells, and their respective α-D-Gal*p*-lacking variants. Both the 3T3 and L-929 cells were highly reactive in ELLA as were the 1.0/L1 cells run as positive controls. The 3T3 B_4^r variants and L-929 Abn^r variants lacking surface α-D-Gal*p* were non-reactive, as were 1.0/anti-B^r cells run as negative controls. The α-D-Gal*p*-lacking variant of the L-929 cells derived in our laboratory (L929/Anti-B^r), was, however, extremely reactive in ELLA. Since fluorescence microscopy with FITC-GS I-B_4 indicated that the L-929/anti-B^r variant did indeed lack surface α-D-Gal*p* further experimentation was conducted using this variant in ELLA. In these further studies, the L-929/anti-B^r cells were exposed to phosphatase substrate in isotonic carbonate buffer, pH 9.6 immediately after harvesting and without exposure to AP-GS I-B_4. An immediate and intense colorimetric reaction occurred indicating the secretion of extremely high levels alkaline phosphatase by these cells.

Fig. 4 illustrates the results obtained by ELLA using AP-GS I-A_4 to detect α-D-GalNAc*p* end groups on human erythrocytes. As can be seen type A erythrocytes, which bear surface α-D-GalNAc*p* (part of the type A determinant) were strongly reactive in this assay, whereas type B and type O erythrocytes were much less reactive.

DISCUSSION

Traditionally, enzyme–lectin conjugates have been used as probes for fixed cells or tissue sections, with detection of binding through the use of insoluble substrates. Fluorescein-conjugated lectins have also commonly been used to detect cell surface carbohydrate units and recently an assay for quantitatively measuring carbohydrates on fixed cells using fluoresceinated lectins has been described by Monsigny and co-workers [13]. The results presented here clearly

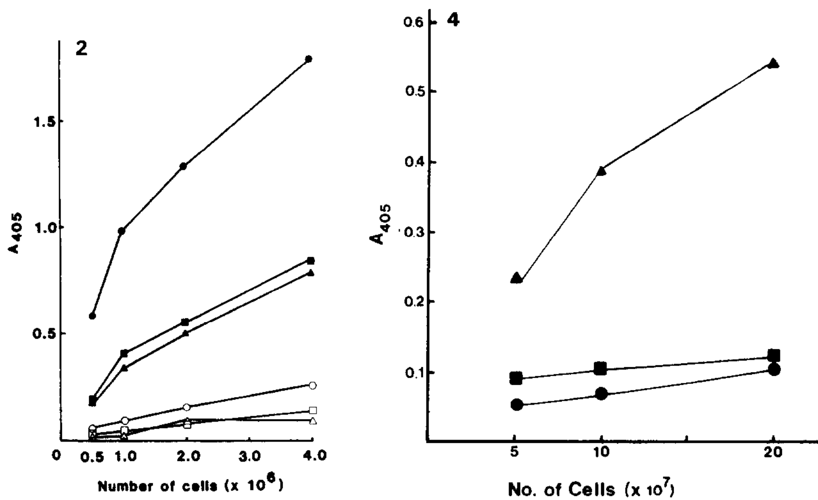
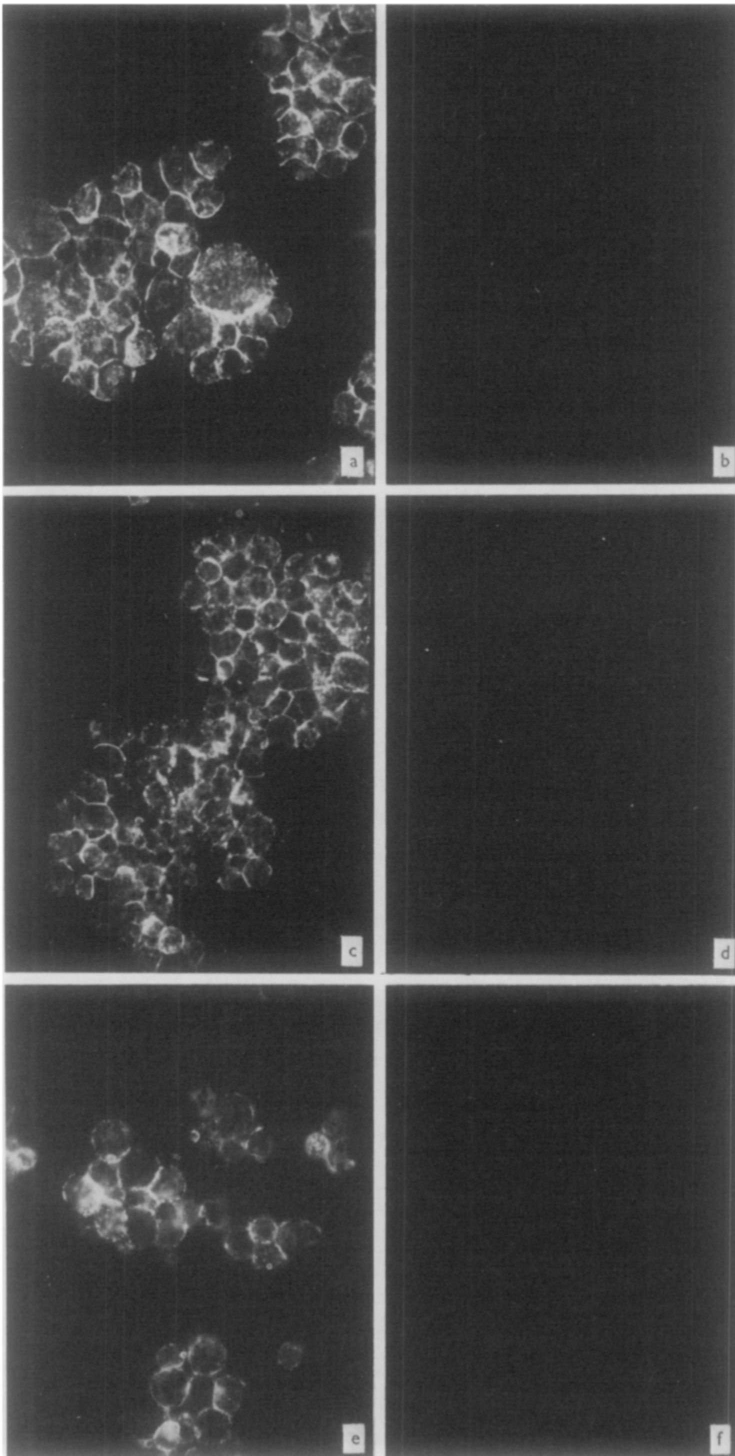


Fig. 2. Reactivity of murine tumor cell lines AP-GS I-B_4. The types of murine tumor cell lines assayed are: ●, Cloned 1.0/L1 high metastatic cells; ○, cloned 1.0/anti-B^r low metastatic cells from the same parental fibrosarcoma as 1.0/L1; ■, uncloned 1.1 fibrosarcoma cells; □, uncloned low malignant cells derived from 1.1, designated 1.1/anti-B^r; ▲, uncloned 1.2 fibrosarcoma cells; △, uncloned low malignant cells derived from 1.2, designated 1.2/anti-B^r. The absorbance at 405nm (A_{405}) was determined after 40 min incubation of the eluted AP-GS I-B_4 with the enzyme substrate.

Fig. 4. Reactivity of human erythrocytes with AP-GS I-A_4. Erythrocytes assayed were ▲, type A; ●, type B; ■, type 0. The alkaline phosphatase-conjugated lectin was eluted from the cells with 20mM GalNA*p* and incubated for 2 h with the substrate.

indicate the feasibility of using an enzyme–lectin conjugate as a specific probe for the detection of carbohydrate groups on the surface of viable cells. The current system utilizes a soluble substrate, thus allowing quantitation of lectin binding by photometric measurements using automated ELISA-plate readers. All of the cell types previously shown to contain surface α-D-Gal*p* end groups reacted with AP-GS I-B_4 in the ELLA, whereas the cell types lacking α-D-Gal*p* end groups on their cell surfaces (except the L-929/anti-B^r) failed to react. Similarly ELLA reactions using AP-GS I-A_4 specifically identified cells bearing surface α-D-GalNAc*p* end groups, i.e. type A erythrocytes. Furthermore, with each positive cell type the intensity of the ELLA reaction was directly proportional to the number of cells assayed. The use of unfixed, and in most instances viable cells, allowed specific measurement of surface membrane components. Physical interference by cells of measurement of the colorimetric reaction is avoided by elution of the enzyme–lectin conjugate from the cells by haptenic sugar. Furthermore, elution of specifically-bound enzyme–lectin conjugate from the cells reduces the

Fig. 3. Immunofluorescence staining of murine tumor cell lines with FITC-GS I-B_4. The cell lines examined were: (*a*) 1.0/L1 high metastatic cells; (*b*) 1.0/anti-B^r low metastatic cells; (*c*) 1.1 fibrosarcoma cells; (*d*) 1.1/anti-B^r variant fibrosarcoma cells; (*e*) 1.2 fibrosarcoma cells; (*f*) 1.2/anti-B^r variant fibrosarcoma cells.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possibility of endogenous phosphatase in the cell interfering with the ELLA assay. We did encounter one cell line, the L-929/anti-B^r, which not only contained endogenous phosphatase, but also secreted this enzyme in enormous quantities. Thus it was not possible to assay these cells for terminal α-D-Gal*p* groups by ELLA using AP-GS I-B_4. Although the other ten cell lines tested contained endogenous phosphatase, none secreted sufficient amounts to interfere with the ELLA assays. However, the endogenous reactivity of the L-929/anti-B^r cells demonstrates the necessity of rigid controls for assays of this nature. In an instance such as this, the obvious solution for assaying cells secreting high levels of a particular endogenous enzyme is to conjugate a different enzyme to the lectin. Nonetheless it remains critical to run the proper control to test for endogenous enzyme activity for any different enzyme–lectin conjugate used. As was evident in fig. 1, methyl α-D-gal*p* was very effective in eluting the enzyme-GS I-B_4 conjugate from the cells, whereas methyl α-D-glucopyranoside was quite inefficient, thus indicating the specificity of the cell–lectin interaction. The transfer of the cells to fresh test tubes during the washing process, and prior to the elution of the lectin, eliminates the possibility that any enzyme–lectin conjugate non-specifically bound to the wall of the test tube will contaminate the material specifically eluted from the cell surface.

Previous work has demonstrated a laminin-like substance on the surface of the high metastatic 1.0/L1 cells and on a large majority of the 1.1 and 1.2 fibrosarcoma cells. The 1.0/anti-B^r, 1.1/anti-B^r and 1.2/anti-B^r cells all lack this laminin-like substance [8, 10]. Other studies have demonstrated α-D-Gal*p* end groups on laminin purified from the EHS sarcoma [14]. Presumably, therefore, the difference in the surface expression of α-D-Gal*p* end groups is due, at least in part, to differential expression of the laminin-like substance on these cells. Since laminin is known to be an attachment factor for many cell types and is used by metastatic tumor cells during secondary colonization [8, 15, 16], the expression of surface laminin, and hence α-D-Gal*p* end groups, on tumor cells may be an index of their metastatic potential. The data shown in fig. 2 would support this hypothesis, since the 1.0/L1 high metastatic clone reacted more intensely than unselected parental fibrosarcoma lines (1.1 and 1.2) and dramatically more intensely than the low metastatic 1.0/anti-B^r cells. Likewise, the unselected parent 1.1 and 1.2 cell lines (which are tumorigenic and metastatic) reacted much more strongly than the variant populations selected from these lines. Like the 1.0/anti-B^r cells, the α-D-Gal*p*-deficient 1.1/anti-B^r and 1.2/anti-B^r lines are of low tumorigenity and non-metastatic. Further investigation into this relationship is clearly warranted and is currently in progress in our laboratory.

One limitation of this ELLA technique is that surface α-D-Gal*p* end units are measured on cell populations, not individual cells. If populations are homogeneous, ELLA reactivity accurately reflects the α-D-Gal*p* content of individual cells. However, if cell populations are extremely heterogeneous, such as found in uncloned tumor lines, ELLA will reflect only the average terminal α-D-Gal*p*

content of the spectrum of cells. This does not pose a problem with uniform cell populations such as erythrocytes or cloned cell lines, which have a more constant expression of surface α-D-Gal*p*. The ELLA methodology currently described could find use in blood banking and experimental cell research to detect cells bearing specific carbohydrate groups. Additionally this methodology should be readily adaptable to microbiology for the detection of carbohydrate groups on fungal and bacterial cells.

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REFERENCES

1. Gonatas, N K & Avrameas, S, J cell biol 59 (1973) 436.
2. Stobo, J D & Rosenthal, A S, Exp cell res 70 (1972) 443.
3. Bernhard, W & Avrameas, S, Exp cell res 64 (1971) 232.
4. McCoy, J P, Varani, J & Goldstein, I J, Anal biochem 130 (1983) 437.
5. Murphy, L A & Goldstein, I J, J biol chem 252 (1977) 4739.
6. Stanley, W S, Peters, B P, Blake, D A, Yep, D, Chu, E H Y & Goldstein, I J, Proc natl acad sci US 76 (1979) 303.
7. Stanley, W S & Chu, E H Y, J cell sci 50 (1981) 79.
8. Varani, J, Lovett, E J, McCoy, J P, Shibata, S, Maddox, D, Goldstein, I J & Wicha, M, Am j pathol 111 (1983) 27.
9. McCoy, J P, Schrier, D, Lovett, E J, Judd, W J & Varani, J, J cell sci 59 (1983) 245.
10. Varani, J, Lovett, E J, Wicha, M, Malinoff, H & McCoy, J P, J natl cancer inst 71 (1983) 1281.
11. Peters, B P & Goldstein, I J, Exp cell res 120 (1979) 321.
12. Watkins, W M, Science 152 (1966) 172.
13. Monsigny, M, Sene, C & Obrenovitch, A, Eur j biochem 96 (1979) 295.
14. Shibata, S, Peters, B P, Roberts, D D, Goldstein, I J & Liotta, L A, FEBS lett 142 (1982) 194.
15. Terranova, V P, Rohrbach, D & Martin, G R, Cell 22 (1980) 719.
16. Terranova, V P, Liotta, L A, Russo, R G & Martin, G R, Cancer res 42 (1982) 2265.

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