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Serum immunoassay of a small cell lung cancer associated ganglioside: development of a sensitive scintillation proximity assay

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We here report an enzyme linked immunosorbent assay (ELISA) and a scintillation proximity assay (SPA) for detection of the ganglioside $FucGM_1$ in sera from small cell lung cancer (SCLC) patients. The SPA was more sensitive and reproducible than the ELISA. In this assay, monoclonal antibodies specific for $FucGM_1$ were bound to SPA particles and incubated with labelled $FucGM_1$ and 100 µl test-serum overnight, and counted in a β-counter. The sensitivity was 0.2 ng. Seven out of twenty sera from SCLC patients were positive, whereas none of twenty sera from healthy individuals were positive for $FucGM_1$. The SPA was more sensitive than the previously reported HPTLC as well as a direct ELISA.

Keywords: SCLC, $FucGM_1$, sera, immunoassay, ELISA, scintillation proximity assay

Abbreviations: MAb, monoclonal antibody; SPA, scintillation proximity assay; HPTLC, high performance thin layer chromatography; SCLC, small cell lung cancer; $FucGM_1$, Fucα1-2Galβ1-3GalNAcβ1-4(NeuAcα2-3)-Galβ1-4Glcβ1-1Cer; ELISA, enzyme linked immunosorbent assay; FCS, foetal calf serum; PBS, phosphate buffered saline.

Cells that undergo malignant transformation often show aberrant glycosylation expressed on the cell surface [1]. Small cell lung cancer (SCLC) cells have been found to express the glycosphingolipid Fucα1-2Galβ1-3GalNAcβ1-4(NeuAcα2-3)-Galβ1-4Glcβ1-1Cer ($FucGM_1$) [2]. Immunohistological studies have demonstrated $FucGM_1$ in up to 90% of human SCLC tumors [3, 4]. Recently, we have shown that this ganglioside is shed from SCLC cells, both *in vitro* and *in vivo*, and that $FucGM_1$ can be detected in the sera of SCLC patients by HPTLC immunostaining [5]. A one step reproducible and sensitive assay of $FucGM_1$ will make it possible to analyse more extensive patient materials. Most radioimmunoassays (RIA) are based upon the competition between labelled and unlabelled antigen, and the separation of bound and unbound antigen. This type of assay has been difficult to apply to lipids, mainly because of mixed micelle formation between the labelled and the unlabelled antigen [6].

Scintillation proximity assays (SPA) have previously been shown to be useful for the assay of molecules of a lipid nature, e.g., prostaglandins, thromboxines and leukotrienes [7]. The assay format of SPA is based on particles that contain a fluor which is excited by radioactive decay. Low energy radiation, such as β-particles emitted by ^3H, is extremely short ranged in water. In practice, the fluor is excited only when the emitted β-particles are brought in proximity to SPA particles by binding to radioisotopic molecules. When the fluor returns from the excited to the normal state, it emits photons, which may be determined directly by scintillation counting [8, 9].

In this paper, we have compared a liposome-inhibition ELISA with the SPA for $FucGM_1$ using serum samples from SCLC patients. We found that the competitive ELISA was hampered by high backgrounds and low assay sensitivity. Both these problems were successfully overcome by the SPA, which provided a simple and sensitive analytical tool which will allow evaluation of the diagnostic and prognostic value of $FucGM_1$ for SCLC.

Materials and methods

Cells and cell cultures

The SCLC cell line GLC-14 [5, 10] was kindly provided by K. Rygaard, State University Hospital, Copenhagen. The

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cells were grown in RPMI-1640 with 10% FCS and sodium pyruvate. The hybridoma cell line 1D7 was established as previously described [5]; 1D7 cells were grown in RPMI-1640 containing 15% FCS and sodium pyruvate. The hybridoma cell line OKT-3 was from the American Type Culture Collection and was grown in Iscove's modified Dulbecco's medium with 20% FCS. All cell culture media were supplemented with L-glutamine, penicillin and streptomycin (200 u ml^{-1}, 25 μg ml^{-1}). Cell cultures were routinely screened for possible mycoplasma contamination at monthly intervals and were negative throughout these experiments.

Monoclonal antibodies

The monoclonal antibody (MAb) 1D7 [5], directed against the antigen $FucGM_1$, was purified from conditioned media. Culture supernatants were adsorbed on a Protein A-Sepharose column (Pharmacia, Uppsala, Sweden), which was washed with 0.15 M NaCl, 10 mM sodium phosphate buffer, pH 7.2 (PBS) and eluted with 0.1 M glycine buffer, pH 3.0, containing 1.5 M NaCl. The eluate was collected in 1.5 M NaCl, 0.1 M sodium phosphate buffer, pH 8.5, dialysed against 1.5 M NaCl, 0.1 M sodium phosphate, pH 7.2, and stored at 4 °C with 0.1% sodium azide. The MAb OKT-3 (anti-human CD3-ε) was purified from ascites fluids on Protein A-Sepharose columns using standard procedures. MAb 1D7 has previously been characterized as an IgG3 isotype and with specificity for $FucGM_1$ [5]. MAb 1D7 did not react with related ganglioside structures such as Fuc-GA_1, GM_1 or asialo-GM_1 [5]. The binding specificity of MAb 1D7 was further tested by analysis of the $FucGM_1$ positive GLC-14 cell line by flow cytometry, showing that 90% of these cells stained positively with MAb 1D7.

Extraction, purification and labelling of $FucGM_1$ from bovine thyroid glands

$FucGM_1$ was extracted from bovine thyroid glands as previously described [11, 12] and purified by reverse phase HPLC [13]. One milligram of purified $FucGM_1$ was custom-labelled with ^3H (Amersham International, UK) by hydrogenation of the double bond in sphingosine with tritium gas. The ^3H-$FucGM_1$ was stored in ethanol at 4 °C. The specific activity of ^3H-$FucGM_1$ was estimated as 45 Ci $mmol^{-1}$.

High performance thin layer chromatography (HPTLC) of $FucGM_1$ and ^3H-$FucGM_1$

The radiochemical purity of ^3H-$FucGM_1$ was tested by HPTLC. $FucGM_1$ and ^3H-$FucGM_1$ were absorbed on HPTLC plates (Merck, Darmstadt, Germany) which were chromatographed in a solvent system of chloroform–methanol–0.5% $CaCl_2$ (50:40:10 by vol) developed with orcinol, and immunostained. For orcinol staining, HPTLC plates were air dried and sprayed with 0.5% orcinol in 10% H_2SO_4. $FucGM_1$ was immunostained as previously described [14]. The plates were developed with 0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyle phosphate (Boehringer Mannheim, Germany) in a glycine buffer (1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 10.4).

Serum samples

The patient material has already been described in detail [5]. Briefly, sera from 20 patients with histologically verified small cell lung cancer (SCLC) were used. Eight patients had localized disease and 12 patients had disseminated cancer involving metastases to other organs. As controls, sera from 20 healthy normal individuals were analysed.

Enzyme-linked immunosorbent assay (ELISA)

Immunoplates were coated with 200 ng $FucGM_1$ antigen dissolved in 100 μl ethanol followed by double dilution. The solvent was evaporated overnight at room temperature. The plate was blocked for 1 h with 1% bovine serum albumin (BSA) in PBS to avoid nonspecific adsorption, followed by incubation with purified MAb 1D7 for 1 h. The plate was washed with PBS and incubated for 1 h with peroxidase-conjugated rabbit anti-mouse Ig diluted 1:1000 (Dako A/S, Glostrup, Denmark). Finally, the plate was washed thoroughly with PBS and incubated with peroxidase substrate containing *o*-phenylenediamine. The colour reaction was stopped with 0.1 M H_2SO_4. The optical density was determined at 492 nm.

Standard scintillation proximity assay (SPA)

Anti-mouse SPA reagent (RPN 141, Amersham International) was reconstituted in PBS to a concentration of 3 mg ml^{-1}. MAb 1D7 was used at a concentration of 1.6 μg ml^{-1} in 1% BSA in PBS. Standards were prepared in glass tubes (Pyrex, UK) from dried $FucGM_1$. One hundred micrograms were sonicated for 10 minutes with 1 ml serum from healthy donors, in a sonicator bath (Metason 200, Branson, USA) at room temperature. From this stock, dilutions were made with healthy donor serum. Labelled antigen was prepared from dried ^3H-$FucGM_1$ by sonication, as described above, to a final concentration of 2 μg ^3H-$FucGM_1$ per ml healthy donor serum. In the standard assay, 100 μl of the SPA reagent were incubated with 100 μl MAb 1D7 in mini-scintillation vials (BN-Instruments, Espergæde, Denmark) under agitation. After 30 min, 100 μl test serum or standard and 400 μl 10 mM EDTA in PBS were added to each sample and it was agitated for 30 min. Finally, the ^3H-$FucGM_1$ was added, the samples were mixed thoroughly, agitated overnight and counted in a scintillation counter. For controls, ganglioside GM_1 was included or MAb OKT-3 (1.6 μg ml^{-1}) replaced MAb 1D7. The SPA reagent was stored dried at −20 °C. After reconstitution with buffer, the maximal storage time was 1 week at 4 °C and 4 weeks at −20 °C. The intra-assay coefficient of variation was below 6% and the interassay coefficient of variation was below 15%.

Results

Inhibition enzyme linked immunosorbent assay (ELISA)

A direct ELISA for $FucGM_1$ using the MAb 1D7 is shown in Fig. 1. In addition an inhibition ELISA was developed for $FucGM_1$. Glycosphingolipids such as $FucGM_1$ are amphiphatic and appear to have a single antigenic epitope. To increase their immunoreactivity, they can be inserted into liposomes, thereby forming multivalent complexes [6]. $FucGM_1$ was incorporated into liposomes by sonication of dried phosphatidylcholine, cholesterol and $FucGM_1$ (5:3:1 by weight) with PBS for 10 min. These reconstructed liposomes were used in an inhibition ELISA, which was more sensitive than a direct ELISA. The incorporation of $FucGM_1$ into liposomes was difficult to control, and the final constitution of the liposomes varied from experiment to experiment. We found that solubilization of $FucGM_1$ in control serum by sonication gave more reproducible results, and increased the interval between minimal and maximal values. The ELISA was useful for quantification of isolated $FucGM_1$, but was not applicable to serum samples, since no differences were observed between $FucGM_1$ positive sera from SCLC patients when compared with control serum samples.

The scintillation proximity assay (SPA)

For developing the SPA, labelled antigen is required. Purified $FucGM_1$ was tritiated by custom synthesis (Amersham International) and the radiochemical purity was analysed in our laboratory. Upon evaporation, 79% of the radioactivity remained in the nonvolatile phase. Sixty percent of the total radiolabelled antigen migrated with a $FucGM_1$ standard in HPTLC. As judged from the ELISA (Fig. 1), labelled $FucGM_1$ retained its reactivity with MAb

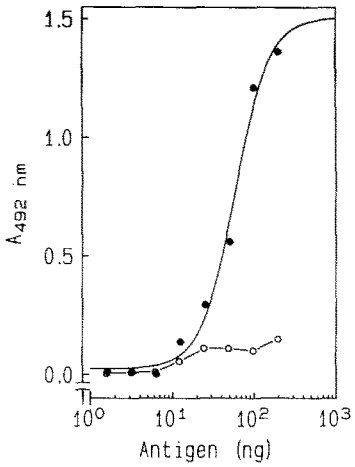

Figure 1. ELISA for ^3H-$FucGM_1$: effect of ^3H-$FucGM_1$ concentration. Immunoplates were coated with ^3H-$FucGM_1$ (filled circles) or GM_1 (open circles). After blocking with 1% BSA, MAb 1D7 (160 ng) was added and the reaction was detected by peroxidase conjugated anti-mouse (1:1000) Ig.

1D7 after ^3H-labelling . The specific activity of labelled $FucGM_1$ was estimated to 45 Ci $mmol^{-1}$.

Several parameters were tested during the development of the SPA, and the conditions referred to as standard (see Materials and methods) are the parameters we found to be optimal in combining high sensitivity, a wide dynamic range and minimal interference from unrelated components in serum. In Fig. 2(a) is shown how the scintillation signal depends on the amount of SPA particles added. In developing a sensitive competition assay, a low number of binding sites on the SPA particles should be combined with a well detectable signal and a low background signal. We found that 0.33 mg SPA per assay gave the optimal combination of these parameters. Theoretically, the optimal concentration of antibody should be the concentration in which a large proportion of antibody is bound to SPA particles and only a small proportion of antibody is in solution; i.e., the highest titre that gives a reproducible signal. In Fig. 2(b) is shown the effect of antibody (1D7) concentration compared with that of an unrelated antibody (OKT-3). At constant limiting concentrations of labelled ligand, the SPA signal reached a maximum when increasing amounts of specific antibody were added. This maximum was achieved at about 700 ng antibody per assay. An antibody concentration of 160 ng per assay (corresponding to 40% of maximal binding) was used in the standard assay conditions.

The effect of different amounts of labelled $FucGM_1$ on the SPA signal is shown in Fig. 2(c). There was an almost linear increase in the SPA signal up to 1.3 μg (78.7×10^6 counts min^{-1}) ^3H-$FucGM_1$ with antibody 1D7. At higher concentrations, it appeared that the binding sites on the SPA became occupied. The background signal from the unrelated antibody increased with the concentration of ^3H-$FucGM_1$. From an additional series of experiments (data not shown) it was found that the best compromise between high sensitivity and low background variation was obtained at 0.03 μg ^3H-$FucGM_1$ (1.83×10^6 counts min^{-1}). This amount of ^3H-labelled antigen occupies only a small fraction of the MAb binding sites on the SPA. The effect of incubation time was determined (data not shown) by counting samples at different incubation times. The SPA signal increased with time: after 20 h it was almost four times the initial signal, and this signal was relatively stable.

The inhibition of the SPA signal by unlabelled $FucGM_1$ and the related ganglioside GM_1 is shown in Fig. 2(d). The 50% inhibition was obtained by 7 ng $FucGM_1$. The related ganglioside GM_1 did not inhibit the SPA signal. From a series of assays we calculated the maximal sensitivity as 0.2 ng $FucGM_1$ (data not shown). Since $FucGM_1$ probably is integrated in lipoproteins in serum *in vivo* [15], we tested whether different ways of solubilizing labelled and unlabelled antigen would increase the sensitivity of the assay. Sonication as well as combinations with several detergents and other agents were tested. We found that the influence

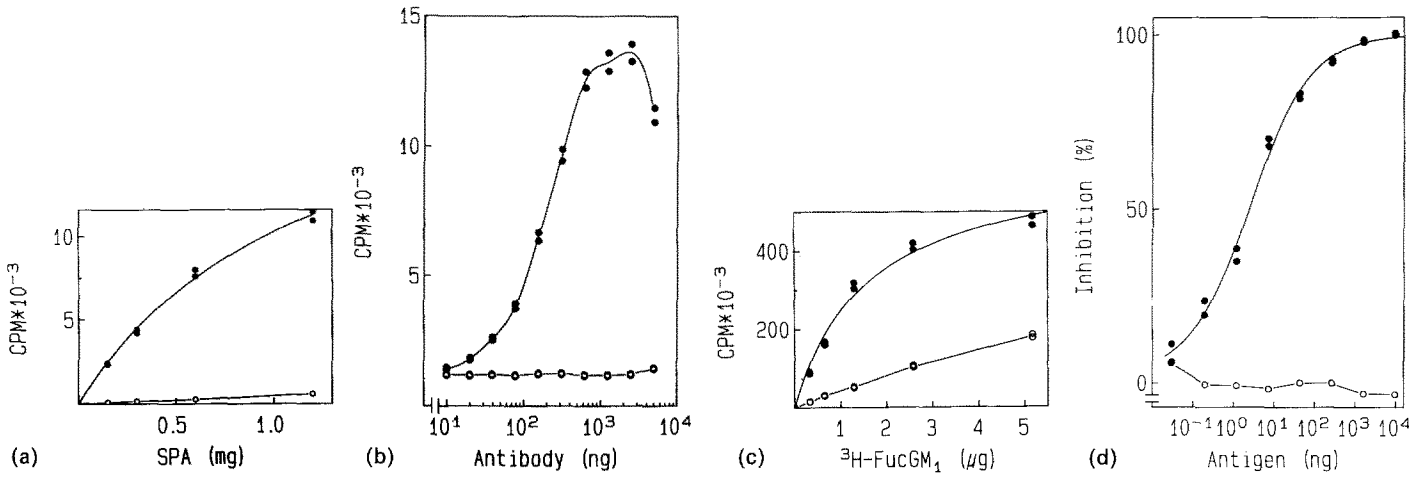

Figure 2. Scintillation proximity assay (SPA) of $FucGM_1$. (a) Effect of amount of SPA beads on the binding of ^3H-$FucGM_1$. Different amounts of SPA beads were incubated with antibody 1D7 (160 ng; filled circles) or nonrelated antibody OKT-3 (open circles) in 200 μl PBS at room temperature for 30 min in scintillation vials. Thirty nanograms (1.83×10^6 counts min^{-1}) ^3H-$FucGM_1$ in 200 μl control serum and 4 μmol of EDTA in 400 μl PBS were added. Samples were incubated overnight and counted in a β-counter. (b) Effect of antibody titre. Different amounts of 1D7 (filled circles) or irrelevant antibody (OKT3; open circles) were added to standard SPA incubation mixtures containing 0.3 mg SPA reagent, 4 μmol EDTA in 500 μl PBS and 30 ng (1.83×10^6 counts min^{-1}) ^3H-$FucGM_1$ in 200 μl control serum. (c) Effect of ^3H-$FucGM_1$ concentration. Different amounts of ^3H-$FucGM_1$ in 100 μl control serum were added to standard incubation mixtures containing antibody 1D7 (160 ng; filled circles) or unrelated control antibody OKT3 (open circles), 0.3 mg SPA reagent, 4 μmol EDTA in 600 μl PBS and 100 μl control serum. (d) Inhibition of ^3H-$FucGM_1$ binding by different amounts of unlabelled $FucGM_1$ (closed circles) and the related ganglioside GM_1 (open circles). The inhibitors were added in 100 μl control serum to incubation mixtures consisting of 30 ng (1.83×10^6 counts min^{-1}) 3H-$FucGM_1$ in 100 μl control serum, 160 ng antibody 1D7, 0.3 mg SPA reagent and 4 μmol EDTA in 600 μl PBS.

of detergent (Tween 20) or of glycerol was minimal on the assay. Unlabelled antigen solubilized in serum from healthy donors had a stronger inhibitory effect than antigen inserted in liposomes, suggesting that the presentation of the antigen is better when dissolved in human serum than when integrated in liposomes. We therefore solubilized both labelled and unlabelled antigen in serum. A series of sera from SCLC patients and from healthy controls were assayed with the SPA method and with the HPTLC/immunostaining method (Table 1). Two different volumes of serum (20 μl and 100 μl) were tested in the SPA, of which 100 μl serum was found to be suitable for the detection of $FucGM_1$ in serum. The amounts of $FucGM_1$ in the sera were determined against a standard curve of isolated $FucGM_1$ suspended in the control serum. Table 1 summarizes the results from 20 patient sera analysed by the semi-quantitative HPTLC method and by the SPA-method. The characteristics of these patients have been described previously [5]. The two different methods correlated well, since all HPTLC-positive sera were also positive by the SPA-method. All the HPTLC-positive sera contained more than 1000 ng ml^{-1} $FucGM_1$ determined by the SPA, except for one patient (No. 13) whose serum contained 38 ng ml^{-1}. However, in the HPTLC this serum was detected only as a faint band, suggesting that this sample contained less $FucGM_1$ than the other positive sera. All control sera were found to be negative by the SPA-method. The sensitivities were calculated

Table 1. Comparison of the HPTLC and the SPA methods for the antigen $FucGM_1$ in sera from SCLC patients.[a]

	HPTLC	*SPA* (ng/ml)
1	+	*
2	−	−
3	−	−
4	(+)	−
5	−	−
6	(+)	−
7	−	40
8	−	−
9	−	−
10	+	3000
11	−	−
12	−	40
13	+	38
14	−	−
15	−	18
16	−	18
17	−	−
18	+	3000
19	−	−
20	−	−

[a] Symbols: +, $FucGM_1$ positive by the HPTLC method; −, $FucGM_1$ negative; (+), weakly $FucGM_1$ positive by the HPTLC method; *, patient died, serum not available.

as 10 ng by the HPTLC method, 4 ng by the ELISA, and 0.2 ng by the SPA method.

Discussion

Serum $FucGM_1$ may be a useful marker for diagnosis of SCLC and for monitoring progression. The only available methods for the analysis of $FucGM_1$ in sera or other body fluids involve extraction in organic solvents and chromatography before immunochemical analysis. The extraction procedures are time-consuming, require large sample volumes and usually result in loss of antigen. In this study, we have established a liposome inhibition ELISA for the detection of the ganglioside $FucGM_1$, but the method was not applicable to serum analysis.

Because SPA methods have proved to be especially useful for immunoassays for molecules of a lipid nature [7], we therefore attempted to develop a SPA method. This method made it possible to detect $FucGM_1$ in the sera of SCLC patients. The method requires only a volume of 100 μl for each assay, and can be performed quickly on a routine basis. An advantage over standard ELISA techniques is that the background signals are low. A disadvantage is that the antigen must be available in ^3H radiolabelled form. A cheaper, and perhaps equally useful, alternative may be ^{125}I labelling of the antigen. ^{125}I also produces short-range excitation of SPA particles. Compared with the HPTLC method, the sensitivity was improved from 10 ng to 0.2 ng, and the SPA required a sample volume which was 10 times less than the amount required for the HPTLC method.

The increased sensitivity of the SPA method was reflected in the present results from the analysis of sera from 20 SCLC-patients. Patients positive by the HPTLC-method were also positive in the SPA method and, in addition, four further patients were positive for $FucGM_1$. The frequency of $FucGM_1$ positive patients in the SCLC material increased from 20 to 40%. None of twenty sera from healthy donors were positive.

The introduction of the SPA method for the detection of $FucGM_1$ in sera from SCLC patients may be useful for assays of other lipids shed from cell surfaces [16, 17], e.g., the detection of disialogangliosides in sera from melanoma patients [18]. We are currently using the SPA method for evaluation of both the diagnostic and prognostic value of the serum-marker $FucGM_1$ in SCLC-patients, and in a follow-up study of patients positive for this marker and subjected to chemotherapy. Recently, Brezicka *et al.* [19] were able to demonstrate tumor specific killing by MAbs against $FucGM_1$ *in vitro* as well as *in vivo*. This observation suggests that the $FucGM_1$ surface marker may serve as a target for antibody mediated lysis (ADDC) of tumor cells. The availability of an easy, rapid and sensitive serum assay for the detection of $FucGM_1$ positive SCLC patients suggests that, if the $FucGM_1$ antigen is present in about 40% of the sera of SCLC patients, immunotherapy with specific antibodies may be considered for these patients.

References

1. Hakomori S (1989) *Cancer Res* **52**: 257–331.
2. Nilsson O, Månsson J-E, Brezicka T, Holmgren J, Lindholm L, Sörenson S, Yngvason F, Svennerholm L (1984) *Glycoconjugate J* **1**: 43–9.
3. Nilsson O, Brezicka FT, Holmgren J, Sörenson S, Svennerholm L, Yngvason F, Lindholm L (1986) *Cancer Res* **46**: 1403–7.
4. Brezicka FT, Olling S, Nilsson O, Bergh J, Holmgren J, Sörenson S, Yngvason F, Lindholm L (1989) *Cancer Res* **49**: 1300–5.
5. Vangsted AJ, Clausen H, Kjeldsen TB, White T, Sweeney B, Hakomori S, Drivsholm L, Zeuthen J (1991) *Cancer Res* **51**: 2879–84.
6. Hakomori S, Kanfer JN (1983) In *Handbook of Lipid Research*, 3rd edition (Hakomori S, Kanfer JN, eds), pp. 381–435. New York: Plenum.
7. Baxendale PM, Martin RC, Hughes KT, Lee DY, Waters NM (1990) *Adv Prostaglandins, Thromboxane, and Leukotriene Res* **21**: 303–6.
8. Nelson N (1986) *Anal Biochem* **165**: 287–93.
9. Bosworth N, Towers P (1989) *Nature* **341**: 167–8.
10. Berendsen HH, Leij L de, Vries EGE de, Mesander G, Mulder NH, Jong B de, Buys CHCM, Postmus PE, Poppema S, Sluiter HJ, The HT (1988) *Cancer Res* **48**: 6891–9.
11. Macher BA, Pacuszka T, Mullin BR, Sweeley CC, Brady RO, Fishman PH (1979) *Biochim Biophys Acta* **588**: 35–43.
12. Van Dessel GAF, Lagrou AR, Hilderson HJJ, Dierick WSH, Lauwers WFJ (1979) *J Biol Chem* **254**: 9305–10.
13. Watanabe K, Arao Y (1981) *J Lipid Res* **22**: 1020–4.
14. Kniep B, Muhlradt PF (1990) *Anal Biochem* **188**: 5–8.
15. Valentino LA, Ladisch S (1992) *Cancer Res* **52**: 810–4.
16. Ladisch S, Kituda S (1987) *J Clin Invest* **79**: 1879–82.
17. Floutsis G, Ulsh L, Ladisch S (1989) *Int J Cancer* **43**: 6–9.
18. Sela B-A, Iliopoulos D, Guerry D, Herlyn D, Koprowski H (1989) *J Natl Cancer Inst* **81**: 1489–92.
19. Brezicka F-T, Holmgren J, Kalies I, Lindholm L (1991) *Int J Cancer* **49**: 911–8.