ABSTRACT

With the aid of a highly specific murine monoclonal antibody, F12, an immunofluorescence method was elaborated that allowed sensitive and specific detection of the ganglioside antigen fucosyl-GM1 (\(\text{Fuc}^{\text{F12}}\text{II}^{\text{Fuc}}\text{II}^{\text{NeuAc}}\text{II}^{\text{Gg0s}}\text{IV}^{\text{Cer}}\)) in different types of human lung cancer and normal tissues. Nineteen of 21 cases of small cell lung cancer were positive with the F12 immunofluorescence method as compared to 2 of 10 squamous epithelial cell lung cancers and 1 of 5 large cell lung cancer specimens. Specimens of lung adenocarcinoma (8 cases) and bronchial carcinoid (3 cases) were all negative, as were 2 examined cases of neuroblastoma. No fucosyl-GM1 could be detected in normal lung and bronchus. However, in thymus, spleen, and lamina propria of the small intestine sparsely distributed clusters of small round cells were stained as well as intramural ganglionic cells of the small intestine and islet cells of the pancreas. All normal other tissues tested were negative. Results obtained with immunofluorescence closely agreed with immunochemical determination of fucosyl-GM1 in lipid extracts of tissues. Our findings suggest that fucosyl-GM1 is strongly associated with small cell cancer of the lung and demonstrate that this tumor-associated antigen can be detected with high sensitivity and specificity with an immunofluorescence method based on the use of the F12 monoclonal antibody.

INTRODUCTION

SCCL\(^3\) is a highly malignant tumor that comprises about 20% of all lung cancer cases (2). It differs from the other major lung cancer types by having a more rapid proliferation and a higher propensity for metastatic spread (3, 4), which in most instances renders it surgically incurable. On the other hand, as compared with SQCL, LCCL, and ADCL, SCCL has a higher sensitivity for cytostatic drugs and is currently treated by combination chemotherapy with or without radiotherapy and prophylactic brain irradiation. However, despite impressive initial responses to such treatment in 50–80% of all patients the long term prognosis of SCCL remains poor with a 5-year survival rate of 5–10% in unselected clinical materials (5).

The hybridoma technique by Köhler and Milstein (6) for the preparation of MAbs has revolutionized the possibilities for the identification of new tumor-associated antigens. MAbs specific for such antigens may be of great value in the diagnosis, treatment, and follow-up of malignant diseases (7). Several SCCL-associated antigens defined by MAbs have recently been described (8–14). One of these is the membrane-bound antigen fucosylsialosylgangliotetraose (\(\text{Fuc}^{\text{1}}\text{Gal}^{\text{2}}\rightarrow 3\text{Gal}^{\text{3}}\rightarrow 4(\text{NeuAc}^{\text{2}}\rightarrow 3)\text{Gal}^{\text{1}}\rightarrow 4\text{Gal}^{\text{1}}\rightarrow 4\text{Glc}\)) as present in fucosyl-GM1 ganglioside (13–15). We observed the presence of a large amount of fucosyl-GM1, with an unusual ceramide portion containing 2-hydroxylated fatty acids in one case of SCCL (13) and demonstrated subsequently by immunochromehemical methods using F-MAbs that this antigen was present in more than 90% of the SCCL specimens tested (14).

We now describe a sensitive and specific immunofluorescence method for the detection of fucosyl-GM1 in tissue sections based on the use of the best of several tested F-MAbs designated F12. Using this method we have examined the distribution of fucosyl-GM1 in normal and lung cancer tissues and compared the results with those obtained in parallel immunochromehemical analyses of fucosyl-GM1 in glycolipid extracts from the same tissue specimens.

MATERIALS AND METHODS

Monoclonal Antibodies. The production of the 7 F-MAbs used in this study, F1, F3, F4, F6, F9, F10, and F12, has been described by us previously (14). The reactivities of these MAbs with a panel of solid-phase immobilized glycolipids of defined structures have been studied (15). The results suggest that these MAbs, which were raised against highly purified fucosyl-GM1, ganglioside, may be divided into 3 classes with regard to specificity: Class 1, specific for fucosyl-GM1 (F12); Class 2, cross-reactive with GM3 (F4); and Class 3, reacting with a variety of glycolipids carrying determinants present on the terminal part of the carbohydrate structure of fucosyl-GM1 (F1, F3, F6, F9, F10). Unless stated otherwise undiluted culture supernatants from hybridomas cloned twice were used. The F12 MAb was concentrated by precipitation of hybridoma culture supernatants from 50% saturated ammonium sulfate solution, the precipitate redissolved in one-tenth of the starting sample volume of 100 >¡. were prepared on glass slides by centrifugation on Lymphoprep (Nyegaard, Oslo, Norway). Preparion of Cells for Immunofluorescence. Cells (IO4) in PBS in a 0.01% (w/v) final concentration aliquots of the concentrated F12 preparation were stored at −70°C until use.

Cell Lines. The rat hepatoma cell line, H4-II-E (American Type Culture Collection, Rockville, MD), was cultured in Iscove's medium supplemented with 10% fetal calf serum, trypsinized, washed, and suspended in PBS. Small cell lung cancer cell lines U-1285, U-1690, U-1906, U-2020, and U-2050 (16, 17) were cultured in suspension or semiadherently and were harvested by pipetting, washed, and suspended in PBS.

Human Cells. RBC were prepared from blood of healthy donors of all ABH blood groups. Freshly prepared suspensions of T- and B-cells and monocytes from heparinized blood samples (18, 19) were a gift from Dr. N. Lycke, Göteborg, Sweden. Nucleated bone marrow cells were prepared from aspirates with a normal cytological appearance by centrifugation on Lymphoprep (Nyegaard, Oslo, Norway).

Preparation of Cells for Immunofluorescence. Cells (10⁶) in PBS in a sample volume of 100 μl were prepared on glass slides by centrifugation in a Cytospin 2 (Shandon) at 1500 rpm for 3 min. Slides were then air dried, stored at −20°C, and used within 1 month.

Human Tissues. Tissue specimens obtained at autopsy were fixed in...
4% formaldehyde in PBS and then frozen in liquid nitrogen and stored at −70°C. Some autopsy and all biopsy specimens were directly frozen without previous formaldehyde fixation. Cryostat sections, 4-6 μm thick, were put on glass slides precoated with 0.3% gelatin (to prevent tissue sections from falling off during the staining procedure). Sections from all tumor tissues were histochemically stained to verify tumor cell presence and diagnoses by comparison with routinely stained diagnostic tissue specimens from the same patients. Lung tumors were classified according to the scheme recommended by WHO (20).

**Immunofluorescence Staining Procedure.** Air-dried cryostat sections of tissues were used. Different fixing agents were tested: acetone, 10 min; 95% ethanol, 30 min; 5% paraformaldehyde in PBS, 30 min; all at room temperature. PBS incubation instead of fixative for 30 min was also tested. Treatment with 7.5% sucrose in PBS after fixation was performed overnight at 4°C. Incubations with MAb followed by RAM-FITC (DAKO; Dakopatts, Copenhagen, Denmark) were done for 30 min each. An additional incubation step with SwAR-FITC (DAKO) for 30 min was also tested. Normal serum homologous to the species of the tissue tested was mixed with the fluorescein-conjugated antisera in equal volumes for adsorption. All dilutions of antisera were made with 0.1% BSA in PBS. Optimal dilutions were determined by checkerboard titrations. Each incubation step during the staining procedure was followed by washing for 15 min with three changes of PBS. Slides were finally mounted with 87% glycerol in PBS.

**Immunofluorescence Scoring.** Immunofluorescence intensity was scored from 0 to 4; 0, total absence of fluorescence; 1, weak; 2, moderate; 3, strong; and 4, very strong. The percentage of the tumor cells that were scored 2–4 was estimated.

**Extraction and Immunoochemical Detection of Fucosyl-GMI.** Total lipids from tumor tissues, normal tissues, and cell lines were extracted twice with 20 volumes of chloroform/methanol/water, 4/8/3 (v/v/v) (21). Monosialogangliosides were isolated by anion exchange chromatography on Spherosil-DEAE-dextran as described previously (22). Detection of fucosyl-GMI in the monosialoganglioside fraction was performed by HPTLC immunostaining as described by Brockhaus et al. (23) using the F12 MAb. Aluminum-backed HPTLC plates, Silica Gel 60, were purchased from Merck, Darmstadt, Federal Republic of Germany. Bound F12 was detected with 125I-labeled goat anti-mouse antiserum (Radiochemical Centre, Amersham, United Kingdom) and visualized by exposure to X-AR5 X-ray film (Eastman-Kodak, Rochester, NY) for 12–24 h. A semi-quantitative determination of fucosyl-GMI, was achieved by comparison of the staining intensity obtained with samples from tissues with that of a standard preparation of fucosyl-GMI tested simultaneously in a series of known concentrations.

**RESULTS**

**Methodological Studies**

**Staining Procedure.** A standard immunofluorescence staining procedure, consisting of paraformaldehyde fixation and subsequent incubation with F12 MAb and RAM-FITC, resulted in staining with only moderate fluorescence intensity (score 2) of sections of a SCCL specimen despite a known high content of fucosyl-GMI in the tissue. In order to raise the sensitivity of the method we tested different fixatives, addition of sucrose incubation, and an extra step of fluorescein-conjugated antiserum. The best fixative turned out to be paraformaldehyde which provided for a homogeneous ring-shaped membrane-associated fluorescence. Ethanol fixation or PBS incubation only for 30 min resulted in a total abolishment of the fluorescence seen with paraformaldehyde fixation. Acetone on the other hand gave a fluorescence intensity equal to that seen with paraformaldehyde fixation, but the pattern was granular instead of ring shaped. The addition of an extra SwAR-FITC step after RAM-FITC clearly enhanced the fluorescence intensity further without any increase in background staining. Sucrose incubation overnight after fixation had only a marginal effect on the staining intensity but enhanced the quality of the staining by making the fluorescing structures more distinct. Whether or not the tissue had been fixed in formaldehyde before freezing and cutting did not influence the intensity or the quality of the staining to an appreciable extent. The final procedure adopted for the immunofluorescence detection of fucosyl-GMI in tissues was as follows: (a) paraformaldehyde fixation (5% in PBS for 30 min at room temperature); (b) sucrose treatment (7.5% in PBS at 4°C overnight); (c) MAb; (d) RAM-FITC; and finally (e) SwAR-FITC incubation (30 min each at room temperature). The dilutions of RAM-FITC and SwAR-FITC used, arrived at after checkerboard titrations, were 1/10 and 1/20, respectively, and the dilution of concentrated F12 MAb was 1/10.

**Reactivity of F-MAbs with H4-II-E Cells.** The rat hepatoma cell line H4-II-E contains large amounts of membrane-associated fucosyl-GMI as determined by HPTLC immunostaining of ganglioside extracts from cell pellets. The ability of the different F-MAbs to detect membrane-associated antigen in these cells was evaluated by immunofluorescence after staining with the optimal method. Two of the 7 tested F-MAbs, F1 and F6, did not react with H4-II-E cells. All the other MAb's stained this cell line, F12 giving the strongest reactions (Fig. 1). No F-MAb gave detectable staining of a fucosyl-GMI-negative colorectal carcinoma cell line, Colo 205 (Fig. 2a), which was included as a negative control. The Colo 205 cell line was strongly stained with MAb C-50 (Fig. 2b) directed against the CA-50 ganglioside antigen (7).

**Reactivity of Different F-MAbs with Human Tissues.** The reactivities of the different F-MAbs with SCCL tissues, small intestine, adrenal gland, and pancreas were then compared in order to select the most sensitive and specific MAb. All the tissues examined except adrenal gland contain fucosyl-GMI as detected by immunoochemical analyses of glycolipid extracts. The results are presented in Table 1. All F-MAbs tested react with fucosyl-GMI in the enzyme-linked immunosorbent assay. MAb F12, with the greatest specificity for fucosyl-GMI as assessed by previous studies (15), gave the most consistent staining reactions with the fucosyl-GMI-containing tissues and was therefore chosen for the following studies.

**Staining of Lung Tumor Tissues**

Both pulmonary lesions and metastases of lung cancer in other organs were tested (Table 2). Of specimens from 21 different patients with SCCL, 19 (90%) were stained by F12. In 10 of these cases all tumor cells were stained (Fig. 3). In 5 cases more than 50% of the tumor was stained whereas in 4.

![Fig. 1. Immunofluorescence staining with MAb F12 of a rat hepatoma cell line, H4-II-E, that has a high expression of the ganglioside antigen fucosyl-GMI.](image-url)
IMMUNOHISTOCHEMICAL DETECTION OF FUCOSYL-GMI

Table 2 Immunofluorescence staining of SCCL tissues using the F12 MAb
For scoring refer to "Materials and Methods."

<table>
<thead>
<tr>
<th>SCCL specimen</th>
<th>Score</th>
<th>Fraction of tumor cells stained (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3003</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>0710</td>
<td>2</td>
<td>&gt;50</td>
</tr>
<tr>
<td>1210</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>0373-84</td>
<td>3</td>
<td>&gt;50</td>
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<tr>
<td>S862-84</td>
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<td></td>
</tr>
<tr>
<td>S950-84</td>
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<tr>
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<td>S375-84</td>
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<td>100</td>
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<td>&gt;50</td>
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<td>4</td>
<td>100</td>
</tr>
<tr>
<td>S185-85</td>
<td>2</td>
<td>&lt;50</td>
</tr>
</tbody>
</table>

Reactivity with Normal Human Tissues

Immunofluorescence studies with F12 indicate that fucosyl-GMI is sparsely distributed in normal tissues outside the central nervous system. Tissues that did not stain with F12 included lung and bronchi, liver, kidney, lymph node, thyroid, parathyroid, adrenal, gastric mucosa and submucosa, large artery, and striated, smooth, and cardiac muscle. Bone marrow and peripheral blood cells, including preparations of B- and T-cells and monocytes, were also negative. However, in spleen, thymus, and small intestine clusters of small round cells were stained by F12. In spleen and thymus these cell populations constituted approximately 1 and 10% of the total cell content, respectively. In the small intestine the cells were located in the lamina propria. In this tissue the submucosal and myenteric ganglionic cells were also stained by F12. In pancreas more than 50% of the cells in each islet were positive.

Fucosyl-GMI in SCCL Cell Lines

Five established SCCL cell lines propagated in vitro were tested for their content of fucosyl-GMI using immunofluorescence with F12. In one cell line, U-2050, all cells were intensely stained with F12 (Fig. 5). In another cell line, (U-1690), 20–30% of the cells were positive. The remaining three cell lines all were completely negative (U-1285, U-1906, and U-2020). We also tested early passages of in vitro cultures from these
Fig. 4. a. In this specimen of a squamous epithelial cell lung cancer no cells are stained with MAb Fl 2 by immunofluorescence. × 100. b. Immunofluorescence staining with MAb C-50 (7) of the same squamous epithelial cell lung cancer specimen as in a. Malignant cells are positive. × 100.

Table 3 Summary of F12 immunofluorescence staining of various tumor tissues

<table>
<thead>
<tr>
<th>Tumor histiotype</th>
<th>No. positive/ no. tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small cell lung cancer</td>
<td>19/21</td>
</tr>
<tr>
<td>Squamous cell lung cancer</td>
<td>2/10</td>
</tr>
<tr>
<td>Lung adenocarcinoma</td>
<td>0/8</td>
</tr>
<tr>
<td>Large cell lung cancer</td>
<td>1/5</td>
</tr>
<tr>
<td>Adenosquamous cell lung cancer</td>
<td>0/1</td>
</tr>
<tr>
<td>Bronchial carcinoid</td>
<td>0/3</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>0/2</td>
</tr>
<tr>
<td>Malignant schwannoma</td>
<td>0/1</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>0/1</td>
</tr>
</tbody>
</table>

DISCUSSION

In the present work we have developed an optimized immunofluorescence method, using the fucosyl-GM₁ specific monoclonal antibody F12, for the specific detection of fucosyl-GM₁ in sections of human lung tumor and normal tissues. The carbohydrate sequence of fucosyl-GM₁ has to date been found only in glycolipids. Like other glycolipids this antigen is readily eluted from paraffin-embedded tissues by organic solvents making it difficult to use this kind of material for the immunohistochemical detection of fucosyl-GM₁. We therefore tested cryostat sections of tissues in combination with various fixatives. In our hands, the best fixative tested was paraformaldehyde. It was also necessary to include an additional secondary step of fluorescein-conjugated antiserum in order to enhance the sensitivity. Introduction of sucrose incubation (24) of tissue sections after paraformaldehyde fixation also improved the quality of the staining.

Using the optimized staining procedure we found that 90% of the SCCL cases tested contained fucosyl-GM₁. In 4 of 19 positive SCCL cases only a minor fraction of the tumor cells was stained indicating a heterogeneity with respect to fucosyl-GM₁ expression between different areas within these tumors. Heterogeneity of tumor-associated antigen expression in tumors has also been reported by others (25). The majority of the SCCL cases studied by us were metastatic lesions presented at advanced stages of disease, and since there may be considerable morphological changes in these tumors (26–28) this may explain the heterogeneity of antigen expression observed. Discrepancies in antigen expression between different tumor locations within one patient have also been observed by us in cases where both pulmonary and hepatic lesions were tested. In one of the cases the lung tumor was positive and the liver metastasis was negative in F12 immunofluorescence, whereas in another case the reverse was found. Heterogeneity of fucosyl-GM₁ expression was also seen in one cell line derived from a clinical case of SCCL, where only 20–30% of the cells were positive. A difference was also seen between the clinical specimens and the cell lines in the percentage of the SCCL cases containing cells expressing fucosyl-GM₁. More than 90% of the clinical tumor
cases but only 2 of 5 cell lines were positive. On the other hand, when early passages of the negative cell lines were tested staining could clearly be seen in 5–10% of the cells in one case. Our results demonstrate that fucosyl-GM₁ is expressed at a high frequency in SCCL, but they also suggest potential instability with loss of this antigen during progressive tumor cell proliferation.

Current views suggest that SCCL and non-SCCL lung cancers represent a differentiation continuum with a common entodermal origin (27, 29–31). Our observation that some SQCL specimens contain foci of F12-positive cells is in accord with a lineage relationship between SCCL and SQCL. Expression of fucosyl-GM₁ in SQCL may thus indicate transition of SQCL to a more malignant state with a worse prognosis than for the fucosyl-GM₁-negative tumor. If this prediction holds true it may have implications for the choice of therapy in these “borderline” cases.

The distribution of fucosyl-GM₁ in normal tissues outside the central nervous system seems to be quite restricted. Stained small round cells were seen in lymphoid tissues such as spleen, thymus and lamina propria of the small intestine. These cells may constitute a distinct subpopulation of lymphocytes with a specific role at their sites of localization, since no positive cells could be found in preparations of peripheral blood or bone marrow samples. Work is in progress to characterize these fucosyl-GM₁-expressing cells and their functions. The other normal tissues showing staining with F12 are represented by the pancreas, where islet cells were stained, and the small intestine, where ganglionic cells also were positive.

In our earlier works we have described that all monoclonal antibodies raised against fucosyl-GM₁ react with purified fucosyl-GM₁ immobilized on plastic surfaces or silica gel plates (14, 15). In our present work we show that several of these F-MAbs (i.e., F1, F3, F6, F9, and F10) did not react with fucosyl-GM₁ in all sections that contained enough of this antigen to be detected with F12. Moreover, F4 does not seem to react with tissue-bound GM₁ ganglioside, which is ubiquitous in most human tissues, the staining with F4 being as restricted as that with F12. These results indicate that the ability of a MAb to detect specific antigens should be defined within each assay system.

The F12 MAb demonstrates a high capability to differentiate SCCL from other lung tumors. Other MAbs that show a preferential reactivity with SCCL have also been described, e.g., SCLC 5023 (8), SM1 (9), B10/12 (10), TSF-4 (11), and NE-25 (12). A principal difference between these MAbs and the F12 MAb in all sections that contained enough of this antigen to be detected with F12. Moreover, F4 does not seem to react with tissue-bound GM₁ ganglioside, which is ubiquitous in most human tissues, the staining with F4 being as restricted as that with F12. These results indicate that the ability of a MAb to detect specific antigens should be defined within each assay system.

In conclusion, fucosyl-GM₁ seems to be an antigen highly associated with SCCL. F12 represents a monoclomal antibody with a high specificity for fucosyl-GM₁ and is able to detect this antigen in tissue sections of SCCL using an immunofluorescence method. Immunohistochemical detection of fucosyl-GM₁ with the aid of the MAb F12 therefore may become a valuable tool in the diagnosis and management of small cell cancer of the lung.

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IMMUNOHISTOCHEMICAL DETECTION OF FUCOSYL-GM₁

Immunohistological Detection of Fucosyl-\(G_m^1\) Ganglioside in Human Lung Cancer and Normal Tissues with Monoclonal Antibodies

Fred-Thomas Brezicka, Sante Olling, Olle Nilsson, et al.


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