Inhibition of subcutaneous growth of Ehrlich ascites carcinoma (EAC) tumor by post–immunization with EAC-cell gangliosides and its anti-idiotype antibody in relation to tumor angiogenesis, apoptosis, cell cycle and infiltration of CD4+, CD8+ lymphocytes, NK cells, suppressor cells and APC-cells in tumor

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Both EAC-tumor associated gangliosides and its anti-idiotype antibody inhibited growth of this tumor significantly. Immuno-histological studies with von Willebrand Factor (vWF) antibody indicated that tumor angiogenesis as determined by expression of vWF decreased in tumors of mice, post-immunized with EAC-cell gangliosides as well as its anti-idiotype antibody. Infiltration of various immune cells of the host in the tumor correlated to some extent with tumor-growth inhibition. Apoptosis study using AnnexinV-FITC and propidium iodide indicated that tumor growth inhibition in mice post-immunized with EAC-gangliosides and its anti-idiotype antibody were due to enhanced apoptosis and cell death. Cell cycle analysis by FACS indicated that EAC-cell associated gangliosides and its anti-idiotype antibody were acting both at the M2 i.e. S and M3 i.e. G2/M phases of the cell cycle to arrest tumor growth.

Keywords: Angiogenesis, Apoptosis, Anti-idiotype, Tumor, Gangliosides

Immunotherapy of human cancer is possible if the right antibodies directed to the right markers are chosen. Immuno-therapy has become a very intensive area of investigation involving various approaches, including vaccines of tumor-cell lysates, proteins, gangliosides, viruses containing tumor associated antigen (TAA) genes, antigen presenting cells (APC’s) sensitized with tumor-derived TAAs in different forms to potentiate its antigenicity. Proper manipulation of active and passive immunization with TAAs and their antibodies may have potential therapeutic effect on cancer patients. Immunization with anti-idiotype antibody may play important role in those cases where antigen is available in very low quantity or is antigenically very weak, specifically complex carbohydrate antigens like gangliosides. Since an anti-idiotype antibody carrying the internal image of a cancer antigen may produce immune response similar to that of cancer antigen and possibly a better response than the antigen itself, the anti-idiotype antibody may serve as a substitute for cancer antigen. An anti-idiotype antibody carrying the internal image of ganglioside GM3, a human melanoma specific antigen was reported by Yamamoto et al. Anti-idiotype antibody mimicking GM3, GD2 and GD3 gangliosides of tumor were used by investigators for immunotherapy of melanoma patients.

Pre-immunization with anti-idiotype antibody to EAC-cell gangliosides could inhibit both ascites and subcutaneous solid tumor growth of the same tumor in animal tumor model. In the present investigation the effect of post-immunization with EAC-cell gangliosides and its anti-idiotype antibody on the growth of same tumor (sc) in vivo in relation to tumor angiogenesis, apoptosis, cell cycle and infiltration of CD4+, CD8+ lymphocytes, NK cells, suppressor cells and APC-cells in the tumor has been studied.

Materials and Methods

Preparation of anti-idiotype antibody to EAC-cell gangliosides—Gangliosides from EAC tumor cells were prepared by solvent extraction and solvent partition following the method of Saha et al. The anti-idiotype antibody against EAC-cell gangliosides was prepared as follows: four Sprague Dawley female

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rats were immunized with EAC-cell gangliosides (100 µg/rat, sc) emulsified with Freund's complete adjuvant followed by two more injections of same dose in incomplete adjuvant at an interval of 14 days. Ten days after the third injection the rats were sacrificed, serum prepared from blood and the total immunoglobulin fraction was separated from the serum by 45% ammonium sulfate precipitation, dialysed against PBS, pH 7.2, protein content was determined and the presence of anti-ganglioside antibody in the fraction was determined by immunoprecipitation against EAC-cell gangliosides. A rabbit was immunized with 100 µg of this first antibody following the same protocol. Ten days after the third injection the rabbit was bled, serum prepared and the IgG fraction of the serum was prepared by 33% ammonium sulfate precipitation. This IgG fraction contained the anti-idiotype antibody against EAC-cell gangliosides and the presence of anti-idiotype antibody was determined by immuno-precipitation against the first antibody.

Preparation of liposome with EAC-cell gangliosides and their anti-idiotype antibody for in vivo experiments—EAC-cell gangliosides (500 µg) in chloroform-methanol (1:1, v/v) mixture was added to a glass vial containing dried 300 µg phosphatidyl choline + 150 µg phosphatidyl serine mixture (PC:PS at a molar ratio of 70:30), and evaporated to dryness. Then 1ml PBS was added to it, mixed on a vortex mixer, added 1ml Freund's adjuvant to it, and mixed again to make emulsion. This liposome was used for post-immunization of mice (0.2 ml/mouse) for in vivo experiments so that each mouse received 50 µg of gangliosides in liposome. Liposome with anti-idiotype antibody to EAC-cell gangliosides was prepared in a similar manner for post immunization of mice.

Preparation of tumor cells from solid tumor—Tumor from sc EAC solid tumor-bearing mouse was harvested, the tumor was finely chopped and then treated with 5 ml enzyme preparation (100 units DNase/ml + 200 µg collagenase/ml + 250 µg hyaluronidase/ml) for 10 min at room temperature. The single cells released by enzyme treatment was removed, washed twice with complete medium and finally suspended in PBS, pH 7.2, counted and then kept in ice until used in different experiments.

DNaseI-from bovine pancreas (Sigma, Cat.No.D5025-15KU), collagenase-from Clostridium hystolyticum, type I (Sigma-Aldrich, Cat.No.C0130), hyaluronidase-typeIV-S, bovine testis (Sigma-Aldrich Cat.No.H3884) were used.

Flow cytometric analysis (FACS) of tumor cells using mouse anti-CD4-FITC (L3T4, cloneGK1.5), anti-CD8-PE (Ly-2, clone53-6.7) anti-CD25-PE (L2Ra.p55, clonePC61.5), anti-CD11c-PE(p150/90, cloneN418) and anti-NK-PE (CD49b, cloneDX5) MAb markers—Tumor cells prepared from solid EAC tumor were suspended in PBS to a concentration of 1×10^6 cells/ml and 1ml cell suspension was dispensed in each tube. The cell suspension was centrifuged, the supernatant discarded and 25 µl of the desired marker (0.25 µg) was added to the cells of a tube in the dark, mixed and then incubated for 30 min in the dark at 4°C. After incubation centrifuged, the supernatant was discarded and the cells were washed twice with 1ml 1% FBS containing PBS, pH 7.2. Then 200 µl paraformaldehyde solution was added to each tube, mixed well and kept in dark at 4°C until FACS. Prior to FACS 250 µl of sheath solution was added to each tube and then analyzed by FACS.

Apoptosis assay using AnnexinV-FITC conjugate (Sigma) and Propidium iodide (PI) (Sigma, Cat.No.P4170) by flow cytometric analysis (FACS)—Assay was performed using procedure described in the technical data sheet of the assay kit purchased from BD Biosciences as follows: the tumor cells prepared from the tumor of EAC tumor-bearing mice were washed twice with cold PBS and then re-suspended in 1x binding buffer supplied with the kit at a concentration of 1×10^6 cells/ml. Then 100 µl of the cell suspension (1x10^5 cells) was transferred to a 5 ml culture tube. Added 25 µl of AnnexinV-FITC (0.25 µg) and/or 25 µl of PI solution to the cell suspension. The cells were gently mixed by vortexing and then incubated for 15 min at room temperature in dark. Then 400 µl of 1x binding buffer was added to each tube and analyzed by FACS within one hour.

Cell cycle analysis of tumor by propidium iodide (PI)—Tumor cells were prepared from solid tumor at a concentration of 1×10^6 cells/ml in PBS. One ml cell suspension was taken in a tube, centrifuged, supernatant discarded, 1ml ammonium chloride red blood cell lysis buffer was added to the pellet, mixed gently, incubated for 2-5 min at 37°C for red blood cell lysis. After incubation centrifuged, supernatant was discarded, the cells were washed twice with 5 ml PBS and then re-suspended in 1 ml ice cold methanol and incubated for 30 min in ice. After incubation centrifuged, the supernatant was discarded, added
25 µl RNase solution (1 mg/ml in PBS), mixed and was incubated for 30 min at room temperature. Then 25 µl PI solution (25 µg) was added to the cells and incubated for 30 min at room temperature in the dark. After incubation 1 ml sheath fluid was added to the tube, mixed well and analyzed by FACS.

**Immunohistochemistry for analysis of tumor vascularization (angiogenesis) by anti-von Willebrand Factor (vWF) antibody**—Tumors were harvested, snap frozen at -80°C, sectioned (4-5 µm) on a freezing microtome and layered on glass slides already layered with egg albumin/glycerol mixture. After layering 2-3 min were allowed for adherence of the sections on the slides and incubated further for 30 min in chilled methanol, air dried and stored at -80°C until staining. Slides containing tissue sections were thawed from -80°C to room temperature before staining. Tissue sections were incubated at room temperature for 20 min in 1-3 drops of 1.5% normal blocking serum (goat serum) in PBS. After incubation serum was aspirated from the slides. Immediately 1-3 drops of pre-diluted (2 µg/ml in 1.5% blocking serum containing PBS) vWF antibody (primary antibody) were added on the tissue sections and incubated for 2 h at room temperature. The slides were rinsed with PBS, washed with PBS twice for two min each on a stir plate. Excess liquid was aspirated from the slides and 1-3 drops of biotinylated secondary antibody (goat anti-rabbit IgG) was added over each section, incubated for 30 min at room temperature and washed as described above. Then 1-3 drops of HRP-streptavidin complex was added over each section and incubated for 30 min at room temperature. Finally, 1-3 drops of HRP-substrate (DAB solution) was added, kept for 30sec, rinsed with deionized water and washed with deionized water on a stir plate for 2 min. The sections were counter stained with hematoxylin for 5-10 sec and immediately washed with deionized water. The sections were dried, dehydrated through alcohols and xylene and 1-2 drops of mounting medium were added, coved with cover slip and brown spots were counted under light microscope at six different fields. The total gave the average number of blood vessels formed. Bound antibody in presence of secondary antibody against the vWF antibody conjugated to an enzyme Horse Radish Peroxidase (HRP) and substrate of the enzyme diaminobenzidine (DAB) gives brown spots which are directly proportional to the number of new blood vessels formed.

**Results**

**Effect of post-immunization with EAC cell gangliosides and its anti-idiotype antibody on the subcutaneous solid tumor growth of the same tumor in vivo**—Three groups of Swiss male mice were inoculated with 5×10^5 EAC-cells/mouse, sc, in 0.5 ml PBS, pH 7.2, on day 0. Group I and Group II mice were post-immunized with 50 µg EAC-cell gangliosides and 50 µg anti-idiotype antibody to EAC-cell gangliosides respectively/mouse, sc, on day+1, day+7 and day+14. Tumor growths of both Gr. I and Gr. II mice were inhibited significantly compared to that of control Gr. III (Table 1). In 2 mice of Gr. I (nos 1 and 5) tumors appeared by day 10, increased in size up to day 15, and then gradually decreased. In one mouse of Gr. I (no. 4) tumor appeared by day 10, increased up to day 15 and then decreased in size and by day 25 there was no palpable tumor. In two other mice of Gr. I (nos 2 and 3) tumor appeared by day 10, gradually increased till day 40 and after then they were still alive with tumor burden. Tumor growth inhibitory effect was much better in Gr. II mice, post-immunized with anti-idiotype antibody to EAC-cell gangliosides compared to that of Gr. I mice, post-immunized with EAC gangliosides. Tumor appeared in 3 (nos 1, 3 and 5) out of 5 mice of Gr. II, decreased in size and disappeared and practically no palpable tumors were found. In 2 other mice (nos 2 and 4) tumor appeared and then gradually decreased in size. In all the 4 mice of control Gr. III tumor appeared by day 10, gradually increased in size and 2 died by day 30 and day 35 respectively with large tumor burden.

**Effect of post-immunization with EAC- gangliosides and its anti-idiotype antibody on tumor angiogenesis as assessed by determining expression of vWF on tumor tissue sections**—On day 30 one tumor-bearing mouse of Gr. I (tumor diameter : 5.5 mm), Gr. II (tumor diameter : 5.0 mm) and Gr. III (tumor diameter : 14.5 mm) respectively of the above experiment (Table 1) was sacrificed, the tumor was harvested and vonWillebrand factor (vWF) was determined in the tumor tissue section using vWF antibody as described under Materials and Methods. The results (Table 2 and Fig. 1) indicated that expression of vWF decreased in both Gr. I and Gr. II mice, post-immunized with EAC-cell gangliosides and anti-idiotype antibody to EAC-cell gangliosides respectively compared to untreated control Gr. III mouse.
Infiltration of CD4+, CD8+ lymphocytes, NK-cells, suppressor cells and APC cells in tumor determined by FACS—On day 25 one tumor-bearing mouse of Gr. I (mouse no. 5: tumor diameter: 6.5 mm), Gr. II (mouse no. 2: tumor diameter: 5.75 mm) and Gr. III (mouse no. 2: tumor diameter: 13.25 mm) respectively of the above experiment (Table 1) was sacrificed, the tumor was harvested, tumor cell suspension was prepared in PBS at a concentration of $1 \times 10^6$/ml and used for immune cell infiltration study.

For immune cell infiltration study $1 \times 10^6$ tumor cells were dispensed in 5 tubes for each tumor. The cells of tube no.1 of each group were labeled with double markers CD4+ and CD8+, cells of tube no. 2 of each group were labeled with NK-cell marker, cells of tube no. 3 were labeled with CD25 marker (suppressor cell marker), cells of tube no. 4 were labeled with CD11c marker (APC marker) and the cells of tube no. 5 was without any label and served as control during FACS. The results of FACS analysis have been shown in Table 3 and Fig. 2. Percent of NK cells infiltration was slightly lower in tumor of Gr. I mouse, post-immunized with EAC-cell gangliosides compared to untreated control tumor of Gr. III. Suppressor cell (CD25) infiltration was much higher in tumor of control Gr. III compared to tumor of Gr. I mice immunized with EAC-cell gangliosides. Infiltration of APC cells (CD11C) was also much higher in control Gr.III tumor compared to tumor of Gr. I. Infiltration of cytotoxic T-lymphocytes (CD8+) was less in tumor of Gr. I compared to control Gr. III tumor. NK cells infiltration was slightly higher in Gr. II tumor of mice post-immunized with anti-idiotype antibody to EAC-cell gangliosides compared to control untreated Gr. III tumor. Suppressor cell (CD25) infiltration was much higher in tumor of control Gr. III compared to tumor of Gr. II mice immunized with anti-idiotype antibody to EAC-cell gangliosides. Infiltration of APC cells (CD11c) was also much higher in control Gr. III tumor compared to
Gr. II tumor. Infiltration of cytotoxic T-lymphocytes (CD8+) was higher in Gr. II tumor compared to control Gr. III tumor.

**Apoptosis assay using AnnexinV-FITC conjugate and propidium iodide (PI) by flow cytometric analysis (FACS)**—The same cell preparation as used above for immune cell infiltration study was also used for apoptosis study. Results have been presented in Table 4 and Fig. 3. Results indicated that early apoptotic cells (AnnexinV-FITC positive cells) increased in tumor of Gr. I mice post-immunized with EAC-cell gangliosides compared to untreated control Gr. III tumor. Though the difference was not much but this was an indication of role of apoptosis in tumor growth inhibition in Gr. I mice. However, percent of PI-positive cells was much higher in Gr. I tumor compared to control untreated tumor, which indicated enhancement in tumor cell death in tumor of mice immunized with EAC-cell gangliosides. Double positive-cells (AnnexinV and PI) were also much higher in tumor of mice immunized with EAC-cell gangliosides compared to control untreated tumor.

Early apoptotic cells also increased in tumor of Gr. II mice post-immunized with anti-idiotype antibody to EAC-cell gangliosides compared to untreated control Gr. III tumor. But percent of PI-positive cells in Gr. II tumor was comparable to that of control Gr. III tumor. However, double-positive cells (AnnexinV and PI) in tumor of Gr. II increased compared to control Gr. III tumor, thus indicating that enhancement in early apoptotic cells in tumor of mice post-immunized with anti-idiotype antibody to EAC-cell gangliosides is contributing to the increase in cell death and consequent tumor growth inhibition.

**Cell cycle analysis of tumor by propidium iodide (PI)**—The cell cycle analysis was also done using the
Results of cell cycle analysis have been presented in Table 5 and Fig. 4. The cells in both M2 and M3 phases of Gr. I and Gr. II tumors were reduced due to inhibitory effect of EAC-cell gangliosides and anti-idiotype antibody to EAC-cell gangliosides compared to tumor of control Gr. III and consequently the cells in the M1 phase increased both in Gr. I and Gr. II tumors compared to control tumor of Gr. III.

Discussion

In studies with Ehrlich’s ascites carcinoma (EAC) tumor model in Swiss mice it has been shown that pre-immunization with tumor associated gangliosides and its anti-idiotype antibody could inhibit both ascites and subcutaneous tumor growth. In the present investigation with the same tumor model it has been shown that post-immunization with EAC-tumor associated gangliosides as well as its anti-idiotype antibody can inhibit subcutaneous growth of this tumor. Moreover, some mechanistic studies have been done to explore the possible target of action of these therapeutic bio-molecules.

vonWillbrand Factor (vWF) is present on endothelial cells. Endothelial cells are involved in new blood vessel formation and their number increases during neo-vascularization i.e. angiogenesis and consequently vWF number increases with new blood vessel formation which is needed by a tumor for its growth and survival. vWF antibody specifically binds to vascular endothelial cells which are involved in the formation of new blood vessels during tumor angiogenesis. Immunohistological studies with vWF indicated that expression of vWF decreased in tumors of mice, post-immunized with EAC-cell gangliosides as well as its anti-idiotype antibody compared to tumors of untreated control mice indicating that tumor angiogenesis decreased in tumors of treated mice and thus correlated tumor angiogenesis with tumor-growth inhibitory effect of gangliosides and its anti-idiotype antibody.

From FACS analysis using monoclonal antibody to CD4+, CD8+, NK , CD25 and CD11c markers it was observed that infiltration of suppressor cells (CD25) and APC-cells (CD11c) were much less in tumor of EAC-cell gangliosides-immunized mice compared to tumor of untreated control mice. Suppressor cells give protection to tumor cells and therefore, decrease in their number in EAC-ganglioside-treated tumor correlated with tumor growth. Infiltration of helper T-lymphocytes (CD4+) was less in tumors of mice post-immunized with EAC-gangliosides compared to control tumor, thus showing correlation with tumor growth inhibition. But infiltration of NK cells and cytotoxic CD8+ T-lymphocytes were slightly lower in tumors of mice post-immunized with EAC-cell gangliosides compared to control tumor, and did not show any correlation with tumor growth inhibition due to EAC-ganglioside treatment.

Infiltration of suppressor cells and APC cells was much less in tumors of mice post-immunized with anti-idiotype antibody to EAC-cell gangliosides compared to tumors of control mice. Infiltration of helper T-lymphocytes (CD4+) was also less in tumors of mice post-immunized with EAC-gangliosides compared to control tumor, thus showing correlation with tumor growth inhibition. But infiltration of NK cells and cytotoxic CD8+ T-lymphocytes were slightly lower in tumors of mice post-immunized with EAC-cell gangliosides compared to control tumor, and did not show any correlation with tumor growth inhibition due to EAC-ganglioside treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Marker positive cells infiltrated in the tumor (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4+</td>
</tr>
<tr>
<td>Gr.I : post-immunized with EAC-cell gangliosides</td>
<td>0.21</td>
</tr>
<tr>
<td>Gr.II : post-Immunized with anti-idiotype antibody to EAC-cell gangliosides</td>
<td>0.40</td>
</tr>
<tr>
<td>Gr.III : no treatment: only inoculated with EAC-tumor cells</td>
<td>0.78</td>
</tr>
</tbody>
</table>
The apoptosis is characterized by certain morphologic features, including loss of plasma membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and intranucleosomal cleavage of DNA. Loss of plasma membrane integrity is one of the earliest event in apoptosis. In apoptotic cells, the membrane phospholipids phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V has high affinity for PS, and binds to the cells with exposed PS. FITC-conjugated Annexin V can thus bind to apoptotic cells and can be identified by FACS. Since externalization of PS occurs in the earlier stages of apoptosis, Annexin V–FITC staining can identify apoptosis at an early stage than assays by PI which is based on nuclear changes such as DNA fragmentation. The cells that are viable are

Table 4—Apoptosis assay of tumors of mice post-immunized with EAC-cell gangliosides and its anti-idiotype antibody by FACS using Annexin V-FITC conjugate and Propidium iodide (PI)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Annexin V-FITC (%)</th>
<th>PI (%)</th>
<th>Annexin V-FITC + PI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr. I : post-immunized with EAC-cell gangliosides</td>
<td>86.40</td>
<td>81.93</td>
<td>86.00</td>
</tr>
<tr>
<td>Gr. II : post-Immunized with anti-idiotype antibody to EAC-cell gangliosides</td>
<td>91.63</td>
<td>60.24</td>
<td>77.61</td>
</tr>
<tr>
<td>Gr. III : no treatment: only inoculated with EAC-tumor cells</td>
<td>83.25</td>
<td>60.35</td>
<td>64.89</td>
</tr>
</tbody>
</table>
Annexin V-FITC and PI negative, cells that are in early apoptosis are Annexin V-FITC-positive and PI negative, and cells that are in late apoptosis or already dead are both Annexin V-FITC and PI positive.

FACS analysis indicated that early apoptotic cells (Annexin V-FITC positive cells), PI-positive cells and double positive cells (Annexin V + PI) increased in tumor of mice post-immunized with EAC-cell gangliosides compared to untreated control tumor, thus indicating acceleration of apoptosis and/or direct cell death by immunization with EAC-cell gangliosides and consequent tumor growth inhibition. Early apoptotic cells and double-positive cells also increased in tumor of mice post-immunized with anti-idiotype antibody to EAC-cell gangliosides compared to untreated control tumor. But percent of PI-positive
cells in tumor of anti-idiotypic antibody-treated mice was comparable to that of control tumor. The results indicated that anti-idiotypic antibody is possibly promoting only early apoptosis and thus contributing to the increase in cell death and consequent tumor growth inhibition.

Results of cell cycle analysis by FACS indicated that cells in M2 and M3 phases of both EAC-cell gangliosides and anti-idiotypic antibody to EAC-cell gangliosides treated tumors were reduced compared to untreated tumor of control group and consequently the cells in the M1, i.e. G0/G1 phase increased in tumor of both the treated groups compared to untreated control tumor, thus indicating that EAC-cell associated gangliosides and its anti-idiotypic antibody are acting both at the M2 i.e. S and M3 i.e. G2/M phases of the cell cycle to arrest tumor growth.

An anti-idiotypic antibody carrying the internal image of ganglioside GM3, a human melanoma specific antigen was reported by Yamamoto et al. An anti-idiotypic antibody mimicking GD2 ganglioside was reported which can act against GD2-positive human neuroectodermal tumors. Anti-idiotypic antibody carrying the internal image of GM3, GD2 and GD3 gangliosides of tumor were used by investigators for immunotherapy of melanoma patients. A phase I clinical trial was conducted in patients with stage III/IV breast cancer with monoclonal anti-idiotypic antibody mimicking...
N-glycolyl-containing gangliosides expressed on human melanoma and breast carcinoma cells. It has been reported that treatment with anti-GD2 monoclonal antibody 3F8 (Ab1) at the time of remission may prolong survival for children with stage 4 neuroblastoma. Despite the high-risk nature of stage 4 neuroblastoma, long-term remission without myeloablative therapy was achieved with 3F8 treatment. Because this response was primarily anti-idiotypic (Ab2), it was postulated that the induction of an idiotypic network that included an elevation of anti-anti-idiotypic (Ab3) and anti-GD2 (Ab3′) antibody titers may be responsible for tumor control. Anti-id vaccines that mimic carbohydrate or glycolipid antigens have shown immunological advantages over the natural antigen. Unlike natural carbohydrate antigens, anti-id vaccines can stimulate cellular in addition to humoral immune response and anti-id vaccines may be more immunogenic than the non-protein antigens they mimic. Several anti-ids have been raised against MoAbs recognizing cell surface tumor targets. Immunization of animals with these anti-ids can generate antibodies (i.e., Ab3′) that recognize the original tumor antigen. In subsequent studies, Ab2 and Ab3 were demonstrated in responding patients. In some reports, specific T-cell-mediated immunity was also found. The potential role of an anti-idiotypic network in colon cancer was first described in patients treated with monoclonal antibody CO-17-1A.

All the earlier studies as well as present investigation indicate that both pre- and post-immunization of tumor-host with tumor-associated gangliosides and its anti-idiotypic antibody have potential therapeutic benefit and should be tried in treatment of human cancers with more emphasis. The mechanistic studies in the present investigation have established the correlation of therapeutic effect observed with tumor angiogenesis and immunological parameters of the tumor host and has also shown that tumor-growth inhibitory effect is due to enhanced apoptosis and/or cell death. Moreover, the study has established the target of action of tumor-associated gangliosides and its anti-idiotypic antibody at the cell cycle level. All these information will be new addition to the existing knowledge in this area of investigation and will definitely help in future planning of immunotherapeutic treatment modalities with gangliosides and similar type of tumor associated antigens and their anti-idiotypic antibody.

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