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Anti-NeuGcGM3 Antibodies, Actively Elicited by Idiotype Vaccination in Nonsmall Cell Lung Cancer Patients, Induce Tumor Cell Death by an Oncosis-Like Mechanism

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1E10 is a murine anti-idiotype mAb specific for an idiotype mAb that reacts with NeuGc-containing gangliosides, sulfatides, and Ags expressed in some human tumors. In melanoma, breast, and lung cancer patients, this anti-idiotype Ab was able to induce a specific Ab response against N-glycolipidated gangliosides, attractive targets for cancer immunotherapy as these glycolipids are not naturally expressed in humans. A clinical study with nonsmall cell lung cancer patients showed encouraging clinical benefits. Immunological studies performed in 20 of these patients suggested a correlation between the induction of Abs against NeuGcGM3 and longer survival times. The induced anti-NeuGcGM3 Abs recognized and directly killed tumor cells expressing the Ag, by a mechanism independent of complement activation. In the present work, we show that this cytotoxicity differs from apoptosis because it is temperature-independent, no chromatin condensation or caspase 3 induction are detected, and the DNA fragmentation induced has a different pattern than the one characteristic for apoptosis. It is a very quick process and involves cytoskeleton reorganization. The Abs induce cellular swelling and the formation of big membrane lesions that allow the leakage of cytoplasm and the loss of the cell membrane integrity. All of these characteristics resemble a process of oncosis necrosis. To our knowledge, this is the first report of the active induction in cancer patients of NeuGcGM3-specific Abs able to induce complement independent oncosis necrosis to tumor cells. These results contribute to reinforcing the therapeutic potential of anti-idiotype vaccines and the importance of NeuGcGM3 ganglioside as antitumor target. The Journal of Immunology, 2011, 186: 3735–3744.

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epite the many efforts dedicated to develop antitumor therapies, neoplasias are still one of the leading death causes all around the world. NeuGc-containing gangliosides are attractive targets for cancer immunotherapy. These glycolipids are not naturally expressed in humans due to a genetic deletion in the gene that codes the CMP-N-acetyl hydroxylase enzyme that catalyzes the conversion of Nacetyl to N-glycolyl sialic acid (1–3). However, both direct and indirect studies have indicated that Neu5Gc is overexpressed in several human tumors (4–6), where they are known to be immunogenic (7). The most accepted theory for this phenomenon is the incorporation of Neu5Gc from dietary sources. Free sialic acids from the medium can be taken up into cells via pinocytosis. The content of the resulting pinocytic vesicles and endosomes would eventually be delivered to the lysosome, where a sialic acid transporter then delivers the molecules into the cytosol (8). The explanation for the differential expression of these Ags in human normal and tumor tissues is that the rapidly growing tumor tissues might be more efficient at scavenging Neu5Gc from dietary sources. Furthermore, it has been proposed that the preferential expression of NeuGc in cancers is closely associated with tumor hypoxia. Hypoxic culture of tumor cells induces expression of a sialic acid transporter, sialin, and enhances the incorporation of nonhuman sialic acid from the external milieu (9).

Anti-idiotype Abs have proved to be able to mimic and induce Ag-specific Ab responses, even against nonprotein tumor-associated Ags like gangliosides. These anti-anti-idiotypic, anti-ganglioside Abs could bind to tumor gangliosides and mediate complement-dependent cell lysis or Ab-dependent cell cytotoxicity, inhibit ganglioside dependent survival cell functions, or block tumor released gangliosides in patient sera, which are known to have immune suppressive activities (10). Furthermore, it has also been proved that mAbs against gangliosides like GM2, GD2, and NeuGcGM3 can bind to and mediate antiproliferative or cytotoxic activities directly against target cells through different mechanisms (11–13). Because of the genetic variability and immune evasion capacity of tumors, Abs with multiple effector mechanisms may be needed to achieve maximal antitumor effects.

We have previously reported the induction of anti-NeuGcGM3 Abs in melanoma, breast, small, and nonsmall cell lung cancer (NSCLC) patients treated with the 1E10 anti-idiotype Ab (Ab2) mAb precipitated on aluminum hydroxide (14–17). This Ab2 was generated from the immunization of BALB/c mice with P3, an idiotype Ab (Ab1) that recognizes NeuGc-containing gangliosides, sulfated glycolipids, and Ags present in different human tumors (18–20). The clinical study performed recently in NSCLC...
patients showed encouraging clinical benefits (21), and immunological studies performed in 20 of these patients suggested a correlation between the induction of Abs against NeuGcGM3 and longer survival times (17). A fraction of nonsuppressible anti-NeuGc–containing ganglioside Abs was demonstrated through adsorption of patients’ sera with 1E10 mAb, suggesting that 1E10 idiotype vaccination might enhance antitumor natural immune response (16, 17).

The induced anti-NeuGcGM3 Abs were able not only to recognize but also to kill tumor cells expressing the Ag by a mechanism independent of complement activation. In the present work, the cytotoxic mechanism mediated by the Abs induced in the 1E10-vaccinated NSCLC patients is characterized. This cytotoxicity seems to differ from apoptosis because it is temperature independent, no chromatin condensation was detected, and the DNA fragmentation induced has a different pattern than the one observed for apoptosis. Furthermore, this cell death was accompanied by cellular swelling, membrane lesions formation, and cytoskeleton activation, resembling an oncosis-like phenomenon. The induction of specific Abs able to mediate tumor necrosis could produce the exposition of new tumor Ags at the same time that it contributes to creating an inflammatory tumor microenvironment, potentiating the cellular antitumor immune responses in cancer patients. To our knowledge, this is the first report of the active induction in cancer patients of NeuGcGM3-specific Abs able to induce complement-independent, oncotic necrosis to tumor cells. These results contribute to reinforce the therapeutic potential of anti-idiotypic vaccines and the importance of NeuGcGM3 ganglioside as antitumor target.

Materials and Methods

Gangliosides and cells

Gangliosides NeuAcGM3 and NeuGcGM3, purified from dog and horse erythrocytes, respectively, as described earlier (22), were provided by Dr. L. E. Fernández (Vaccine Department, Center of Molecular Immunology, Havana, Cuba). Murine lymphoid leukemia cell line L1210 and murine myeloma cell line X63, in which NeuGcGM3 is the major ganglioside expressed on the cell membranes (11), and the human small cell lung carcinoma U1906, and H82 (17), the undifferentiated large cell lung carcinoma cell line U1810, or the murine Lewis lung carcinoma (3LL), which do not express NeuGcGM3, were purchased from the American Type Culture Collection. Cells were grown in DMEM (Life Technologies) which do not express NeuGcGM3, were purchased from the American Type Culture Collection. Cells were grown in DMEM (Life Technologies) supplemented with 1% FCS, at 37˚C or on ice, for the indicated times. The cell death induction was detected by the addition of propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO) at a final concentration of 10 μg/ml and analyzed by flow cytometry. Similar experiments were performed with patients samples previously heated 30 min at 56˚C for complement inactivation. To determine if the induced anti-NeuGcGM3 Abs were mediating the cytotoxic effect of patients’ sera, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP; Matreya), an effective inhibitor of the glucosyltransferase that affects glycolipidsynthesis, was added to the culture medium at 10 μM for 3 d. With this same objective, before cell death induction, patient sera were incubated with 1 or 5 μg NeuGcGM3, previously air dried, resuspended in PBS, and sonicated, to block the anti-NeuGcGM3 Abs. To determine the importance of cytoskeleton organization in the death mechanism, the cells were treated with 10 μg/ml cytochalasine-D (Sigma-Aldrich) during death induction. As a control of apoptosis induction, L1210 cells were cultured overnight at 37˚C or 4˚C with 10 μM cisplatin (Sigma-Aldrich), 20 min with 10 μM CI973 300 (25), a fast and strong apoptosis inducer kindly provided by Dr. Perera from the Centre of Genetic Engineering and Biotechnology, or with 5 μM H2O2; 2 h at 37˚C.

Flow cytometry analysis of external phosphatidyl serine exposure with the Annexin V/PI assay

After the incubation of L1210 cells with patients’ sera at the indicated times and temperatures, 10^5 cells were double stained with an apoptosis detection kit supplemented with FITC-conjugated Annexin V and PI, for 10 min, at room temperature, following the manufacturer’s instructions (Annexin V-FITC Apoptosis Detection Kit; Sigma-Aldrich). The cells were immediately analyzed on the flow cytometer in their staining solution. Percent of positive stained cells was determined in a FACScan instrument (BD Biosciences). The WinMDI 2.9 program was used to analyze a total of 10^4 cells acquired on every FACS assay. Cells analyzed included living cells with normal forward light scatter (FSC)/side scatter (SSC) parameters and dying cells with altered FSC/SSC. Cell debris characterized by a low FSC/SSC and an Annexin VFITC 1^+ phenotype were excluded from analysis.

Fast halo assay

For the assessment of DNA single-strand breakage at the single-cell level, we performed the fast halo assay (24). Briefly, after 2 h incubation with pre- and hyperimmune patient sera, cells were resuspended in ice-cold PBS containing 5 mM EDTA. This cell suspension was diluted with an equal volume of 2.0% low-melting agarose in PBS and immediately sandwiched between an agarose coated slide and a coverslip. After complete gelling at 4˚C, the coverslips were removed, and the slides were immersed in 0.3 M NaOH for 15 min at room temperature. The slides were then washed with distilled water and dehydrated with ethanol. After dehydration, the slides were silver stained using the Silver Stain Plus kit (Bio-Rad) following the manufacturer’s instructions. The frequency of cells with diffuse DNA among 1000 nucleoids was visually scored using a microscope (OLYMPUS BH-2, Olympus; Tokyo, Japan) at original magnification ×10 and ×40.

Activated caspase 3 detection assay

To study if the cell death mechanism was mediated by caspase activation, L1210 cells were incubated for 2 h with the pre- and hyperimmune patients’ sera. Next, the cells were labeled using the Sulforhodamine FLICA Apoptosis detection kit (Molecular Probes) following the manufacturer’s instructions. The cells were visualized on a fluorescence microscope (OLYMPUS BH-2; Olympus).

Flow cytometry detection of patients’ Ab binding to L1210 tumor cells

L1210 cells were blocked in PBS containing 1% BSA for 20 min on ice. Patient serum, diluted 1/10, was incubated with 10^5 cells for 30 min on ice. After washing with PBS, the cells were incubated with FITC-conjugated goat anti-human IgG (Jackson Immunoresearch Laboratories), diluted 1/ 400 for 30 min on ice. Percent of positive stained cells were determined in a FACScan instrument (BD Biosciences). The WinMDI 2.9 program was used to analyze a total of 10^4 cells acquired on every FACS assay.

Induction of tumor cell death

Patients’ sera, diluted 1/10, were incubated with 10^5 L1210 and U9106 tumor cells or healthy donor PBMCs in 100 μl RPMI 1640 culture medium supplemented with 1% FCS, at 37˚C or on ice, for the indicated times. The viability of cultured cells was determined by the addition of propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO) at a final concentration of 10 μg/ml and analyzed by flow cytometry. Similar experiments were performed with patients samples previously heated 30 min at 56˚C for complement inactivation. To determine if the induced anti-NeuGcGM3 Abs were mediating the cytotoxic effect of patients’ sera, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP; Matreya), an effective inhibitor of the glucosyltransferase that affects glycolipid synthesis, was added to the culture medium at 10 μM for 3 d. With this same objective, before cell death induction, patient sera were incubated with 1 or 5 μg NeuGcGM3, previously air dried, resuspended in PBS, and sonicated, to block the anti-NeuGcGM3 Abs. To determine the importance of cytoskeleton organization in the death mechanism, the cells were treated with 10 μg/ml cytochalasine-D (Sigma-Aldrich) during death induction. As a control of apoptosis induction, L1210 cells were cultured overnight at 37˚C or 4˚C with 10 μM cisplatin (Sigma-Aldrich), 20 min with 10 μM CI973 300 (25), a fast and strong apoptosis inducer kindly provided by Dr. Perera from the Centre of Genetic Engineering and Biotechnology, or with 5 μM H2O2; 2 h at 37˚C.
Optical microscopy
To determine the nuclear and membrane morphology, after the incubation with the patients’ sera during the indicated times, L1210 cells were dried on microscope slides, fixed with 4% formaldehyde, stained with Giemsa’s azure eosin methylene blue solution (Merck KGaA, Darmstadt, Germany) and Vectashield Hardmount mounting media with DAPI (Vector Laboratories, Burlingame, CA), and visualized on a fluorescence microscope (OLYMPUS BH-2, Olympus). Cell death with nuclear condensation and cell shrinkage was regarded as apoptosis, whereas oncosis cell death was defined by nuclear and cellular swelling and the loss of plasma membrane integrity (24). To visualize the binding of the Abs to the cell membrane, the cells fixed on the microscope slides were blocked with PBS containing 1% FCS, incubated with FITC-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories) 30 min at room temperature in the dark, and washed with PBS. Coverslips were mounted using Vectashield Hardmount mounting media with DAPI (Vector Laboratories, Burlingame, CA) and immediately visualized on a fluorescence microscope (OLYMPUS BH-2, Olympus).

Scanning electron microscopy
L1210 cells in 100 μl RPMI 1640 culture medium supplemented with 1% FCS were incubated with patients’ sera for 2 h at 37˚C, washed three times with PBS, and then fixed with 3.2% glutaraldehyde in 0.1 mol/l sodium phosphate buffer (pH 7.4) at 4˚C for 1 h. These cells were postfixed for 1 h in 1% OsO4, washed three times with PBS, and dehydrated in ethyl alcohol. Cells were mounted onto a metallic stub and gold coated for 2 min before analyzed by TESCAN Vega TS 5130 SB Scanning Electron Microscope (Tescan).

Results
We have previously reported the immune response against NeuGcGM3 induced in patients of NSCLC due to the immunization with the anti-idiotypic mAb 1E10 (17). The treatment of these NSCLC patients with 1E10 mAb elicited anti-NeuGcGM3 Abs of both IgM and IgG isotypes. The Ab response against NeuGcGM3 ganglioside increased with the course of vaccination, reaching a peak after patients received the fourth or fifth doses of the Ab. The presence of these Ag-specific Abs was demonstrated by direct binding to the purified ganglioside assessed by ELISA, TLC immunostaining, and their recognition of the NeuGcGM3-positive myeloma cell line X63 by flow cytometry. Furthermore, most of the patient sera tested killed these myeloma cells by a complement-independent mechanism (17) (Supplemental Fig 1).

To characterize the cell death mechanism mediated by the Abs induced in the NSCLC patients immunized with the 1E10 mAb anti-idiotypic vaccine, four of these patients were selected due to their high cytotoxicity against NeuGcGM3-expressing tumor cells. Firstly, the capacity of the patients’ sera was tested to recognize and induce a complement-independent cell death to a different cell line, L1210, lymphocytic leukemia cells that also express high levels of NeuGcGM3 (11). As is shown in Fig. 1, hyperimmune patients’ sera recognized L1210 cells (Fig. 1A) and, after 2 h incubation at 37˚C, were able to induce cell death, which was measured by the PI incorporation assay by flow cytometry (Fig. 1B). This cell death was independent of the complement activation, because the heating of patients’ sera at 56˚C for 30 min did not affect the cytotoxicity (Fig. 1B). No cytotoxicity was detected against normal human PBMCs or the small cell lung carcinoma U1906, which do not express N-glycolylated Ags (Fig. 1C). These patients’ hyperimmune sera did not recognize the human small cell lung cancer cell line H82 (17), the undifferentiated large cell lung carcinoma cell line U1810 or the murine Lewis lung carcinoma (3LL) cells, which do not express NeuGcGM3 (Supplemental Fig. 2). Interestingly, forward scatter plots showed that the size of the cells increased after the incubation with hyperimmune patients’ sera, suggesting that the recognition by the Abs induced cell swelling (Fig. 1D).

To study the kinetics of the cell death induction, L1210 cells were incubated for 30 min, 2, and 4 h with patients’ sera. It was observed that this cytotoxic effect is very quick, because most of the cell death was already detected after 30 min of incubation with the patients’ hyperimmune sera (Fig. 2).

With the purpose to define whether the anti N-glycolyl Abs induced by 1E10 mAb vaccination specifically mediate this cytotoxic effect, the L1210 cells were cultured in the presence of PDMP, an inhibitor of glycosphingolipids’ synthesis. As is shown in Fig. 3A for patient 8, this treatment affected by 48% the induction of cell death by the patient’s hyperimmune serum. Furthermore, when the patients’ sera were preincubated with 1 or 5 μg NeuGcGM3, the cytotoxic effect was specifically inhibited in a manner dependent on the NeuGcGM3 concentration, whereas no inhibition was observed preincubating the serum with NeuAcGM3 (Fig. 3B).

We subsequently proceeded to identify the mechanism mediating the death induced by the patients’ sera. In contrast with oncosis and other types of cell death, apoptosis is an energy-dependent mechanism (25). So, first, the effect of the temperature on the cytotoxicity of the induced Abs was studied. L1210 myeloma cells were incubated with pre- and hyperimmune patients’ sera at 37˚C and 4˚C during 2 h and analyzed by flow cytometry for PI uptake. As is shown in Fig. 4, there were no differences in the PI uptake when the incubation took place at 4˚C or 37˚C in contrast with the inhibition at 4˚C of the cytotoxicity elicited by the uptake with cisplatin and CIGB300, apoptosis-inducer drugs. The nondependency on the temperature and the rapidity of the Abs action suggested that active metabolic processes, like the ones involved in apoptosis, were not required for this cell death. Next, the exposition of phosphatidyl serine on the outer surface of cell membranes, an early event described during apoptosis induction, was detected by Annexin V staining. Performing a double staining with PI and Annexin V, as early as 30 min after incubation, a high percentage of double-stained cells, a hallmark of necrosis, was found, as is shown in Fig. 5 for a representative patient. However, not only cells double stained with both markers but also a percentage of cells only stained with Annexin V was observed (Fig. 5A; Supplemental Fig. 3). Interestingly, this increase in Annexin V staining did not precede PI incorporation but occurred simultaneously. Furthermore, it was not inhibited when the incubation took place at 4˚C as opposed to what was observed for CIGB300, used as apoptosis-positive control (Fig. 5B).

The activation of caspase 3, another major hallmark of apoptosis induction, was also measured. No significant caspase 3 activation was detected in the cells incubated with the hyperimmune patient’s sera after 2 h incubation, the time at which a high percentage of the cells are already positive for PI staining. As is shown in Supplemental Fig. 4, we could not detect any caspase 3 activation in the L1210 cells incubated with hyperimmune sera from patients 7 and 15, whereas only 1.6 and 6% active caspase 3-positive cells were detected for patients 1 and 8, respectively. The cells incubated with CIGB300, used as apoptosis induction-positive control in our work, showed 100% of caspase 3 activation.

It has been proved that DNA fragmentation can be detected not only as a late event during apoptosis, but also as a consequence of extensive necrosis (26). To study the induction of DNA fragmentation during the death process, the fast halo assay was chosen. This is a method to determine DNA single-strand breaks in single cells that has been reported to be able to qualitatively identify the different kinds of DNA fragmentation induced during apoptosis and necrosis (27). This method is based on the observation that single-stranded DNA fragments, osmotically driven,
diffuse radially from the nuclear cage and generate an image that resembles a halo concentric to the nuclear remnants: the area of the halo is a direct function of the extent of DNA strand scission. Apoptotic cells, according to the intense autolysis of genomic DNA, are characterized by nuclear remnants resembling pinheads surrounded by very large DNA halos. Necrotic cells are characterized by circular, faint halos surrounding a well-conserved nuclear remnant. The diffused DNA can be detected by silver staining and monitored with a light microscope. As is shown in Fig. 6A for patients 1 and 8, damaged cells, obtained after 2 h incubation with hyperimmune sera, unlike control cells incubated with preimmune sera, displayed necrotic type DNA halos surrounding the nuclei. The number of necrotic or apoptoticlike halos was counted from 1000 cells analyzed in randomly selected microscope fields under the microscope. There were 652 cells with necrotic DNA (65%) versus 187 cells (18%) showing apoptoticlike halo for patient 1 and 529 necrotic cells (52%) and 56 apoptoticlike halos (5%) for patient 8. The preimmune sample showed 163 necrotic cells (16%) and 27 apoptoticlike cells (2.6%), whereas for H2O2-treated cells, used as apoptosis-positive control, 465 were apoptotic (46%) and 429 were necrotic (42.8%) (Fig. 6B).

It was previously shown that the induction of cell death by the recognition of NeuGcGM3 by a mAb depends on cytoskeleton organization (11). To study the importance of actin polymerization in the death mechanism induced by patient Abs, the cells were coincubated with the patients’ sera and cytochalasin D, a potent inhibitor of actin polymerization. As is shown in Fig. 7, cytochalasin D treatment strongly affected the induction of cell death. Finally, microscopy observations were conducted to characterize the morphology of the affected cells. First, the binding of the Abs to L1210 cells was detected by fluorescent microscopy. As can be observed in Fig. 8A for a representative patient, the hyperimmune Abs attached to the cell surface, displaying greater concentrations...
on some regions of the cell membrane, and induced membrane rupture. Next, after the incubation with the sera, the cells were stained with Giemsa and visualized with a light microscope. This examination revealed that after 2 h incubation, 65% of the cells had the morphology of oncotic necrotic cells with very important swelling, membrane disruption, and release of cytoplasmic content before a visible nuclear breakdown (Fig. 8B). No chromatin condensation, a hallmark of apoptosis, was detected in the Giemsa-stained cell nucleus after the incubation with hyperimmune sera, a result that was confirmed by DAPI staining of the nucleus (data not shown).

To further investigate the effects of the cytotoxic Abs on the cell membranes, L1210 cells were incubated with the patients’ sera for 2 h and examined for surface structural changes by scanning electron microscopy. Although the cells incubated with the pre-immune sera have membrane contours and microvilli typical of lymphoid cells (Fig. 9A), the cells incubated with hyperimmune sera have a convoluted surface and are covered by debris (Fig. 9B–D). Hyperimmune patients’ sera induced large membrane lesions on the surface of the target cells. Most usually, a single giant lesion was observed on individual cells (Fig. 9B–D), although smaller holes were also observed in variable numbers (Fig. 9C). The induction of cell swelling was further confirmed (Fig. 9D).

Discussion

It was previously shown that the anti-idiotypic Ab 1E10 was able to induce both IgM and IgG anti-NeuGcGM3 Abs in advanced NSCLC patients treated with this idiotypic vaccine (17). Interestingly, these Abs are able to recognize and kill tumor cells expressing NeuGcGM3, like the myeloma cells X63 and L1210 lymphocytic leukemia cells. These Abs do not recognize tumor cell lines that do not express this ganglioside, like small cell lung cancer cell line H82 (17), the small cell lung carcinoma U1906, the undifferentiated large cell lung carcinoma cell line U1810, or the murine Lewis lung carcinoma (3LL) cells. Specially, they have no cytotoxic effect over normal human lymphocytes, also negative for NeuGcGM3 expression, which could have serious adverse effects for the treated patients. In this work, the cytotoxic mechanism mediated by the anti-NeuGcGM3 Abs induced in NSCLC patients against NeuGcGM3 expressing L1210 lymphocytic leukemia cells is described. The detected cell death was mediated by Abs induced after the vaccination, because preimmune sera did not show cytotoxicity against this cell line, at least at the dilutions used in our experiments. The presence of N-glycolilated Ags in the cell membrane was necessary for this cytotoxic effect: 1) although hyperimmune patients’ sera were highly cytotoxic against L1210, they did not affect normal human lymphocytes, where these Ags are not naturally expressed; 2) the lysis was affected when the cells were cultured in the presence of PDMP, an inhibitor of glycosphingolipids’ synthesis, and 3) the cytotoxicity was specifically inhibited when patients’ hyperimmune sera were previously blocked.
with saturating amounts of NeuGcGM3, whereas the preincubation with NeuAcGM3 did not cause any effect. The cell death occurred very quickly after the incubation with the patients’ hyperimmune sera; 30 min of incubation was enough to obtain >30% of dead cells. This process depended on the cytoskeleton rearrangement, because it was affected when actine polymerization was inhibited by treating the cells with cytochalasin D.

The induced Abs killed the tumor cells by a mechanism other than complement-mediated necrosis, because the cytotoxicity was not inhibited after the preheating of hyperimmune patients’ sera at 56˚C for 30 min. It also seems to differ from energy-dependent apoptosis because it was not affected when the incubation of the cells with the patients’ sera took place at 4˚C, in contrast with the inhibition at this temperature of the death induced by cisplatin or CIGB300, known apoptosis inducers. Caspase 3 activation, a hallmark for apoptosis induction, could not be detected. Furthermore, Giemsa staining of the nucleus showed the chromatin in small irregular aggregates distributed throughout the nucleus, but not the formation of peripheral, sharply delineated masses of condensed chromatin or apoptotic bodies, which are characteristic structural features of apoptosis. In contrast, an increase in the exposure of phosphatidyl serine in the outer cell membrane, an event usually associated with early apoptosis (28), was detected by Annexin V staining in the flow cytometer. However, it has been proved that Annexin V staining is not an event exclusively associated with apoptosis induction. In a previously reported study, primary necrosis or apoptosis were induced in several cell types, and phosphatidyl serine residue translocation was analyzed by the Annexin V/PI assay, whereas the cell morphology associated with the different types of cell death was confirmed by optical and electron microscopy examination. Their results revealed that in both types of cell death Annexin V staining can be detected (27). Furthermore, during apoptosis, Annexin V binding precedes PI uptake and is affected when the induction takes place at 4˚C, as was observed for L1210 cells incubated with CIGB 300, used as a positive control for apoptosis induction. But during necrosis, the plasma membrane ruptures, and PI is taken up in the absence of or prior to Annexin V binding. When L1210 cells were incubated with patients’ sera, both events took place simultaneously and were not affected when the incubation took place at 4˚C, being the double stain with PI and Annexin V detectable already 30 min postincubation with patients’ hyperimmune sera.
DNA fragmentation occurs in the course of apoptotic and necrotic cell death (24). During apoptosis, an extensive autolytic DNA fragmentation produces kilobase-sized (50 kb) double-stranded fragments, which can be revealed by pulsed-field gel electrophoresis (29), and smaller ones producing the typical ladder pattern following conventional DNA electrophoresis (30), which is considered a hallmark of this type of cell death (31). In contrast, DNA fragments produced during necrosis are relatively large (≥0.2 Mb) (29) and do not diffuse as much as smaller apoptotic fragments (≤50 kb). As a consequence, single-stranded DNA fragments, osmotically driven from a single cell, diffuse radially and generate a halo concentric to the nuclear remnants, which is a direct function of the extent of DNA strand scission. Apoptotic cells are characterized by the presence of large DNA halos, and the disproportion between the size of nuclei and that of corresponding halos has been interpreted as a sensitive index of apoptotic-type DNA fragmentation (32, 33). These were the type of halos obtained when L1210 cells were treated with 5 μM H2O2. However, most of the cells incubated with patients’ hyperimmune sera showed double, smaller, fainter, and perfectly circular halos, typical of necrotic cells.

Microscopy analysis of the morphology of the cells showed that the cells incubated with hyperimmune sera showed swelling and membrane leakage and/or rupture following treatment. Scanning electron microscopy analysis showed that hyperimmune patients’ sera induced the formation of lesions that resemble holes in the

![FIGURE 7](image1.png)

**FIGURE 7.** Cytoskeleton activation is required for cytotoxicity. L1210 cells were incubated with preimmune (pI) and hyperimmune (hI) sera in the presence or not of 10 μg/ml cytochalasin B. Cell death was analyzed by PI uptake.

![FIGURE 8](image2.png)

**FIGURE 8.** Hyperimmune patients’ sera induced cell death involves morphologic changes. A. L1210 cells were incubated with preimmune (pI) and hyperimmune (hI) sera during 30 min. The cells were visualized by FITC-conjugated goat anti-human Igs in a fluorescence microscope. B. Morphologic changes in L1210 cells after 2 h incubation with preimmune (pI) and hyperimmune (hI) sera visualized by Giemsa staining: oncotic necrotic cells with swelling, membrane disruption, and release of cytoplasmic content (black arrows).
cell membranes. The cell membranes lost the contours typical of lymphoid cells and were covered by debris, probably product of the cytoplasm leakage from the holes.

Thus, anti-NeuGcGM3 Abs induced in NSCLC patients immunized with the anti-idiotypic Ab 1E10 have a direct cytotoxic effect over tumor cells expressing this Ag. This cytotoxicity is mediated by a mechanism that depends on the expression of the Ag on the cell membrane; it is very quick, independent of the temperature, and involves cytoskeleton reorganization. Furthermore, there is DNA degradation, cellular swelling, and the formation of big membrane lesions that produce the leakage of cytoplasm and ends up on the loss of the cell membrane integrity. All of these characteristics resemble a process of oncocytic necrosis, first defined by Majno and Joris in 1995 (25) and often caused by ischemia, toxic agents that interrupt the ionic pumps of the plasma membrane, oxidative stress, inhibitors of ATP synthesis, or heat shock (25, 34–36).

The capacity of the anti-NeuGcGM3 mAb called 14F7, a murine IgG highly specific for NeuGcGM3, to induce oncocytic cell death to tumor cells expressing this Ag has been previously reported. This Ab induced a tumor cell death that was accompanied by cellular swelling, membrane lesion formation, and cytoskeleton activation (11).

Besides 14F7 mAb, other examples of Abs that mediate cell death resembling oncosis have been reported: the RE2 Ab, cytotoxic to T and B lymphocytes (37); anti-porimin mAb, a type I transmembrane protein with extensive O- and N-linked glycosylation sites characteristic of a mucin, which induced oncosis-like cell death in Jurkat cells (38); mAbs 216 and A6(H4C5) that bind specifically to a human B lymphocyte surface carbohydrate Ag (39); RAV12 that recognizes an N-linked carbohydrate Ag (RAAG12), strongly expressed on multiple solid organ cancers (40); and mAb 84, which binds to podocalyxin-like protein-1, a highly glycosylated sialomucin, on human undifferentiated embryonic stem cells (41). Interestingly, these Abs bind to carbohydrate Ags or highly glycosilated proteins. It has been found that deglycosylation of affinity purified porimin protein from Jurkat cells resulted in loss of reactivity with the anti-porimin mAb, which suggested that anti-porimin mAb also reacts with a carbohydrate epitope (42).

It had been reported that healthy human sera contain detectable levels of anti-N-glycolylated ganglioside Abs, even of IgG isotype (7, 43, 44). These Abs were able to kill human leukemic cells and activated T cells that were exogenously fed with NeuGc, but in these studies, the detected cell death was mediated only by a complement-mediated mechanism (7). The affinity and the title in the sera could determine the differences in the type of cytotoxicity produced by the anti-NeuGcGM3 Abs in healthy donors and the ones induced by 1E10 vaccination. 14F7 mAb, a hypermutated IgG, recognizes NeuGcGM3 with higher affinity than P3 mAb, a germline origin IgM. Both recognize NeuGcGM3-expressing cell lines, but whereas 14F7 mAb induces oncosis, P3 mAb induces complement-mediated cytotoxicity (11).

Presently, we are conducting studies on the influence of the affinity maturation of the anti-NeuGcGM3 Abs produced in the sera of the NSCLC patients, along the immunization protocol, on their capacity to induce oncocytic death to tumor cells.

The induction of cytotoxic Abs could potentiate the cellular immunity against the tumor cells. Cell death confers immunogenic properties to tumor cells (45–47). In particular, necrotic cell death has long been regarded as immunogenic due to the release of damage-associated molecular patterns, like heat shock protein 70, high mobility group box 1 (48, 49), and lipid membranes (50). In addition, several mitochondrial Ags, characteristic of their bacterial ancestors, like N-formyl peptides (51, 52), mitochondrial transcription factor A (53, 54), and mitochondrial DNA (55), rich in cytosine-phosphate-guanosine sites dinucleotides, are exposed and recognized by intracellular TLR9 in specific immune cells (56). These released Ags serve as potent chemotactractors for neutrophils (51), promote proinflammatory macrophage responses (57), and induce monocyte and dendritic cell (DC) maturation and DC Ag cross presentation (58). Moreover, cells dying by necrosis actively secrete inflammatory cytokines such as IL-6, and are characterized by NF-kB and p38 MAPK activation (57, 59).

Recently, it has been discovered that chemotherapy-induced cell death can elicit a cellular immune response against dying tumor cells (49, 60, 61) and that this immune response is actually required for an optimal therapeutic effect of antitumor chemotherapy. On gastric and renal carcinoma cell lines, high-dose UVC was associated with increased immunogenicity and damage-associated molecular pattern release, and the death treatments reversed the inherent suppressive activity of the tumors on CTL cross-priming (62). Necrosis caused by thermal (63, 64) and photodynamic (65) therapies induces the release of SP70, thus speeding up the tumor-associated Ags’ delivery to the DC cross presentation pathway. In another in vivo model, hyperthermia combined with intratumoral injection of autologous DC induced a CTL response through the release of heat shock protein 70 and reverted local and systemic recurrence of a mouse tumor (66). Furthermore, the exposure of tumor cell debris in the context of an inflammatory environment could induce the generation of T cell responses against Ags other than those targeted by the Ab, and this might help prevent the emergence of therapeutic resistance caused by tumor cells losing the original target Ag (67).

The induction of specific Abs able to mediate tumor cell death by oncotic necrosis could produce the exposition of new tumor Ags and at the same time contribute to create an inflammatory tumor microenvironment that could enhance antitumor immune responses in cancer patients.

Many therapeutic Abs have been developed for the treatment of cancer; however, only a small subset of these Abs have shown
to elicit direct cytotoxic activity over tumor cells by different mechanisms (11–13, 37–40, 68, 69). All of these Abs have proved their cytotoxic potential as passive treatments in preclinical models. To our knowledge, this is the first report of the active induction in cancer patients of these kind of anti-NeuGcGM3 cytotoxic Abs, able to induce oncocytic necrosis to tumor cells. Several experiments to confirm the capacity of idiotypically induced anti-NeuGcGM3 Abs to exert this antitumor cytotoxicity and to prove the immunogenicity of this cell death, in vivo, are currently ongoing.

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Disclosures

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References

Supplementary Figure 1. 1E10 mAb treated NSCLC patients’ hyper-immune sera recognize and induce the death of the myeloma cell line P3-X63-Ag8.653. A. Patients pre-immune (pl) and hyper-immune (hl) sera, diluted 1/10, were incubated with the NeuGcGM3 expressing myeloma cell line P3-X63-Ag8.653. The reaction was developed with PE-conjugated anti-human IgG+IgM. The numbers represent the percentage of pre and hyper-immune sera-reacting cells. B. X63 cells were incubated for 2h at 37°C with patients 1, 7, 8 and 15 preimmune (pl) and hyperimmune (hl) sera, diluted 1/10. The percentage of cell death was determined by the PI incorporation assay.

Supplementary Figure 2. 1E10 mAb treated NSCLC patients’ hyper-immune sera do not recognize NeuGcGM3 negative cell lines. A. Patients preimmune (pl) and hyperimmune (hl) sera, diluted 1/10, were incubated with the cell lines 3LL, U1810, U1906 and H82. The reaction was developed with PE-conjugated anti-human IgG+IgM.

Supplementary Figure 3. Annexin V/PI double staining of L1210 cells incubated with patient’s sera. Patients’ pre (pl) and hyper-immune (hl) sera were incubated with L1210 cells for the indicated times at 37°C.

Supplementary Figure 4. The incubation of L1210 cells with patient’s hyper-immune sera did not induce caspase 3 activation. L1210 cells were incubated during 2h at 37°C with pre or hyper-immune patient’s sera. Propidium iodide incorporation was detected by flow cytometry and activated caspase 3 was detected with Sulforhodamine labeled DEVD-FMK caspase inhibitor peptide. The cell nucleuses were labeled with Hoecht. The fluorescent cells were visualized on a fluorescence microscope (20X amplification). The graphics represent the respective percentage for PI incorporation measured by flow cytometry and activated caspase 3 positive cells counted from 500 cells analyzed in randomly selected microscope fields under the microscope.