High-Performance Immunoaffinity Chromatographic Detection of Immunoregulatory Anti-Idiotypic Antibodies in Cancer Patients Receiving Immunotherapy

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Anti-idiotypic antibodies are regulatory antibodies responsible for the shutdown of active immune responses against growing tumor cells. In an attempt to study these antibodies, a technique for isolating specific anti-idiotypic antibodies by immunoaffinity chromatography was devised. Human anti-tumor antibodies were isolated by affinity absorption to fixed autologous tumor cells. These antibodies were biotinylated, immobilized on streptavidin-coated beads, and used as a ligand to isolate reactive anti-idiotypes from the plasma of patients during periods when immune reactivity against their tumors could not be detected. The isolated anti-idiotypes demonstrated the ability to react with the original antibodies and to inhibit their binding to autologous tumor cells. Thus functional anti-idiotypic antibodies can be isolated by immunoaffinity chromatography with the original idiotype as the ligand. This technique can be used to monitor regulatory antibodies in cancer patients receiving immunotherapy.

Additional Keyphrases: biotin-avidin ligands • immune response to tumors

Anti-idiotypic antibodies are an essential part of the regulatory pathways of the immune system and are responsible for both enhancing and suppressing humoral and cellular immune responses (1–5). They are also involved in the down-regulation of the immune system in cancer patients and are thought to be responsible for the shutdown of active immune surveillance against growing tumor cells (6–8). This leads to a loss of host control over the growing neoplasm and is thought by some investigators to be the first step that leads to the metastatic spread of the cancer (6–9).

Anti-idiotypic antibodies react with sites in the hypervariable or antigen-binding regions of the target idiotype and inhibit it from reacting with the antigen (10). Similarly, anti-idiotypic antibodies can react with idiotypic receptors on activated T cells and inhibit clonal expansion (11). Either mechanism usually results in the shutdown of active immune reactions against the specific antigen or cell carrying the antigen. Although these inhibitory antibodies can be detected by antigen-binding-inhibition studies with the original idiotype and its reactive antigen, isolation and measurement of the anti-idiotypes is often difficult. The use of solid-phase immunoglobulin G (IgG) molecules has been tried, but this technique often isolates other anti-antibodies, especially rheumatoid factor (12). The use of digested IgG overcomes this problem but presents other problems in attachment of fragments to matrix.

In an attempt to further study the involvement of inhibitory anti-idiotypes in the regulation of immune responses in cancer patients undergoing active immunotherapy, a technique for isolating and purifying specific anti-idiotypic antibodies has been developed. This technique involves "high-performance" immunoaffinity chromatography, using the patient's own tumor-antigen-directed idiotypic antibodies as the immobilized ligand. In this way reactive anti-idiotypes can be isolated, quantified, and recovered in a biologically active form for use in other studies.

Materials and Methods

Materials

Patients' samples. Blood samples were obtained weekly from 36 melanoma patients who were undergoing immunotherapy with vaccines prepared from isolated autologous tumor cell membranes. Autologous tumors, obtained by surgical removal, were prepared as single-cell suspensions and stored in vapor-phase liquid nitrogen.

Reagents. "Glycophase" controlled-pore glass beads, with derivatized carbonyl diimidazole-reactive side chains attached to their surfaces, and purified streptavidin were obtained from Pierce Chemical Co., Rockford, IL. Radiolabels (51Cr and 125I) were obtained from Amersham Corp., Arlington Heights, IL. Immunodiffusion plates and the human IgG standards were obtained from Kallestad Diagnostics, Austin, TX. The chaoetric ions used to elute bound materials from the immunoaffinity columns were purchased from Sigma Chemical Co., St. Louis, MO.

Standard solutions. The stock solution of the chaoetric ion elution buffer was prepared by dissolving sodium thiosulphate (202.67 g) in 1 L of distilled water and filtering under reduced pressure through a 0.22-µm (pore size) filter. The human IgG standards (2470, 1220, and 350 mg/L) were prepared by dissolving the appropriate amount of lyophilized powder in 1 mL of doubly distilled water and centrifuging at 10 000 × g for 10 min.

Apparatus

Immunoaffinity chromatography was performed with a Model 340 isocratic HPLC system (Beckman Instruments, Palo Alto, CA) comprising a Model 112 pump, a Model 160 ultraviolet detector set at 280 nm, and a Shimadzu C-RIB peak integrator (Shimadzu Scientific Instruments, Columbia, MD). Elution control was performed by programming a Model III OPG/S solvent selector (Autochrom, Milford, MA).

The columns and end fittings used to construct the immunoaffinity columns were purchased from Bio-Rad Laboratories, Rockville Centre, NY.

A Beckman 4000 gamma counter (Beckman Instruments) was used to measure 51Cr and 125I radiolabels.

Procedures

Monitoring antitumor responses. The patients' serum samples were checked for the presence of cytotoxic anti-tumor antibodies by a chromium release assay (13), with use of 51Cr-labeled autologous tumor cells.

Isolating tumor-reactive idiotypes. Reactive anti-tumor antibodies were isolated by incubating the patients' serum
overnight at 4 °C with a pellet of autologous cells fixed in 50 mL/L glutaraldehyde reagent. After extensive washing to remove unreacted serum, the tumor cells were incubated in 1 mol/L sodium thiocyanate reagent for 30 min at room temperature, then centrifuged at 500 × g for 20 min. The supernate was then dialyzed overnight against phosphate buffer, 10 mmol/L, and further purified by cross absorption against a panel of melanoma cell lines. The purified antibodies were checked by immunoelectrophoresis and measured by radial immunodiffusion.

Western blot analysis of the anti-tumor antibodies. To check the specificity of the isolated antibodies, we reacted them against polyacrylamide gel maps of solubilized autologous and allogenic melanoma cell membranes. This was performed by a standard Western blot technique (14) with gel maps transferred to nitrocellulose paper.

High-performance immunoaffinity isolation of auto-antiidiotypic antibodies. The idiotypic anti-tumor antibodies were biotinylated via the carbohydrate component of the Fc portion by reacting them with hydrazide-derivatized biotin (15). The biotinylated antibodies were immobilized on glass beads that were previously coated with streptavidin (2.5 mg of streptavidin was incubated with 10 g of the derivatized glass beads overnight in 0.5 mol/L carbonate buffer, pH 8.5) (16). The antibody/streptavidin bead mixture was suspended in 2 mL of 10 mmol/L phosphate buffer, pH 7, in a 5-mL capped tube and incubated for 1 h on a rotator. The antibody-coated beads were slurry-packed into a 15 cm × 4.6 mm (i.d.) HPLC column and attached to the HPLC system. One hundred microliters of patients' serum was injected into the system, and the column washed at 1 mL/min for 15 min with 10 mmol/L phosphate buffer as the mobile phase. The bound antibodies were then recovered by introducing a chaotropic sodium thiocyanate ion gradient (from 0 to 2.5 mol/L) over a 10-min run. The absorbance of the eluted peaks was monitored at 280 nm with a detector sensitivity setting of 0.08 absorbance, full-scale; 200-μL fractions of the eluted peaks were collected. The column was kept at 4 °C.

Inhibition studies. The specificity of the immunoaffinity-purified anti-idiotypic antibodies was tested by studying the inhibition of antigen-binding. Solubilized membranes from the autologous tumor cells were labeled with 125I by the lactoperoxidase technique (17) and incubated with either the anti-tumor antibody or a mixture (pre-incubated for 30 min at room temperature) of anti-tumor antibody and anti-idiotypic antibody. Samples collected after 0, 5, 10, 20, 40, 80, and 160 min of incubation were precipitated with 100% saturated ammonium sulfate, and the precipitates were analyzed for the presence of radiolabeled antigen.

Results

Monitoring anti-tumor activity. Monitoring the patients for the presence of cytotoxic anti-tumor antibodies during therapy revealed that 32 of 35 patients demonstrated complement-binding antibodies 21 days after vaccination. These responses generally lasted for five to six weeks before there was a sharp decline in the detectable concentrations of antibodies. Re-immunization produced a second peak of cytotoxic anti-tumor activity that lasted for three to five weeks; during this time, circulating immune complexes containing anti-antibodies could be demonstrated. In addition, the concentrations of detectable suppressive anti-idiotypic antibodies were increasing. Figure 1 illustrates these findings in the 32 responsive patients.

Isolation of tumor-reactive antibodies. Isolation of the patients' anti-tumor antibodies by absorption to fixed autologous tumor cells and cross absorption with four melanoma cell lines gave an average result of 42 ng/mL for IgG antibody. These antibodies reacted only with a 125-kDa band derived from the autologous tumor when reacted against Western blots of whole-tumor-membrane preparations. Although several bands could be detected by silver staining of the membrane preparation, these isolated antibodies failed to react with any band other than the 125-kDa band or with Western blots taken from the membranes of the other patients' tumor cells (Table 1).

Isolation of anti-idiotypic antibodies. Biotinylation of the anti-tumor IgG with the hydrazide-derived biotin inhibited the binding of anti-Fc antibodies to the anti-tumor antibodies and allowed attachment of the antibody via the Fc portion. This placed the hypervariable or antigen-binding region in the liquid phase of the column and increased the chance of its being recognized by anti-idiotypic antibodies. The reactive anti-idiotypic antibodies were isolated as a
Table 1. Binding Specificity of the Anti-Tumor Antibodies against Western Blots of Tumor Antigens

<table>
<thead>
<tr>
<th>Blotted antigen</th>
<th>125I-labeled antibody binding, counts/min*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autologous tumor</td>
<td></td>
</tr>
<tr>
<td>125-kDa band</td>
<td>29045 ± 1377</td>
</tr>
<tr>
<td>92-kDa band</td>
<td>286 ± 209</td>
</tr>
<tr>
<td>51-kDa band</td>
<td>486 ± 515</td>
</tr>
<tr>
<td>21-kDa band</td>
<td>390 ± 508</td>
</tr>
<tr>
<td>Melanoma cells, 125-kDa band</td>
<td></td>
</tr>
<tr>
<td>Cell line I</td>
<td>538 ± 287</td>
</tr>
<tr>
<td>Cell line II</td>
<td>205 ± 315</td>
</tr>
<tr>
<td>Cell line III</td>
<td>600 ± 499</td>
</tr>
<tr>
<td>Cell line IV</td>
<td>452 ± 627</td>
</tr>
<tr>
<td>Allogeneic tumor, 125-kDa band</td>
<td></td>
</tr>
<tr>
<td>Tumor 1</td>
<td>707 ± 361</td>
</tr>
<tr>
<td>Tumor 2</td>
<td>1042 ± 633</td>
</tr>
</tbody>
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*Mean (minus background) ± SEM. n = 32 each.

Patients' anti-tumor antibodies were reacted overnight at 4°C against Western blots of the major protein bands of the autologous tumor and against the 125-kDa band isolated from two allogeneic tumors and from four melanoma cell lines. Each band was cut from the nitrocellulose paper, washed in 10 mmol/L phosphate buffer, and its radioactivity was measured only against the 125-kDa band of the autologous tumor indicate the restricted nature of the anti-tumor activity.

second peak, produced after the initial unreacted material had been washed through the immunoaffinity column. This peak eluted at 25 min into the chromatography run (Figure 2).

Immunoelectrophoretic and immunodiffusion studies demonstrated that only IgG was contained in the eluted peak and that, depending on the patient and the time post-vaccination, immunoaffinity chromatography isolated between 5 and 12 ng of the anti-idiotypic per milliliter. The antibody concentrations were measured by integration of the area under the second peak and calibrated against the areas under the IgG standards run under identical conditions. These concentrations were checked by immunodiffusion.

Monitoring the suppressive effects of the anti-idiotypic antibodies. Antigen-binding inhibition experiments demonstrated that the anti-idiotypic antibodies isolated by immunoaffinity greatly reduced the original antigen-binding capacity of the anti-tumor antibodies. This effect increased proportionally with the concentration of detectable anti-idiotypic antibodies (Figure 3), which also correlated with loss of detectable cytotoxic anti-tumor activity in all of the patients monitored. In a limited number of experiments, in which patients' lymphocytes were incubated with the isolated anti-idiotypic antibodies prior to co-culture with the autologous tumor cells, the amount of cell-mediated cytotoxicity decreased. Chromium-release studies demonstrated a decrease of 25–58% in the cytotoxic activity of the anti-tumor antibodies, after incubation with the autologous anti-idiotypic. This decrease in tumor cell kill was not present when the anti-tumor antibodies were pre-incubated with anti-idiotypes from other patients.

Discussion

Success in the treatment of cancer by immunotherapy has been mixed, ranging from ineffective (18) to producing short-term responses (19–21). Several mechanisms have been suggested to explain relative states of unresponsiveness, including excess of shed tumor antigen (22), suppressive circulating immune complexes (23), and inhibitory anti-antibodies (24). Although these mechanisms have been described, few studies have included all of these possibilities in the monitoring protocols. The presence of regulatory idiotypic antibodies is difficult to detect and their concentrations are often extremely low, making routine screening difficult.

In this study, patients on immunotherapy protocols were screened for several different immunological variables, including immune reactivity against their autologous tumors and the presence of circulating immune complexes and regulatory anti-idiotypes. The specificity of the isolated anti-tumor antibodies demonstrated a restricted reactivity against only their autologous tumor cells; this reactivity identifies these antibodies as idio- types. The isolation of these original idiotypes provided a suitable ligand for the development of the immunoaffinity screening technique for monitoring the presence of reactive anti-idiotypic. With this technique, one can measure the amount of bound material and also provide active antibodies for use in other assays. The isolation of these antibodies allows for further investigation of their exact role in the suppression of the immune response against the tumor.

The presence of suppressive anti-idiotypes has been described in several different tumor systems, especially malignant melanoma (24) and lymphatic leukemia (25). In the latter, the use of anti-idiotypic antibodies was suggested as
a therapeutic procedure (25). If the system proposed by Jerne (26) is correct, then isolation of suppressive anti-idiotypes could be of value as a second vaccine, acting to stimulate enhancing anti-idiotypes in cases where the immune monitoring demonstrates that a loss of the original idiotypic response is taking place.

Experimental work in animals has shown that the injection of idiotypic antibodies complexed with rheumatoid factor can elicit the formation of anti-idiotypic antibodies (27). Further, injection of these suppressive anti-idiotypes (in the form of rheumatoid/anti-idiotype immune complexes) into immune-suppressed animals can elicit the restoration of the original idiotype (28). If this system can be transposed to human studies, then isolation of suppressive anti-idiotypes may prove a useful technique for providing material for re-injection, as a specific idiotypic vaccine. When coupled with the detection and removal of free tumor antigen, idiotypic vaccines may provide the ideal technique for immunotherapy.

However, the presence of suppressive anti-idiotypic antibodies must be monitored in patients receiving immunomodulatory therapy. The use of high-performance immunoaffinity chromatography provides a technique for detecting, measuring, and isolating anti-idiotypic antibodies in <1 h. This technique can be used in any facility that has a simple HPLC system and can be converted to batch isolation for research purposes. Once made, the immobilized-antibody columns remain viable for 50–75 runs and are usable, when stored refrigerated, for up to one year.

References