Isolation of anti-idiotypic antibodies by immunoaffinity chromatography on Affinichrom beads

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ABSTRACT

Anti-idiotypic antibodies are important regulators of the immune system but they are difficult to isolate and monitor. We have developed a technique for isolating specific auto-anti-idiotypic antibodies by high-performance immunoaffinity chromatography using immobilized autologous anti-tumor antibodies as the affinity ligand. The isolated anti-idiotypes demonstrated the ability to react with the original anti-tumor antibodies and inhibit their reactivity against autologous tumor cells. This technique can be used to monitor regulatory antibodies in cancer patients receiving immune modulation therapy.

INTRODUCTION

Anti-idiotypic antibodies are part of the regulatory pathways of the immune system and are responsible for regulating both humoral and cellular immune responses[1-5]. They are actively involved in the down-regulation of the immune system in cancer patients and are thought to be responsible for the suppression 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 hyper-variable or antigen-binding regions of the target antibody (idiotype) and inhibit it from reacting with the antigen[10]. In a similar manner, suppressive anti-idiotypic antibodies can interact with specific antigen receptors on activated T cells and suppress clonal expansion, even in the presence of antigen[11]. Although these inhibitory antibodies can be detected by antigen binding inhibition studies using the original idiotype and its reactive antigen, isolation and measurement of reactive anti-idiotypes is difficult. Affinity chromatography or immunoprecipitation techniques, using solid-phase or cross-linked im-

munoglobulin G (IgG) molecules have been used to isolate anti-idiotypes, but these techniques have been shown to isolate other anti-antibodies, such as anti-F(Ab)$'_2$ and rheumatoid factor[12].

In an attempt to study the involvement of inhibitory anti-idiotypes in the suppression of immune responses in cancer patients receiving immunomodulatory therapy, we developed a high-performance immunoaffinity chromatography (HPIAC) technique for isolating specific auto-anti-idiotypic antibodies in a biologically active form.

EXPERIMENTAL

Materials

Solid glass beads (diameter, 1 mm) were obtained from Kontes (Vineland, NJ, U.S.A.). Purified streptavidin was purchased as a lyophilized, pure product from Bethesda Research Labs (Gaithersburg, MD, U.S.A.) and reconstituted in 0.5 M carbonate buffer, pH 9.0. The hydrazine biotin and the laboratory chemicals including the 3-aminopropyltriethoxysilane and 1,1'-carbonyldiimidazole (CDI) were obtained from Sigma (St. Louis, MO, U.S.A.). All columns and column fittings were purchased from Alltech (Deerfield, IL, U.S.A.). Radiolabels (^{51}Cr and ^{125}I) were obtained from Amersham (Arlington Heights, IL, U.S.A.). Immunodiffusion plates and the human IgG standards were obtained from Kallestad (Austin, TX, U.S.A.).

Blood samples from 50 patients with malignant melanoma, on active immunotherapy were obtained weekly. Autologous tumor material was obtained following surgical resection and scissor-minced into a single cell suspension and following controlled-rate freezing, the suspensions were stored at $-136°C$ in vapor-phase liquid nitrogen.

Detection and isolation of anti-tumor antibodies

The patients' serum samples were checked for the presence of cytotoxic anti-tumor antibodies by a chromium release assay[13], with use of ^{51}Cr-labeled autologous tumor cells. Samples which exhibited specific autologous anti-tumor activity were selected for further purification. 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 of 2% glutaraldehyde. After extensive washing to remove unreacted serum, the tumor cells were incubated in 2 M sodium thiocyanate, for 30 min at room temperature, then centrifuged at 500 g for 20 min. The supernate was dialyzed overnight against 0.1 M phosphate buffer, pH 7.2 and further purified by cross-absorption against a panel of allogeneic melanoma cell lines. The isolated antibody preparations were checked by immunoelectrophoresis and the immunoglobulin content measured by radial immunodiffusion.

Western blot analysis of the anti-tumor antibodies

The specificity of the isolated anti-tumor antibodies was checked by performing a Western blot[14] against polyacrylamide gel maps of sodium dodecyl sulfate-solubilized autologous and allogeneic melanoma cell membranes. Briefly, 100 μl of each patient's anti-tumor antibodies were reacted against western blots, of the major protein bands of the patient's autologous tumor and against the 125 000-dalton band

isolated from two allogeneic tumors and from six melanoma cell lines, overnight at 4°C. The bound antibodies were detected by reacting the blot with ^{125}I-labelled anti-human IgG for 2 h at room temperature. Each band was cut from the nitrocellulose paper, washed in 0.1 M phosphate buffer and counted.

Affinichrom streptavidin immunoaffinity beads

The glass beads were cleaned, silanized and activated with carbonyldiimidazole as previously described[15–17]. The beads were air-dried and used immediately for immobilization of the streptavidin. A 10-g amount of the CDI-derivatized beads was suspended in 5 ml of doubly distilled water prior to the addition of 5 ml of 0.5 M carbonate buffer containing 2.5 mg of streptavidin. The mixture was placed into a 15-ml capped glass tube and incubated for 18 h at 4°C in an overhead mixer. Following this incubation, the beads were allowed to settle and washed ten times in 0.01 M phosphate buffer, pH 7.2 by sedimentation and decantation. Attachment of the streptavidin to the beads was checked by incubating a drop of the bead suspension, obtained from the last wash, with fluorescein-labelled biotin and examining 100 beads under a fluorescence microscope.

Construction of the immunoaffinity column

The idiotypic anti-tumor antibodies were biotinylated via their carbohydrate component of the fragment crystalline (Fc) portion by reacting them with hydrazide-derivatized biotin as previously described[15–18]. The biotinylated antibodies were immobilized on the streptavidin-coated glass beads by incubating 1 ml of the antibody solution (adjusted to 150 μg/ml in 0.5 M carbonate buffer, pH 8.5) with 10 g of the streptavidin-coated glass beads overnight at 4°C. The beads were then washed five times in 0.1 M phosphate buffer, pH 7.2 and slurry-packed into a 5 cm × 4.6 mm (I.D.) HPLC column and attached to the HPLC system.

HPIAC isolation of auto-anti-idiotypic antibodies

HPIAC was performed with a Beckman Model 340 isocratic high-performance liquid chromatography (HPLC) system (Beckman, Palo Alto, CA, U.S.A.), comprising a Model 112 pump, a Model 160 ultraviolet detector set at 280 nm and a Shimadzu C-R1B peak integrator (Shimadzu, Columbia, MD, U.S.A.). Elution control was performed by programming an Autochrom Model III OPG/S solvent selector (Autochrom, Milford, MA, U.S.A.).

A 100-μl volume of the patient's serum was injected into the system, through an Altex 210 injection port and the column isocratically developed at 1 ml/min for 15 min with 0.1 M phosphate buffer, pH 7.0 as the mobile phase. Throughout the entire run, the column temperature was maintained at 4°C by a glass column jacket, attached to a recycling ice-bath.

Following the initial 15-min run, during which the auto-anti-idiotypic antibodies reacted with the immobilized ligand, an elution recovery phase was started A chaotropic ion gradient was developed by adding 0 to 2.5 M sodium thiocyanate to the running buffer, over a further 15 min and maintained at the high level for 5 min before recycling the column back to the original running buffer. Fractions of the eluted material were collected in 500-μl Beckman Microfuge tubes, in a modified ISCO Cygnet fraction collector (ISCO, Lincoln, NB, U.S.A.) and dialyzed overnight at 4°C against 0.01 M phosphate, pH 7.2.

Inhibition studies

The specificity of the immunoaffinity purified anti-idiotypic antibodies was tested by studying their ability to inhibit the antigen-binding capacity of the original anti-tumor antibodies. The tumor cells were thawed at 37°C and following a cell count, adjusted to $1 \cdot 10^6$ cells/ml in RPMI 1640 medium prior to freezing and thawing three times. The disrupted cells were then sonicated for 2 min at maximum power. The sonicated pellet was resuspended in 2 ml of 0.01 M phosphate buffer and the membrane fraction was isolated by centrifugation of 100 000 g for 60 min. The membrane-enriched supernatant from the autologous tumor cells were labeled with ^{125}I by the lactoperoxidase technique[19] and incubated with either the anti-tumor antibody or a mixture (preincubated 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 radiolabelled antigen.

RESULTS

The presence of autologous cytotoxic anti-tumor antibodies was demonstrated in 41 of the 50 patients studied and was shown to be strongest at 21 days post-treatment. This anti-tumor activity was shown to last for five to six week before there was a sharp decline in the antibody concentrations. Repeat treatments produced a second peak of cytotoxic anti-tumor activity, that lasted for three to five weeks before returning to baseline.

Isolation of the anti-tumor antibodies from the positive samples, by absorption to fixed autologous tumor cells yielded an average IgG anti-tumor antibody concentration of 120–270 ng/ml but following cross-absorption against six melanoma cell lines resulted in a reduction of the anti-tumor antibody levels to 60–85 ng/ml. Western blot analysis demonstrated that the anti-tumor antibodies reacted only with

TABLE I

BINDING SPECIFICITY OF THE ANTI-TUMOR ANTIBODIES AGAINST WESTERN BLOTS OF TUMOR ANTIGENS

Blotted antigen	*Band (molecular weight, dalton)*	^{125}I-*labeled antibody binding (counts/min)*[a]
Autologous tumor	125 000	35199 ± 1587
Autologous tumor	92 000	305 ± 221
Autologous tumor	51 000	499 ± 515
Autologous tumor	21 000	428 ± 364
Allogeneic tumor I	125 000	914 ± 400
Allogeneic tumor II	125 000	1245 ± 704
Melanoma cell line 1	125 000	608 ± 627
Melanoma cell line 2	125 000	455 ± 309
Melanoma cell line 3	125 000	617 ± 300
Melanoma cell line 4	125 000	511 ± 363
Melanoma cell line 5	125 000	490 ± 388
Melanoma cell line 6	125 000	639 ± 426

[a] Counts expressed as mean minus backgroup ± standard error of the mean ($n = 41$).
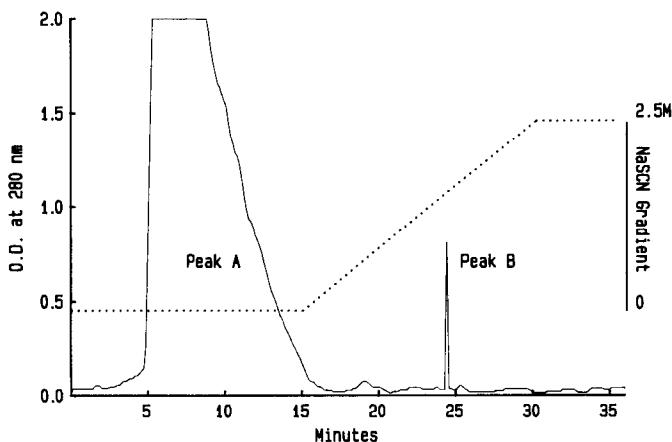
Fig. 1. HPIAC isolation of anti-idiotypic antibodies. Peaks: A = unreacted material; B = the anti-idiotypic antibody fraction. The dotted line represents the sodium thiocyanate gradient.

a 125 000-dalton band derived from the autologous tumor. The antibodies failed to react with any other blotted band, although several bands could be detected by silver staining of the membrane preparation. A lack of reactivity was also observed when the antibodies were reacted against Western blots of the membranes of the other patients tumor cells. These results are shown in Table I.

HPIAC isolation of the reactive anti-idiotypic antibodies produced the chromatogram shown in Fig. 1. Following the development of the primary peak (A) which contained non-specific serum products, the anti-idiotypic antibodies were isolated as a sharp, well-defined second peak (B), eluted at 24.5 min into the chromatography run. Immunoelectrophoretic and immunodiffusion studies demonstrated that only IgG was contained in the second peak at a concentration of 5–12 ng/ml. In twelve of the
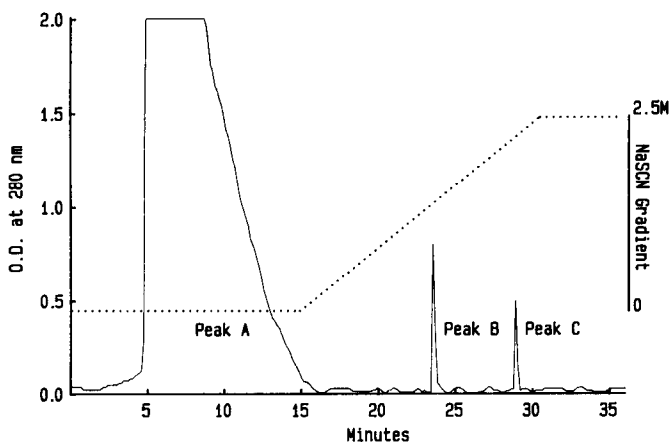

Fig. 2. HPIAC isolation of anti-idiotypic antibodies. Peaks: A = unreacted material; B = the anti-idiotypic antibody fraction; C = an anti-F(Ab)$'_2$ fraction. The dotted line represents the sodium thiocyanate gradient.
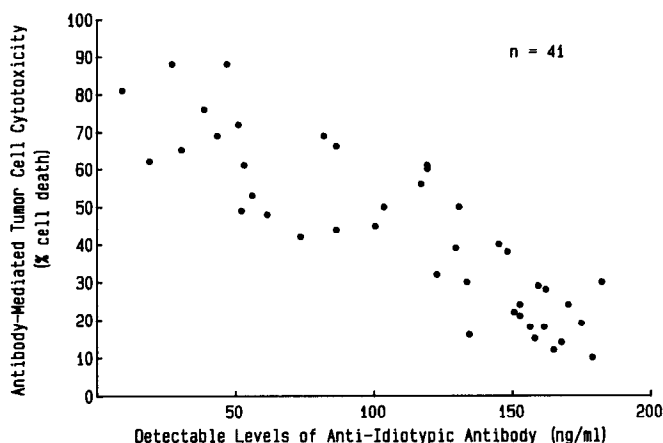
Fig. 3. Correlation between the concentrations of anti-idiotypic antibodies, as detected by immunoaffinity chromatography, and the concentrations of detectable anti-tumor antibody.

anti-tumor antibody positive patients, a different chromatographic pattern was seen (Fig. 2). Following the development of the primary peak (A), two distinct peaks were developed during the elution phase. The second peak (B) eluted at 24 min into the run and was followed by the third peak (C) at 28.5 min. Analysis of these two peaks demonstrated that both peaks contained IgG at concentrations of 5–15 ng/ml and 30–100 ng/ml, respectively.

Experiments on the effects of the isolated anti-idiotypes on the antigen-binding capacity of the original anti-tumor antibodies demonstrated that the antibodies isolated in the second peak (peak B) of the HPIAC runs possessed the ability to greatly reduce the activity of the anti-tumor antibodies. This effect could be shown to increase proportionally with the concentration of detectable anti-idiotype. The antibodies contained in the third peak (peak C) did not demonstrate any ability to inhibit the antigen-binding capacity of the original anti-tumor antibodies. Further studies, using Western blots of enzyme digests of the original antibodies demonstrated that the antibodies isolated in peak C reacted with the hinge region of the original anti-tumor antibodies and appears to be an anti-F(Ab)$'_2$ class of anti-antibodies.

The levels of anti-idiotype also correlated with a loss of cytotoxic anti-tumor activity in all of the patients monitored (Fig. 3). Chromium-release studies demonstrated that a decrease of 20–90% in the original cytotoxic reactivity of the anti-tumor antibodies could be demonstrated following incubation with the autologous anti-idiotype. This reduction in tumor cell kill was not present when the anti-tumor antibodies were pre-incubated with anti-idiotypes from other patients.

DISCUSSION

Several mechanisms have been put forward to explain the relative state of immunological unresponsiveness in cancer patients[20-22]. However, few studies have monitored the presence of regulatory idiotypic antibodies; possibly due to the difficulty involved in isolating and monitoring these highly specific antibodies. In an

attempt to overcome these difficulties, we have used the patient's autologous anti-tumor antibodies as immobilized ligands for isolating reactive anti-idiotypes by immunoaffinity chromatography. Isolation of reactive anti-idiotypes in an active form allows further investigation of their exact role in the suppression of the immune responses against growing tumors to be performed.

The presence of suppressive anti-idiotypes has been described in several different tumor systems, especially malignant melanoma[9,22,23] and lymphatic leukemia[24]. In the former case, the presence of anti-antibodies have been shown to correlate with the loss of active immune surveillance against the growing tumor. Studies on the effects of the anti-antibodies in the inhibition of tumoricidal activity has been shown to be proportional to the level of anti-antibodies detected in the patient serum[23]. In lymphatic leukemia, the use of laboratory-made anti-idiotypic antibodies, directed against idiotypic antibodies on the tumor cell membrane, has been suggested as a therapeutic procedure[24].

If the system forwarded by Jerne[25] is correct, then isolation of suppressive anti-idiotypes could have value in providing material for a vaccine capable of stimulating enhancing anti-idiotypes in cases where the immune monitoring demonstrates that a loss of the original idiotypic response is taking place. Animal studies have shown that anti-idiotypic antibodies can be elicited when a vaccine of idiotypic antibodies complexed with rheumatoid factor is injected into normal animals[26].

In this study we have demonstrated that specific anti-idiotypic antibodies can be isolated by HPIAC using the original idiotype as the ligand. Biotinylation of the original idiotypes by the hydrazine technique appeared to inhibit the binding of anti-Fc antibodies but could not inhibit the binding of anti-F(Ab)$'_2$ antibodies. However, the anti-idiotypic antibodies consistently eluted before the anti-F(Ab)$'_2$ antibodies, and this prevented contamination of the anti-idiotypic fractions with other types of anti-antibodies. In this way we feel that HPIAC is a reasonable technique for the isolation of regulatory anti-antibodies, especially when the ligand is coupled to streptavidin-coated glass beads. This form of attachment prevents binding of anti-Fc antibodies which are a major source of contamination in auto-anti-idiotypic antibody isolations. Streptavidin-coated Affinichrom solid glass beads[15-17] are a stable immunoaffinity packing media which can be used to immobilize antibodies for the immunoaffinity isolation of anti-antibodies. The packing is stable for 50–70 cycles and can be run at relatively high flow-rates with little deterioration. Immunoaffinity techniques using Affinichrom beads can isolate anti-idiotypes in under 30 min with no apparent loss of biological activity.

CONCLUSIONS

The use of HPIAC provides a technique which can detect, measure and isolate anti-idiotypic antibodies in under 30 min. This technique can be used in any facility that has a simple HPLC system and can be converted to batch isolations for research purposes. Once made, the immobilized antibody columns remain viable for 50–70 runs and are storable under refrigerated conditions for up to one year.

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