Use of Anticytokeratin Monoclonal Anti-Idiotypic Antibodies to Improve Tumor:Nontumor Ratio in Experimental Radioimmunolocalization

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Abstract

A syngeneic, high-affinity, anti-idiotypic monoclonal antibody (MAb; oTS1) raised against an anticytokeratin monoclonal antibody (TS1) was evaluated as a second antibody to promote the rapid clearance of radio-labeled TS1 from the blood during experimental radioimmunolocalization. By using a novel biosensor technology (BIAcore), association rate dissociation rate, and affinity constants between the idiotype and the anti-idiotypic could be determined. The in vivo results in nude mice carrying HeLa Hep 2 tumors demonstrate the possibility of selectively regulating the amount of the idiotypic 125I-labeled circulating MAb by in vivo injection of this high-affinity, anti-idiotypic antibody. Injection of the anti-idiotypic in a molar ratio of 0.75 to the idiotype cleared the blood pool from circulating radiolabeled idiotypic within 24 h, with a concomitant rapid excretion of 125I in urine. The total amount of remaining radioactivity in the animals decreased to 15–20% during these 24 h, with the tumors still retaining 60–65% of their initial radioactivity. This approach, using syngeneic primary and secondary MAbs with minimized immunogenicity, significantly improves the tumor:nontumor ratio, thus improving efficiency in experimental radioimmunolocalization and radioimmunotherapy, leaving the endogenous antibody repertoire of the host unaffected.

Introduction

RIT3 and RIL are developing and promising technologies to diagnose and treat tumors by the use of radiolabeled antibodies targeting tumor-specific antigens (1). The major reason why RIL and RIT are not efficient enough is the comparatively low accumulation of radiolabeled antibodies in the tumors. Irrespective of the antigen-antibody system used, the maximum tumor uptake is limited to less than 0.1% of the total injected dose (2–4), with a significant isotope load remaining in the blood pool and extravascular fluid.

Several approaches have been elaborated to increase the low tumor uptake and increase the tumor:nontumor ratio. For diagnostic purposes, Goldenberg et al. (5) developed a dual-isotope subtraction technique in which albumin was labeled with [99mTc]pertechnetate and injected in addition to the 131I-labeled antibody. By a computerized subtraction technique, detection of human tumors was improved. Other approaches have used fragmented MAbs, i.e., F(ab')2 and F(ab') fragments, single-chain Fv, or recombinant antibodies (6). Generally, fragments have been shown to be cleared rapidly from blood with less total tumor uptake and initially a larger kidney uptake compared with intact MAbs, making these techniques more useful for imaging than therapeutic purposes.

Other techniques to improve the tumor:nontumor ratio include extracorporeal IgG adsorption using MAbs (7) or two- or three-step approaches, Goldenberg et al. (5) developed a dual-isotope subtraction technique, detection of human tumors was improved. Other approaches have used fragmented MAbs, i.e., F(ab')2 and F(ab') fragments, single-chain Fv, or recombinant antibodies (6). Generally, fragments have been shown to be cleared rapidly from blood with less total tumor uptake and initially a larger kidney uptake compared with intact MAbs, making these techniques more useful for imaging than therapeutic purposes.

Inhibition ELISA for Anti-idiotypic Antibodies

One precipitating anticytokeratin 8 MAb (TS1) of the IgGlK subclass, earlier demonstrated to be efficient in RIL and RIT (18, 19), was used to generate anti-idiotypic MAbs. The TS1 MAb, at a concentration of 4.1 mg/ml, was conjugated for 1 h with KLH (Sigma Chemical Co.; 0.5 mg/ml) with 0.2% glutaraldehyde. After incubation, the conjugate was dialyzed 24 h against PBS (pH 7.4). Six- to 8-week-old female BALB/c mice (Bombolgaard, Ry, Denmark) were injected i.p. with 100 μg KLH-TS1 conjugate in Freund’s complete adjuvant. Six weeks later, the animals were boosted terminally by repetitive i.p. injections of 75 μg TS1-KLH on 3 consecutive days. On day 4, the spleen cells were fused with SP2/0 myeloma cells, as described earlier (1), at a cell ratio of 1:0.7. Detection of anti-idiotypic antibodies was performed in a specific inhibition ELISA (see below). One hybridoma clone was selected and recloned. MAbs were obtained from ascites and purified using Staphylococcal Protein A-Sepharose, as described earlier (20).

Materials and Methods

Generation of Anti-idiotypic Antibodies

One precipitating anticytokeratin 8 MAb (TS1) of the IgG1κ subclass, earlier demonstrated to be efficient in RIL and RIT (18, 19), was used to generate anti-idiotypic MAbs. The TS1 MAb, at a concentration of 4.1 mg/ml, was conjugated for 1 h with KLH (Sigma Chemical Co.; 0.5 mg/ml) with 0.2% glutaraldehyde. After incubation, the conjugate was dialyzed 24 h against PBS (pH 7.4). Six- to 8-week-old female BALB/c mice (Bombolgaard, Ry, Denmark) were injected i.p. with 100 μg KLH-TS1 conjugate in Freund’s complete adjuvant. Six weeks later, the animals were boosted terminally by repetitive i.p. injections of 75 μg TS1-KLH on 3 consecutive days. On day 4, the spleen cells were fused with SP2/0 myeloma cells, as described earlier (1), at a cell ratio of 1:0.7. Detection of anti-idiotypic antibodies was performed in a specific inhibition ELISA (see below). One hybridoma clone was selected and recloned. MAbs were obtained from ascites and purified using Protein A-Sepharose, as described earlier (20).

Inhibition ELISA for Anti-idiotypic Antibodies

The inhibition ELISA is illustrated schematically in Fig. 1. Microtiter plates were coated with a mixture of purified antigens and cytokeratins 8, 18, and 19 (tissue polypeptide antigen; 2 μg/ml in 0.1 M sodium bicarbonate buffer) and washed. Horseradish peroxidase conjugated TS1 mixed with supernatants from

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3 The abbreviations used are: RIT, radioimmunotherapy; RIL, radioimmunolocalization; MAb, monoclonal antibody; KLH, keyhole limpet hemocyanin; SA, secondary antibody.
The MAb (TS1) was for the in vivo experiments radiolabeled with $^{125}$I (New England Nuclear) to a specific activity of 40–130 megabecquerels/mg using the chloramine-T method (25). Free iodine was removed by gel filtration on a Sephadex G50 column (Pharmacia).

**In Vivo Experiments**

The in vivo effects of $^{125}$I-labeled TS1 and unlabeled αTS1 were examined in three separate experiments. The first, experiment A, was set up to study the total body radioactivity following injections of $^{125}$I-labeled TS1 and αTS1. In the second experiment, B, $^{125}$I levels in the blood pool and in the urine were examined, and in experiment C, finally, the clearance effect of non-tumor-targeted $^{125}$I-labeled TS1 by the use of αTS1 in experimental RIL was evaluated. In all experiments, the animals had free access to fresh water supplemented with 10 mm NaHCO$_3$ and 1 mg/ml KI (Lugol’s solution).

**Antibody Kinetics**

Six- to 8-week-old healthy female mice (BALB/c; Bomhoolgaard) were divided into two groups of six animals. All animals in both groups were given injections i.p. with 100 μg $^{125}$I-labeled TS1. Twenty-four hours later, one of the groups received an additional injection of 75 μg unlabeled αTS1 (molar ratio, αTS1:TS1, 0.75:1). The total radioactivity of each mouse was followed by repetitive measurements every 24 h with a gamma camera (General Electric Porte camera type II C) for 2 weeks.

**Evaluation of Kinetic Constants.** The $k_a$ and $k_d$ constants were determined by using the BIAcore kinetic evaluation software. The $k_a$ was taken as the ratio $k_a/k_d$. The amount of bound analyte ($R_a$) to the ligand, as well as the reaction rate ($dR_a/dt$) at specified time intervals, are given by the software. $R_{max}$ is the maximal amount of bound analyte to the ligand, and $C$ is the concentration of the analyte. The equation of the $dR_a/dt$ as a function of $R_a$ can be written as follows:

$$
dR_a/dt = k_a R_{max} C - R_a (k_a C + k_d).
$$

To calculate $k_a$, parts of the $dR_a/dt$ versus $R_a$ curve that reflect association are chosen. The slope of the $dR_a/dt$ versus $R_a$ curve at each concentration is plotted versus the concentrations in use to a straight line. This new slope gives the $k_a$. The $k_d$ was calculated from a specified time frame ($t_1 - t_2$) in the dissociation phase. The slope of the plot $\ln(R_a/I/R_s)$ versus $t_1 - t_2$ gives $k_d$.

**Fig. 1. Outline of the inhibition assay for anti-idiotypic antibodies. Left, interaction between only TS1-HRP and TPA, an interaction that generates substrate conversion and high absorbance. Center, nonspecific MAb allowed to interfere in the system, not affecting the TS1-HRP/TPA interaction. Right, anti-idiotypic antibody interferes with the TS1-HRP/TPA interaction by forming complexes with the idiotype, inhibiting substrate conversion (low absorbance). Bottom, final inhibition curve.**

The affinity of the idiotypic-anti-idiotypic interaction was measured using the BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden; 23). The molecular interactions occur on the surface of a CM 5 sensor chip (24). The sensor chip consists of a glass slide covered with a thin gold film bound to a carboxylated matrix of the dextran. One of the reactants, i.e., the ligand, is coupled covalently to the dextran matrix, whereas one or several reactants, i.e., the analytes, are allowed to pass over the surface. Optical phenomena of surface plasmon resonance are used to detect changes in optical properties as the concentration of molecules on the surface is modified. The changes in the resonance signal are referred to as resonance units. Monitoring this process continuously in real time generates a sensorgram, from which the association rate constant ($k_a$) and dissociation rate constant ($k_d$), as well as the affinity constant ($K_a$), can be determined by the Biosensor software.

**Equipment and Reagents.** The BIAcore system, CM5 sensor chip, P20 surfactant, and the amine-coupling kit, containing N-hydroxysuccimide, N-ethyl-N’-(3-diethylaminopropyl)carbodiimide, and ethanolamine-hydrochloride, were purchased from Pharmacia Biosensor AB. The MAb TS1 was obtained from InRo Biomedtek (Umeå, Sweden).

**Preparation of Sensor Surfaces.** The TS1 antibody was immobilized via free primary amino groups to the carboxylated matrix, according to standard procedures. To obtain an optimal capturing level, TS1 was dissolved in Na-acetate coupling buffer (pH 4.75) to a concentration of 100 μg/ml, and the time of activation by the N-ethyl-N’-(3-diethylaminopropyl)-carbodiimide/ N-hydroxysuccimide mixture was set to 60 s. The immobilization procedure was carried out at a continuous flow rate of the running HBS buffer (pH 7.4; HEPES, 10 mM; NaCl, 150 mM; EDTA, 3.4 mM and P20 surfactant, 0.05%) of 5 μm/min, whereas the real-time analysis of the idiotypic-anti-idiotypic interaction was carried out at a flow rate of 10 μl/min.

**Gel Electrophoresis**

Nondenaturing PAGE was performed according to Laemmli (22) in 25 mM Tris/190 mM glycerol (pH 8.6) in Mini-Protean 2 electrophoresis cells (Bio-Rad).

**Isoelectric Focusing**

Isoelectric focusing of TS1 and αTS1 was performed by the use of Pharmacia premade ampholyte plates according to the instructions from the manufacturer.

**BIAcore Biosensor System**

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**Radionlabeling**

The MAb (TS1) was for the in vivo experiments radiolabeled with $^{125}$I (New England Nuclear) to a specific activity of 40–130 megabecquerels/mg using the chloramine-T method (25). Free iodine was removed by gel filtration on a Sephadex G50 column (Pharmacia).

**In Vivo Experiments**

The in vivo effects of $^{125}$I-labeled TS1 and unlabeled αTS1 were examined in three separate experiments. The first, experiment A, was set up to study the total body radioactivity following injections of $^{125}$I-labeled TS1 and αTS1. In the second experiment, B, $^{125}$I levels in the blood pool and in the urine were examined, and in experiment C, finally, the clearance effect of non-tumor-targeted $^{125}$I-labeled TS1 by the use of αTS1 in experimental RIL was evaluated. In all experiments, the animals had free access to fresh water supplemented with 10 mm NaHCO$_3$ and 1 mg/ml KI (Lugol’s solution).

**Antibody Kinetics**

Six- to 8-week-old healthy female mice (BALB/c; Bomhoolgaard) were divided into two groups of six animals. All animals in both groups were given injections i.p. with 100 μg $^{125}$I-labeled TS1. Twenty-four hours later, one of the groups received an additional injection of 75 μg unlabeled αTS1 (molar ratio, αTS1:TS1, 0.75:1). The total radioactivity of each mouse was followed by repetitive measurements every 24 h with a gamma camera (General Electric Porte camera type II C) for 2 weeks.

**Fig. 2. Generation of anti-idiotypic MAb against the anticytokeratin 8 MAb TS1, using an ELISA inhibition assay. One anti-idiotypic antibody, named αTS1, was identified and selected for further studies.**
Results

Generation and Characterization of the Anti-idiotypic Antibody

In the fourth fusion, one single clone was able to cause significant inhibition in the ELISA. As seen in Fig. 2, the supernatant from this clone had activity to the same extent as that of the positive control serum obtained from the immunized mouse before fusion, containing polyclonal anti-idiotypic antibodies. This clone, named αTS1, was isolated and subcloned several times. It was isotype classified as an IgG1κ by the use of the Bio-Rad isotype characterization kit. At isoelectric focusing, αTS1 presented bands with an average isoelectric point of 7.4, distinct from TS1 (IP, 6.25).

Real-time Biospecific Interaction Analysis of the TS1-αTS1 Interaction. To optimize the conditions for a kinetic analysis, a low concentration of the immobilized ligand and a comparatively high flow rate are necessary. Due to the low solubility properties of the target antigens, i.e., cytokeratins, only the idiotypic-anti-idiotypic interaction was analyzed. The TS1 MAb was immobilized to approximately 1800 resonance units. The interaction between TS1 and αTS1 was found to be a high-affinity interaction \( (8.6 \times 10^{10} \text{M}^{-1}) \). The overlaid curve presented in Fig. 3 represents a typical kinetic run, with αTS1 interacting with TS1 at increasing concentrations. The high affinity is caused mainly by a very slow dissociation \( (i.e., \text{low } k_d) \) and not by a remarkably fast association \( (i.e., \text{high } k_a) \). Table 1 summarizes the \( k_a, k_d, \) and \( K_A \) as calculated by use of the Biosensor software. The correlation of the slopes in the \( \frac{dR_a}{dt} \) versus \( R_a \) curves and in the \( k_a, k_d \) versus the concentration curves varied between 0.997 and 0.999, indicating a high reliability of the experimental system. To verify that the slow dissociation not was a result of rebinding of dissociating αTS1 molecules, TS1 was injected (in much higher concentration than immobilized) during the dissociation phase. The TS1 injection during the dissociation of αTS1 did not affect the dissociation rate of αTS1, confirming the true slow dissociation (results not presented).

Nondenaturing PAGE of the TS1-αTS1 Interaction. To characterize the interaction between TS1 and αTS1, a 5% native PAGE was performed. Fig. 4 shows in Lanes 1 and 2 that TS1 and αTS1 have clearly different mobilities. In Lane 3, in which TS1 and αTS1 were mixed in equimolar amounts and left overnight, high molecular complexes were formed, which were permitted to enter the separation part of the gel only partly.
**In Vivo Experiments**

**Antibody Kinetics.** The results in Fig. 5, in which total-body radioactivity was measured, demonstrate the effects of injection of αTS1 in molar ratio of 0.75 compared with TS1. Twenty-four h after the αTS1 injection, a rapid decrease was observed, and approximately 15–20% of the total-body radioactivity remained in the group receiving the anti-idiotype, compared with the group receiving only 125I-labeled TS1. The major effects of the anti-idiotype were observed within the first 24 h after the injection. Approximately 48 h after the αTS1 injection, the curves of the 125I-labeled TS1, respectively, the 125I-labeled TS1- and αTS1-treated groups, were parallel.

**Total 125I Levels in Blood and Urine.** The changes of total 125I in the urine during the treatment of 125I-labeled TS1 and 125I-labeled TS1 plus αTS1 are visualized in Fig. 6. Following injection of αTS1, a significant increase during the next 50 h of 125I excreted in the urine can be observed compared with the group receiving no anti-idiotype. Also, a small initial increase in 125I in the urine following the injection of 125I-labeled TS1 can be observed.

In Fig. 7 serum samples from two representative mice, one from each group, are compared on PAGE and corresponding autoradiography. Lanes A, B, and C demonstrate that the administration of only radiolabeled idiotype (125I-labeled TS1) yields persistant high levels of circulating antibody, whereas, as seen in Lanes D, E, and F this is not the case when the anti-idiotype (αTS1) is used. As seen in Lane f in the autoradiogram, in the mouse treated with 125I-labeled TS1 and αTS1 24 h later, the radioactive band corresponding to 125I-labeled TS1 is missing. This confirms that the formed complexes between 125I-labeled TS1 and αTS1 are rapidly degraded rapidly and cleared from circulation 24 h after the αTS1 injection, as indicated in in vivo experiment A.

**Experimental RIL.** The effects of injection of the anti-idiotypic MAbs are seen in Fig. 8. The animals, when treated with 125I-labeled TS1, demonstrated activity in the tumor and also, to a significant degree, in other tissues. Following injection of αTS1, a clear demarcation of tumor to nontumor tissue was seen, and the remaining activity in the nontumor tissues was lower. The amount of activity was determined in 10 different tissues from each animal in the two experimental groups. The organs were dissected and weighed, and radioactivity was determined. The results are presented in Figs. 9 and 10. In Fig. 9, the amount of remaining radioactivity in the organs from the group treated with 125I-labeled TS1 and αTS1 was compared with the remaining activity in the group treated with only 125I-labeled TS1.
IMPROVED TUMOR TARGETING BY MONOCLONAL ANTI-IDIOTYPES

Fig. 8. Scintigraphic evaluation of two mice injected with $^{125}$I-labeled TS1 24 h after injection and prior to injection of αTS1 (top). The effect of injection of half-equimolar amounts of the anti-idiotype (αTS1) 48 h later (i.e., 72 h after the initial injection) is seen (bottom).

In Fig. 10, the remaining organ radioactivity was compared with the tumor tissue. All animals that received αTS1 displayed significantly lower levels of radioactivity in the selected organs compared with animals that received no αTS1. The largest decreases were observed for the blood, spleen, lungs, and kidneys, which retained only approximately 15–30% of their activity compared with the group that did not receive αTS1. The smallest decrease was observed in the tumor tissue, which retained approximately 60–65% of its activity, significantly higher than observed in all other organs. Organs such as skin and muscle also displayed small decreases, which are related to the lower absolute radioactivities, as seen in Fig. 10. These differences in activity reduction increase the tumor:nontumor ratio in the group treated with αTS1 compared with the untreated group 2–3-fold.

Discussion

The present investigation confirms the possibility of regulating the amount of one single, defined circulating radiolabeled monoclonal antibody selectively by in vivo injection of a high-affinity, anti-idiotypic secondary antibody. This enables an initial saturation of target antigens on the tumor cells by administration of a surplus of the radiolabeled primary idiotype. The target antigen in the present experimental system was cytokeratin 8. This antigen has several advantages, i.e., it is expressed in significant amounts in epithelial cells; furthermore, it appears in 70–80% of all human carcinomas (17, 26). During degradation of tumor tissues, it is released in a partially degraded form and is used as a tumor marker for carcinomas (27).

Earlier investigations have demonstrated that i.p.-injected idiotypes are transferred rapidly to the blood pool and reach their highest levels in the tumor tissue approximately 24 h after the initial injection (19, 28). We chose to inject the anti-idiotype i.p. 24 h after the idiotype injection to clear redundant circulating antibodies rapidly. By using i.p. and not i.v. injection of the anti-idiotype, the potential formation of high amounts of immunoprecipitates that may be harmful is reduced. The selected procedure causes a significant reduction in total body radioactivity, as visualized in Fig. 4, with the absence of circulating levels of radiolabeled idiotype on the autoradiogram. As the nonspecific uptake is reduced, this contributes to a higher tumor:nontumor ratio, which in RIL would give larger discriminatory and diagnostic potential.

Due to the specific targeting to the tumor, the relative decrease of radioactivity by injection of the anti-idiotype of radioactivity in the tumor is not of the same order of magnitude as seen in the other organs (Fig. 9). Approximately 60–65% of the activity remains in the tumor tissue, compared with 12–45% in other organs, increasing the tumor:nontumor ratio at least 2–3-fold. The partial decrease in the tumor may depend on the high affinity in the idiotypic-anti-idiotypic interaction; i.e., a competition between the target antigen and the anti-idiotype may occur. By selection of anti-idiotypes with optimal affinities (which might be lower or higher) and balanced injected

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Fig. 9. Remaining radioactivity expressed in percentages in different tissues in the group treated with $^{125}$I-labeled TS1 and αTS1 compared with the group receiving only $^{125}$I-labeled TS1. Remain, remaining tissues.
does, an even larger retention of the first antibody could be obtained. The BIACore technology provides possibilities of selecting such antibodies in vitro by evaluating the kinetic characteristics of the idiotypic-anti-idiotypic interaction. It should be emphasized that in vitro evaluations regarding the complex formation of isotype combinations of idiotypes and anti-idiotypes should be tested in vivo also, because differences in biodistribution have been reported by Pimm et al. (30, 31).

In earlier investigations regarding the use of anti-idiotypic MAbS to reduce the non-tumor-targeted antibody, a 100-fold molar excess of SA had to be used in order to be efficient (15). The use of a high-affinity anti-idiotypic in this investigation could reduce the amount of antibody needed to be injected compared with earlier investigations (11, 15) and could be balanced against the idiotype in a more controllable way. The processing of the in vivo-formed immune complexes is fast, because no or only trace amounts of high-molecular-weight idiotype-anti-idiotype complexes can be identified by autoradiography, assuming similar kinetics in the transfer of the idiotype and anti-idiotype from the peritoneal cavity to the circulation. The use of $^{131I}$ as a radionuclide is favorable, because uptake of free $^{125I}$ is limited to the thyroid, which can be blocked, as in this investigation. Other radionuclides, such as $^{99m}$Tc and $^{90}$Y, may cause accumulation in organs such as the liver, kidney, and bone marrow. Furthermore, the required time for absorption of the anti-idiotype from the peritoneal cavity, formation of immune complexes, phagocytic Fc-mediated uptake by macrophages, degradation, and excretion in the urine was estimated to be 12 h and is complete 50 h later. Similar kinetics, with low excretion in treated and untreated groups, can be seen in the later phases. This rapid and controllable excretion of a redundant isotope load in RIL (a decrease in total body radioactivity of 80–85%), using syngeneic primary MAbS and SAs with minimized immunogenicity, is the major reason why the approach of using anti-idiotypic antibodies for clearance is promising. Furthermore, only one single antibody (i.e., the idiotype) and not the endogenous antibody repertoire is affected, leaving the host with an intact humoral immune defense system.

References

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