Idiotypic-Anti-idiotype Antibody Interactions in Experimental Radioimmunotargeting

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Abstract

Idiotypic-anti-idiotype antibody interactions can be used in vivo to regulate the serum levels of specific radiolabeled antibodies. Anti-idiotype antibodies can also be used as clearing agents for radiolabeled antibodies in radioimmunolocalization and radioimmunotherapy. The present study describes the immunochemical interactions between the monoclonal idiotype (H7) and three generated monoclonal anti-idiotype antibodies (oLH7:1, oLH7:35, and oLH7:38). An unexpected variability in complex formation could be demonstrated in vitro, revealing three different stable complex patterns, i.e., low molecular weight 1:1 complexes, ladder formation with oligomeric, consecutively added constituents, and large linear polymeric complexes of high molecular weight. Within 24 h, the anti-idiotypes were able to cause a significant decrease in total body radioactivity, and the antibody generating a ladder formation (oLH7:38) was found to be the most efficient at removing radiolabeled idiotypes from the circulation. It is concluded that monoclonal anti-idiotype antibodies may be valuable tools in improving radioimmunolocalization and radioimmunotargeting.

Introduction

Major obstacles for radioimmunotherapy include the low accumulation of injected radiolabeled antibodies in the tumor and the presence of redundant nontargeted antibodies, which remain in the circulation for a considerable time, causing negative side effects in radiosensitive organs (1). Today, radiation doses needed to sterilize solid tumors are difficult to reach due to this toxicity. If the relative proportion of injected antibodies reaching the tumor could be increased, e.g., by clearing of nontargeted antibodies in the circulation, the therapeutic effects would be greatly enhanced.

Several strategies to achieve the clearing of nontargeted antibodies have been pursued. One approach is to use smaller antibody fragments such as F(ab')2, Fab, minibodies, and ScFvs (2–5), all of which clear rapidly due to their size but usually also demonstrate low accumulation. Two-step or multistep procedures based on the high-affinity (1015 M−1) avidin-biotin interaction have also been proposed to enhance efficacy. These procedures involve a tumor-specific mAb1 conjugated to biotin that is administered to the patients. The mAb-biotin complex administered in vivo accumulates in the tumor and is given time to disappear from the circulation. Radiolabeled avidin is then administered, which localizes to the biotinylated mAb accumulated in the tumor (6, 7).

A different approach to reduce the severe toxic effects of radiation is to administer recombinant cytokines such as granulocyte colony-stimulating factor and granulocyte macrophage colony-stimulating factor that promote granulocyte/macrophage proliferation and make it possible to reduce myelotoxicity by alleviating its manifestations (8–12). This strategy, however, does not address the issue of low tumor accumulation.

Another strategy to increase the T:N dose ratio is to reduce the number of circulating nontargeted radiolabeled antibodies in vivo. This can be achieved by immunophoresis or extracorporeal immunoadsorption, techniques in which the patient’s blood is cleared from radiolabeled antibodies using an online (continuous) antibody adsorption column (10, 13–16).

Anti-idiotype antibodies can be used to rapidly and specifically clear the radiolabeled idiotype from the circulation (17–23), increasing the T:N ratio up to 10 times. By using monoclonal, syngeneic, high-affinity anti-idiotype antibodies against anticytoketatin mAb TS1, we have recently shown that the total body radioactivity could be rapidly reduced by 80–85% with a 2–3-fold increase in the T:N ratio (23).

The stoichiometry of idiotype-anti-idiotype complexes has been studied previously by the use of electron microscopy (24–29), but the way we have pursued the study of these complexes has, to our knowledge, not been reported previously. mAb H7 against the oncofetal antigen PLAP has been used and proven effective in several experimental radioimmunotargeting studies (30–33). We have now generated three monoclonal anti-idiotype antibodies against mAb H7: (a) αH7:1, (b) αH7:35; and (c) αH7:38 (34, 35). One of these, αH7:1, has been shown to effectively reduce the levels of H7 in nude mice (35).

The aim of the present study is to investigate the immunochemical interaction patterns in vitro and to evaluate the efficacy of these three different anti-idiotypes (αH7:1, αH7:35, and αH7:38) as clearing agents for the radiolabeled idiotype H7 in mice.

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3 The abbreviations used are: mAb, monoclonal antibody; PLAP, placental alkaline phosphatase.
Materials and Methods

**mAbs.** A mAb against PLAP, H7 (36), and three monoclonal anti-idiotypes [αH7:1 (35), αH7:35, and αH7:38 (34)] were used (InRo Biomedtek AB, Umeå, Sweden). H7 is of the isotype IgG2A-κ, and αH7:1, αH7:35, and αH7:38 are of the isotype IgG1-κ. They were purified from mouse ascites on protein A-Sepharose (37).

**Gel Electrophoresis.** Gel electrophoresis was performed according to Laemmli (38) in mini-Protean 2 electrophoresis cells from Bio-Rad. Nondenaturing PAGE (4% gel) was performed at 5°C in 25 mM Tris/190 mM glycine (pH 8.6).

**Antibody Complex Formation in Vitro.** The interactions between the antibodies were studied to determine the complex patterns and their dependency on concentration and reaction time. The variability in the patterns formed between the idiotype, H7, and the three anti-idiotypes (Fig. 1) were studied by adding 5 μg of H7 to a test tube containing 5 μg of the respective anti-idiotype. The antibodies were incubated at room temperature for 10 min and then loaded on a nondenaturing PAGE gel. The importance of time for complex formation (Fig. 2) was investigated by incubating equimolar amounts of H7 and αH7:1 for different time periods (1, 5, 10, 20, 40, 90, and 180 min) before they were loaded on the nondenaturing PAGE gels.

The concentration dependency of the formation of the complexes between H7 and αH7:38 was studied by preparing an equimolar mixture (5 μg) with an equimolar mixture (10 μg) for 24 h in a moist chamber and then washed in 0.9% sodium chloride solution for 2 h before staining with Coomassie Brilliant Blue.

**Gel Precipitation.** Twenty ml of a 1% agarose solution were cast on an 85 × 95-mm glass plate. After 30 min, the wells were punched out, and a 9-μl sample containing 2 μg of antibody was added to each well. Equimolar amounts of the antibodies were used. Samples in the gel were allowed to react for 24 h in a moist chamber and then washed in 0.9% sodium chloride solution for 2 h before staining with Coomassie Brilliant Blue.

**Radiolabeling.** Purified H7 was labeled with 125I (New England Nuclear) using the chloramine-T method to a specific activity of 158 MBq/mg (39). Free iodine was removed on a Sephadex G-50 (Pharmacia) column.

**In Vivo Clearing of Radiolabeled Idiotype Antibody with the Anti-idiotypic Antibodies.** Sixteen female nude mice (nu/nu; Bomholtgaard, Denmark) were divided into four groups, groups A–D. All mice received a 38-μg i.p. injection of the idiotype mAb H7 labeled with 125I to a specific activity of 158 MBq/mg. On average, 6.01 MBq were injected into each mouse. At 24 h after the injection of the idiotype, a new injection with anti-idiotypic antibody was given i.p. to each mouse as follows: Group A, 20 μg of αH7:1; Group B, 20 μg of αH7:35; and Group C, 20 μg of αH7:38. The dose ratio between idiotype and anti-idiotypic antibody was 1:0.6. The injection volume was 60 μl in this experiment. The animals were sacrificed 5 h after the injection of the anti-idiotypic antibody and subsequently dissected, and the internal organs were weighed. The activity of the internal organs and the blood was measured using a gamma counter (1282 Compugamma; Upplands Väsby, Sweden).

Results

**PAGE Analyses.**

The interactions between idiotype and anti-idiotypes were investigated using native PAGE, in which the intrinsic charge of the antibody makes it possible to identify each antibody by its mobility in the gel.

When H7 and αH7:1 were mixed in equimolar amounts, characteristic heterogeneous precipitates were formed, as seen in Fig. 1 (Lane 5). Two different high molecular weight complexes can be seen: (a) one of higher molecular weight, which did not enter the gel; and (b) the other of lower molecular weight, which did enter the gel. Both mAbs were almost completely consumed.

A completely different type of complex formation was seen.
Antibody complex formation variability over time

Fig. 2 Nondenaturing 4% PAGE of the complex between H7 and αH7:1. Lanes 1–7, complex of H7 and αH7:1, with different incubation times before loading on the gel. (1, 2–5, 3–10, 4–20, 5–40, 6–90, and 7–180 min, respectively). Lane 8, H7 only; Lane 9, αH7:1 only.

Concentration dependency of antibody complex formation

Fig. 3 Nondenaturing 4% PAGE of the complex between H7 and αH7:38. Lane 1, equimolar conditions; Lane 2, surplus of H7; Lane 3, H7 only; Lane 4, αH7:38 only.

Idiotype–anti-idiotype precipitates

Fig. 4 Double immunodiffusion of equimolar amounts of idiotype and anti-idiotype antibodies in a 1% agarose gel for 24 h.

for H7 and αH7:38, which formed a typical ladder with at least six distinguishable bands with decreasing intensity (Fig. 1, Lane 6). There was no high molecular weight complex at the top of the gel, as compared with αH7:1.

A third pattern of reactivity was seen for H7 with αH7:35. This anti-idiotype, as seen in Fig. 1 (Lane 7), formed mainly a low molecular weight complex, with no formation of high molecular weight complexes seen at the top of the gel. The charge of this complex was intermediate as compared with its parent molecules, which indicates that this band consists of a 1:1 complex of H7-αH7:35.

The kinetics of the in vitro reactions were tested by PAGE as seen in Fig. 2, using H7 and αH7:1. The antibodies were mixed and incubated for periods of time ranging from 1 min to 3 h, demonstrating an almost immediate formation of complexes that were unaffected by longer incubation times. Similar stable patterns over time were also seen for the other anti-idiotypic antibodies, which were incubated in the same manner as αH7:1 for up to 3 h, with no conversion between the bands once they are formed (data not shown).

Unequimolar conditions gave the same bands with the surplus antibody, H7, as an extra band at the same position as when it is run alone (Fig. 3, Lane 2). When competitive studies were performed on PAGE, i.e., two anti-idiotypes were allowed to react simultaneously with H7, interesting patterns emerged. When αH7:35 and αH7:1 were allowed to react with H7, only complexes with αH7:35 were obtained. However, when αH7:38 competed with αH7:35, only αH7:38 formed complexes with H7, and when all three antibodies were allowed to react with H7, the entire complex formation was due to H7-αH7:38 complexes, as judged from the mobility of the complexes in the gel (data not shown). These results are in complete agreement with the affinity constants for these interactions (34).

Gel Precipitation

Fig. 4 illustrates the results of the double immunodiffusion performed with idiotype H7 together with each of the three anti-idiotypes. The strongest precipitate was seen between H7 and αH7:38. αH7:35 also gave a clear precipitate when reacting with H7, whereas there was a much weaker precipitate with αH7:1.

Animal Experiments

In Vivo Clearing of Radiolabeled Idiotype Antibody with the Anti-idiotypic Antibodies. The results of this experiment are presented in Fig. 5. Group A, the control group, showed a stable decrease of activity over time, whereas the three other groups showed a significant reduction of activity over the 20 h after the injection of the anti-idiotypic antibodies. After 20 h, about 30% of the activity had been cleared from the...
animals in group B (αH7:1), 25% of the activity had been cleared in group C (αH7:35), and as much as 75% of the activity had been cleared in group D (αH7:38). After 25 h, the decline rate of activity in all experimental groups was similar to that of the controls.

Measurements of the Activity Uptake in Different Organs during the Clearing of Radiolabeled Idiotype with the Anti-idiotype Antibodies. As can be seen in Fig. 6A, the average activity/gram of tissue in the different groups followed the same overall pattern for all the organs. The mean relative activity of the organs compared with the activity of the blood of each animal is presented in Fig. 6B; the results are similar, but the SEs are smaller. The group that received the fast clearing αH7:38 tends to present relatively lower levels of isotope in the liver and higher levels of isotope in the kidney as compared with the other groups (Fig. 6B).

Discussion

The selection of generated anti-idiotypes using an inhibition assay favors antibodies with an affinity high enough to compete with the interaction between the original antigen and the idiotype, in this case, PLAP-H7 binding. This interaction is known to be of high affinity, $10^9 \text{ M}^{-1}$ (35), and, as would be expected, the affinity constants for the idiotypic-anti-idiotypic interactions are also high [$10^7-10^9 \text{ M}^{-1}$ (34)].

It seems obvious from the present data using three different monoclonal anti-idiotypes that the interaction patterns between idiotype and anti-idiotype antibodies demonstrate a considerable variability (Fig. 1). With the three selected anti-idiotype antibodies, at least three different patterns can be demonstrated: (a) the formation of high molecular weight complexes; (b) ladder formation starting with dimeric complexes and then progressively larger and more weakly stained bands, forming, in this case, at least six different types of complexes; and (c) low molecular weight (dimeric) complexes between one idiotype and one anti-idiotype.

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**Fig. 5** Changes of the average whole-body activity of the different experimental groups, as measured with a gamma camera. Injection of radiolabeled idiotype H7 at −25 h was followed by an anti-idiotype injection to respective groups at time 0. The activity is expressed as a percentage of the activity of the respective group at time 0. The SD is shown.

**Fig. 6** A, absolute radioactivity of the blood and the internal organs (kcounts/min X gram). B, relative radioactivity, counts/(min X gram tissue) divided by counts/(min X gram) of the blood. Animals were sacrificed 5 h after anti-idiotype injection and dissected. The internal organs were weighed, and their activity was measured. Mean values and SEs are shown.
This intriguing pattern formation was unexpected, although some reports in the literature have documented related findings (25–29, 40), and the mechanisms controlling the generation of these complexes may only be speculated upon. The high molecular weight complexes (H7–αH7:1) may be formed by consecutive repetitive pairs of mAb1–mAb2, resulting in large linear complexes not entering the gel at PAGE (Fig. 1, Lane 5). One additional high molecular weight band, however, penetrates the gel. The molecular reasons for this are unknown. The equimolar consumption of the participating components during complex formation supports a ratio of H7:αH7:1 of 1.0 in the complexes.

We describe the second complex pattern observed, H7–αH7:38, as a ladder formation. These 10 different complexes are stable over time and do not seem to grow bigger once they have been assembled. This complex formation also seems to be independent of the relative concentration of the two mAbs (Fig. 3). The distribution of the stained band intensities indicates, when charge and size estimates have been made, the consecutive addition of two new mAbs for each new step in the ladder. The stability over time may indicate closed circles of antibodies rather than open chains. The homogeneity of the bands also support this concept, because if they were open chains, there would be a new band for every added antibody. Furthermore, because the charge of the anti-idiotype is different from that of the idiotype, the exact position of this complex would vary depending on the antibody added, and the bands would become heterogeneous.

The third type of pattern is a 1:1 complex of idiotype-anti-idiotypic complex. The charge of this complex is intermediate to that of its parent molecules (Fig. 1, Lane 7). The major reason for the absence of linear polymerization of this complex may be due to sterical factors. A monovalent interaction between idiotype-anti-idiotype may sterically block a second, bivalent interaction with another antibody or antibody complex. The idiotype-anti-idiotypic interaction may also simply be monogamously bivalent, a type of closed circle complex that has been observed with electron microscopy (25) and is thought to reflect an anti-idiotypic recognition of idiotopes located laterally on the idiotypic variable domain (27).

It has been reported previously (29) that an equimolar mixture of mutually reactive mAbs will, for thermodynamic reasons, preferentially form ring-shaped complexes with the fewest possible components. Ring dimers will therefore dominate when two fully flexible antibodies interact, but any limitations of this flexibility and/or steric hindrance will force the equilibrium to shift toward rings composed of four components or, if severely restricted, six components or even larger multiples of two (26, 28, 40–42). This agrees completely with the third type of complex (H7–αH7:35) that we could identify in this investigation. The second interaction pattern, the ladder formation, indicates that large complexes can indeed be formed, despite the thermodynamic force to generate complexes with the smallest number of components.

It is tempting to speculate on the putative biological implications of this repertoire of different complexes. Evidently, as judged from the present results, one of the anti-idiotypes (αH7:38) is twice as efficient at removing radiolabeled idiotypes from the circulation as the other anti-idiotypes (Fig. 5). This anti-idiotypic also exhibits strong immunoprecipitation (Fig. 4) and a high-affinity interaction with its idiotope (34) and presents a ladder formation when these complexes are examined by PAGE (Fig. 1). Whether or not any of these properties are related to the efficient clearing mechanisms remains a matter of speculation. It might technically be possible for one anti-idiotypic to bind two idiotypes, but there are no clear indications that this would be the case. Furthermore, the formation of cyclic aggregates necessarily includes equimolar contributions of both idiotype and anti-idiotypic.

The organ distribution of activity obtained 5 h after the injection of the anti-idiotypic reveals no obvious differences (Fig. 6A). The fast-clearing antibody tends to present relatively lower levels of isotope in the liver and higher levels of isotope in the kidney if the activity uptake of each organ is related to the blood activity of the respective group (Fig. 6B), which might be a reflection of a more rapid turnover.

References
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