Radiolysis of radioimmunoconjugates. Reduction in antigen-binding ability by α-particle radiation.

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Summary.

The potential of delivering a high and localized radiation dose using α-particle-emitting radioimmunoconjugates (RIC's) is investigated as treatment for some types of cancer. High specific activity of the RIC may for some uses be desirable, allowing enrichment of the radionuclide on membrane antigens on target cells. Radiation exposure during the radiolabelling procedures may, however, reduce the antigen binding ability of RIC's of high specific activity. The influence of α-particle dose on the cell binding fraction was therefore investigated for two monoclonal antibodies, i.e., TP-1 F(ab')$_2$ and TP-3 IgG. Samples of ^{125}I-labelled MoAb were added different amounts of [^{211}At]astatide to give accumulated doses ranging from 50 to 50,000 Gy. After the ^{211}At had decayed 15 half-lives, the cell binding fractions were measured. The

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results show that [^{211}At] astatide activities up to 25 kBq/μl, corresponding to an accumulated α-particle radiation dose of approximately 1,000 Gy, could be tolerated without significantly reducing the cell binding fraction, whereas higher doses gradually reduced the immunoreactivity. Although there may be differences in dose sensitivity of different radioimmunoconjugate due to differences in radionuclide conjugation methods, size of the antibody and structure of the antigen binding portion, the results presented here indicate that it is possible to produce radioimmunoconjugates of high specific activity with currently used methods of preparation. The potential problem of radiolysis should, however, be taken into consideration when planning procedures for preparation and use of α-particle-emitting radioimmunoconjugates.

Key words: Radiolysis; α-particles; radioimmunoconjugates; antigen-binding ability.

Introduction.

Radiolabelled monoclonal antibodies (MoAbs) have shown a clinical potential in treatment of cancers (1-3). The physical and chemical properties of the radionuclide, as well as the biochemical properties of the carrier-protein, define the therapeutic potential of a radioimmunoconjugate (RIC) (4,5). In treatment of minimal disease *e.g.*, small quantities of single tumor cells and micrometastases, MoAbs carrying α-particle-emitters may be particularly effective, due to the high linear energy transfer of the radiation, implying a few disintegrations on a cell surface are sufficient to cause cell inactivation (6-12).

A high specific activity may be desireable for a RIC, ensuring a sufficient number of radioactive atoms to disintegrate on the cell surface to give a high probability of cell-inactivation (7,13). The high ionization density of α-particles may, however, impose problems to the production and storage of RIC's. Radiolysis of the MoAbs may occur, particularly with RIC's of high specific activity.

We therefore present a study addressing the problem of α-particle radiolysis of RIC's. Radiolabelling and storage procedures for α-particle-emitting radioimmunoconjugates may thereby be adjusted to avoid decrease of quality due to radiolysis.

Materials and methods.

Monoclonal Antibodies

The two MoAbs TP-1 (IgG2a) and TP-3 (IgG2b) were obtained by fusion of X-63 Ag 865.3 mouse myeloma cells with spleen cells from mice immunized with human osteosarcoma cells and purified as described previously (14). $F(ab')_2$ fragment of TP-1 was prepared by treatment with pepsin (15).

Radiolabelling Procedure

The MoAbs were labelled with ^{125}I using the reagent N-succinimidyl-3-(trimethylstannyl)benzoate (NSTB). This reagent was synthesized and used, with minor modifications, according to a previously published method (16,17). Briefly, a 2 ml reaction vial containing 100 μl of chloroform was added 2 μl of glacial acetic acid, 0.15 μmol of NSTB, 30 μmol of *tert*-butylhydroperoxide, and finally, 5-15 μl of $Na^{125}I$ in NaOH (pH 7-10, Amersham). The vial was stirred for 20 min on a shaker and after that the organic layer was transfered to a new vial and evaporated to dryness with a stream of N_2-gas. The residue was resolved in 50 μl of chloroform and transferred to a Sep-pak silicagel cartridge (Waters) which was eluted with 30 ml of hexane, 30 ml of 8% ethylacetate in hexane, and finally 15 ml of 30% ethylacetate in hexane. The 30% ethylacetate eluted was collected in fractions of 1 ml. Fractions 4-6 contained 75% to 90% of the applied radioactivity as N-succinimidyl[^{125}I]iodobenzoate and were pooled. The solution was evaporated to dryness in a 2 ml vial, the residue added to 100 μl of MoAb solution (2 mg/ml in borate, pH 9) and shaken gently for 20 min. Finally 0.3 ml 0.2 M glycine (in borate, pH 9) was added and the reaction vial shaken for additional 5 min. Finally the radiolabelled MoAb was purified by elution through a Sephadex G-25 PD 10 column (Pharmacia). Radioimmunoconjugates with specific activity ranging from 30 to 80 MBq/mg were produced in this manner.

Cell Lines

The human osteosarcoma cell line OHS (18) was used for measuring the immunoreactive fractions of the MoAbs. The antigen expressed on this cell line is a monomeric polypeptide with

a molecular weight of approximately 80,000 (19). The human small-cell lung cancer cell line H 146 did not bind TP-1 or TP-3 significantly and was used as a control of nonspecific binding. Cells were cultured in monolayer in 80 cm^2 tissue culture flasks containing RPMI 1640 (Gibco) supplemented with 10% fetal calf serum.

Production of ^{211}At

^{211}At (half-life of 7.2 h) was produced at the Scanditronix MC-35 cyclotron at Oslo University using the $^{209}Bi(\alpha,2n)^{211}At$ reaction. A beam current of 10 to 12 μA and an α-particle energy of 28 MeV was used. The production yield was approximately 10 MBq/(μA/h) when a target thickness of 0.2 mm was used.

^{211}At was separated from the bismuth target by dry-distillation at approximately 660 °C (20). Volatile ^{211}At was led from the still using argon carrier-gas and through a dry, 2 ml, ice-cooled gas-washing bottle which acted as a cold-finger. Compared to the initial target radioactivity 40% to 70% was recovered after 1 h of distillation. In one experiment the condensed [^{211}At]astatide was made by washing the gas-washing bottle with phosphate buffered saline (PBS, pH 7.4, 0.1 M). In two other experiments condensed ^{211}At was dissolved in methanol and transferred to a 1/2 dram vial. The methanol was evaporated carefully with a stream of nitrogen and the ^{211}At disolved in 20 to 50 μl of PBS buffer. This last method was used to prepare more concentrated [^{211}At]astatide solutions, since it was difficult to wash the astatine off the condensing unit with very small volumes of PBS buffer due to adsorption of water on the glass surfaces. Dilutions with different concentrations of [^{211}At]astatide were made to obtain doses varying from 50 to 50,000 Gy in the following experiments.

α-Particle Radiation Exposure of Radioimmunoconjugates

To the tip of 2 ml plastic vials (Eppendorf) were carefully added 5 μl of the solution containing ^{125}I-labelled MoAb (1-2 μg/μl) and 5-15 μl of one of the solutions containing [^{211}At]astatide using a 25 μl HPLC syringe (Hamilton). The two solutions were mixed thoroughly without any spill outside of the droplet. The vials were stored at 5 °C for 48 h (to more than 99% of the ^{211}At had decayed) and after that in a freezer at −20 °C for three days.

Immunoreactive Fraction Measurements

For each dose level, three 2 ml test tubes containing 2.5 •10^6 cells in 0.25 ml tissue culture medium were added approximately 10 ng of MoAb in 10 μl of PBS. Thereafter the tubes were incubated for 2 h, counted for radioactivity, washed three times and finally counted for cell bound radioactivity. Both the antigen-positive and the antigen-negative MoAbs were tested. The cell binding of the exposed antibodies could thereby be compared to the untreated control to determine the retained cell binding ability after exposure.

The retained cell binding ability (RCBA) was calculated as follows:

RCBA = immunoreactive fraction of exposed RIC / immunoreactive fraction of unexposed RIC

Radiation dose calculation

The added activity value units were converted into radiation dose units by assuming that all the added ^{211}At disintegrated in the solutions, since in a 10 mm^3 aqueous sphere more than 95% of the α-particles emitted would be completely absorbed within the sphere. Assuming that an energy of average 6.8 MeV was absorbed in the solutions per transformation (21,22) the following equations could be derived:

1. Number of ^{211}At atoms = $N = D / \lambda = D \times t_{1/2} / \ln 2 = D\,(Bq) \times t_{1/2}\,(sec) / \ln 2$

2. Absorbed Dose = $E_{abs} / m = N \times 6.8$ MeV $\times$ $(1.60 \times 10^{-13}$ J / MeV)/m (kg of solution)

D = the number of nuclear transformation in Bq of ^{211}At at the start of the exposure;

$t_{1/2}$ = the halflife of ^{211}At = 7.2 h = 25920 s; m = mass of solution in kg.

Results.

The immunoreactive fractions of the ^{125}I-labelled TP-1 F(ab')$_2$ and TP-3 IgG, as measured by the one point assay using OHS cells, were in the range of 50% to 60% for the unexposed

controls. This corresponds to an immunoreactivity of 70% to 80% in the Lindmo plot where the values are extrapolated to infinite antigen excess (17,23).

Figure 1 presents the plots of added ^{211}At radioactivity versus retained cell binding ability and Figure 2 presents the plots of ^{211}At α-particle radiation dose versus retained cell binding ability for the two radioiodinated monoclonal antibodies and the OHS cells. Measurements were done after the added [^{211}At]astatide had disintegrated completely. The plots indicate a significant decrease in retained cell binding ability at doses above 1,000 Gy.

Nonspecific binding to the H 146 cell line was on the average approximately 5% (ranging from 3% to 9 %), and was not influenced significantly by α-particle exposure.

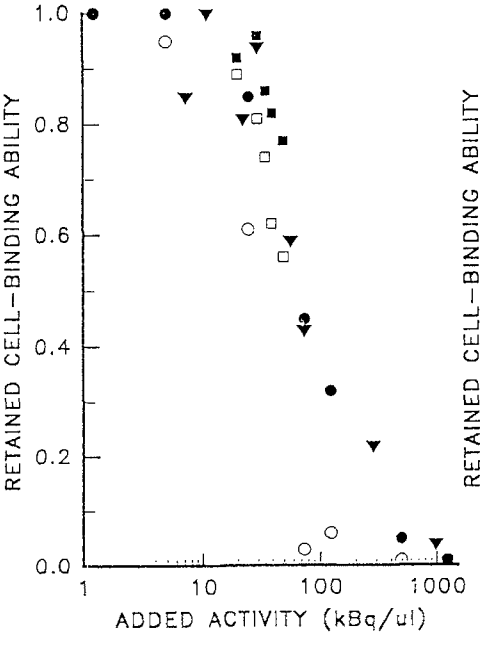

Figure 1. Retained cell binding ability of ^{125}I-labelled TP-1 F(ab')$_2$ (open symbols) and TP-3 IgG (closed symbols) measured after 15 half-lives decay of various amounts of added ^{211}At[astatide]. Different symbols indicate different experiments. Each point represents the mean value of a triplicate.

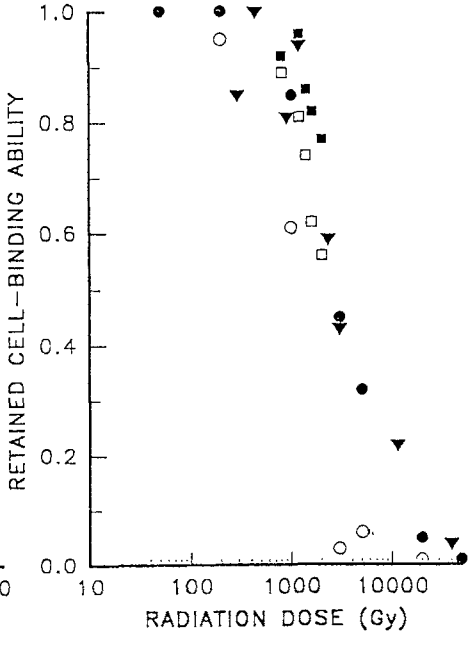

Figure 2. Retained cell binding ability expressed as a function of α-particle radiation dose. The doses were calculated using 6.8 MeV as the average energy absorbed in the solutions for each ^{211}At transformation. ^{125}I-labelled TP-1 F(ab')$_2$: Open symbols; ^{125}I-labelled TP-3 IgG: Closed symbols.

Discussion.

The work presented here represents, to the authors knowledge, the first study on the potential problem of self-radiolysis of radiolabelled monoclonal antibodies. The technical implications of α-particle induced radiolysis are focused rather then adressing molecular mechanism governing the effects observed. However, reduced immunoreactivity is likely to be caused by one or a combination of the three following molecular changes: 1) fragmentation of the MoAbs; 2) oxidation or reactions of radicals with chemical structures of the MoAbs; 3) release of radionuclide from the RICs due to direct radiolytic cleavage of the halogen-carbon bond or the benzoate peptide bond; 4) substitution of the halogen on the benzoate by radiolytic created radicals.

Radiolysis of different proteins in aqueous solutions has previously been studied extensively (24-26). Under anarobic conditions aggregation caused by covalent and noncovalent interactions between protein molecules was the main effect observed, while for aerobic conditions, as in the presented study, protein fragmentation was the main effect observed (27-29). In these previous studies significant radiolytic degradation was observed for γ- and x-ray doses as low as 450 Gy which agrees quite well with the findings of α-particle sensitivity in this investigation.

With the labelling methodology presently used for preparation of α-particle-emitting RICs, a very low fraction of the MoAb-molecules is actually radiolabelled (typically less than 1 in 500). Multiple labelling of a molecule is therefore highly unlikely. Although, intramolecular damage has a high probability to occur during radioactive transformation of a covalently bound α-emitter, the damaged MoAb molecule would have lost its only conjugated radioactive atom and would therefore not be of significant importance in radioimmunotargeting of cancer cells or counted in immunoreactivity measurements based on radioactivity detection. Intermolecular damage (*i.e.* an intact RIC exposed to radiation emitted from surrounding molecules) can, however, influence the antigen-binding fraction detected in the immunoreactivity assay. The methodology chosen in the present study was therefore to expose ^{125}I-labelled MoAbs to [^{211}At]astatide and thereby simulate the intermolecular radiolysis that may occur in concentrated

solutions of α-particle emitting RICs. Alternatively, different batches of ^{211}At-labelled MoAbs with varying specific activity and varying radiation dose exposure could have been prepared. The advantages of the chosen methodology were 1) batch to batch variations for different production runs were eliminated for the different dose levels within each experiment 2) higher radiation doses could be studied because loss of ^{211}At during the MoAb labelling procedure was avoided and longer exposure time was possible. It should also be noted that the labelling technique used for ^{125}I is similar for ^{211}At and utilize the same structure (*i.e.* mainly the lysine groups) on the MoAb. The lysine groups are also used for labelling with the other α-particle emitter preclinically studied for radioimmunotherapy, ^{212}Bi (11).

A slight reduction in immunoreactivity (approximately 15 % lower as measured by the Lindmo method) due to preparation and storage has previously been observed for actual ^{211}At-TP-3 preparations (13). During conjugation of N-succinimidyl-3-[^{211}At]astatobenzoate to the antibody, solutions of 300 μg of MoAb in 50 μl of buffer were exposed to approximately 50 MBq for 20 min. After purification by gelfiltration, 20 MBq of RIC in 750 μl was stored for 4 h (to the specific activity was reduced to approximately 50 MBq/mg) before immunoreactivity was measured. This procedure corresponds to an accumulated dose of approximately 1,700 Gy.

The results in this study indicate that α-particle radiolysis may significantly reduce the quality of a RIC at doses above 1,000 Gy. Although possible differences in radiation dose sensitivity of different RICs due to differences in radionuclide conjugation methods, size of the MoAb and structure of the antigen binding portion may occur, these results may be used to indicate an approximate dose level which may be tolerated for RICs. The conditions for preparation and storing of RICs should therefore be predetermined to keep the accumulated radiation dose significantly below this level. Measures taken to reduce the risk of radiolysis may include using a maximal dilution of MoAb and a minimal reaction time during conjugation of the radionuclide to the MoAb, a maximal dilution of the RIC immediately after the conjugation step, and to utilize the preparation as quickly as possible after purification. If this is done, potential clinically useful α-particle emitting RIC with high specific activity can be produced with at present available conjugation-methods.

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