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Bi-labelled antibody and Bi-labelled streptavidin. Comparison of targeting efficacy of a lymphoma cell line *in vitro.*

Gjermund Henriksen [1], Steinar Funderud [2] and Per Hoff [1]

[1] Department of Chemistry, University of Oslo, P.O box 1033 Blindern, 0315 Oslo, Norway, [2] Section for Immunology, The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway.

Summary

An anti-lymphoma antibody (HH-1) and streptavidin were conjugated with the chelator CHX-A DTPA and subsequently labelled with ^{205,206}Bi. The immunoreactivity of the antibody to the target antigen and the binding ability of streptavidin to antigen-bound biotinylated HH-1 were then investigated at different degrees of chelator content. Streptavidin was shown to be more tolerant than HH-1 towards the chelator modification. While the binding reactivity of HH-1 decreased to 60% at an average of 3.5 chelators per molecule, the 60 % level was obtained at an average of 6.5 chelators per streptavidin molecule. Streptavidin may therefore possibly be used to obtain ^{212,213}Bi-labelled compounds for α-particle radiotherapy with higher specific activity.

Key words: streptavidin; MoAbs ; targeting efficacy; α-particle radiotherapy.

Introduction

The potential of α-particle-emitting compounds in targeted radiotherapy has been widely recognized (1-3), but so far barely used clinically. Compared to the radiation modalities currently used in radiotherapy, α-particles may have significantly higher therapeutic effect on certain malignancies (4). Because of the high linear energy transfer (LET) and short pathlength relative to electrons, an α-particle is considerably more likely to inactivate a target cell by a direct mechanism in a single interaction with the cell-nucleus (5), while low-LET radiation require multiple interactions with the cellular DNA to inactivate a cell (6). Furthermore, the biological effect of low-LET radiation clearly depends on the chemical status of the tissue (7), while this effect is negligible for high-LET radiation. Finally, the α-particle has a significant ability to inactivate the same cell to which the α-emitter was bound (8,9). Therefore, this radiation mode may be advantageous for the treatment of strongly disseminated disease. For this situation targeted radiotherapy with β-emitters should be inferior because cross-fire is essential for obtaining sufficient effect.

^{212}Bi ($t_{1/2}$ = 60.6 min) and ^{213}Bi ($t_{1/2}$= 45.3 min) are considered among the most promising candidates for clinical use of α-emitters in radiotherapy. The preparation of monoclonal antibodies (MoAbs) labelled with these radioisotopes has been facilitated through the development of

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bifunctional chelating agents containing a stable binding site for bismuth and a functional group for binding covalently to MoAbs (10). As a consequence of the rather short radionuclide half-lives, the use of ^{212,213}Bi-labelled compounds is limited to tumours showing rapid enrichment of the α-active compound. Due to the rather unfavourable dynamics of antibodies in solid tissue (11), it is important to consider alternatives to direct Bi-labelling of MoAbs. One such alternative is the *in vivo* labelling of MoAbs by the use of the biotin-avidin system in a two-step procedure, reported to give improved conditions compared to direct use of MoAbs for tumour-diagnostic applications (12-14). This two-step procedure involves pretargeting of the tumour with biotinylated antibody followed by administration of radiolabelled (strept)avidin at a time when the ratio tumour:normal tissue of antibody is at a maximum. Because (strept)avidin is tetravalent for biotin, it might be expected that the binding affinity of (strept)avidin to biotin is less sensitive to the modification imposed by a chelator than the binding affinity of a MoAb to the corresponding antigen. If so, the acceptable number of chelators would be higher. This in turn would imply that the specific activity of (strept)avidin could be superior to that of certain MoAbs. For this strategy to be useful for ^{213,213}Bi, the amount of radioactive Bi binding to tumour cells must increase compared to direct targeting with MoAbs. It is an obvious prerequisite that this condition is fulfilled also in simple *in vitro* assays. In the present work, we have therefore used a well-characterized MoAb-antigen system and first investigated the sensitivity towards chelator-conjugation and Bi-labelling of the MoAb. Furthermore, the binding ability of Bi-labelled streptavidin to biotinylated MoAb on tumour cells was measured. These measurements facilitated a comparison of the tolerance of an anti-lymphoma antibody and of streptavidin to the attachment of the chelator CHX-A DTPA (15). A derivative of the DTPA ligand is used in clinical trials with β^--emitters (16) and the CHX-A DTPA will probably be the chelator of choice for the clinical use of ^{213,213}Bi-labelled MoAbs.

Materials and methods

Monoclonal antibodies and tumour-cell system

HH-1 (IgG1) recognizes a 45 kDa glycoprotein, CD37 antigen, expressed on the surface of Raji-Burkitt lymphoma cells. The average antigen expression per cell is $1{\cdot}10^5$ and the affinity constant, K_a, is $4.8{\cdot}10^8$ (17). The production and purification of HH-1 has been described elsewhere (18). The anti-osteosarcoma antibody TP-1 (IgG2a) was used as a negative control on the Raji-cells (19).

Conjugation of the chelator

To reduce the content of contaminating metal ions in the solutions used in the preparations of chelator-conjugates and in the radiolabelling procedures, all buffer solutions were deionized prior to use by passage through a 2x10cm column of Chelex-100, 100-200 mesh, Na^+-form cation exchange resin (Bio-Rad).

Conjugation of the chelator CHX-A DTPA was performed according to previously published procedures (20). Briefly, the desired quantity of MoAbs or streptavidin (Sigma) in borate solution (0.1M borate, 0.15 M KCl, adjusted to pH 9 with 0.1M NaOH), was allowed to react with 20-200 times molar excess of CHX-A DTPA for 24h at 25°C. Unconjugated chelator was then removed from chelator conjugates by 1:10 dilution with MES buffer (20mM 2-morpholinoethanesulphonic acid, 0.15M NaCl, 0.05% NaN_3) followed by centrifugation at 8000g. Micro-centrifuge tube filters (Whatman) with a molecular cut-off value of 20 kDa were used. To efficiently remove unreacted or free chelator, this step was repeated 4-5 times.

Quantification of chelator content

Determination of the amount of CHX-A DTPA bound to streptavidin and MoAbs was done from spectrophotometrical readings by titration with the 1:2 yttrium(III) complex of arsenazo III (21). Here, 4-5 aliquots of conjugates containing 0.3-0.5 nmoles of streptavidin- or MoAb were added serially to 3.0ml of the $Y^{III}(AAIII)_2$ solution. The changes in absorbance at 652nm were recorded after each addition. After correction for MoAb or streptavidin dilution, the mole ratios of CHX-A DTPA to MoAbs or streptavidin were determined by comparision to a calibration curve prepared by addition of 0.2-2 nmoles from an aqueous solution of the chelator.

Biotinylation of HH-1

N-hydroxysuccinimidyl 6-(biotinamido) hexanoate (Vector) in DMSO was added to HH-1 (5mg/ml) in 0.1M borate solution, pH 8.5 in a molar ratio of 10:1. The solution was placed on a shaker and the reaction was allowed to proceed for 15 h at 25°C. Free biotin was then removed using size-exclusion chromatography on a Sephadex-G25 Nap-5 column (Pharmacia) with PBS (0.1M phosphate, 0.15M saline) as eluent. The degree of biotinylation was measured spectrophotometrically according to Green (22). This method makes use of the change in absorbance at 500nm resulting from the interaction of (strept)avidin saturated with HABA ((2-4 hydroxyazobenzene)-benzoic acid) with free biotin. Samples of the biotin-conjugated antibody were digested with 1% pronase (Sigma) (23) for 12 h at 25°C prior to addition to the avidin/HABA solution (Sigma). A calibration curve obtained from three independently prepared biotin solutions was used as reference. To assay the availability of the biotin on HH-1, the conjugate was incubated with Raji cells and subsequently labelled with phycoerythrin-streptavidin conjugate (Vector Laboratories). Thereafter, the fluorescence intensity was analysed by a flow cytometer (Bio-Rad Bryte HS).

Production of $^{205,206}Bi$

^{205}Bi($t_{1/2}$= 15.3d) and ^{206}Bi($t_{1/2}$= 6.2d) were used as tracers in this work. These Bi- isotopes were produced from (p,xn) reactions on lead targets using the cyclotron (Scanditronix SC 35) at the Department of Physics, University of Oslo. The lead targets were irradiated with 5-12μA·h of 29-30MeV protons. The radiobismuth isotopes were separated from macro amounts of lead using a combination of lead precipitation and a lead-selective extraction-chromatographic resin as described elsewhere (24, 25).

Bismuth-labelling of MoAbs and avidin

The solution containing $^{205,206}Bi$ in 0.1M HNO_3 after the extraction-chromatographic step was loaded onto a 3x30mm AG50X4 (200-400 mesh), H^+ form resin (Bio-Rad). After passing through 0.3 ml of 0.1M HNO_3 and 0.3 ml deionized water, $^{205,206}Bi$ was eluted with 0.15 ml of 0.1M HI. pH was then adjusted to 4-4.5 with 3M Na-acetate and chelator-conjugated MoAbs or streptavidin in MES-buffer was added. After 15 min, the reaction was quenched by raising pH to 6 with 3M Na-acetate followed by 10μl of a $1 \cdot 10^{-2}$M DTPA solution to scavenge any free bismuth. Thereafter, labeled conjugate was separated from the free $^{205,206}Bi$ using a Sephadex G-25 NAP-5 column or a HPLC TSK G3000 SW_{XL} column (Toso Haas) with PBS as eluant.

The effect of the concentration of conjugated antibody and streptavidin on specific activity was measured by the addition of a constant amount of $^{205,206}Bi$ to various concentrations of CHX-A DTPA conjugates of either HH-1 or streptavidin. An energy and efficacy calibrated HPGe-detector (Canberra) was used to quantify the ^{205}Bi and ^{206}Bi. The concentration of Bi-atoms used in this assay was chosen to be equal to that of ^{212}Bi depositing approximately $1 \cdot 10^3$ Gy in the solution during the labeling procedure. This dose was assumed as an upper limit for this antibody to avoid significant decrease in immunoreactivity due to radiolysis from the α-emitters during labelling (26).

Immunoreactivity of biotinylated HH-1

The reactivity of the biotinylated HH-1 with Raji-cells was evaluated with an indirect immunofluorescence assay by flow cytometry. Raji cells at a fixed concentration were incubated with varying amounts of either HH-1-biotin or unconjugated HH-1 for comparison. The cell-bound antibody was detected by phycoerythrin-conjugated goat anti-mouse antibody (Southern Biotech). Analysis of the fluorescence intensity and the degree of positive staining were performed using flow cytometry.

Immunoreactivity of $^{205,206}Bi$-labelled MoAbs

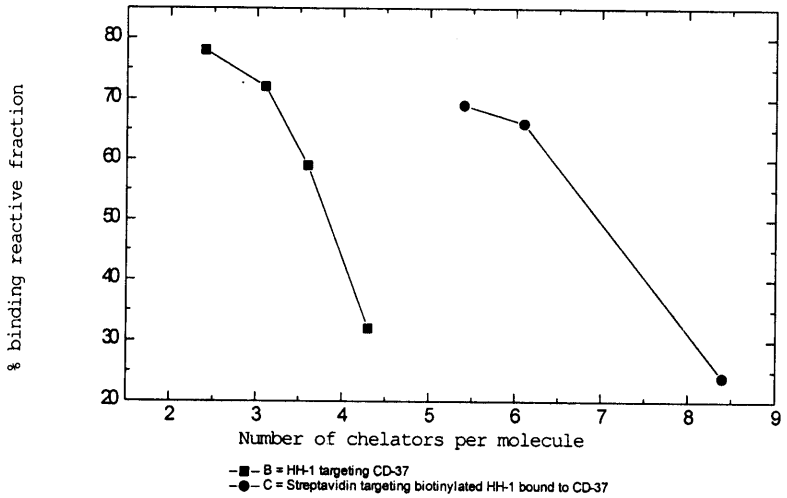
The immunoreactive fraction of CHX-A DTPA conjugated HH-1 was determined on Raji-cells using previously published procedures (27). Briefly, 6 different concentrations of cells ranging from $2 \cdot 10^7$ to $6 \cdot 10^5$cells/ml in a total volume of 0.2ml were added fixed amounts of $^{205,206}Bi$-labelled MoAb conjugates. After 1h the incubation was quenched, and the total activity in each vial was measured. To remove unbound antibody conjugates, the cell-suspensions were centrifuged at 200g for 10min and the cells were washed three times with PBS. Thereafter, the cell-bound activity was measured. The value used as the reactive fraction of the antibody was obtained by linear extrapolation of the regression line derived from the mean values of triplicates to infinite antigen excess.

Biotin-binding ability of streptavidin conjugates
The Raji-cells were incubated with a tenfold excess of biotinylated HH-1 for 30min. The cells were washed three times and resuspended in 2% BSA in PBS. Fixed amounts of ^{205,206}Bi-labelled streptavidin was added to various concentrations of preincubated cells and the reactive fraction was found using the same method as described above.

Results

There was no detectable reduction in immunoreactivity with an average of 1.7±0.3 (mean ± s.d, n=3) biotin per HH-1 molecule compared to the unmodified antibody, as measured by flow cytometry. Incubation of the biotinylated antibody with phycoerythrin conjugated streptavidin and the goat-anti-mouse antibody gave, like the unconjugated control, 97% positive staining which indicates sufficiently high availability of the conjugated biotin on this MoAb.

Figure 1
Binding reactivity of CHX-A DTPA conjugated HH-1 and streptavidin



[1]The chelator content was determined spectrophotometrically by titration of the three independently prepared conjugates with $Y(AAIII)_2$. See text for details.
[2] The immunoreactivities of HH-1 conjugates and the biotin-reactive fraction of streptavidin conjugates were determined as described in reference 27.

The immunoreactive fraction of HH-1 and the biotin-binding fraction of streptavidin are shown in Figure 1, as a function of the average amount of CHX-A DTPA conjugated. The CD-37 antigen reactivity of HH-1 was reduced to levels which are too low for obtaining sufficient tumour selectivity of the antibody when more than 3 molecules of this chelator was bound to it. In contrast, streptavidin was shown to have a greater tolerance for the chelator modification than this MoAb (Table 1). A sufficient binding reactivity of streptavidin measured on Raji-cells preincubated with biotinylated HH-1 was achieved with as much as 6 chelators per streptavidin molecule.

The unspecific binding of the TP-1 control antibody was less than 1%, showing high specific binding of Bi-labelled HH-1. A somewhat higher unspecific binding of streptavidin was obtained, as 4-5% of the initial activity was retained on Raji-cells which had not been preincubated with the biotinylated lymphoma antibody.

Table 2. Concentration dependence of CHX-A DTPA-conjugated HH-1 and streptavidin on the radiolabelling yield at constant ^{205,206}Bi-activity

HH-1			
Molar concentration[1] of conjugated HH-1[3]	Initial ratio of Bi atoms to antibody molecules	Radiolabelling yield %	Final ratio of Bi-atoms [2] to antibody molecules
0.0 [4]	1: 400	<0.1	$< 1{:}4.7{\cdot}10^5$
$1.3{\cdot}10^{-6}$	1: 47	12.9	$1{:}4.6{\cdot}10^2$
$2.6{\cdot}10^{-6}$	1: 94	25.1	$1{:}4.7{\cdot}10^2$
$5.2{\cdot}10^{-6}$	1: 188	53.7	$1{:}4.4{\cdot}10^2$
$1.3{\cdot}10^{-5}$	1: 470	63.8	$1{:}9.4{\cdot}10^2$
$2.6{\cdot}10^{-5}$	1: 940	68.8	$1{:}1.7{\cdot}10^3$

Streptavidin			
Molar concentration[1] of conjugated streptavidin [5]	Initial ratio of Bi atoms to streptavidin molecules	Radiolabelling yield %	Final ratio of Bi-atoms [2] to streptavidin molecules
[6] 0.0	1:668	<0.5	$<1{:}133{\cdot}10^3$
$5.0{\cdot}10^{-8}$	1:7	7.1	$1{:}1.0{\cdot}10^2$
$1.0{\cdot}10^{-7}$	1:14	17.1	$1{:}0.8{\cdot}10^2$
$1.6{\cdot}10^{-7}$	1:24	43.3	$1{:}0.5{\cdot}10^2$
$4.1{\cdot}10^{-7}$	1:60	61.1	$1{:}0.9{\cdot}10^2$
$8.2{\cdot}10^{-7}$	1:120	65.0	$1{:}1.8{\cdot}10^2$

The solutions used in the labelling experiments were of 250µl volume. Labelling time: 15 minutes.

[1] Based on absorbance values of the used protein stock-solutions at 280nm.
[2] The ^{205}Bi and^{206}Bi were quantified by γ-spectroscopy using a HPGe -detector. A constant amount of these radionuclides were added to the various HH-1 or streptavidin aliquots.
[3] All conjugated HH-1 used had on the average 3.1 chelators per antibody molecule, determined from spectrophotometry by titration of the conjugate against the 1:2 complex of Y^{III} with arsenazo III. Details are given in materials and methods.
[4] 100µg unconjugated HH-1.
[5] All streptavidin used had on the average 6.1 chelators per molecule.
[6] 70µg unconjugated streptavidin

Discussion

The biological effects of α-particles are considered to be dominated by a direct interaction with the cell nucleus. For a single-cell situation this implies that the degree of specific cell inactivation depends on the number of α-emitters bound to each cell. Therefore, for a given antigen expression, the specific activity of the labelled compound is largely determining the therapeutic efficacy (3). Radiolytic effects caused by disintegration of the radionuclide during labelling is likely to reduce the binding reactive fraction of the compound. This effect limits the achievable level of specific activity. Antibodies of the IgG-type labelled with α-emitters have previously been reported to fully retain their immunoreactivity after receiving doses of $1{\cdot}10^3$ Gy (26) to $2{\cdot}10^3$ Gy (4) from ^{211}At and ^{212}Bi, respectively. A direct extrapolation to the HH-1 antibody is not feasible, as its sensitivity may be different from that of other antibodies of the same class (26). An upper level of $1{\cdot}10^3$ Gy was assumed in this study. At these

corresponding conditions, the chelator concentration is seen to determine the radiolabelling yield (Table 1). This means that a high chelator content is crucial for obtaining high specific activity. The substantial loss of immunoreactivty resulting from the introduction of more than 3 chelators per HH-1 shows that this is a limiting factor. With the experimental conditions used here, labelling of the HH-1 antibody with a chelator content that is interpreted to sufficiently pertain immunoreactivity resulted in a maximum Bi:antibody ratio of 1:450 (Table 2). This specific labelling ratio implies that an average of approximately 250 Bi atoms could be bound to each cell at saturation of the CD-37 antigen by HH-1 on Raji-cells. Considering that the biotin on HH-1 is shown highly available for streptavidin, the higher specific labelling of streptavidin compared to the MoAb indicates that increased specific cell-inactivation may be obtained with this two-step procedure. Moreover, for tumour cells expressing several antigens, the administration of a mixture of MoAbs with affinity for different antigens should enhance the targeting efficiency. After preadministration of several biotinylated antibodies, Bi-labelled streptavidin could then function as common targeting molecule, facilitating the use of MoAb cocktails appropriate for the tumour cells in question.

For the two-step targeting procedure, circulating biotinylated antibodies and endogenous biotin could possibly represent a problem as they may interfere with the tumour targeting of streptavidin. Although the severity of the possible interference may depend on the tumour system, the two-step strategy used for diagnostic applications has been shown to give increased tumour uptake of radioactivity and improved tumour/non-tumour ratios compared to radiolabelled MoAbs (12-14). Furthermore, administration of unlabelled avidin prior to radiolabelled streptavidin to decrease the levels of circulating biotinylated MoAbs and endogenous biotin has been done without significantly decreasing tumour accumulation of streptavidin (28).

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