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IMMUNOCONJUGATE STABILITY IN VIVO MEASURED BY LABEL RELEASE FROM ^{125}I OR ^{99m}Tc-ANTIBODY COATED CELLS KEPT IN INTRAPERITONEAL DIFFUSION CHAMBERS IN MICE

JAN FJELD, BJØRN BRORSON, GRETHE KARIN MARTINUSSEN, HAAKON BENESTAD and KJELL NUSTAD

The present work demonstrates how intraperitoneal (i.p.) diffusion chambers (DC) can be used to investigate the in vivo stability of the bond between an antibody and its radioactive label. A monoclonal antibody (MoAb) was labelled with ^{125}I or ^{99m}Tc. The ^{125}I-labelled preparation showed high stability in vitro, since little radioactivity eluted from incubated DC containing ^{125}I-MoAb bound to specific, fixed target cells. Similarly, when we evaluated the ^{125}I-MoAb in vivo by using the i.p. DC the stability was intact. The ^{99m}Tc-MoAb was also stable in vitro, with only about 10% of the radioactivity lost after 48 h. However, when tested in vivo, about 50% of the ^{99m}Tc label was lost after 1 day, increasing to 60% after 2 days. Hence, by carrying out preclinical in vivo stability testing with i.p. DC method we discovered that an immunoconjugate with high stability as tested in vitro, in fact was unstable in vivo and probably unsuited for clinical use.

Radiolabelled monoclonal antibodies are used both in vitro in various immunoassays, and in vivo for immunotargeting purposes. The quality of the conjugates is a crucial factor, and standard assay procedures are available for in vitro analysis of immunoreactivity, avidity, and stability of the conjugate. When used in vivo, the characteristics of the conjugates may be quite different from what has been found in vitro. It is, however, difficult to obtain quantitative data on the detrimental effects of the in vivo conditions.

Tumour models can be applied to answer many of the questions that arise when labelled MoAbs are used in radioimmunotargeting (1). We have previously presented an artificial tumour model system, based on an intraperitoneal (i.p.) diffusion chamber (DC) technique. The DC consists of a plastic ring, with a micropore membrane wall on both sides of the ring. These chambers, filled with antigen-coated polymer particles (2) or viable or fixed tumour cells (3, 4), have previously proved useful for immunotargeting purposes. The DCs are implanted in the peritoneal cavity of experimental animals; they are permeable to proteins, including immunoglobulins, whereas the target cells of antigen-coated particles are kept within the DC. We have shown that this standardized, artificial 'tumour' can be used to quantify, under controlled conditions, the specific target uptake of intact antibodies or their fragments (4). Moreover, the antibody avidity in vivo could be estimated (5).

Good labelling techniques are crucial to the outcome of immunotargeting, and there is continuous search for new methods, with the purpose of exploiting new radionuclides or improving the conjugation methods for previously used labels. A novel and improved method for radiohalogenation has been developed (6). This method may represent a significant contribution to radioimmunotherapy, since it has opened the possibility for improved labelling with the

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From the Department of Clinical Chemistry, Rikehospitalet (JG Fjeld), Inst. of Basic Medical Sciences, Dept. of Physiology, University of Oslo (HB Benestad) and Central Laboratory, The Norwegian Radium Hospital (K. Nustad), Oslo, and Institutt for Energiteknikk, Kjeller (B Brorson, GK Martinussen), Norway.
Correspondence to: Dr Jan Fjeld, Dept. of Clinical Chemistry, Rikshospitalet, N-0027 Oslo, Norway.
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alpha-emitting radionuclide ^{211}At which is well-suited for localized radiation therapy (7). Other promising developments include a series of chelator systens (8), indirect methods based either on a double layer of antibodies, biotin-avidin (9), or bifunctional antibodies (10).

One of the most important achievements in diagnostic immunotargeting is the direct coupling of ^{99m}Tc to antibodies without the use of chelators (11–16). In the present paper we have used the i.p. DC technique to evaluate the in vivo stability of a MoAb labelled directly with either ^{99m}T or ^{125}I, and the results were compared with in vitro stability experiments.

Material and Methods

Diffusion chambers (DC). The chambers were constructed from 2 mm thick acrylic plastic rings with an outer diameter of 13 mm, and micropore filtration membranes (Millipore GSWP), mean pore diameter 0.22 μm. The membranes were heat-sealed to both sides of the ring. A suspension of antibody-coated cells was inoculated through an orifice in the ring, and a conical plastic plug was used to close the chamber (17).

Radiolabelled monoclonal antibody preparations. The murine MoAb K13 (IgG1) is specific for human light chains (18). The IgG of K13 was iodinated with ^{125}I, with iodogen (Pierce, U.S.A.) as oxidant, according to the procedure described by Fraker & Speck (19), as modified by Paus et al. (20). Procedures for reduction-mediated direct ^{99m}Tc-labelling of antibodies have been published (11–16). The IgG of K13 was labelled with ^{99m}Tc, with our own modification of the direct labelling procedure previously described by Mather & Ellison (14). The IgG of K13 was reduced by addition of 2-mercaptoethanol (Merck, Germany), molar ratio 1 : 10 000, in a 0.01 M PBS buffer, pH 7.4. After 30 min at room temperature, the preparation was purified by gel filtration (Sephadex G-25M, Pharmacia, Sweden). The number of thiols was determined at 326 nm after reduction with 4,4′-dithiodipyridine (Sigma, USA), and the reduced IgG of K13 was stored at −20°C until labelling. Labelling with ^{99m}Tc was performed by first adding 5–10 μl of a tinchloride solution (0.6 mg $SnCl_2 \cdot H_2O$ in 0.01 M HCl) to 0.5 ml of the reduced IgG (0.1–0.5 mg protein) in 0.01 M PBS, and then 300 MBq ^{99m}Tc-pertechnetate in 0.5 ml elution volume from an IFETEC ^{99m}Tc-generator (Institutt for Energiteknikk. Norway). The reactants were gently mixed, and the ^{99m}Tc-IgG was ready for use after 15 min reaction time.

Target tumour cells in the DC. The human lymphoma cell line Daudi produced immunoglobulin (IgG, IgM) with κ light chains (21) and could therefore be used as target cells for the κ light chain specific MoAb K13. The cells were fixed with 3% formaldehyde in PBS, washed 3 times, and resuspended in PBS with 0.1% BSA and 0.01% azide.

The fixed cells (10^7 cells/ml) were incubated at room temperature for about 1 h with the radiolabelled MoAb in excess, washed 4 times, resuspended in PBS with 0.1% BSA, and filled into the DC ($8 \cdot 10^5$ cells/160 μl).

Animals. The DCs were implanted i.p. in immunocompetent and randomly bred female mice (NMRI/Bom), 8–12 weeks of age and 20–25 g in weight.

Measurement of radioactivity release from the DC in vitro. The DC with radiolabelled antibody-coated cells were incubated in vitro at 37° in test tubes. Each tube contained 7 ml PBS with 0.1% BSA and 0.01% axide, as well as two DCs; one with ^{125}I-K13-coated cells and a second filled with the cells coated with ^{99m}Tc-K13. The tubes were submitted to continuous end-over-end rotation (10 cycles/min). The volume of 7 ml in the test tubes was chosen since this is close to the antibody distribution volume in a mouse (4). The amount of radioactivity remaining in the DC after various periods of time was measured in a multi-well gamma counter.

Measurement of radioactivity release from the DC in vivo. Each mouse was implanted i.p. with two DC, one filled with ^{125}I-K13-coated cells and a second with the cells coated with ^{99m}Tc-K13. The operation was performed under anaesthesia with a mixture of Dormicum (Roche, Switzerland) and Hypnorm (Janssen, Belgium), administered i.p. The animals were killed with ether overdosage after various periods of time, and radioactivity of the DC harvested from the peritoneal cavity was recorded as for the in vitro incubated DC.

Results

The immunoreactivity of the two different immunoconjugates, as tested in vitro, was found to be similar and good (results not shown).

Cells from the lymphoma cell line Daudi were incubated with the conjugates, washed, and filled into DC. In general, radioactivity loss from these immunoconjugate-coated cells may not be a consequence of label release alone, but antigen shedding or antigen-antibody dissociation may also contribute. To overcome this problem with the interpretation, experiments with two different labels was compared, while the antibody and the specific target cell were the same. Hence, in addition to the ^{99m}Tc, we also used ^{125}I for preparation of a second conjugate with the MoAb K13.

The DC with ^{125}I-K13-coated cells lost only about 10% radioactivity during the first 2 days, and this was the case both in vitro and in vivo (Figure).

In the experiments with the cells coated with the ^{99m}Tc-K13, the in vitro stability corresponded to the results with the ^{125}I-K13-coated cells, i.e. only about 10% loss after 2 days. In contrast, when the DCs containing the ^{99m}Tc-K13-coated cells were implanted i.p., as much as 60% radioactivity was lost during the same 2-day period (Figure).

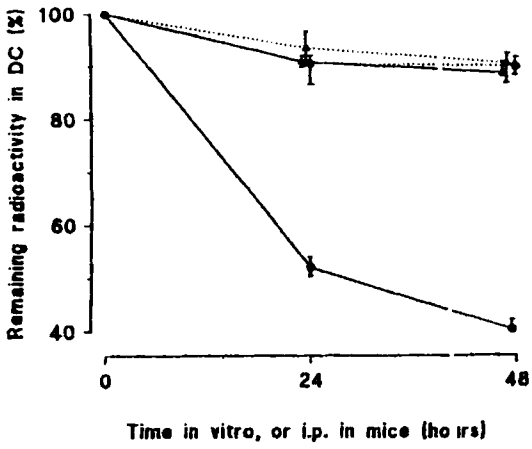

Figure. In vivo (——) and in vitro (· · ·) release of radioactivity from DC containing $8 \cdot 10^5$ fixed Daudi cells, coated with the specific monoclonal antibody K13 labelled with ^{125}I (▲) or ^{99m}Tc (●). Median and range for 4 DC in vitro and 3 DC in vivo.

Discussion

The artificial tumour model here applied can be used to obtain answers to several fundamental questions in radioimmunotargeting (2–5). The topic studied in the present paper, i.e. the effect of the in vivo conditions on the stability of radiolabelled antibodies, is difficult to predict from results obtained in vitro, and impossible to analyze in quantitative terms in patients or in experiments with tumour-carrying animals. In contrast, with artificial tumour model systems such as the i.p. DCs, exact measurement of the leakage of target antigen, bound antibody, or label from the antibody is possible. The last of these variables reflects the stability of the conjugate.

In the present work we showed that our ^{99m}Tc-labelled antibody was very stable in vitro, whereas an unexpected loss of ^{99m}Tc occurred in vivo, when the conjugate was exposed to extracellular, i.e. peritoneal fluid. Hence, the factors responsible for this loss were presumably of host origin. Thus this experiment proves that the stability of the ^{99m}Tc-conjugate should be improved before it is used in patient trials. Since low and equal amounts of radioactivity were lost both in vivo and in vitro from the ^{125}I-K13-coated cells, the only plausible explanation of the large radioactivity loss in vivo from the ^{99m}Tc-K13-coated cells is conjugate instability, i.e. dissociation of the ^{99m}Tc from the thiol groups on the immunoglobulin.

Though not representing a real tumour situation, our model experiments nevertheless indicate that in vivo loss of radioactive label may be a significant and unexpected problem in immunotargeting, at least when more than a few hours elapse between injection and detection. In vivo instability of directly labelled ^{99m}Tc-immunoconjugates have been reported (22). Our result with about 50% release during the first 24 h period does not support the suggestion by Hnatowich (23) that in vivo instability of immunoconjugates may not be pronounced during the first 5–24 h, which is the period normally required for adequate target localization and blood clearance.

In conclusion, our findings clearly demonstrated that in vitro tests for stability may not be representative for the situation in vivo. The mechanism of the in vitro vs. in vivo discrepancy remains to be clarified. The labelling method here presented was certainly not optimal with respect to the in vivo stability of the conjugate. Moreover, other monoclonals may give different stability with the same labelling method. However, the primary goal of the present paper was not to present an optimal and fully characterized conjugation procedure, but rather to demonstrate how the i.p. diffusion chamber method can be utilized to detect in vivo instability, when the same preparation seems stable as tested in vitro. Work to improve the conjugate stability is in progress.

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