CONSTRUCTION AND BIOLOGICAL *IN VITRO* EVALUATION OF IMMUNOTOXINS BY LINKING OF MONOCLONAL ANTIBODIES WITH A HAEMOLYTIC TOXIN FROM A SEA ANEMONE

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Summary

The immunotoxins (IT) are bifunctional proteins which are obtained by conjugation of monoclonal antibodies (MAb) and toxins. This kind of molecules has the monoclonal specificity and the toxic properties are conferred by the toxin component. The cell which carried the appropriate receptor will selectively killed by the immunotoxin. In the present work we show the construction of IT using chemical methods and its *in vitro* biological evaluation. The hybrid molecules were built by covalent linking between the hemolytic toxin and the 17-1A and IOR-R3 monoclonal antibodies that recognize a 37kDa protein expressed in colorectal human carcinoma (17-1A MAb) and the human Epidermal Growth Factor receptor (hEGFr) (IOR R3 MAb), respectively. The toxin was conjugated to the MAbs using the heterobifunctional reagent N-succinimidyl-3(2-piridilditio) propionate (SPDP). The thiolated and reduced HT and the thiolated MAb were mixed in a molar ratio 1:1 and 7:1 for IOR-R3 MAb and 17-1A MAb, respectively. The reaction time was 72 hours at 4°C. Toxicity *in vitro* for IOR R3-HT for was measured in H125 cell line (human lung adenocarcinoma) which carried the hEGFr and on U1906 cell line (small cell lung carcinoma) which did not carry the hEGFr. Toxicity *in vitro* for 171A-HT for was measured in SW1116 cell line (human colon carcinoma) which carried the antigen and on MW14 cell line (human melanome) which did not express the antigen. A hybrid molecule was obtained in both cases. The biological activity of IT´s components were preserved as it was demonstrated by the selective citotoxicity experiments with cell lines. Membrane-active molecules of these kinds could be good choice for building up immunotoxin comprising antibodies towards antigen which not need endocytic internalization like carcinoma-associated antigens.

KEY WORDS: monoclonal antibody, immunotoxin, molecular hybrids, cancer.

Introduction

Chemotherapy treatment has some major problems in cancer therapy, due to the lack of selectivity of most anticancer drugs, the difficulty of accessing the tumour issue, and the dynamic biology of the tumour growth.(1). In the last years, one approach has been explored to improve selectivity involving the conjugation of drugs and toxins to cell-surface directed antibodies. The availability of monoclonal antibodies has stimulated an intensive search for antibodies that recognizes specific cells including cancer cells. A number of monoclonal antibodies against tumour cells have been coupled to a variety of toxin derivates. The resulting of antibody-toxin conjugates is named immunotoxins (2). These are a group of artificially-made molecules which are cytotoxic for the targeting of cancer cells, and may be a new option for the treatment of malignancies resistant to conventional treatments (3,4). The most common agents employed for the construction of immunotoxins by chemical methods have been plant and bacterial toxins that inhibits protein synthesis at ribosome level by internalization of the antigen-antibody complex (5,6), which is usually the rate-limiting step in cell-killing kinetics (7). We have tried to bypass this difficulty by using a hemolytic toxin active at the cell membrane level, which can be covalently linked to monoclonal antibodies by artificial disulphide bridges. (8,9). In the present paper we shows the preparation and *in vitro* evaluation of two immunotoxins by linking an hemolytic toxin to a monoclonal antibody that recognizes the epidermal growth factor receptor (hR3) and another monoclonal antibody towards to a protein of 37kDa expressed in carcinoma human colorectal (17-1A Mab).

Methods

Monoclonal antibody (Mab): The monoclonal antibody IOR-R3 (IOR R3 MAb) was kindly supplied for the Center of Molecular Immunology of Havana, and was generated by fusion of murine myeloma SP2/O with splenocytes obtained from mice that were immunized with a semipurified fraction of human placenta hREGF rich (10). It is an IgG2a subclass and it recognizes the H125 cell line, a lung adenocarcinoma that express hEGF-R and it doesn't recognize U1906 cell line, a carcinoma of small cells of lung (11). The 17-1A Mab was kindly supplied by Karolinska Hospital of Stockholm, Sweden. The murine 17-1A Mab is secreted by hybridomas obtained from the fusion of the P3X63Ag8 myeloma (12) with spleen cells of animals immunized with SW1083 cells. This is an IgG2a Mab that identifies an antigen, a 40kDa protein, selectively expressed on human colorectal carcinomas (13). It recognizes the SW1116 cell line, a human colon carcinoma that express the 37 kDa protein and it doesn't recognize the MW14 cell line, a melanoma cell lines that don’t express the 37 kDa protein.

Hemolytic toxin: The hemolytic toxin (HT) was isolated from a saline extract of the sea anemone *Stichodactyla helianthus* (before Stoichactis helianthus).

Purification was performed by acetone precipitation, gel filtration (Sephadex G-50) and cation exchange (CM-Sephadex C-50) (14). The HT shows an apparent molecular weight of about 18 kDa and isoelectric point to pH 9.8 produces complete lysis of human erythrocytes at concentrations above 0.6 μg/ml. (14). It exhibits a potent citolytic activity. (15).

Hemolytic activity: The qualitative identification of hemolytic activity in the chromatographic fractions was done by adding 20μL from the fraction to 100μL of a 1% saline-washed human erythrocytes and incubating during 10 min, at room temperature. After centrifugation (500g, 10 min, 40^0C) the released hemoglobin was estimated at 540nm.

Preparation of immunotoxins: The toxin was conjugated to the monoclonal antibodies using the heterobifunctional reagent N-succinimidyl-3(2-pyridildithio) propionate (SPDP, Pharmacia, Uppsala, Sweden) following the manufacturer instructions (16). The modified HT and monoclonal antibodies were obtained by reaction with an excess of SPDP of 3 and 70, for 17-1A and IOR-R3 Mabs, respectively. The extent of thiolation was determined by the variation of the optical density at 343nm due to the liberation of groups pyridine 2-thione, after the reduction with 100mM dithiothreitol (DTT). The reduced toxin was mixed with the pyridyl disulphide derivate at 1:1 and 7:1 molar ratio for IOR R3 MAb and 17-1A Mab, respectively during 72 hours, at 4^0C. The reaction mixture was fractionated on an Ultrogel AcA44 column (1.5x74 cm) for 17-1A-HT immunotoxin and Sephacryl S-200 column (2.2x33cm) of the IOR-R3-HT immunotoxin. The extent of conjugation was estimated following the release of pyridine 2-thione at 343nm, and by measuring the hemolytic activity released after reduction of the conjugated.

In vitro cytotoxicity: A citotoxicity assay for the 17-1A-HT immunotoxin was performed on the SW1116 and MW14 cell line by Neutral Red dye incorporation. Cells were distributed in a round bottomed 96well tissue culture plate ($2x10^4$cells/well) incubated for 24 hours at 37^0C with the fraction to be tested. After incubation, the supernatants were removed and the optical density measured at 540nm. Control cultures were incubated with medium only. For IOR R3-HT immunotoxin, a citotoxicity assay was performed by measuring the inhibition of tumor cell proliferation by [^{3}H] thymidine incorporation. The tumor cells (H125 and U1906) were added in a round-bottomed 96-well tissue culture plate ($1x10^4$cells/well) and incubated overnight at 37^oC in 5% CO_2(g) atmosphere. Culture medium was removed and the cells were incubated with the samples (100μL) for 48 hours. [^{3}H] thymidine (1mCi/well) was added for 7 hours at 37^0C. After trypsinization, the cells were harvested and the radioactivity was counted. All counts were expressed as the percentage of [^{3}H] thymidine incorporation in the test cells versus control cells.

Stability study of IOR-R3-HT immunotoxin: The IOR R3-HT immunotoxin, the free IOR-R3 Mab and the free HT were stored at 45º, 53º and 60ºC during 2 to 15 days in order to predict the immunotoxin stability during long periods of storage. The Arrhenius' expression was used to predict the stability of the compound.

Results

The chromatogram of the coupling reaction mixture showed two peaks, followed by optical density at 280 nm. The fractions from the first peak, corresponding to the immunotoxin, didn't show hemolytic activity. However, after reduction conditions with DTT (50mM, 40 min, 25^0C) the hemolytic activity was recovered and was coincident with the first peak, near the void volume (V_0) After the conjugation reaction, the hybrid molecule did not exhibit hemolytic activity and after reduction with DTT, the hemolytic activity was recovered. The estimated conjugation ratio of toxin per molecule of antibody was 2.5 and 3 for IOR R3-TH and 17-1A-TH, respectively (Fig.1).

Because the higher expression of the EGF-R in H125 cell line, the IOR R3-HT immunotoxin was more toxic in this type of cells, comparing with U1906. The immunotoxin inhibited the cellular proliferation after exposition to reducing conditions in a similar way to free HT in H125 and U1906 cell lines Unconjugated IOR R3 Mab was nontoxic for either kind of cell while the free toxin was toxic for both. (Fig.2a).The 17-1A-HT immunotoxin was toxic for SW1116 cells, and nontoxic for MW14 cells. The addition of an excess of free 17-1A Mab protected the SW1116 cells from the toxicity of the immunotoxin (Fig.2b). Unconjugated 17-1A Mab was nontoxic for either kind of cell while the free toxin was toxic for both.

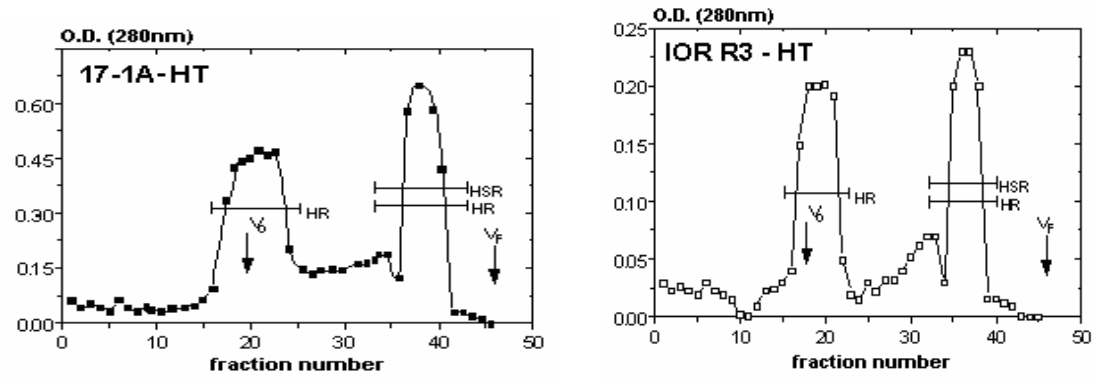

Figure 1. Gel filtration chromatography of the conjugation mixture. Arrows indicate the void volume (V_o) and the total volume of the column (V_f) **HR :** Hemolytic activity after reduced with DTT 50mM **HSR :** Hemolytic activity without reducing with DTT 50mM.

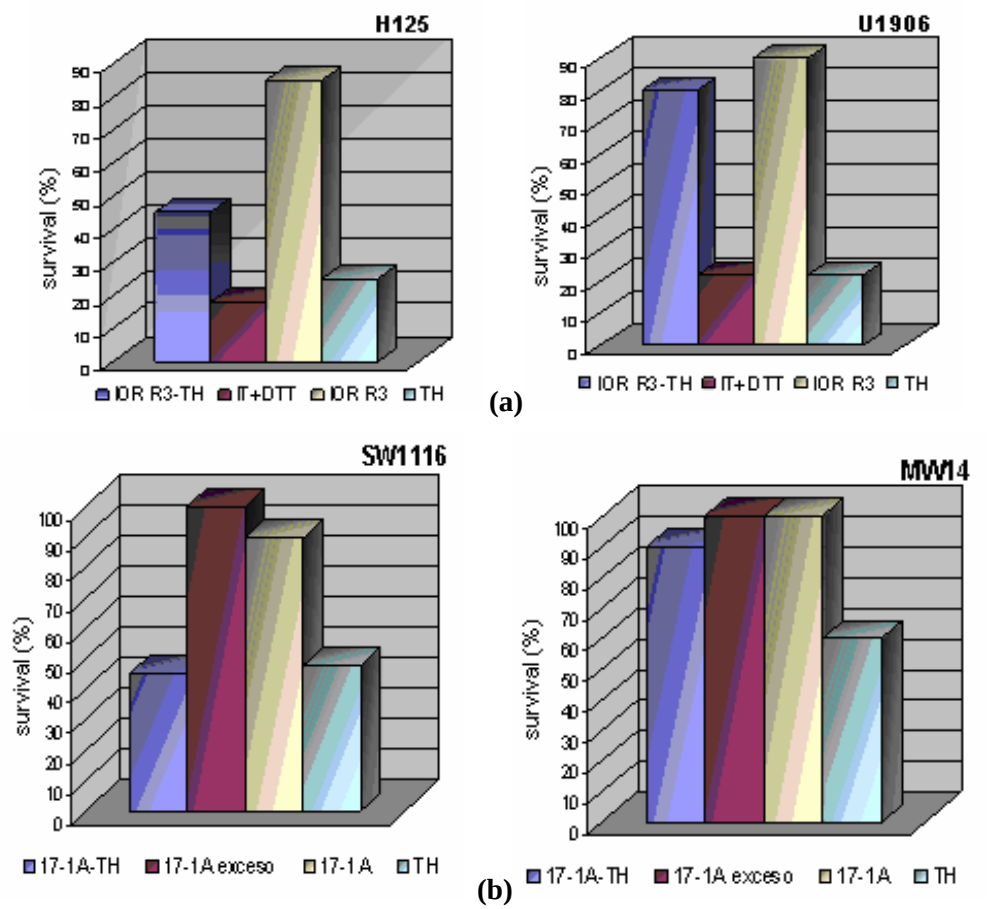

Figure 2. Cytotoxicity studies of the immunotoxins IOR R3 –HT on H125 and U1906 cell lines by measuring the inhibition of tumor cell proliferation by [^{3}H] thymidine incorporation and 17-1A-HT on SW1116 and MW14 cell lines by Neutral Red dye incorporation.

Thermostability of the IOR R3-HT immunotoxin.

The stability of the conjugated IOR R3-TH (Fig. 3) was studied by means of the lost of the biological activity for each components in the **IT** for separate as well as the whole conjugate in a accelerated heat-degradation process. The Arrhenius' expression was used to predict the stability of the compound stored at 4^0C. HT was highly resistant to heat degradation (data not shown) in the temperature interval we studied here. At 60^0C the biological activity was severely damaged for the three molecules. According to these results the IT should lose a 50% of its properties after 1.5 years, stored at 4^0C.

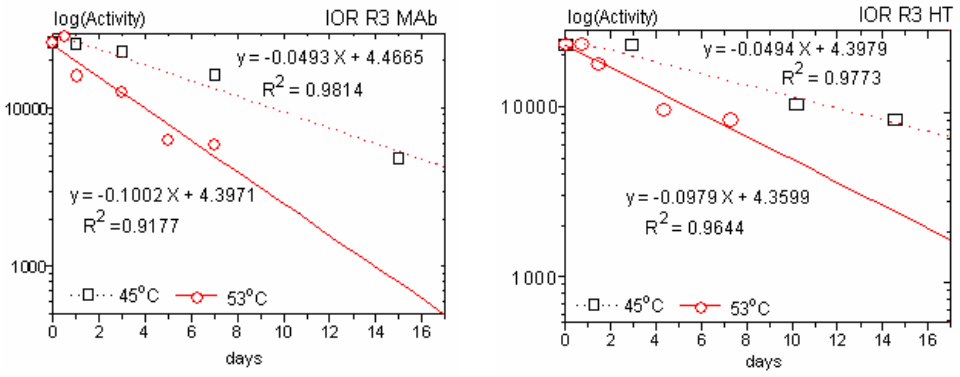

Figure 3. Thermo stability of the IOR R3-HT immunotoxin. The IOR R3-HT, free IOR-R3 Mab were stored at 45º, 53º during 2 to 15 days in order to predict the immunotoxin stability during long periods of storage.

Discussion

The most interesting properties of both IOR-R3-HT and 17-1A-HT immunotoxins are the "lost" of hemolytic activity which is recovered under reduction conditions and the toxic component is released from the conjugate. It is very important for in vivo experiments in the future. The lack of the hemolytic activity might be due to some steric hindrance introduced by the larger immunotoxin molecules. An *in vivo* reduction of the hybrid molecule implies a loss of specificity and the appearance of the toxic effect, however it is demonstrated that in mammals there is not an important system of reduction for disulphide bridges in plasma (17). Nevertheless, the stability of the disulphide bridge should be carefully checked in vivo before carrying out any clinical trials. Recombinant immunotoxins may be circumvents this problem. The preliminary study about the thermostability of IOR R3-HT immunotoxin predicted that a half of biological activity (recognizing MAb and toxin hemolytic abilities) would be lost after 1.5 years storing at 4^0C. Although these results are a rough estimation, they showed that the IOR R3-HT is a stable molecule. It is an important issue for patient applications in general.

Conclusions

The results show that it is possible the linking of IOR-R3 and 17-1A monoclonal antibodies to the hemolytic toxin obtained from *S. helianthus* by means of disulphide linkage. The selective toxicity of the IOR R3-HT immunotoxin on H125 cell line (lung adenocarcinoma) becomes this molecule in an interesting alternative for the treatment of this kind of lung cancer that is a resistant pathology to chemotherapy and radiotherapy treatments.

Similarly this ability could be used with the 17-1A-HT immunotoxin which was selectively toxic on SW1116 cell line, a human colon carcinoma becoming this molecule in an attractive choice for treatments of these tumours, keeping in mind that the most carcinoma-antigens the internalization into the cell is not needed. We have presented here two immunotoxins involving a hemolytic toxin that is active on the cell membrane as a system that does not need go into into the cells. This feature gives an advantage over ricin-immunotoxins and could be a good choice for building immunotoxins using antibodies towards antigens in which there is not endocytic internalization, as it is happens with many kinds of carcinoma-antigens like lung and head & neck cancer.

References

1. Vasir JK, Labhasetwar V. Targeted drug delivery in cancer therapy. Technol Cancer Res Treat. 2005; 4:363-74.
2. Li Z, Yu T, Zhao P, et al. Immunotoxins and cancer therapy. Cell Mol Immunol. 2005; 2:106-12.
3. Frankel AE, Neville DM, Bugge TA, et al. Immunotoxin therapy of hematologic malignancies. Semin Oncol. 2003; 30:545-57.
4. MacDonald GC, Glover N. Effective tumor targeting: strategies for the delivery of Armed Antibodies.Curr Opin Drug Discov Devel. 2005; 8:177-83.
5. Youn YS, Na DH, Yoo SD, et al.: Carbohydrate-specifically polyethylene glycol-modified ricin A-chain with improved therapeutic potential. Int J Biochem Cell Biol. 2005; 37:1525-33.
6. Kreitman RJ and Pastan I: Chimeric fusion proteins-Pseudomonas exotoxin-based. Curr Opin Invest. Drugs. 2001; 2:1282-1293.
7. Carriere D, Casellas P, Richer G, et.al: Endocytosis of an antibody ricin A-chain conjugate (immuno-A toxin) absorbed on colloidal gold. Effect of ammonium chloride and monensin. Exp Cell Res. 1985; 156:327-340.
8. Avila AD, Mateo de Acosta C and Lage A: A new immunotoxin built by linking a hemolityc toxin to a monoclonal antibody specific for immature T lymphocytes. Int. J Cancer. 1988; 42:568.
9. Avila AD, Mateo de Acosta C and Lage A. A carcinoembryogenic antigen-direct immunotoxin built by linking a monoclonal antibody to hemolytic toxin. Int J. Cancer. 1989, (43):926.
10. Fernández A, Spitezr E, Pérez R, et. al. Sistema de producción del anticuerpo monoclonal IOR R3. J. Cell Biochem. 1992; 49:157-167.
11. Suarez Pestana E, Greoser U, Sánchez B, et. al. Growth inhibition of human lung adenocarcinoma cells by antibodies against epidermal growht factor receptor and ganglioside GM_3. Br J Cancer 1997; 75:213-220.
12. Balzar M, Winter MJ, de Boer CJ, Litvinov SV. The biology of the 17-1A antigen (Ep-CAM). J Mol Med.1999; 77:699-712.

13. Herlyn D., Herlyn M., Stepleweski Z. and Koprowski H: Monoclonal antibodies in cell-mediated cytotoxicity against human melanoma and colorectal carcinoma. Europ. J. Immunol. 1979; 9:657-659.
14. Avila A.D, Mateo de Acosta C y Lage A. Purificación de una toxina a partir de la anémona de mar Stoichactis helianthus. Revista Cubana Oncología. 1989; 5:107-121.
15. Alvarez C., Casallenovo F., Shida CS., et.al. Binding of sea anemone pore-forming toxins sticholysins I and II to interfaces-modulation of conformation and activity, and lipid-protein interaction. Chem. Phys. Lipids, 2003; 122: 97-105.
16. Carlsson J, H. Drevin and Axen R. Protein thiolation and reversible protein-protein conjugation. N-succinimidyl 3-(2-pyridildithio)propinate, a new heterobifuntional reagent. Biochem. J.1978; 173:723-73.
17. Beutler E., Red Cell metabolism. In: A manual of biochemical methods. Grume and Stratton, New York and London, 1971.