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HIGH SPECIFIC CYTOTOXICITY OF ANTIBODY-TOXIN HYBRID MOLECULES (IMMUNOTOXINS) FOR TARGET CELLS

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1. Summary

In an attempt to develop highly efficient antibody–drug conjugates for passive immunotherapy of cancer the A-chain of the potent toxin, ricin, was coupled to antibodies in order to render them specifically cytotoxic for target cells without the participation of complement. The antibody–toxin conjugates (immunotoxins) showed no loss of antibody or A-chain activity. In vitro, highly purified A-chain was about 5000 times less toxic on HeLa cells than whole ricin. Unconjugated A-chain made no difference between TNP-HeLa and HeLa cells but when coupled to anti-DPN antibodies it became about 500 times more cytotoxic to TNP-HeLa cells than to HeLa cells. In vivo A-chain (LD_{50}: 20 mg/kg) was about 3000 times less toxic than whole ricin and treatment with immunotoxin significantly inhibited tumor take and tumor growth of TNP-HeLa cells in nude mice.

2. Introduction

Antibody–toxin conjugates should combine the specificity of antibodies with the efficiency of potent toxins in order to selectively destroy the target cells. Since they do not need complement their application in passive immunotherapy of cancer may be highly advantageous.

The first antibody–toxin conjugates containing diphtheria toxin or ricin by Moolten et al. [1,2] were only marginally specific. Thorpe et al. [3], by using a less denaturating coupling method, obtained hybrid molecules which were more toxic for target cells than for non-target cells, but which possessed nevertheless non-specific toxicity. This may be explained by the binding of the toxin part in the hybrid molecules to almost all cell types. Polypeptide toxins such as diphtheria toxin, abrin and ricin consist of two domains or chains with different functions: the A-domain enzymatically inhibits protein synthesis, while the B-domain binds to cell surface constituents and appears to mediate the entry of the A-domain into the cell [4]. Recent attempts have been made to replace the B-domain of various toxins by other protein molecules capable of binding to cell membrane receptors, in order to trigger internalization of the toxic A-moiety. For instance, when the toxic A-fragment of diphtheria toxin was coupled to whole ricin, it utilized the receptor-mediated transport system of ricin which induced its entry into the cytoplasm [5]. Instead of whole ricin, lectins have been used to the same end, i.e. concanavalin A, but the resulting internalization was less efficient [6–8].

On the other hand, if the B-domain was substituted with hormones or antibodies, higher selectivity could be expected. In fact the A-fragment of diphtheria toxin was coupled to human placental lactogen [9] or the A-chain of ricin to human chorionic gona-

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dotropin [10] and both resulting hybrid molecules did show specificity for cells bearing the hormone receptors. The use of antibodies appears more attractive, because specific antibodies directed against most antigens characterizing tissues or cell subpopulations can be induced. In a first attempt, however, Olsnes [11] reported that he was unable to demonstrate specific cytotoxicity using a conjugate composed of abrin A-chain and antibodies against cell-bound trinitrophenyl (TNP) groups. Masuho et al. [12] prepared a conjugate by coupling the A-fragment of diphtheria toxin to antibodies directed against a mouse leukemia. This conjugate showed high cytotoxicity, as compared to diphtheria toxin alone. However, since these cells are completely insensitive to the action of the B-domain of whole diphtheria toxin, the specificity of the conjugate could not be proven. In a toxin-sensitive cell system, the slightest contamination by the B-domain or the whole toxin could considerably diminish the specificity of cytotoxicity. Furthermore, diphtheria toxin encounters difficulties when administered to humans because almost everybody is vaccinated against it. Several years ago, we began a study of these hybrid molecules using the purified A-chain of ricin coupled to different antibodies*. Here we describe results obtained with anti-DPN antibodies. These conjugates were found to kill TNP-coated HeLa cells (target cells) with high specificity. The prerequisites for obtaining such results were: (a) highly purified A-chain; (b) purified anti-DNP antibodies; (c) conjugates prepared by linking A-chain to antibody via a disulphide bond thus mimicking the natural bond between the A- and B-chains of ricin; and (d) an optimal amount of TNP on target cells.

3. Materials and methods

3.1. *Antibodies*

Goat-anti-DNP antibodies were obtained by hyperimmunization for several months with 5 mg DNP_{49} BGG in CFA resulting in 4–5 mg/ml of specific antibodies. They were purified by immunoadsorption performed in antibody excess in order to diminish contaminations. Antibodies eluted with 2,4-dinitrophenol were pure since no contaminations could be demonstrated in immunoelectrophoresis and albumin was not detectable by electrophoresis at the 0.1% level of sensitivity. The average affinity measured by the method of Nahm et al. [13] with [^{3}H]DNP-lysine corresponded to an affinity constant $K_0 \sim 2 \times 10^7$ M^{-1} and a heterogeneity index a ~0.65. $F(ab')_2$ fragments were obtained by classical peptic digestion [14]. The binding constants of the anti-DNP antibodies on TNP-HeLa cells [15] were measured by the method of Steck and Wallach [16] with antibodies radio-iodinated using chloramine-T [17].

*French patent no. 78 27 838 (Sept. 1978); US patent no. 79441 (Sept. 1979).

3.2. *Toxin*

Ricin was extracted from ground and defatted Castor beans and then purified by affinity chromatography on agarose using a stepwise elution with galactose. After cleavage of the disulphide bridge with 2-mercaptoethanol, the A-chain was isolated on DEAE-Sepharose 6B and concentrated on CM-Sepharose 6B. At concentrations >10 mg/ml crystals of A-chain were obtained.

3.3. *Coupling procedure*

In order to obtain a disulphide-linked conjugate of the antibody and the A-chain, mixed disulphide radicals were introduced into the antibodies by reacting them with 3-(2-pyridyldithio)-propionic acid in the presence of a carbodiimide. Following dialysis the modified antibodies were allowed to react with the free thiol group of A-chain. At the end of the reaction, monitored by measuring 2-thiopyridone (2-TP) absorbance at 343 nm, the mixture was centrifuged and the supernatant applied to a Sephadex G-200 column equilibrated with 125 nM phosphate buffer (pH 7.0) containing 1 mM Na_2EDTA. The effluent was monitored by measuring the absorbance at 280 nm and by assaying the inhibitory effect of A-chain in a cell-free protein synthesizing system from rat liver (Fig.1). The fractions in the first peak with the highest conjugated A-chain concentrations, partially depleted of free antibodies, were pooled and constituted the immunotoxin preparation (fractions 115–195). The second and third peaks, containing free (non-conjugated) A-chain and 2-TP respectively, were discarded.

3.4. *In vitro assay*

To test the effect of various substances on intact cells, TNP-HeLa cells were incubated with immunotoxin or its controls and then pulsed with [^{14}C]leucine. HeLa cells were suspended in Earle's medium supplemented with amino acids except for leucine and isoleucine, and 30% inactivated foetal calf serum. TNP-labelling was performed at 4°C, by incubating HeLa cells (2×10^6 per ml) in phosphate-buffered saline (PBS) with an equal volume of a solution of sodium trinitrobenzene sulphonate (10 mg/ml). The reaction was stopped after 15 sec by addition of excess L-lysine, and cells were washed. 500 µl of appropriate dilutions of immunotoxin, ricin or A-chain were added to each tube containing 10^5 cells in 500 µl. Incubation was continued for 16 hours at 37°C in a horizontal shaker in a 5% CO_2 atmosphere. Cultures were then pulsed for 90 min with 0.5 µCi [^{14}C]leucine. Cells were harvested with a Titerteck cell harvester. Radioactivity uptake was measured in a liquid scintillation counter and results were expressed as a percentage of the uptake by untreated control cells. To compare the effects of different products, the concentration that produced 50% inhibition of [^{14}C]leucine uptake, which was verified to correspond to 50% protein synthesis inhibition (PI_{50}), was chosen.

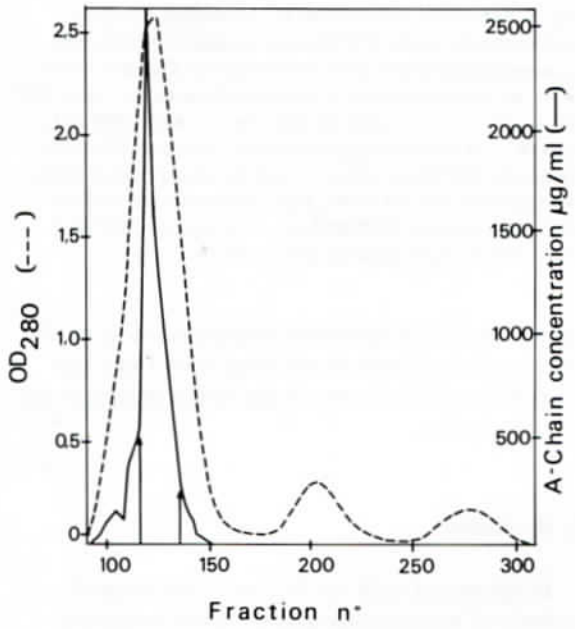

Fig. 1. Isolation of immunotoxin from the coupling reaction mixture on Sephadex G-200. The effluent of the column was monitored by the absorbance at 280 nm (– – –) and by the inhibitory activity of A-chain in a cell-free protein synthesizing system prepared from rat liver (——) (represented for fractions 90–150 only).

3.5. *In vivo assay*

The in vivo biological activity was assessed by the appearance of tumors following injection of THP-HeLa cells into nude mice and subsequent i.p. treatment with immunotoxin. TNP-HeLa cells (2×10^6 per mouse) labelled as described earlier were inoculated i.p. into Balb/c nu/nu Bom mice, 10 weeks old, followed or not by immunotoxin injection within one hour of the injection of cells, at a dose of 36 µg conjugated A-chain/mouse (corresponding to $<1/10$ of the LD_{50} of the conjugate). Mice were killed 25 days later and the presence of tumors in the peritoneal cavity verified. Tumors, (when present), were excised, weighed and their values and geometric means represented. The smallest visible tumor weighed 5 mg; therefore, the absence of tumors was considered as tumors weighing less than 5 mg.

4. Results

4.1. *Conjugates*

Coupling of antibodies to ricin A-chain resulted in hybrid molecules composed of about one molecule of A-chain chemically bound per molecule of antibody (Fig. 1). They showed no alteration of antibody or toxin activity as measured by radioimmunoassay with the hapten DNP-lysine for antibody activity and inhibition of cell-free protein synthesis for A-chain activity.

4.2. *In vitro activity*

DNFB, originally intented for modification of HeLa cells, had to be replaced by TNBS which was more compatible with cell survival. HeLa cells modified with TNBS under the special conditions mentioned above showed perfect viability during almost 24 h of culture. With the method of Stech and Wallach [16] the number of accessible membrane bound TNP haptens to anti-DNP antibodies was 7×10^7 per cell and their affinity to antibodies 10^7 M^{-1} [15]. Ricin at the concentration of 10^{-10} M caused at 50% inhibition of [^{14}C]leucine uptake by TNP-HeLa cells

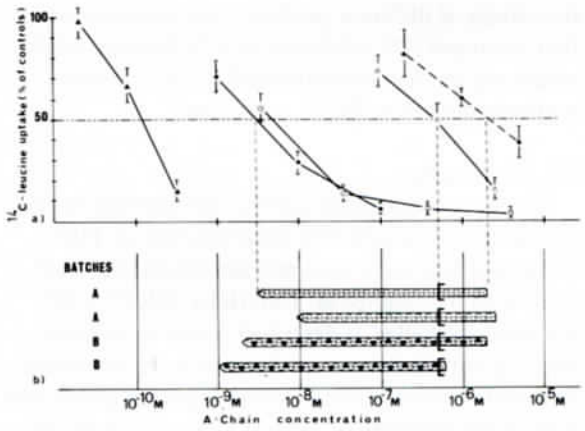

Fig. 2. The effect of immunotoxin and controls on [^{14}C]leucine uptake of viable cells. a: immunotoxin (anti-DNP IgG coupled to A-chain) on TNP-HeLa cells (●——●) or HeLa cells (●– – –●); ricin on TNP-HeLa (▲——▲); A-chain on TNP-HeLa cells (△——△); immunotoxin prepared with the F(ab')$_2$ fragment of goat-anti-DNP IgG on TNP-HeLa cells (□——□). b: horizontal arrows represent 'specificity factors', being the ratio between the PI_{50} (in M) for immunotoxin on TNP-HeLa cells and the PI_{50} (in M) for immunotoxin on HeLa cells without TNP. Each arrow corresponds to a different experiment. Two different batches were tested. The brackets represent the PI_{50} of free A-chain on TNP-HeLa cells.

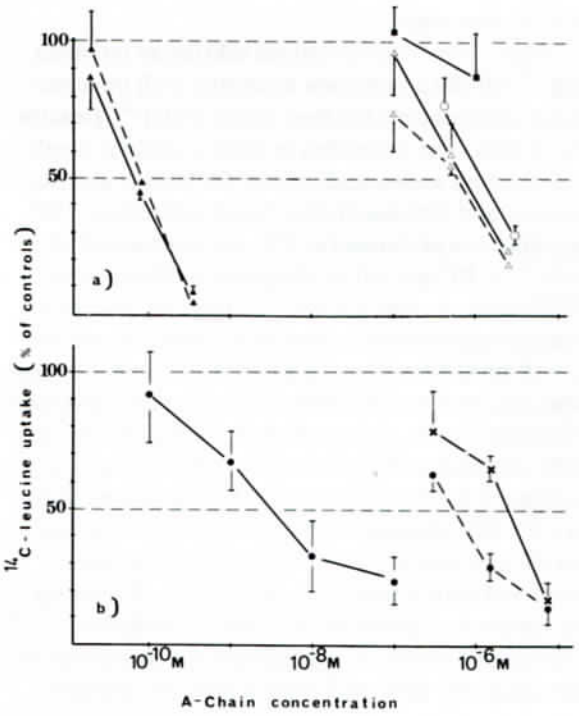

Fig. 3. Specificity controls for the inhibitory effect of immunotoxin on the [^{14}C]leucine uptake by viable cells. a: non-conjugated anti-DNP antibodies on TNP-HeLa cells (■——■), non-conjugated A-chain on HeLa (△– – –△) or TNP-HeLa cells (△——△); ricin on HeLa (▲– – –▲) or TNP-HeLa cells (▲——▲); non-conjugated A-chain plus anti DNP antibodies on TNP-HeLa cells (○——○). b: absorption of immunotoxin with 100 nM $DNP_{19}BSA$: immunotoxin on TNP-HeLa cells without DNP-BSA (●——●) or with DNP-BSA (×——×), A-chain alone on HeLa cells (●– – –●).

whereas highly purified A-chain, with a PI_{50} of about 5×10^{-7} M, was 5000 times less active. Different preparations of immunotoxin including F(ab')$_2$–toxin conjugates, with a PI_{50} of about 10^{-9} to 10^{-8} M had an intermediate activity (Fig. 2). Non-conjugated antibodies plus free A-chain behaved as free A-chain. When incubated in the presence of DNP-labelled bovine serum albumin ($DNP_{19}BSA$), the immunotoxin had the same activity as free A-chain. The activity of ricin on HeLa and TNP-HeLa cells was similar. A-chain did not discriminate between either system (Fig. 3).

4.3. *In vivo activity*

In the mouse the LD_{50} of purified ricin was 7.5 μg/kg and that of A-chain 20 mg/kg, A-chain being about 3000 times less toxic than whole ricin. Based on its content in A-chain, immunotoxin had the same LD_{50} as free A-chain. Immunotoxin-treated animals presented tumors in only one out of 15 mice (7%), while 14 out of 15 untreated controls (93%) presented tumors 25 days after inoculation (Fig. 4). Preliminary experiments showed that both HeLa and TNP-HeLa cells had very similar tumor incidence and growth pattern.

5. Discussion

In agreement with the literature, the isolated A-chain of ricin has low toxicity when tested in a cellular system while it remains as toxic as whole ricin when tested in a cell-free system. In fact, we have obtained a crystallized purified A-chain possessing much lower toxicity for whole cells than that reported by others [4,18]. Such a weakly toxic subunit is an

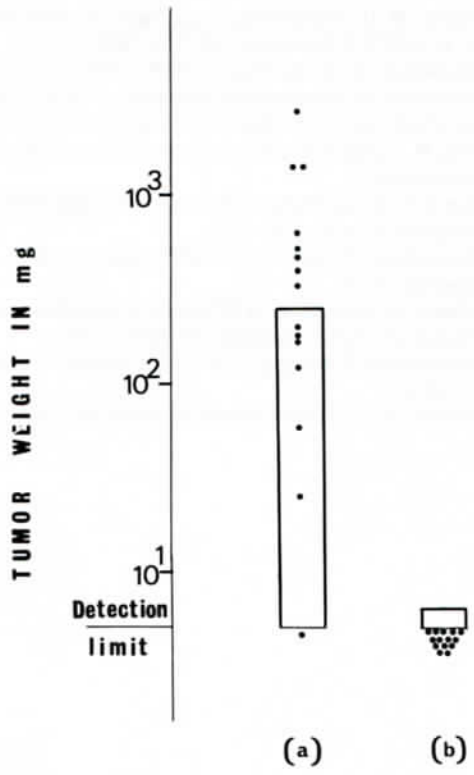

Fig. 4. The effect of immunotoxin on the appearance and growth of HeLa tumors in nude mice. Tumor weight 25 days after i.p. inoculation of 2×10^6 TNP-HeLa cells into nude mice: (a) without injection of immunotoxin; (b) after i.p. injection of 36 μg conjugated A-chain/mouse within one hour of tumor cell inoculation.

ideal agent for a drug–antibody conjugate because the drug would be non-toxic during transport in the circulation and then, when the antibody binds to the target cell, the A-chain would become internalized and exert its toxic effect on protein synthesis. All depends, however, on the capacity of an antibody to replace the B-chain. Our results clearly demonstrate that such replacement can be effected by an antibody. Since the anti-DNP antibodies, as shown in this study, and the B-chain of ricin, according to Olsnes [18], showed very similar affinity for the hapten or for the B-chain receptor respectively ($K_o \sim 3-7 \times 10^7$ M^{-1}) and since accessible haptens (7×10^7/cell) [15] or B-chain receptors (3×10^7/cell) [18] appear to have similar surface density, comparison between the efficiency of ricin and immunotoxin is valid. Immunotoxin, however, did not attain the PI_{50} of whole ricin, and the slopes of the respective log-dose–response curves were different. This may reflect an additional function of the B-chain of ricin as compared to an antibody [18,19]. Therefore the antibodies seem only to replace one function of the B-chain of ricin, their binding to membrane receptors, thus effecting a concentration of A-chain on the cell surface. This function, however, could be more efficiently replaced if antibodies of higher affinity or avidity, as compared to ricin B-chain, could be used. Such high affinity specific antibodies can be expected with natural membrane antigens. Although artificial antigens like TNP-haptens may not reflect the cell distribution, density and structure of natural membrane antigens, this artificial model is of great importance, since it allows to: (a) replace the B-chain by an antibody of similar binding functions in respect to affinity and antigen density; (b) to vary the antigen density on the same cell line and to study its influence; and (c) to vary the antibody affinity maintaining their specificity. In respect to tumor cells it may be hoped that the low density of tumor specific membrane antigens, may be compensated for by high avidity antibodies. Therefore conjugates composed of potent toxin subunits and antibodies specific for tumor membrane antigens are expected to efficiently destroy tumor cells without the interaction of complement.

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