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Production of anti-CD3 and anti-CD7 ricin A-immunotoxins for a clinical pilot study

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Abstract

This report describes the preparation of an immunotoxin-combination, consisting of an anti-CD3 and anti-CD7 monoclonal antibody (MoAb) both conjugated to the A-chain of plant toxin ricin, for the experimental treatment of graft-versus-host disease. MoAbs and toxin were conjugated by conventional biochemical and chromatographic techniques. Raw materials, intermediate and final products were evaluated in accordance with the relevant 'points to consider' of the FDA. Yields, purity and sterility of the two final products were all satisfactory. Preservation of MoAb-affinity and toxin-activity were confirmed in biological assays. The LD50, 25–45 mg immunotoxin-combination/kg mouse, equalled that of similar immunotoxins already in clinical use. Because in vitro cross-reactivity screening revealed an unexpected binding of the CD3-MoAb to the esophagus epithelium, human doses of immunotoxin-combination were administered to two cynomolgus monkeys. Clinically relevant serum concentrations were obtained without irreversible toxicities occurring. The $T_{1/2}$ varied between ~ 6 and 9 h and the C_{max} ranged from 1.8 to 3.9 μg/ml. The main side effect was a transient rise of serum creatine kinase. Importantly, neither damage nor binding of the CD3-immunotoxin to the monkey esophagus epithelium could be demonstrated. It was concluded that sufficient material of proper quality and with an acceptable toxicity profile was produced, warranting the evaluation in a clinical pilot-study.

Keywords: Immunotoxin; Quality control; Antigens CD3 & CD7; Mice BALB/C; Cynomolgus monkey; Ricin

1. Introduction

Monoclonal antibodies (MoAbs) conjugated to bacterial or plant-derived toxins (e.g. pseu-

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domonas exotoxin or the deglycosylated A-chain of plant toxin ricin), so-called immunotoxins, form attractive agents for the selective elimination of disease causing cells (reviewed in Vitetta et al., 1993; Vallera, 1994; Kreitman, 1999). Within the last two decades, various immunotoxins have been tested in clinical studies for the treatment of life-threatening disorders, including solid and diffuse tumors and immunological disorders (Byers et al., 1990; Vitetta et al., 1991; Engert et al., 1997; Lynch et al., 1997; Kreitman et al., 2000; van Oosterhout et al., 2000). Despite differences in specificity and efficacy, their mechanism of action is basically comparable. The MoAb moiety binds specifically to an antigen expressed on the surface of target cells, whereafter the entire antigen–immunotoxin complex is internalized. Once inside the cell, the toxin-subunit is translocated to the cytosol where it inhibits protein synthesis irreversibly by means of a catalytic reaction culminating in the cell's death. The potency of a given immunotoxin depends in essence on the number of immunotoxin molecules internalized, and on the intracellular handling following internalization (Preijers et al., 1988; Chignola et al., 1990; May et al., 1991; van Oosterhout et al., 1994; van Horssen et al., 1995). Recently, we demonstrated in laboratory experiments that a combination of two deglycosylated ricin A-chain (dgA) based immunotoxins, directed against lymphocyte differentiation antigens CD3 and CD7, acts synergistically in killing activated T cells (van Oosterhout et al., 2000). This makes this particular immunotoxin-combination potentially valuable to control certain immunological disorders, like transplantation-related rejection and several auto-immune diseases. To explore its therapeutic potential, a clinical pilot study was set up for the treatment of patients with severe graft-versus-host disease, a serious complication of hematological stem cell transplantation initiated by donor-derived T cells (van Oosterhout et al., 2000). This document describes the semi-large scale laboratory production and quality control of the clinical grade immunotoxin-combination used for the pilot-study.

2. Material and methods

2.1. Processes, facilities and documentation

The manufacturing processes and the production facilities were designed to minimize the risk of contamination of raw materials, intermediates and the two final products. Prior to the production, the formulae, manufacturing method and specifications of all (intermediate) products were established and documented. The documentation included instructions for operation of equipment, the manufacturing, packaging and storage.

2.2. MoAb and dgA

Hybridoma cells producing murine MoAb SPV-T3a (αCD3, IgG2bκ) (Spits et al., 1983) and WT1 (αCD7, IgG2aλ) (Tax et al., 1983) were kindly provided by Drs H. Spits (NKI, Amsterdam, The Netherlands) and W. Tax (UMC St Radboud, Nijmegen, The Netherlands), respectively. From both hybridomas, a master cell bank (MCB) and manufacturing working cell bank (MWCB) were prepared. The production of MoAb WT1 was performed at the institutional Central Hematology Laboratory. The production of MoAb SPV-T3a, as well as the protein A purification of both MoAb, was performed by IQ Corporation (Groningen, The Netherlands).

For antibody production, hybridoma WT1 was grown in 162 cm^2 culture flasks in RPMI1640 medium (Flow, Irvine, UK) supplemented with 8% fetal calf serum (FCS) (Integro, Zaandam, The Netherlands), glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified incubator with 5% CO_2 in air at 37°C. Hybridoma SPV-T3a was cultured under the same conditions in RPMI1640 supplemented with 8% FCS, D(+)-glucose (2.5 g/l), sodium hydrogencarbonate (2.0 g/l), L-glutamine (0.4 g/l), gentamycine (0.04 g/l), and HybridoCult supplement (IQ Corporation). Cells were first expanded in log-phase and then cultured for another 2 weeks without medium changes, resulting in a final viability of about 10%. Subsequently, cell cultures were centrifuged, and the supernatants were filter sterilized (crude harvest) and stored at −20°C

(SPV-T3a) or 4°C (WT1). For MoAb purification, the crude harvest was 1:1 diluted with 'bindings-buffer', containing 1.5 M glycine and 3.0 M NaCl (pH 8.9), and applied to a protein A column. Affinity bound MoAbs were eluted with citrate buffer (0.1 M citrate, pH 2.8–3.0) and directly neutralized with 0.1 vol. 1 M Tris–HCl. Subsequently, purified MoAbs were dialyzed against 0.01 M phosphate buffered saline (pH 7.4) (PBS), filter sterilized and stored at a concentration of approximately 4 mg/ml at 4°C.

Clinical grade deglycosylated ricin A (dgA) was purchased from Inland Laboratories (Austin, TX). The dgA contained the two naturally occurring A-chain forms, of 30 and 32 kDa, found in the seeds of *Ricinus communis* (Fulton et al., 1986; Thorpe et al., 1988). The dgA was stored at a concentration of 3.06 mg/ml in phosphate buffered saline (pH 7.2) containing 50% glycerol at −20°C.

2.3. MoAb–dgA conjugation

Preparation of MoAb–dgA conjugates was based on the method described by Ghetie et al. (1991). Sterile and endotoxin-free solutions were prepared by the Department of Clinical Pharmacy. The preparative chromatographic columns were set up as a closed system except for the inlet and outlet which were positioned in a class 100 laminar flow cabinet. Chromatographic media and columns (all Amersham Pharmacia Biotech, Uppsala, Sweden) were sanitized for every conjugation according to the supplier's 'product support files'. The conjugation procedure started with the derivatization of MoAb with an 8- to 12-fold molar excess of crosslinker 4-succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)-toluene (SMPT) (Thorpe et al., 1988) (Pierce, Rockford, IL), and the reduction of dgA with 5 mM DTT. Both reactions were carried out at 37°C for 1 h. Excess SMPT and DTT were subsequently removed by gel filtration on Sephadex G-25 (XK100/50 column) equilibrated with PBS containing 0.05% Tween-20 (PBST). Average MoAb-MPT loading was determined spectrophotometrically, as described elsewhere (Carlsson et al., 1978). For the actual conjugation reaction, MoAb-MPT was then mixed with a 2.5-fold molar excess of freshly reduced dgA-SH. Subsequently, 0.2 vol. 85% glycerol was added and the conjugation mixture was gently stirred and the reaction was allowed to proceed at 20–25°C for 48 h. The resulting crude conjugate was purified by affinity chromatography on Blue-Sepharose (XK50/60 column) for removal of unconjugated MoAb (The Sepharose-conjugated Cybacron Blue has a high affinity for dgA). In case of WT1-dgA, the Blue-Sepharose was equilibrated with PBST. For SPV-T3a-dgA, the Na^+ concentration of the reaction mixture and loading buffer had to be adjusted to 300 mM in order to prevent affinity-binding of unconjugated SPV-T3a. Affinity-bound dgA and MoAb–dgA conjugates were eluted with PBST containing 1 M NaCl. High molecular weight material and free dgA were subsequently removed by size exclusion chromatography on Sephacryl S-300 HR (K100/100 column) equilibrated with PBST (formulation buffer). The resulting immunotoxins were concentrated to approximately 1 mg/ml and stored as infusion concentrates in sterile 5-ml glass vials at −20°C at the Department of Clinical Pharmacy. The immunotoxin-combination is prepared directly for use by diluting equal amounts (w/w) of SPV-T3a-dgA and WT1-dgA in formulation buffer.

2.4. Immunotoxin composition and biological activity

The composition of purified MoAb–dgA conjugates was analyzed by nonreducing SDS–PAGE using 4–15% gradient gels (PhastSystem, Amersham Pharmacia Biotech). The ratio of different reaction products was determined by densitometric scanning of the separated product bands of Coomassie blue stained gels. Samples containing a 1:1 mixture of unconjugated dgA and MoAb were used for compensation of variance in dye-uptake between both proteins.

Binding capacity of the MoAb before and after conjugation to dgA was examined by a standard flowcytometric titration assay using an antigen-positive cell line and a fluorescent la-

beled goat-anti-mouse antibody to detect cell-bound MoAb. Preservation of ricin A toxicity was determined by measurement of protein synthesis inhibition in a cell-free reticulocyte assay (Press et al., 1986).

These analyses were periodically repeated and compared to baseline values to monitor the chemical stability and biological activity of the infusion concentrates during storage.

2.5. Sterility, endotoxins and adventitious agents

The FCS added to the culture medium was derived from healthy calves housed in a BSE-free environment, and tested negative for Bovine Diarrhoea virus, Infectious Bovine Rhinotracheitis, Parainfluenza and Bovine Polyoma virus. The MWCB of SPV-T3a and WT1 were tested for bacterial and fungal contamination and for mycoplasm. An 'extended S^+L^- focus assay' and 'extended XC-plaque assay' were performed by Inveresk Research (Tranent, UK) for detection of xenotropic and ecotropic retroviruses, respectively. Species specific viruses were tested by the institutional Department of Virology using a mouse antibody production test (MAP-assay), detecting 16 different exogenous mouse viruses including sendai virus, reovirus type 3, Mouse rotavirus (EDIM), hantaan virus, and lymphocytic choriomeningitis virus. The presence of adventitious viruses was tested by the Department of Virology using eight different indicator cell lines (including three human) and by inoculation of embryonated chicken eggs. Purified MoAb were tested for endotoxins, sterility and mycoplasma. The final immunotoxins were tested for the presence of hybridoma DNA by Inveresk Research. The leakage of affinity chromatography media was assayed using commercial enzyme immunoassays according to the manufacturer's instructions: Protein A using a kit from Cygnus Technologies (Wrentheam, MA, USA), Cybacron Blue with a kit from Affinity Chromatography Ltd (Girton, Cambridge, UK). The stored infusion concentrates were tested for endotoxins, sterility and mycoplasma.

2.6. Cross-reactivity with human tissues

Potential cross-reactivity of MoAb SPV-T3a and WT1 was tested by immunostaining of the following panel of quickly frozen human tissue sections derived from the tissue bank at the Department of Pathology (# donors): heart (3), lymph node (3), spleen (3), lung (3), esophagus (3), stomach (3), ileum (3), colon (3), liver (3), pancreas (3), testis (3), prostate (3), bladder (3), renal pelvis (3), kidney (3), ovary (3), Fallopian tube (2), uterus (3), breast (3), cerebrum (3), cerebellum (3), spinal cord (1), skin (3), striated muscle (3), thyroid (3), parathyroid (2), adrenal gland (3), pituitary (2), eyeball (1). Reactivity against hematopoietic cell lines and stem cells has been tested extensively during the initial characterization of the MoAbs. Potential cross-reactivity against embryonic/fetal tissues has not been determined yet since pregnant women are no candidates for allogeneic stem cell transplantation.

2.7. In vitro dissociation in human plasma

Dissociation of MoAb and dgA during incubation in human plasma at 37°C was monitored using enzyme immunoassays which detect intact MoAb–dgA conjugates (van Oosterhout et al., 2000).

2.8. LD50 mice

All animal experiments were performed by personnel of the Central University Animal Laboratory in accordance with the 'Principles of Laboratory Animal Care' (NIH publication # 85-23, revised 1985). The LD50 experiment was performed with male BALB/C mice of 8 weeks weighing $\sim$17 g, as described by Weil (1952). Four doses were administered: 96, 193, 385 and 770 μg immunotoxin-combination per mouse, in a final volume of 200 μl. Administration was intravenously through one of the tail veins. Upon administration, mice were weighed daily and followed for survival for 2 weeks. The control group was injected with formulation buffer only.

2.9. Administration to cynomolgus monkeys

The monkeys received two doses of immunotoxin-combination, administered 48 h apart as 100-ml infusions over a period of 4 h. Prior to infusion, the monkeys were sedated (ketamine 10 mg/kg, atropine 0.5 mg) and subsequently anesthetized (ratio oxygen and nitrous oxide 1:2, enflurane 1.5%). The immunotoxin-combination was administered via a subclavian (first infusion) or femoral (second infusion) central venous catheter. For detection of any esophagus toxicity, endoscopy was performed on day -7, 4 and 9 (day 1 being the first infusion day). During endoscopy, biopsies were taken for microscopic analysis of potential tissue damage. Moreover, biopsy-derived tissue was stained with a peroxidase-labeled anti-mouse-IgG2b antibody for detection of SPV-T3a-dgA. Tissue pre-incubated in vitro with saturating amounts of SPV-T3a-dgA served as positive control. In addition, both monkeys underwent regular physical examinations and blood chemistries, complete blood counts and leukocyte differentials were obtained for general safety assessment. One of the monkeys (receiving 0.1 and 0.2 mg/kg immunotoxin-combination) suffered from diabetes mellitus, but showed no physical disorders which were expected to influence the outcome of the experiment.

3. Results

3.1. Immunotoxin production

The crude harvest of SPV-T3a and WT1 contained approximately 50 μg/ml MoAb. The recovery following Protein A purification was $\sim 85\%$, about 1 g of purified SPV-T3a and WT1 were produced. The subsequent conjugation process is schematically depicted in Fig. 1. To reduce the risk of losing all material by a technical failure, MoAbs were divided in two fractions which were conjugated separately to dgA and subsequently partly purified by BlueSepharose. The MoAb–dgA containing BlueSepharose eluates of each MoAb were then pooled and applied as one fraction to the Sephacryl S-300 column. The final MoAb-recovery following conjugation was 34 and 26% for SPV-T3a and WT1, respectively. Fig. 2 displays a representative non-reducing SDS–PAGE analysis of an SPV-T3a-dgA conjugation.

Table 1 summarizes the main characteristics of the final products, the left column displaying the proposed release criteria. Ideally, the final product consists entirely of MoAb conjugated to one or two dgA molecules. In practice, the final product contains some unconjugated MoAb, and MoAb bound to three or more dgA molecules, as well. The composition of WT1-dgA did not strictly meet the (arbitrary) release criteria as for its relatively high amount of free MoAb (23% instead of $< 10\%$). However, the acceptance of WT1-dgA in its present form was justified by the absence of 'contamination' with MoAb conjugated to three or more dgA-molecules, as well as by the high preservation of antigen-binding activity (90%) and effective in vitro killing capacity (van Oosterhout et al., 2000). Another point of attention was the presence of genomic DNA. The IT-combination contains < 10 pg DNA/mg protein, the average of SPV-T3a-dgA and WT1-dgA. The relevant FDAs 'Points to Consider in the Manufacture

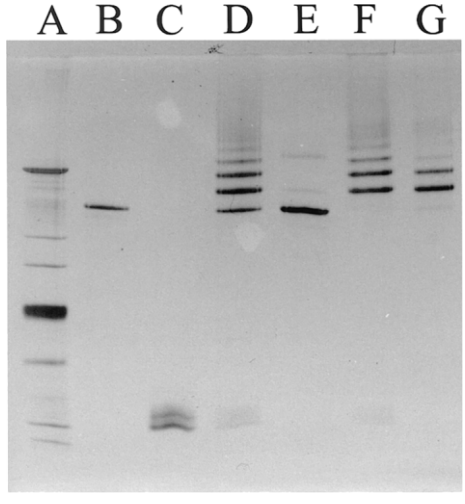

Fig. 2. SDS–PAGE analysis of SPV-T3a-dgA conjugation. (A) High molecular weight marker; (B) SPV-T3a (IgG2a); (C) reduced dgA; (D) crude conjugate; (E) run-through fraction BlueSepharose; (F) high-affinity eluate BlueSepharose; (G) pooled fractions of Sephacryl S300HR eluate containing SPV-T3a-dgA.

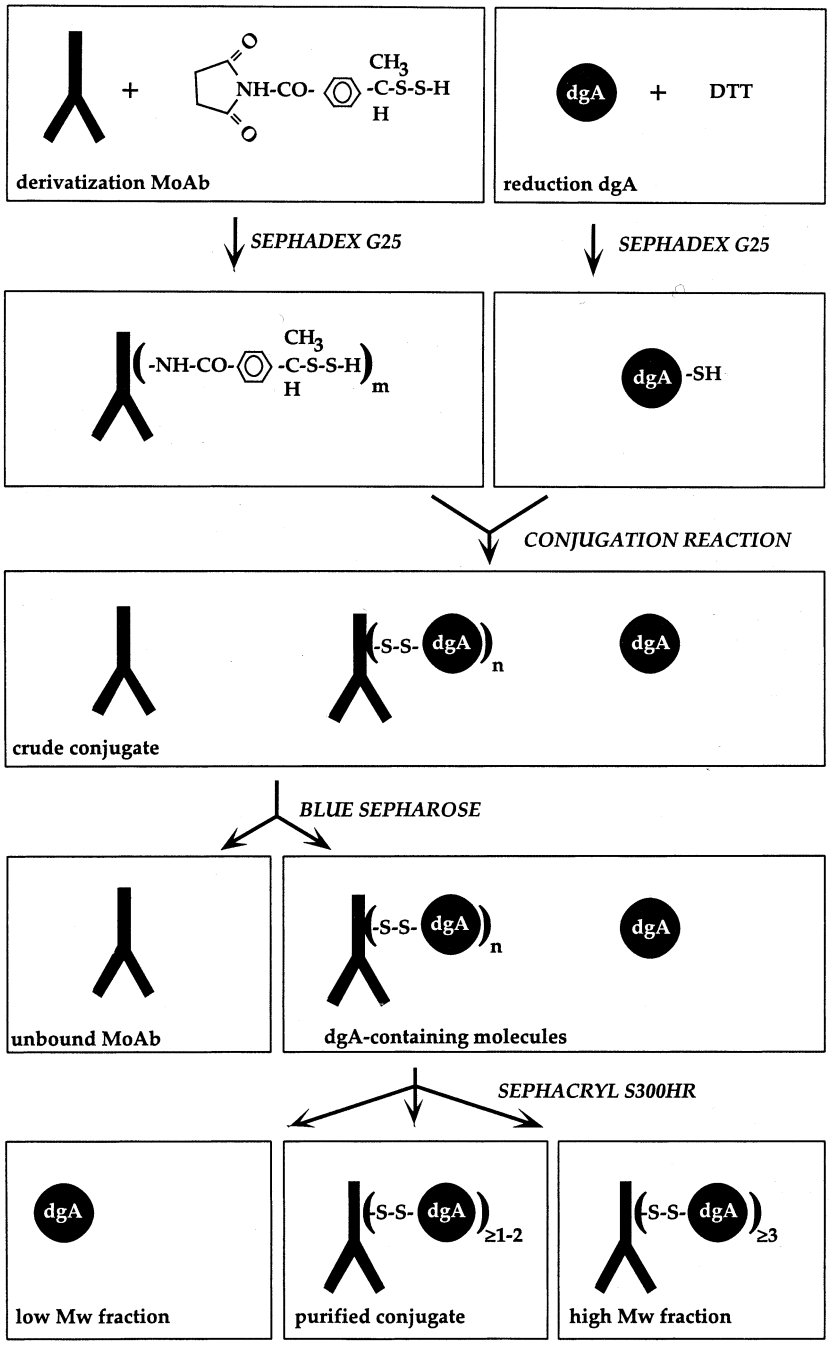

Fig. 1. Immunotoxin preparation. MoAb and dgA are derivatized with SMPT (m-fold, with $m = 0$, 1, 2, etc.) and reduced with DTT, respectively. The purified reaction mixtures (Sephadex G25) are mixed and incubated at room temperature for 48 h. The resulting crude conjugate, containing protein aggregates, MoAb conjugated to dgA (n-fold, with $n \leq m$), and non-conjugated molecules, are then separated by BlueSepharose-affinity chromatography and S300-size exclusion chromatography.

Table 1
Product specifications

	Proposed release criteria	SPV-T3a-dgA (CD3)	WT1-dgA (CD7)
MoAb-Isoform	IgG2bκ/IgG2aλ	IgG2bκ	IgG2aλ
Composition (%)			
$MoAb(dgA)_{\geq 3}$	<10	6	0
$MoAb(dgA)_{1\text{-}2}$	>80	88	78
MoAb	<10	6	23
dgA	<3	<1 (detection limit)	<1
Sterility	Sterile	Sterile	Sterile
Endotoxins (EU/mg protein)	<20	<0.8	<0.7
DNA content (pg/mg protein)	<5	<11	<9
Protein A (ng/mg protein)	<10	1.1 ± 0.3	2.7 ± 0.8
Cybacron Blue (nM)	<10 (detection limit)	<10	<10
A-chain activity (% native dgA)	>75	84	91
Binding activity (% native MoAb)	>50	70	90
Dissociation in vitro (% in 24 h)	~15	10	10
In vitro cross-reactivity	Absent. If not, relevant animal studies should follow	Esophagus epithelium (++); Smooth muscle cells (+/−)	Kupffer cells (+/−)
LD50 in mice (μg/g)	~14 μg/g mouse	25–45 μg/g mouse for the IT-combination	
Toxicity for cynomolgus monkey	No severe irreversible toxicities	Reversible toxicity to skeletal muscles after 0.1–0.25 mg/kg IT-combination	

and Testing of Monoclonal Antibody Products for Human Use' suggests that, whenever possible, the final product contains no more than 100 pg cellular DNA per dose. This implies that total human doses of 10 mg and higher (equalizing ~5–7 mg/m^2) require additional testing (e.g. with concentrated immunotoxin to lower the detection limit).

The stored infusion concentrates of SPV-T3a-dgA and WT1-dgA have been periodically tested for stability. No changes could be detected in composition (SDS–PAGE), MoAb binding capacity (flow cytometric titration assay), or ricin A toxicity (cell free reticulocyte assay), after storage at −20°C for a period of 4 years.

3.2. Cross-reactivity with human tissues

MoAb SPV-T3a showed two unexpected cross-reactivities: against (1) smooth muscle cells, and (2) basal epithelial cells of the esophagus. The staining of smooth muscle cells was weak (+/−) but consistently found throughout various tissues. The diffuse staining pattern suggested a cytoplasmatic binding of physically disrupted cells. This is supported by the observation that SPV-T3a-dgA (10^{-8} M for 24 h) appeared to be non-toxic to cultured intact human leptomeningial smooth muscle cells (data not shown). The staining intensity of SPV-T3a with the esophagus-basal epithelium was as strong (++) as observed with positive lymphocytes, and appeared to be charac-

teristic for SPV-T3a since it was not observed with any of the other CD3 MoAbs tested (OKT3, UCHT and WT32). The in vivo consequence of this cross-reactivity will depend on the local concentration of SPV-T3a-dgA, and whether SPV-T3a-dgA will be internalized (a prerequisite for toxicity). To gain more insight into these parameters, it was decided to administer the immunotoxin-combination to cynomolgus monkeys, demonstrating in vitro cross-reactivity of comparable intensity and localization.

The screening with MoAb WT1 revealed a weak staining (+/−) of some Kupffer cells located in the liver sinus wall. This staining pattern was not observed during previous preclinical tissue screenings performed with WT1 (Hertler et al., 1989). No further unexpected cross-reactivities were observed.

3.3. LD50 mice

The LD50 in mice was determined for comparison with reference immunotoxin RFB4-dgA, a comparable immunotoxin already tested in FDA-approved phase I/II trials, having a LD50 of 14 mg/kg (Amlot et al., 1993). Three of the four mice treated with the highest dose immunotoxin-combination (770 μg/mouse) died within the follow-up period of 2 weeks. One mouse already died during injection, probably due to an administration-related toxicity (dgA-related deaths are normally first seen after 1–2 days). All mice treated with 385 μg immunotoxin-combination or less survived. Based on average initial weight, this implies an LD50 of 25–45 mg/kg mouse.

Apart from the survival score, mice were daily weighed and observed for behavior. Administration of IT combination caused weight loss in all groups, which was most pronounced after 9 days (Fig. 3). The extent and duration correlated well with the dosage. Besides weight-loss, mice treated with 770 or 385 μg IT-combination demonstrated lethargy, lack of appetite and a neglected fur. The mice treated with the two lower doses could not be distinguished visually from the control group.

3.4. Administration to cynomolgus monkeys

The immunotoxin-combination was administered to two male cynomolgus monkeys. As the administration was performed under general anesthesia, the animals had to be deprived of nutrition before each infusion. Since this may negatively impact their physical condition, the administration was restricted to two doses at a 48-h interval (patients in the pilot-study were supposed to receive four doses at the same interval; van Oosterhout et al., 2000). This decision was justified by observations of Amlot et al. (1993) who demonstrated that the toxicity of a dgA-based immunotoxin mainly depends on the strength of the individual doses and not on the number of doses administered. Both monkeys received two of the higher individual doses of the proposed human dose escalation scheme (ranging from 2 to 10 mg/m^2), translated to mg/kg. One monkey (about 12 years and 7.6 kg) received 0.1 and 0.2 mg/kg, the other monkey (4 years and 4.1 kg), received 0.2 and 0.25 mg/kg (equalizing human doses of 4, 8 and 10 mg/m^2). In human, the immunotoxin-combination will be given as a 100-ml infusion over 4 h. For the monkeys, this was changed to 20 ml administered in 1 h because of their smaller blood volume.

Fig. 4 shows the plasma concentrations of the immunotoxin-combination. The clearance curves best fitted a two-compartment model for both SPV-T3a-dgA and WT1-dgA individually and given in combination. The pharmacokinetic

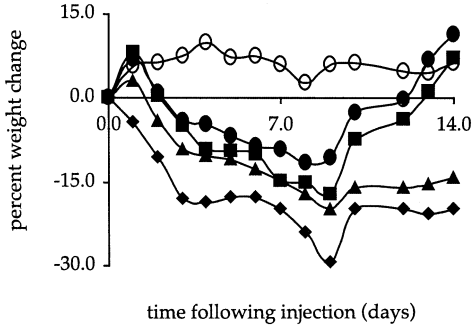

Fig. 3. Average weight-changes of mice treated with formulation buffer (○) or with 96 μg (●), 193 μg (■), 385 μg (▲) or 770 μg immunotoxin-combination/mouse (◆).

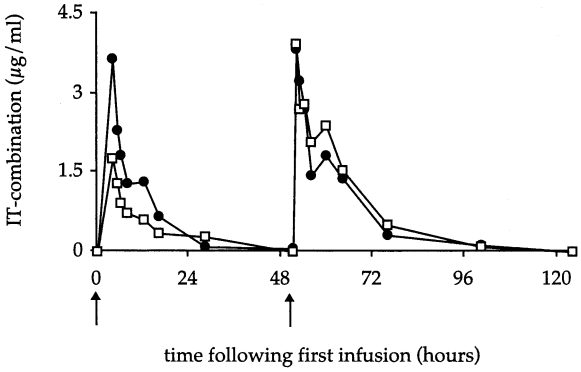

Fig. 4. Immunotoxin-combination plasma concentrations. Two male cynomolgus monkeys were each given two doses immunotoxin-combination with an interval of 48 h (arrows beneath the *x*-axis). One monkey received 0.1 and 0.2 mg/kg (□), the other 0.2 and 0.25 mg/kg (●).

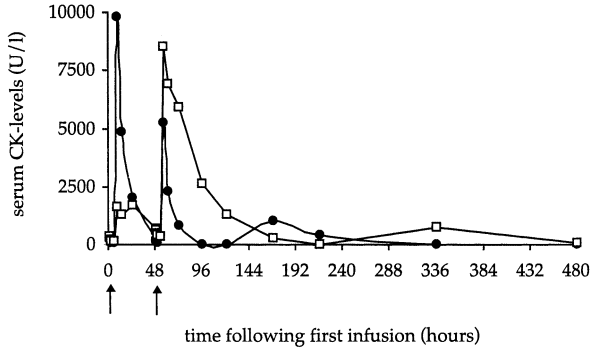

Fig. 5. Serum CK-levels following administration of the immunotoxin-combination to the cynomolgus monkeys. One monkey received 0.1 and 0.2 mg/kg (□), the other 0.2 and 0.25 mg/kg (●), separated by a 48-h interval.

parameters are listed in Table 2. The $T_{1/2}$ of the immunotoxin-combination was 9.3 and 5.7 h in monkeys A and B, respectively. Notably the $T_{1/2}$ of SPV-T3a and WT1 in 'monkey B' differed considerably, being approximately 9 and 2 h, respectively. This may be explained by the capture of WT1-dgA by the CD7-antigen expressed on T and NK cells (virtually all these cells being $CD7^+$ in 'monkey B'). 'Monkey A' with only $\sim 30\%$ of its T/NK cells being $CD7^+$, demonstrated equal half lives for SPV-T3a-dgA and WT1-dgA (~ 9 h). It should be noted that SPV-T3a-dgA is not influenced by expression of the CD3-antigen as SPV-T3a does not bind 'monkey-CD3'. Peak plasma levels were attained directly following each infusion and decreased (nearly) to baseline level in about 48 h. The C_{max} of the immunotoxin-combination ranged from 1.8 to 3.9 μg/ml and strongly correlated with the dose administered. In general, plasma levels of ~ 1.8 μg/ml (10^{-8} M) are considered therapeutic concentrations (Ghetie and Vitetta, 1994). In both monkeys, these plasma concentrations were maintained for 8–10 h following the second infusion.

Regarding the cross-reactivity of SPV-T3a, neither macroscopic nor microscopic examinations revealed any evidence of toxicity towards the esophagus. Moreover, immunostaining of biopsy-derived tissue could not demonstrate any in vivo binding of SPV-T3a-dgA to the esophagus epithelium. Blood chemistry analyses revealed a transient drug-related toxicity. Each administration was followed by a sharp increase in serum creatine kinase (CK), reaching a maximum at 7 h after infusion and normalizing to baseline within

Table 2
Pharmacokinetic parameters ($\pm$S.D.)[a]

Monkey (dose, mg/kg)	Immunotoxin	$T_{1/2\beta}$ (h)	AUC (μg h/ml)	Cl (ml/h)	V_d (ml)	C_{max} (ng/ml)
A (0.1 and 0.2)	SPV-T3a-dgA	9.1 ± 0.6	9.4 ± 1.6	26.5 ± 4.5	347.3 ± 29.1	1.830
	WT1-dgA	9.4 ± 1.6	12.8 ± 1.7	19.6 ± 2.6	265.8 ± 17.5	2.090
	Combination	9.3 ± 1.7	22.3 ± 3.1	4.5 ± 0.6	60.3 ± 4.2	3.920
B (0.2 and 0.25)	SPV-T3a-dgA	9.3 ± 2.0	23.5 ± 3.8	8.5 ± 1.4	113.6 ± 9.2	2.280
	WT1-dgA	2.2 ± 0.2	4.7 ± 0.3	42.9 ± 3.1	133.7 ± 7.4	1.450
	Combination	5.7 ± 1.0	24.8 ± 3.3	8.1 ± 1.1	65.8 ± 4.6	3.840

[a] Abbreviations: $T_{1/2\beta}$, plasma half-life; AUC, area under the concentration versus time curve; Cl, clearance; V_d, volume of distribution; and C_{max}, maximum plasma concentration.

2–7 days (Fig. 5). Following the second peak, both monkeys demonstrated a third, more modest, rise of CK-levels that might be explained by a secondary release of dgA. The increase in CK levels is indicative for destruction of striated muscles and/or heart muscle. Damage of the heart muscle could be excluded by measurement of heart-muscle isomer CKMB. In accordance with the destruction of striated muscle cells, a concomitant (modest) increase in LD, ASAT and ALAT serum levels could be noted. None of the test results were indicative for VLS: no drop in serum albumin, weight gain or edema could be noted. Four weeks after completion of the study all biochemical parameters had returned to baseline levels. Complete blood counts and leukocyte differentials displayed a normal distribution. Immediately after the infusions, both monkeys seemed a bit weakened. Aside from the general anesthesia, this may have resulted from the destruction of striated muscle tissue. One day after the second dose, both monkeys ate almost as normal again. Within a week, both monkeys functioned as before treatment.

4. Discussion

This report describes the laboratory preparation and control of two dgA-based immunotoxins that are currently under evaluation in a clinical pilot-study. The design of this study was strongly inspired by earlier reports on RFB4-dgA, a comparable immunotoxin for the treatment of Non-Hodgkin's lymphoma (Amlot et al., 1993; Sausville et al., 1995). The murine LD50 of the immunotoxin-combination compared favorably with that of RFB4-dgA (25–45 and 14 mg per kg mouse, respectively) (Ghetie et al., 1991). Moreover, the monkey experiment demonstrated pharmacokinetics resembling those previously observed with RFB4-dgA and comparable immunotoxins in man. Importantly, clinically significant plasma concentrations were reached for several hours without being accompanied by acute irreversible toxicities. With respect to the potential cross-reactivity of SPV-T3a-dgA, it was particularly of interest that no binding or toxicity to the esophagus was observed. The main toxicity observed was a clear but transient rise in CK-levels. This is in accordance with myalgias, sometimes associated with rhabdomyolysis, being the second dose-limiting toxicity associated with dgA-immunotoxins in man (after vascular leak syndrome, VLS) (Ghetie and Vitetta, 1994). Consistent with the human studies, this toxicity resolved upon ending the administration of immunotoxin. It should be noted, though, that the animal studies do not eliminate all uncertainties with regard to clinical administration of the immunotoxin-combination. The esophagus of patients with severe graft-versus-host disease may be more accessible for SPV-T3a-dgA (e.g. due to disease-associated lesions) than those of healthy cynomolgus monkeys. Another concern is that monkeys are hardly vulnerable for dgA-induced VLS, the main toxicity reported in man. However, as these issues cannot be clarified by animal studies, the current data were considered maximally supportive for performing the clinical pilot-study.

So far, the preliminary results of the clinical pilot-study are very encouraging and do warrant the set up of multi-center Phase I/II studies. Consequently, in addition to a further scaling-up of production, product-specifications will probably have to meet stricter criteria. A point for improvement is the reduction of the relatively high amount of unconjugated WT1. Moreover, stricter guidelines might ask for future MoAb production to be performed in serum-free medium. This inevitably will result in repeated hybridoma cell banking and subsequent virus testing, as changes in growing conditions might provoke the expression of different latent viruses. As an additional requirement, the actual viral removing capacity of the production process will need to be demonstrated by virus spiking using a scale-down model. Though the FDA foresees the human testing of (modified) MoAb combinations, it is anticipated that the required animal toxicity studies (e.g. acute/subchronic toxicity and reproduction toxicity studies) will have to be performed with both immunotoxins separately.

Notwithstanding the foreseen adaptations, the current batch is very well suited for its intended use: the evaluation in a clinical pilot study for the

treatment of a life-threatening disease. As such, the described production forms a stepping stone towards the future productions as required for the actual registration process. It provided sufficient quantities of proper quality to enable the clinical evaluation in an academic setting. Especially for niche indications like severe graft-versus-host disease, some clinical proof of concept appears a prerequisite for further development by the pharmaceutical industry. The in-house production and clinical evaluation of biological therapeutics by academic centers might therefore contribute increasingly to the development of new drugs within this area.

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