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SENSITIVITY OF FRESH LEUKEMIC CELLS TO T101 RICIN A-CHAIN IMMUNOTOXIN: A COMPARATIVE STUDY BETWEEN Fab FRAGMENT AND WHOLE Ig CONJUGATES*

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Abstract—We compared the cell killing potency of a whole Ig ricin A-chain immunotoxin (T101 IgG-RTA) against its Fab fragment counterpart (T101 Fab-RTA) on both CEM cells and fresh malignant lymphoid cells. A dye exclusion assay (DEA), was used to evaluate the kinetics of leukaemia cell viability mediated *in vitro* by each immunotoxin (IT). This study found that in the absence of ammonium chloride (NH_4Cl), used as an enhancer agent, T101 Fab-RTA was significantly more toxic to both CEM and fresh leukaemia cells than T101 IgG-RTA. In the presence of NH_4Cl (10^{-2} M), while no differences could be found between the two IT on CEM cells, T101 Fab-RTA was clearly superior to T101 IgG-RTA on fresh leukaemia cells. These results suggest that T101 Fab-RTA may offer an excellent alternative to T101 IgG-RTA for IT treatment of CD5 positive leukaemia patients.

Key words: T101 immunotoxin, fresh leukemic cells.

INTRODUCTION

RICIN immunotoxins directed against lymphoid differentiation antigens have been found *in vitro* to be specific, and generally highly effective, cytotoxic agents to lymphoid leukaemia cells [1–6]. However, depending on their construction, ricin-IT present very distinct clinical potentials. While intact ricin-IT are highly potent cytotoxic agents for purging bone marrow [7], their *in-vivo* application is very limited by the tremendous non-specific cytotoxicity of native ricin. In contrast, ricin A-chain subunit-IT (RTA-IT) represent a promising approach in the perspective of a systemic administration in humans since RTA-IT show little non-specific binding to non-target cells [1].

The absence of the B chain, however, which plays a role in facilitating the translocation of the A chain from the endosome into the cytosol greatly decreases the *in-vitro* activity of RTA-IT; several activating agents were used to increase this activity: lysosomotropic amines [8], carboxylic ionophores [8], and free or bound B chain [9]. In the presence of these enhancer agents RTA-IT showed similar if not higher efficacy than intact ricin [8, 10]. This lead to the use of RTA-IT in association with lysosomotropic amines for *ex-vivo* bone marrow purging in both autologous and allogeneic bone marrow transplantation [11, 12].

In order to further improve the clinical applications of RTA-IT administered by i.v. route, another possibility may simply consist in selecting, for a given target antigen, the most activator-independent RTA-IT. As an illustration of this concept Derocq *et al.* recently showed that both T101 Fab and T101 $F(ab')_2$ fragments coupled to the ricin A chain were, in the absence of activator, significantly more cytotoxic than T101 whole Ig conjugates on CEM cells [13].

Similarly, the aim of our study was to evaluate the respective cell killing efficacy of T101 whole IgG-RTA (T101 IgG-RTA) and T101 Fab fragment-RTA

* This work was carried out with the help of the "Conseil Scientifique de la Faculté de Toulouse-Purpan".

Abbreviations: IT, immunotoxin; *RTA*, ricin toxin A chain; *DEA*, dye exclusion assay; *PI*, propidium iodide; *FDA*, fluorescein diacetate; *CLL*, chronic lymphocytic leukaemia; *BMT*, bone marrow transplantation; *MoAb*, monoclonal antibody.

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(T101 Fab-RTA), with or without ammonium chloride (NH_4Cl) as a potentiating agent, on both CEM cells and fresh chronic lymphocytic leukaemia B cells (B-CLL) by using a previously described dye exclusion assay [14]. The findings reported herein show that T101 Fab-RTA induced a higher and more reproducible specific cytotoxic effect with or without NH_4Cl than T101 IgG-RTA on leukaemia cells.

MATERIALS AND METHODS

CEM cell lines

CEM III were subcloned from CEM, a human CD5 positive leukaemia cell line, using a FACS IV cell sorter (Becton–Dickinson, Mountain View, CA) equipped with a single-cell deposition system. Cells were maintained by serial passage in RPMI 1640 medium (Mérieux, Lyon, France) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratory, McLean, VA), 20 mM glutamine, and antibiotics (Streptomycin (100 μg/ml) and penicillin (100 U/ml)). CEM III expressed a mean CD5 density of 30,000 molecules per cell [14].

Patient selection

Ten B-CLL patients with white blood cell counts over 80,000 per mm^3, and who had not received chemotherapy or corticosteroide for at least one month, were selected for this study.

Fresh leukaemia cells

Heparinized peripheral blood samples obtained from leukaemia patients were separated by Ficoll–Hypaque density centrifugation. Mononuclear cells were then washed twice and resuspended at a final concentration of 10^6 cells/ml in RPMI + 20% fetal calf serum.

Immunofluorescence analysis

After Ficoll separation, two washes, and resuspension in RPMI-1640 (SeraMed, France), mononuclear cells were processed for immunophenotypic study by standard indirect immunofluorescence. The monoclonal antibodies (MoAb) used included: T101 (CD5), (Hybritech Inc.), OKT3 (CD3) (Ortho Diagnostic Systems), B1 (CD20) (Coulter, France), SB4 (CD19) and 2G5 (monomorphic anti HLA-DR-DP), both of which were produced in our laboratory (Sanofi, Montpellier, France). In brief, 10^6 cells/100 μl were incubated with various concentrations of MoAb for 30 min in a 4°C ice bath. After incubation, cells were washed twice in cold PBS-BSA and incubated with a secondary antibody, fluorescein isothiocyanate (FITC) conjugated sheep $F(ab')_2$ antimouse IgG (FITC-GAM) (New England Nuclear, Boston MA), at 40 μg/ml for 45 min at 4°C. Cells were then washed twice in cold PBS-BSA containing 0.1% sodium azide and analysed in a fluorescence activated cell sorter (EPICS-C, Coulter Hialey, FL). Percentage of positive cells were calculated (Immuno computer program) after substraction of negative control fluorescence which was determined by using a non immune mouse Ig fraction followed by FITC-GAM.

Preparation of IT

T101 monoclonal antibody, a mouse IgG2a anti CD5 antibody was purchased from Hybritech Inc. (San Diego, CA). For the preparation of Fab fragment, T101 whole IgG was digested with papain and purified by gel filtration or ion exchange chromatography as described [15]. Analysis of Fab fragments by SDS-PAGE revealed undetectable levels of whole antibody. Ricin A-chain immunotoxins were prepared according to the method previously reported [16]. An average value of two ricin A-chain molecules were linked per antibody or Fab fragment. IT concentrations were expressed as molar concentrations of bound A-chain.

Treatment of target cells

One million cells were incubated with IT at a final concentration of 10^{-8} M, with or without NH_4Cl (10^{-2} M), during 4 h in a total volume of 1 ml in 2 ml macroplates (Linbro Flow Laboratories) at 37°C in a humidified atmosphere of 5% CO_2 (pH = 7.4). Controls consisted of cell samples treated with ricin A-chain alone (10^{-8} M) or with NH_4Cl (10^{-2} M).

Colony assay

Cloning assay was performed as previously described [1]. Briefly, 1–10^6 cells were seeded in double layer agarose and incubated at 37°C for 15–20 days. Colonies were score using an automatic colony counter. Results were expressed as an absolute number of surviving cells extrapolated from the cloning frequency [1].

Dye exclusion assay (DEA)

Sensitivity of malignant cells to IT was performed using DEA as previously described [14]. Briefly, after incubation with IT, treated cells were washed once and resuspended in RPMI + 20% FCS. Then, at different times of culture (24, 48 and 72 h) treated cells were stained by propidium iodide (PI) (Sigma) and fluorescein diacetate (FDA) (Sigma) at a final concentration of 10 μg/ml and 1.5 μg/ml respectively. FDA-stained living cells were enumerated using a fluorescent microscope. Each experiment was performed in triplicate. A minimum of 5×10^5 residual viable cells per control was warranted to validate each experiment at different times of culture. Results were expressed either in percentage of relevant controls or in absolute number of residual viable cells.

Cytofluorometric quantification analysis

Mean density of CD5 antigen was performed by indirect immunofluorescence according to the technique described by Poncelet *et al.* [17]. Briefly, for each experiment an internal standard curve was established by measuring mean fluorescence intensity of different CEM subclones whose T101 IgG binding levels (respectively 500, 10,000, 30,000, 44,000 and 95,000 molecules per cell) had been previously determined by saturation experiments using radiolabelled T101 MoAb. Mean fluorescence intensity of these cell lines has been shown to be proportional to the number of T101 molecules bound per cell. Thus, the evaluation of mean fluorescence intensity obtained from T101 IgG reacting at saturation dose (10 μg/ml) with the relevant leukaemia cells permitted, using the standard curve, to calculate the number of CD5 molecules on the cell membrane. For the construction of the standard curve a correlation index higher than 0.99 was warranted to validate each experiment.

Stem cell assay

Normal committed (CFU-GM, BFU-E) and pluripotent (CFU-GEMM) bone marrow precursor cells were obtained

TABLE 1. PATIENTS' CHARACTERISTICS

Patient	WBC (per mm^3)	lymphocytosis (%)	T101 (*)	B_1 (*)	SB_4 (*)	DR (*)	OKT_3 (*)	mean CD5 density (†)
1	98,000	90	87	48	97	85	3	10,000
2	280,000	98	79	71	98	93	1	5500
3	87,900	90	98	81	93	90	5	35,000
4	86,000	93	98	84	97	91	3	2500
5	88,000	91	95	85	87	85	5	6000
6	140,000	91	86	89	96	95	2	9000
7	430,000	96	89	92	72	76	2	11,000
8	350,000	98	99	52	88	92	1	7000
9	88,100	91	93	93	88	NT	2	8000
10	181,800	92	99	NT	98	98	2	9000
mean	183,000	93	92.3	77.2	91.4	89.4	2.6	10,300

* Percentage of positive cells.
† Absolute number of CD5 molecules per cell.

by aspiration from the posterior iliac crests of healthy donors and collected in RPMI 1640 with 100 U/ml preservative free heparin. Mononuclear cells were obtained by centrifugation over a Ficoll–Hypaque gradient under sterile conditions.

CFU-GM colony assays were performed as follows: to bone marrow mononuclear cells was added 30% FCS, 5% medium conditioned by phytohemagglutinin stimulated leukocytes (PHA-LCM), 5% CM 5637 cell line supernatant, 2×10^{-5} M mercaptoethanol, 20% IMDM medium (Gibco), and 40% methyl cellulose 2.2% (Fisher Scientific, Fair Lawn, NJ). One milliliter of cell suspension containing 7.5×10^4 cells were distributed into quadruplicate 35×10 mm Petri dishes (Falcon, France) and cultured at 37°C in a humidified atmosphere of 5% CO_2. CFU-GM colonies, defined as granulocytic, monocytic or eosinophilic aggregates of more than 20 cells were scored on day 14 under an inverted microscope.

For BFU-E and CFU-GEMM colonies, bone marrow cells, were incubated with 30% FCS, 5% PHA-LCM, 5% CM 5637 cell line supernatant, 2×10^{-5} mercaptoethanol, 20% IMDM medium, 40% methyl cellulose 2.2% and 1 U/ml of erythropoietin (Connaught, Toronto, Step III). 7.5×10^4 marrow cells were plated in quadruplicates. BFU-E and CFU-GEMM colonies were scored at day 14.

In these experiments, bone marrow cells were treated with various concentrations of T101 IgG-RTA and T101 Fab-RTA ranging from 10^{-10} M to 10^{-7} M during 4 h at 37°C with or without NH_4Cl (10^{-2} M). Controls consisted of untreated cells and cells treated with ricin A chain alone or mixed with unconjugated T101 Fab fragments at various concentrations (0.1, 1, 10 μg/ml).

Statistical analysis

Comparison between T101 IgG-RTA and T101 Fab-RTA killing efficacy on B-CLL samples was performed by using paired Student's *t* test.

RESULTS

Patients characteristics

Ten B-CLL patients were selected for this study. Each of them presented a high white blood cell count (mean = 183,000 WBC per mm^3) and hyperlymphocytosis (mean = 93%). After Ficoll separation, leukaemia cells were positive for T101 (mean = 92.3%), B1 (mean = 77.2%), SB4 (mean = 91.4%), HLA-DR (mean = 89.4%). In each case, CD_3 (OKT3) positive cells were below 5% (mean = 2.6%). Mean CD5 antigen density was measured by cytofluorometric quantification analysis using T101 IgG MoAb. A mean value of 10,300 CD5 molecules per cell was found with a range at 2500–35,000 as shown in Table 1.

T101-RTA cell killing efficacy on CEM III cells

Cloning assay was used to evaluate both T101 IgG-RTA and T101 Fab-RTA cell killing efficacy on CEM

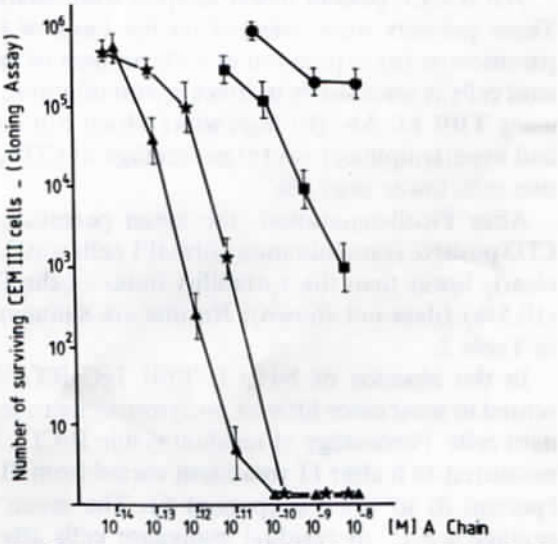

FIG. 1. Cloning assay on CEM III cells: T101 IgG-RTA + NH_4Cl (▲—▲), T101 Fab-RTA + NH_4Cl (★—★), T101 Fab-RTA (■—■), T101 IgG-RTA (●—●).

III cells (see Fig. 1). While in the absence of NH_4Cl, T101 IgG-RTA was almost non effective, T101 Fab-RTA induced, at saturating dose (10^{-8} M), a 3-log kill on CEM III cells. In the presence of NH_4Cl (10^{-2} M) T101 IgG-RTA and T101 Fab-RTA produced a similar dose-dependent effect. At the concentration of 10^{-8} M both the two IT induced a 6-log cytoreduction but no effect was found on CEM III cells treated with RTA alone (10^{-8} M) or with NH_4Cl (10^{-2} M).

Dye exclusion assay (DEA) was also performed in order to compare the respective susceptibility of CEM III cells and B-CLL cells using the same screening system. In four distinct experiments residual viable CEM III cells were enumerated in quadruplicates, 48 h after treatment with IT concentrations varying from 10^{-14} M to 10^{-8} M. For each concentration the mean of absolute numbers of residual viable CEM III cells was calculated. Results were expressed as percentage of CEM III cells treated with RTA alone (10^{-8} M) or with NH_4Cl (10^{-2} M). As shown in Fig. 2, in the absence of NH_4Cl, DEA showed that T101 Fab-RTA (10^{-8} M) displayed a significantly higher cytoreduction with a mean value of 48.1 ± 7.1 (45.2–51) vs the 74.5 ± 5.2 (67–79) obtained with T101 IgG-RTA (10^{-8} M) ($p = 0.01$). In the presence of NH_4Cl (10^{-2} M), DEA showed that T101 IgG-RTA and T101 Fab-RTA displayed similar dose-effects, and no difference was found at the saturating dose of 10^{-8} M. These results were consistent with those obtained from cloning assay (see above).

T101-RTA cell killing efficacy on fresh B-CLL cells

Ten B-CLL patient blood samples were analysed. These patients were selected on the basis of three parameters: (a) expression of CD5 antigen of malignant cells as assessed by indirect immunofluorescence using T101 MoAb; (b) high white blood cell counts and hyperlymphocytosis (c) percentage of CD3 positive cells lower than 5%.

After Ficoll-separation, the mean percentage of CD3 positive contaminating normal T cells was 2.6%, clearly lower than the variability index of the DEA (10.5%) (data not shown). Results are summarized in Table 2.

In the absence of NH_4Cl, T101 IgG-RTA presented in most cases little or no cytotoxicity to malignant cells. Percentage of residual viable B-CLL cells measured 48 h after IT treatment varied from 71.3% (patient 8) to 139.5% (patient 6). The mean percentage ± S.E. of residual malignant cells after IT treatment was 103.4 ± 14.3, 97.4 ± 12.2 and 84.5 ± 16 at 24, 48 and 72 h of culture respectively. Student's *t* test showed no significant difference with

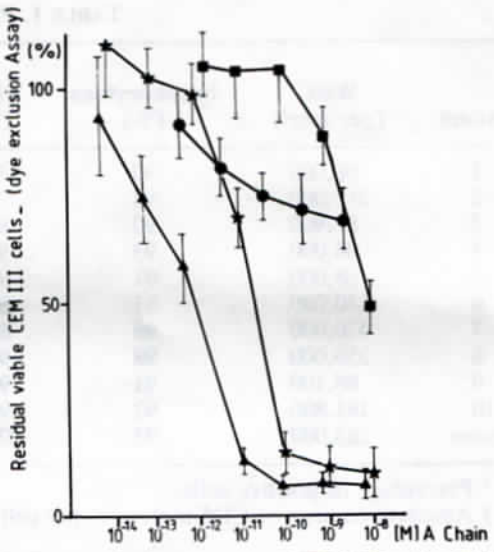

FIG. 2. Dye exclusion assay on CEM III cells: T101 IgG RTA + NH_4Cl (▲—▲), T101 Fab-RTA + NH_4Cl (★—★), T101 Fab-RTA (■—■), T101 IgG-RTA (●—●).

controls (RTA alone 10^{-8} M). T101 Fab-RTA induced in each case a higher cytoreduction with percentages of residual viable B-CLL cells varying at 48 h from 36.7% (patient 4) to 78.0% (patient 2). Mean percentage of residual leukaemia cells after treatment was 90.1 ± 10.3, 49.4 ± 6.2, 39.2 ± 9.4 at 24, 48 and 72 h respectively. Student's *t* test showed a significant difference with controls (RTA alone 10^{-8} M) with $p = 0.01$. These results are depicted in Fig. 3.

In the presence of NH_4Cl (10^{-2} M), T101 IgG-RTA induced a wide range of cytoreduction on B-CLL cells. The percentage of residual malignant cells measured at 48 h varied from 25.5% (patient 4) to 113.7% (patient 2). The mean percentage of residual B-CLL cells after T101 IgG-RTA treatment was 95.5 ± 5.4, 57.9 ± 6.8, 41.9 ± 9.9 at 24, 48 and 72 h respectively. T101 Fab-RTA was significantly more toxic with percentages of residual viable B-CLL cells measured at 48 h varied from 0.7% (patient 6) to 41.0% (patient 10). The mean percentage of residual B-CLL cells after T101 Fab-RTA was 69.3 ± 11.6, 18.9 ± 4.8, 5.1 ± 1.4 at 24, 48 and 72 h respectively. These results are depicted in Fig. 4.

Comparison between T101 IgG-RTA and T101 Fab-RTA cell killing efficacy on B-CLL cells

Paired Student's *t* test was used to analyse the overall differences of activity of the two ITs on fresh leukaemia cells. The results are summarized in Table 3. In the absence of NH_4Cl, T101 Fab-RTA induced

TABLE 2. INDIVIDUAL SUSCEPTIBILITY OF FRESH B-CLL CELLS TO T101 Fab-RTA (Fab) AND T101 IgG-RTA (Ig)

Patient	IT	IT with NH_4Cl (*)	IT without NH_4Cl (*)	mean density of CD5 antigen
1	Fab	5.7	53.9	10,000
	Ig	57.2	93.0	
2	Fab	39.6	78.0	5500
	Ig	113.7	134.0	
3	Fab	15.0	43.5	35,000
	Ig	69.7	107.8	
4	Fab	27.9	36.7	2500
	Ig	25.5	107.6	
5	Fab	16.2	100.0	6000
	Ig	52.4	101.5	
6	Fab	0.7	38.5	9000
	Ig	64.1	139.5	
7	Fab	4.2	48.3	11,000
	Ig	56.2	101.2	
8	Fab	6.8	56.4	7000
	Ig	36.2	71.3	
9	Fab	19.2	56.8	8000
	Ig	88.6	108.0	
10	Fab	41.0	71.2	9000
	Ig	65.3	102.0	

* Percentage of residual viable leukaemia cells 48 h after IT treatment.

a significantly higher cytotoxicity than T101 IgG-RTA ($p = 0.01$). In the presence of NH_4Cl, T101 Fab-RTA was more active than T101 IgG-RTA ($p = 0.01$). Both T101 IgG-RTA and T101 Fab-RTA were potentiated by NH_4Cl ($p = 0.01$).

Comparison between CEM III cell and B-CLL cell susceptibility to T101-RTA

In the absence of NH_4Cl, while CEM III and B-CLL cell viability was not altered after treatment with T101 IgG-RTA, T101 Fab-RTA induced in both CEM III and B-CLL cells an equally significant cytoreduction (48.1 ± 7.1% vs 49.4 ± 6.2% as measured at 48 h of culture). In the same treatment conditions cloning assay showed a 3-log cytoreduction on CEM III cells. In the presence of NH_4Cl, T101 Fab-RTA was as efficiently cytotoxic on both CEM and fresh leukaemia cells in contrast to T101 IgG-RTA which did very little to the latter as depicted in Fig. 5.

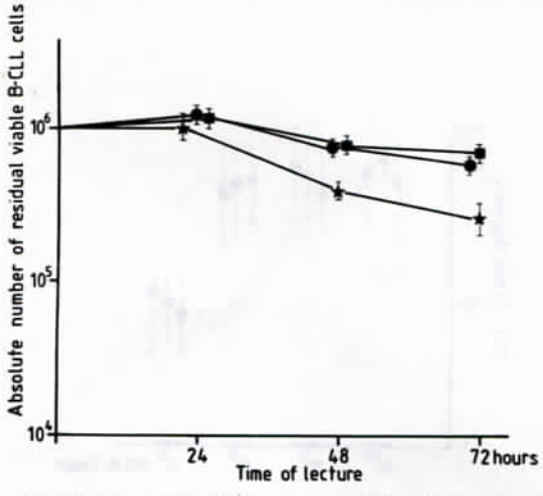

FIG. 3. Kinetics of B-CLL cells viability after treatment with ricin-A-chain alone (■—■), T101 IgG-RTA (●—●), T101 Fab-RTA (★—★) (concentration of 10^{-8} M).

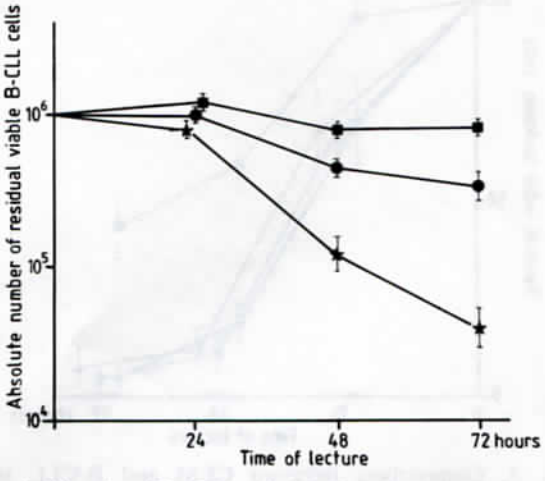

FIG. 4. Kinetics of B-CLL cell viability after treatment with NH_4Cl (10^{-2}M) and ricin-A-chain alone (■—■), T101 IgG-RTA (●—●), T101 Fab-RTA (★—★) (concentration of 10^{-8}M).

TABLE 3. COMPARISON BETWEEN T101 Fab-RTA AND T101 IgG-RTA EFFICACY ON B-CLL CELLS: STATISTICAL ANALYSIS (STUDENT'S *t* TEST)

Time of lecture	24 h	48 h	72 h
RPMI vs RTA RPMI vs RTA + NH RTA vs RTA + NH	NS	NS	NS
Ig RTA vs RTA	NS	NS	$p = 0.05$
Fab RTA vs RTA	NS	Fab > RTA $p = 0.01$	Fab > RTA $p = 0.001$
Fab RTA vs Ig RTA	NS	Fab > Ig $p = 0.01$	Fab > Ig $p = 0.01$
Fab RTA + NH vs Ig RTA + NH	Fab > Ig $p = 0.01$	Fab > Ig $p = 0.01$	Fab > Ig $p = 0.001$
Fab RTA vs Fab RTA + NH	NH > $p = 0.05$	NH > $p = 0.01$	NH > $p = 0.01$
Ig RTA vs Ig RTA + NH	NS	NH > $p = 0.01$	NH > $p = 0.01$
Ig RTA + NH vs MD	NS	NS	NS
Fab RTA + NH vs MD	NS	NS	NS

Abbreviations used are: vs (versus), RPMI (cells incubated with medium), RTA (cells incubated with ricin A-chain alone 10^{-8}M), NH (NH_4Cl 10^{-2}M), Ig RTA (T101 IgG-RTA), Fab RTA (T101 Fab-RTA), MD (mean density of CD5 antigen).

Comparison between mean density of T101 antigen and T101-RTA cell killing efficacy

No correlation was found between the mean density of CD5 and cell killing efficacy of T101 IgG-RTA and T101 Fab-RTA with or without NH_4Cl as shown in Table 2.

T101-IT cytotoxicity on haematopoietic progenitor cells

Bone marrow cells were cultured with various doses of T101 IgG-RTA and T101 Fab-RTA with or without NH_4Cl. No toxicity was found after treatment with any of the two T101-RTA with or without NH_4Cl (10^{-2} M) for concentrations equal or lower than 10^{-8} M in both CFU-GM, BFU-E (data not shown) and CFU-GEMM assays (see Fig. 6).

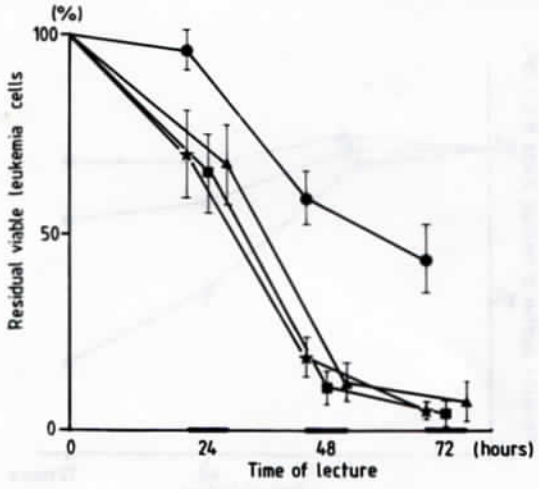

FIG. 5. Comparison between CEM and B-CLL susceptibility in the presence of NH_4Cl(10^{-2}M) to T101-RTA: T101 IgG on B-CLL cells (●—●), T101 Fab on B-CLL cells (★—★), T101 IgG on CEM cells (▲—▲) and T101 Fab on CEM cells (■—■).

DISCUSSION

T101-IT-RTA represents an interesting approach for both *in-vivo* and *ex-vivo* treatment of CD5 expressing malignant chronic and acute lymphoid neoplasias. However, a number of preclinical and

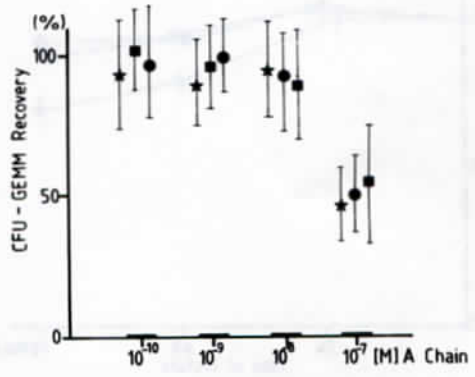

FIG. 6. Toxicity of ricin-A-chain alone (■—■), T101 IgG-RTA (●—●) and T101 Fab-RTA (★—★), with adjunction of NH_4Cl 10^{-2}M, on CFU-GEMM hematopoietic cell progenitors.

clinical studies pointed out several obstacles for both *in-vivo* and *ex-vivo* T101-IT therapy. Systemic administration of T101 whole Ig-RTA (T101 IgG-RTA) in B-CLL patients showed little or no benefit [18, 19] due, at least in part to the poor cell killing efficacy of T101 IgG-RTA in the absence of an enhancer agent as it has been shown *in vitro* on continuous cell lines ([1] this study). *Ex-vivo* treatment of bone marrow with T101 IgG-RTA before autologous BMT might be more effective since IT induced cytotoxicity can be enhanced by the adjunction of potentiating agents such as NH_4Cl [1, 3, 8, 20]. However, in a previous study we found that even if the presence of NH_4Cl, both fresh leukaemia cells and different CEM subclones displayed *in vitro* a wide range of individual susceptibility to T101 IgG-RTA [14].

These findings prompted us to select an anti CD5-RTA which without the adjonction of activating agents can induce a high cytotoxicity on malignant cells, and a more reproducible cytotoxic effect on leukaemia cells in the presence of enhancers. Recently, Derocq *et al.* showed that both T101 Fab and $F(ab')_2$ fragments coupled to ricin A chain are considerably more active on CEM cells than its whole IgG-IT counterpart in the absence of activating agents [13]. The present study aimed to extend this comparison to fresh leukaemia cells and to evaluate the clinical potential of T101 fragment-RTA.

Dye exclusion assay was selected for this study despite the reticence of a decade ago about *in-vitro* drug sensitivity tests based on cell viability. We have previously shown that DEA represents an easy, rapid, and reliable procedure which was found to be, on continuous cell lines, as informative as protein synthesis inhibition assays [14]. Moreover, unstimulated fresh leukaemia cells, especially chronic lymphoid neoplastic cells, usually incorporate very low amounts of radiolabelled leucine or thymidine which limits the use of radionucleotide assays. Stimulation of B-CLL cells is possible by using B-CLL growth factors, lectins or cytochalasin. But stimulation of target cells may notably modify their sensitivity to immunotoxins, as it has been shown with mature T cells [21]. In our study the findings given by DEA on CEM III cells were consistent with those obtained from cloning assay which is currently considered as the most sensitive assay for evaluation of IT activity [14].

In the absence of NH_4Cl, T101 IgG-RTA was practically non effective on both CEM III and B-CLL cells. These results underline the poor clinical potential of T101 IgG-RTA for *in-vivo* treatment of leukaemia patients. T101 Fab-RTA, on the other hand, induced a higher, although moderate, cytotoxicity on B-CLL cells. Through DEA we found that, *in vitro*, CEM III cells and B-CLL cells were equally susceptible to T101 Fab-RTA (10^{-8} M). This finding is encouraging since the cloning assays, done in the same treatment conditions presented a 3-log cytoreduction on CEM III cells. However, the clinical potential of T101 Fab- RTA for *in-vivo* therapy remains uncertain because of other parameters such as unfavorable pharmacokinetics and low affinity.

Enhancer agents such as lysosomotropic amines or carboxylic ionophores were found to dramatically improve RTA-IT cytotoxicity to leukaemia cells [1, 3, 8] as well as to mature T-cells [20]. Thus, NH_4Cl has been extensively used at concentrations varying from 6 to 20 mM as an enhancer agent for *ex-vivo* bone marrow purging with RTA-IT before autologous (elimination of residual malignant cells) [11, 12] or allogeneic (mature T-cell depletion for prevention of graft-vs-host disease) BMT in leukaemia patients [22]. The present study found, as expected from previous experiments performed with clonogenic leukaemia cells, that in most cases NH_4Cl significantly increased the cell killing efficacy of both T101 Fab and T101 IgG-RTA on fresh leukaemia cells. However, in the presence of NH_4Cl, fresh leukaemia cells displayed a significantly higher sensitivity to T101 Fab-RTA than to T101 IgG-RTA; a difference which was not observed on CEM cells. Furthermore T101 Fab-RTA (up to 10^{-8} M) in the presence of NH_4Cl (10^{-2} M) did not hamper CFU-GM, BFU-E and CFU-GEMM hematopoietic progenitor cell recovery of healthy donor bone marrow. Thus, the present study suggests that T101 Fab-RTA in association with NH_4Cl might present a higher clinical potential than T101 IgG-RTA for *ex-vivo* bone marrow purging, of CD_5 positive leukaemia or lymphoma patients before autologous BMT, even if T101 Fab-RTA induced cell killing efficacy remained variable from one patient to another.

The higher activity of T101 Fab-RTA is surprising since T101 Fab antibody displayed a 7- to 13-fold lower affinity compared to the whole T101-IgG [13]. Moreover, Derocq *et al.* showed that 50% of Fab IT had lost their binding capacity likely due to A chains linked to or close to the single antigen recognition site [13]. One could argue that the number of accessible CD_5 Ag per cell is higher with the monovalent fragment. In the same study we ruled out such a possibility since on CEM cells the number of active monovalent T101 IT molecules bound per cell at the IC_{50} was found to be five fold lower than that of T101-IgG-RTA. The higher activity of T101 fragment coupled to ricin A chain compared to its whole IgG counterpart, may be more likely related to a distinct kinetics of internalization or selective routing of IT through cytosol and subsequent degradation.

Thus, Casellas *et al.* recently showed that in the absence of NH_4Cl, after binding to mature T lymphocytes, T101 IgG-RTA is rapidly internalized and subsequently degraded while T101 $F(ab')_2$-RTA translocation and degradation proceeds much more slowly [23].

In the presence of NH_4Cl, T101 IgG-RTA and T101 Fab-RTA induced a similar cytotoxic effect on CEM cells while T101 Fab-IT was much more active on fresh leukaemia cells. It could be possible that the residual monocytes after Ficoll separation played an indirect role by enhancing the modulation of CD5 antigen through cross linking of T101 Ig [24] and hence increase the degradation processes.

Finally, this study found that T101 Fab-RTA offers an excellent alternative to its whole IgG counterpart for *ex-vivo* bone marrow purging and could represent a promising approach to improve the *in-vivo* clinical efficacy of T101-RTA.

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REFERENCES

1. Casellas P., Canat X., Fauser A. A., Gros O., Laurent G., Poncelet P. & Jansen F. K. (1985) Optimal elimination of leukemic T-cells from human bone marrow with T101-ricin-A-chain immunotoxins. *Blood* **65,** 289.
2. Muirhead M., Martin P. J., Torok-Storb B., Uhr J. W. & Vitetta E. S. (1983) Use of an antibody ricin-A-chain conjugate to deplete neoplastic B-cells from human bone marrow. *Blood* **62,** 327.
3. Myers C. C., Thorpe P. E., Ross W. C. J., Cumber A. J., Katz F. E., Tax W. & Greaves M. F. (1984) An immunotoxin with therapeutic potential in T-cell acute leukemia: WT_1-ricin A. *Blood* **63,** 1178.
4. Stong R. C., Youle R. J. & Vallera D. A. (1984) Elimination of clonogenic T-leukemic cells from human bone marrow using anti-Mr 65,000 protein immunotoxins. *Cancer Res.* **44,** 3000.
5. Uckun F. M., Ramakrishnan S. & Houston L. L. (1985) Increased efficiency in selective elimination of leukemia cells by a combination of a stable derivative of cyclophosphamide and a human B-cell specific immunotoxin containing pokeweed anti-viral protein. *Cancer Res.* **45,** 69.
6. Wiels J., Junqua S., Dujardin P., Le Pecq J. B. & Tursz T. (1984) Properties of immunotoxins against a glycolipid antigen associated with Burkitt's lymphoma. *Cancer Res.* **44,** 129.
7. Filipovich A. H., Youle R. J., Neville D. M., Vallera D. A., Quinones R. R. & Kersey J. H. (1984) *Ex-vivo* treatment of donor bone marrow with anti-T-cell immunotoxins for prevention of graft-vs-host disease. *Lancet* **i,** 469.
8. Casellas P., Bourrie B., Gros P. & Jansen F. K. (1984) Kinetics of cytotoxicity induced by immunotoxins. Enhancement by lysosomotropic amines and carboxyl ionophores. *J. biol. Chem.* **259,** 9359.
9. Youle R. J. & Neville D. M. (1982) Kinetics of protein synthesis inactivation by ricin-anti Thy 1.1 monoclonal antibody hybrids. Role of the ricin B subunit demonstrated by reconstitution. *J. biol. Chem.* **257,** 1598.
10. Weil-Hillman G., Runge W., Jansen F. & Vallera D. (1985) Cytotoxic effect of anti-Mr 67,000 protein immunotoxins on human tumors in a nude mouse model. *Cancer Res.* **45,** 1328.
11. Maraninchi D., Novakovitch G., Laurent G., Mascret B., Tubiana N., Derocq J. M., Casellas P., Gastaut J. A., Perrimond H. & Carcassonne Y. (1985) Bone marrow transplantation after *in vitro* purging with T101-ricin-A-chain-immunotoxins: a phase I clinical study. In *Autologous Bone Marrow Transplantation. Proceedings of the First International Symposium,* (Dicke K. A., Spitzer G., Zander A. R., Eds), pp. 401–407. The University of Texas M.D. Anderson Hospital and Tumor Institute, Houston.
12. Gorin N. C., Douay L., Laporte J. P., Lopez M., Zittoun R., Rio B., David R., Stachowiak J., Jansen F. K., Casellas P., Poncelet P., Liance M. C., Voisin G. A., Salmon C., Leblanc G., Deloux J., Najman A. & Duhamel G. (1985) Autologous bone marrow transplantation with marrow decontaminated by immunotoxin T101 in the treatment of leukemia and lymphoma: first clinical observations. *Cancer Treat. Report* **69,** 953.
13. Derocq J. M., Casellas P., Laurent G., Ravel S., Vidal H. & Jansen F. K. (1988) Comparison of the cytotoxic potency of T101 Fab, $F(ab')_2$ and whole IgG immunotoxins. *J. Immun.* **141,** 2837.
14. Laurent G., Kuhlein E., Casellas P., Canat X., Carayon P., Poncelet P., Correls S., Rigal F. & Jansen F. K. (1986) Determination of sensitivity of fresh leukemia cells to immunotoxins. *Cancer Res.* **46,** 2289.
15. Stanworth D. R. & Turner M. W. (1973) In *Handbook of Experimental Immunology* (Weir D. M., Ed.), Vol. 1. Blackwell Scientific, London.
16. Gros O., Gros P., Jansen F. K. & Vidal H. (1985) Biochemical aspects of immunotoxin preparation. *J. Immun. Methods* **81,** 283.
17. Poncelet P. & Carayon P. (1985) Cytofluorometric quantification of cell surface antigens by indirect immunofluorescence using monoclonal antibodies. *J. Immun. Methods* **85,** 65.
18. Laurent G., Pris J., Farcet J. P., Carayon P., Blythman H., Casellas P., Poncelet P. & Jansen F. K. (1986) Effects of therapy with T101 ricin-A-chain immunotoxin in two leukemia patients. *Blood* **67,** 1680.
19. Hertler A. A., Schlossman D. M., Borowitz M. J., Laurent G., Jansen F. K., Schmidt C. & Frankel A. (1988) A phase I study of T101-ricin-A-chain immunotoxin in refractory chronic lymphocytic leukaemia. *J. Biol. Response Modif.* **7,** 97.
20. Derocq J. M., Laurent G., Casellas P., Vidal H., Poncelet P., Fauser A., Demur C. & Jansen F. K. (1987) Rationale for the selection of ricin-A-chain anti-T immunotoxins for mature T-cell depletion. *Transplantn.* **44,** 763.
21. Preijers F. W. M. B., Tax W. J. M., Wessels J. M. C., Capel P. J. A., Dewitte T. & Haanen C. (1988) Different susceptibilities of normal T cells and T-cell lines to immunotoxins. *Scand. J. Immun.* **27,** 533.
22. Blythman H. E., Laurent G., Derocq J. M., Schneider

P. & Jansen F. K. (1988) Treatment of donor bone marrow with Fab T101-ricin-A-chain immunotoxin for the prevention of graft-vs-host disease after allogeneic bone marrow transplantation. In *Lymphocyte Activation and Differentiation* (Mani J. C. & Dornand J., Eds), p. 831. Walter de Cruytez, Berlin, New York.
23. Casellas P., Ravel S., Bourrie B. J. P., Derocq J. M., Jansen F. K., Laurent G. & Gros P. (1988) Entry of T101-ricin-A-chain immunotoxin into human T-lymphocytes. *Blood* **72,** 1197.
24. Schroff R. W., Klein R. A., Farrell M. & Stevenson H. C. (1984) Enhancing effects of monocytes on modulation of a lymphocyte membrane antigen. *J. Immun.* **133,** 2270.