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Induction and increase of HLA-DR antigen expression by immune interferon on ML-3 cell line enhances the anti-HLA-DR immunotoxin activity

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SUMMARY

In order to evaluate the impact of induction and increase target antigen expression on immunotoxin potency, we measured the potentiating effect of recombinant immune interferon-gamma (rIFN-γ) on the cytotoxicity of an anti HLA-DR ricin A-chain immunotoxin (2G5 RTA-IT) on the myeloid cell line ML-3. After 48 h of incubation with rIFN-γ (500 U/ml) the percentage of 2G5-positive cells increased from 40% to 79%, and the 2G5 mean density was enhanced by 10-fold (11 000 *versus* 110 000 molecules/cell). Concurrently, rIFN-γ pretreatment induced a dramatic improvement of 2G5 RTA-IT dose–effect cytotoxicity, as well as immunotoxin cytotoxicity kinetics. When 2G5 RTA-IT was used at the optimal dose of 10^{-8} M (the maximum dose which avoided non-specific ricin A-chain cytotoxicity), the immunotoxin-induced cell kill increased with the percentage of DR-positive ML-3 cells according to a similar linear–logarithmic function of rIFN-γ concentration. Moreover, in the same range of rIFN-γ concentrations, the killing values and the percentage of DR-positive ML-3 cells were similar if not identical. These findings imply that the enhancement of 2G5 RTA-IT cytotoxicity by rIFN-γ is mainly related to the rIFN-γ 2G5 antigen induction on HLA-DR negative cells when immunotoxin was used at 10^{-8} M. Furthermore, 2G5 RTA-IT dose–effect cytotoxicity on DR-expressing ML-3 cells, when used at lower concentrations, was also increased by rIFN-γ in a dose-dependent manner. This result suggests that for immunotoxin concentrations close to the limiting membrane saturation dose (10^{-10} M), rIFN-γ may not solely act by inducing HLA-DR expression on DR-negative ML-3 subpopulation but also by increasing individual cellular DR density on DR expressing ML-3 cells. Finally, our study showed that immunotoxin potency on malignant cell populations which display an heterogeneous antigen expression, could be greatly improved by the use of rIFN-γ.

Keywords immune interferon HLA-DR antigens anti-HLA-DR immunotoxin cytotoxic activity

INTRODUCTION

Individual sensitivity of malignant cells sharing the same target antigen to a given relevant immunotoxin has been well established on both continuous cell lines and fresh malignant cells (Casellas *et al.*, 1985; Laurent *et al.*, 1986; Uckun *et al.*, 1986; Preijers *et al.*, 1988). Several factors may account for such differences in immunotoxin sensitivity including distinct internalization rates, intracellular routing, degradation processes, and target antigen expression.

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Recently, we investigated the influence of target antigen expression on immunotoxin potency (Laurent *et al.*, 1986). These studies suggested that for a given tumour cell population, target antigen-negative cells significantly affect ricin toxin A-chain immunotoxin (RTA-IT) cytotoxicity whereas the quantitative expression of the target antigen on antigen-expressing cells, when above a minimum threshold, should not significantly modify immunotoxin potency. If true, this would mean that in most clinical situations where great heterogeneity in target antigen expression is expected, the efficacy of immunoconjugates could be hampered by the presence of a target antigen-negative subpopulation.

In order to overcome this limit, one approach involves pretreating neoplastic cells with interferons which have been

clearly identified as biological agents capable of modulating the expression of a number of tumour-associated antigens (Giacomini *et al.*, 1984; Greiner *et al.*, 1984; Herlyn, Guerry & Koprowsky, 1985; Taramelli *et al.*, 1986; Borden, 1988; Matsui *et al.*, 1988; Marth *et al.*, 1989).

To explore the influence of interferon on the efficacy of immunotoxin used against malignant cell populations which display heterogenous expression of target antigen, we selected among various cellular models the ML-3 cell line. This myeloid cell line presents a heterogeneous expression of HLA-DR antigen which can be greatly modulated by immune recombinant interferon-gamma (rIFN-γ) (Amatruda *et al.*, 1987). Therefore, we designed a study in which the cytotoxicity of an anti HLA-DR RTA-IT was evaluated after pretreatment of ML-3 cells with rIFN-γ.

We report here that rIFN-γ dramatically enhances the potency of an anti-HLA-DR RTA-IT in a dose-dependent manner by reducing the proportion of escaping HLA-DR-negative cells and by increasing cellular antigen density.

MATERIALS AND METHODS

Cells

The human myeloid cell line ML-3 (a gift from Dr P. Mannoni, Institut Paoli-Calmettes, Marseille) was grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 200 U/ml penicillin and 100 μg/ml streptomycin. ML-3m$^+$ subclone was obtained by limiting dilution and selected for highest 2G5 (HLA class II, see below) antigen expression.

Immune interferon

Human rIFN-γ was purified from *Escherichia coli*, with a purity greater than 99·5% and a titre of 10^7 U/mg protein (a generous gift of Roussel Uclaf, Romainville, France). ML-3 cells (10^5/ml) were incubated in the presence of rIFN-γ at various concentrations (1, 10, 100, 250, 500, and 1000 U/ml) for 48 h in complete medium. For all rIFN-γ concentrations used, treated ML-3 cells displayed the same growth rate and viability as untreated ML-3 cells.

Preparation of 2G5 RTA-IT

Monomorphic anti-HLA-DR 2G5 monoclonal antibody (MoAb) which reacted with DR alpha–DR beta but not with DQ alpha–DQ beta determinants, was prepared as previously described (Demur *et al.*, 1989). Purified 2G5 IgG1 MoAb was conjugated to ricin A-chain according to the technique previously described (Gros *et al.*, 1985). Activated disulphide radicals were introduced into the 2G5 MoAb by treatment with the heterobifunctional SPDP reagent. Following dialysis, activated MoAbs were reacted with excess RTA which resulted in the formation of a disulphide linkage between the two proteins. Immunotoxin was purified by gel filtration chromatography. The immunotoxin preparations obtained contained an average of 1·5-coupled A-chains, 20% of antibody molecules which had not reacted with A-chain and 20% antibody molecules which had more than two A-chains. Immunotoxin concentrations are expressed in A-chain molarity.

2G5 RTA-IT efficacy evaluation by dose-dependent cytotoxicity and kinetics

2G5 RTA-IT efficacy was evaluated by the inhibition of ^{3}H-leucine incorporation, as described previously (Derocq *et al.*, 1987). Briefly, 10^6 cells/ml in log phase, pretreated with or without rIFN-γ, were incubated for 24 h at 37°C in complete medium with various concentrations of toxin or immunotoxin and, where specified, in the presence of the potentiating agent NH_4Cl (10^{-2} M) in order to optimalize RTA-IT cytotoxicity. ^{3}H-leucine was added six h before the end of treatment. Washed cells were harvested on filter paper (Titertek, Flow Laboratories) and radioactivity was measured with a liquid scintillation beta counter. The background uptake of ^{3}H-leucine was obtained using cells treated with 10^{-9} M ricin which ensured a complete inhibition. The background level was always low and represented less than 1% of the control values. The control values of ^{3}H-leucine uptake usually represented more than 10000 ct/min and were not modified by pretreatment with rIFN-γ.

For the cytotoxicity kinetics, the cells were treated with a single dose of immunotoxin (10^{-8} M) during specified times and harvested as above. The T_{50} values, which represent the time required to reduce protein synthesis by 50% were calculated by least-squares regression without considering lag time in a first order process (Casellas *et al.*, 1984).

The results (expressed as mean ± s.d. of quadruplicate experiments) were calculated according to the following formulae:

$$\text{Percentage of } ^3\text{H-leucine incorporation} = \frac{\text{Experimental} - \text{Background incorporation}}{\text{Control} - \text{Background incorporation}} \times 100$$

$$\text{Cell kill} = \%\text{ incorporation (control)} - \%\text{ incorporation (experimental)}$$

$$\text{Survival fraction of DR-positive cells} = \frac{\%\text{ Incorporation (experimental)}}{\%\text{ of DR-positive cells (experimental)}} \times 100$$

The percentage of DR-positive cells was evaluated by flow cytometry as described below.

Immunofluorescence study

The reactivity of 2G5 MoAb with ML-3 cell line was assessed by standard immunofluorescence staining. One-million cells untreated or pretreated with rIFN-γ, were incubated with 2G5 MoAb at the concentration of 10 μg/ml for 60 min in a 4°C ice bath. After incubation, cells were washed twice in ice-cold phosphate-buffered saline (PBS)/bovine serum albumin (BSA) and incubated with a secondary antibody, FITC-conjugated sheep F(ab')$_2$ anti-mouse IgG (New England Nuclear, Boston) for 45 min at 4°C. After two further washes in PBS, cells were immediately analysed by flow cytometry. The negative control consisted in an irrevelant IgG1 anti-CD7 (8H8 MoAb, a gift from Dr C. Mawas, Marseille) as primary MoAb. For each experiment, 10000 viable cells, gated on the basis of forward and perpendicular light scattering, were analysed using a Coulter EPICS-C cytometer. The proportion of positively stained cells against the control was measured by a linked INTEL8086 computer. To evaluate quantitative changes, the results were expressed in arbitrary units based on calibrated

polystyrene fluorescent microspheres or in mean number of DR antigen per cell evaluated by quantitative flow cytometry (Poncelet & Carayon, 1985).

Modulation study

Modulation of 2G5 antigen in the presence of 2G5 RTA-IT (10^{-8} M) was assessed in triplicate on untreated ML-3 cells or cells pretreated with rIFN-γ (500 U/ml). The cell suspensions (10^6/ml) were incubated with immunotoxin at 4°C for 1 h, washed three times to remove unbound immunotoxin and resuspended at 37°C for the indicated times. At the end of the allotted times 10^6 cells were washed twice and resuspended in PBS/BSA with FITC-labelled goat anti-ricin A-chain MoAb for 1 h. The cells were then washed and resuspended in PBS alone for flow cytometry analysis. The negative control was obtained by using 8H8 antibody coupled to ricin A-chain (8H8 RTA-IT); the blocking control, by pre-incubating ML-3 cells at 4°C with 2G5 MoAb (10 μg/ml) 2 h before the beginning of the experiment. The results were expressed in arbitrary units (see above).

^{125}I-radiolabelled 2G5 RTA-IT studies

Labelling of 2G5 RTA-IT. RTA-IT was labelled with ^{125}I (Amersham) in the presence of chloramine T. After 1 min of incubation, sodium metabisulphite, followed by potassium iodine, were added. This preparation was deposited on a PO_4^{2-}-buffered, Sephadex G-25 M, PD-10 column (Pharmacia). The collected fractions which contained labelled protein were pooled and the radioactivity measured on a gamma counter. The resultant iodinated protein preparation had a specific activity of 8 Ci/g.

Saturation curve. The saturation curve was established by incubating rIFN-γ-treated or untreated ML-3 cells (10^6/ml) at 4°C for 1 h with various doses of radiolabelled 2G5 RTA-IT. After three ice-cold washes the radioactivity was measured.

Internalization of 2G5 RTA-IT into ML-3 cells. For internalization and degradation studies, ML-3 cells (10^6/ml) were incubated for 1 h at 4°C with ^{125}I-labelled 2G5 RTA-IT (10^{-8} M), washed, and thereafter warmed to 37°C for the length of the various incubation times. At the end of the allotted times, the cellular suspensions were washed twice. Cell-associated radioactivity was measured in the cell pellet; ligand degradation and shedding was assessed in the medium by TCA-soluble and precipitable radioactivity, respectively. The total of these three radioactivity measurements represented the total radioactivity (100% of ^{125}I ligand) and remained constant throughout the experiment. The negative control was determined by incubating the cells at 4°C with cold 2G5 MoAb (10 μg/ml) 2 h before the addition of ^{125}I 2G5 RTA-IT.

RESULTS

Influence of rIFN-γ on 2G5 RTA-IT immunotoxin efficacy

In the absence of rIFN-γ, ML-3 cells were poorly susceptible to 2G5 RTA-IT cytotoxicity (Fig. 1). The immunotoxin concentration of 10^{-10} M which was determined as the cell membrane binding saturating dose (data not shown) inhibited protein synthesis by less than 10%. Increasing 2G5 RTA-IT concentration by 100-fold did not greatly modify its cytotoxicity. The adjunction of NH_4Cl (10^{-2} M) produced only a small increase in

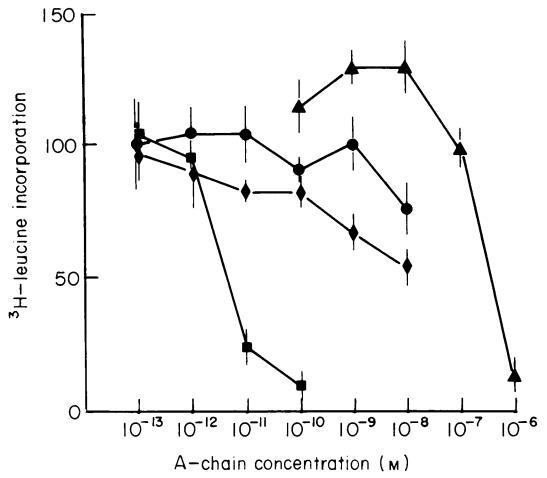

Fig. 1. Toxins and 2G5 RTA-IT dose-dependent cytotoxicity on ML-3 cell line 2G5 RTA-IT (●); ricin α-chain (▲); ricin (■); and 2G5 RTA-IT + NH_4Cl (10^{-2} M) (◆). Means ± s.d.

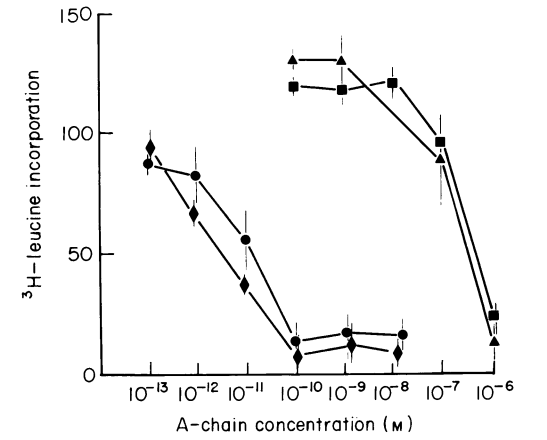

Fig. 2. Toxins and 2G5 RTA-IT dose-dependent cytotoxicity on ML-3 cell line pretreated with rIFN-γ (500 U/ml) during 48 h: 2G5 RTA-IT (●); ricin α-chain (▲); 8H8 (anti-CD7) RTA-IT (■); and 2G5 RTA-IT + NH_4Cl (10^{-2} M) (◆). Means ± s.d.

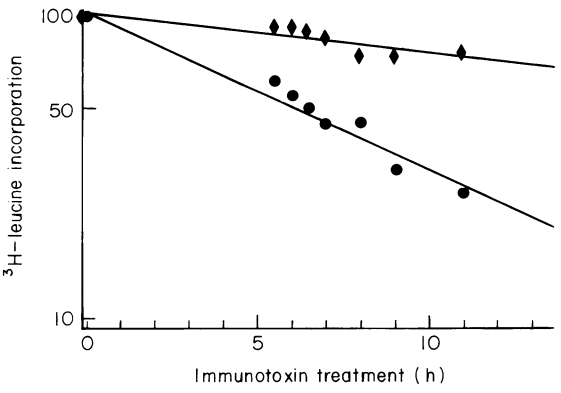

Fig. 3. 2G5 RTA-IT kinetics on ML-3 cells: ML-3 cells (◆); and rIFN-γ-pretreated ML-3 cells (●). Mean %; s.d. < 10%.

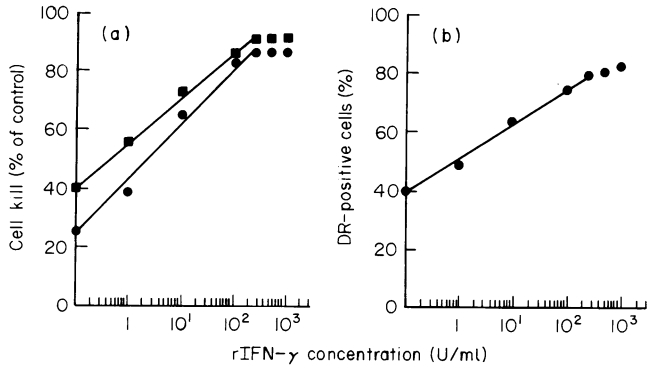

Fig. 4. Dose-effect of rIFN-γ on the cell kill induced by 2G5 RTA-IT (a) and the percentage of DR positive cells (b) on ML-3 cell line. The cytotoxicity of 2G5 RTA-IT (10^{-8} M) was evaluated by ^{3}H-leucine incorporation without NH_4Cl (●) and with NH_4Cl 10^{-2} M (■) and the percentage of DR positive cells was determined by flow cytometry after 48 h of incubation with rIFN-γ. Mean of four replicates; s.d. were always less than 10%.

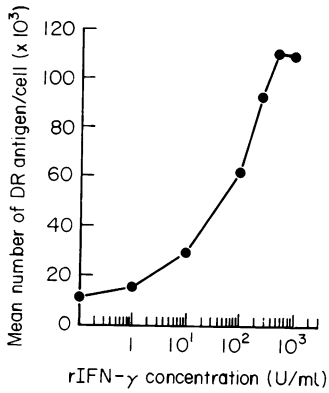

Fig. 5. Effect of rIFN-γ on the mean number of DR antigen per cell, evaluated by quantitative flow cytometry. Mean of three experiments; s.d. were less than 10%.

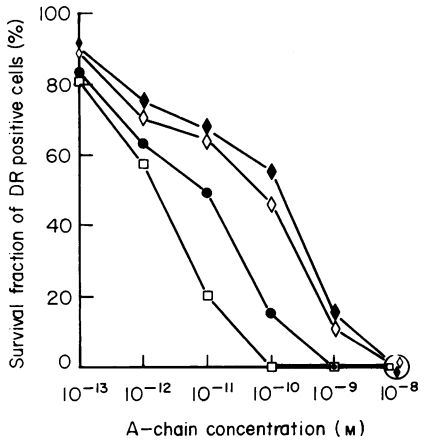

Fig. 6. Survival fraction of DR-positive ML-3 subpopulation pretreated during 48 h with rIFN-γ at 10 U/ml (◇); 100 U/ml (●); 500 U/ml (□); or not pretreated (◆). ML-3 cells were incubated with 2G5 RTA-IT for 24 h in the presence of NH_4Cl (10^{-2} M). Results are evaluated as described in Materials and Methods.

cytotoxicity resulting in 40% inhibition of ^{3}H-leucine incorporation at 10^{-8} M of 2G5 RTA-IT .

ML-3 cells treated with a single concentration of rIFN-γ (500 U/ml) during 48 h displayed an extremely high sensitivity to 2G5 RTA-IT, as depicted in Fig. 2: the immunotoxin concentration required to obtain 50% leucine incorporation inhibition (IC_{50}) was $1{\cdot}3 \times 10^{-11}$ M; 2G5 RTA-IT efficacy was only slightly increased in the presence of NH_4Cl ($IC_{50} = 3{\cdot}4 \times 10^{-12}$ M). A plateau of 85% inhibition of ^{3}H-leucine incorporation was reached at 10^{-10} M of 2G5 RTA-IT, which also corresponded to the saturating dose of 2G5 RTA-IT on rIFN-γ-pretreated cells (data not shown). In these experiments neither ricin A-chain toxicity ($IC_{50} = 3{\cdot}1\ 10^{-7}$ M) nor irrelevant 8H8 RTA-IT activity were increased by rIFN-γ as shown in Fig. 2. Hence an optimal dose of 2G5 RTA-IT of 10^{-8} was used for the remaining investigations.

2G5 RTA-IT kinetics was dramatically improved, as shown in Fig. 3. T_{50} (length of immunotoxin treatment needed to obtain 50% leucine incorporation inhibition) was 6 h and 30 min for rIFN-γ-pretreated ML-3 cells; at the same time period, rIFN-γ-untreated cells showed only 12% of ^{3}H-leucine incorporation inhibition.

2G5 RTA-IT efficacy was dose dependently improved by rIFN-γ. The cell kill induced by 2G5 RTA-IT (10^{-8} M) increased in a linear fashion as the rIFN-γ concentrations rose logarithmically in a range 0–250 U/ml. The slope of the semi-logarithmic dose–response curve was fitted by linear least-squares regression (see Fig. 4a; $r = 0{\cdot}988$). For concentrations higher than 250 U/ml of rIFN-γ, the cell kill was not modified. Similar results were obtained with NH_4Cl 10^{-2} M (see Fig. 4a; $r = 0{\cdot}993$).

Impact of rIFN-γ on HLA-DR expression of ML-3 cells

Modulation of HLA-DR antigen expression was evaluated by measuring the percentage of positive cells and analysing cytofluorometric quantification of mean 2G5 antigen density of ML-3 cell line.

The percentage of 2G5-positive cells (varying from 40% to 79%) increased in a linear fashion as the rIFN-γ concentrations (ranging from 0 to 250 U/ml) rose logarithmically (see Fig. 4b; $r = 0{\cdot}998$). At higher concentrations of rIFN-γ, a plateau was reached and the percentage of 2G5 positive cells was stabilized.

rIFN-γ induced a dose–effect increase in the mean DR antigen density of ML-3 cell line (Fig. 5). A rIFN-γ-dose escalation of up to 500 U/ml increased the 2G5 mean density from 11 000 to 110 000 molecules of 2G5 antigen per cell. At higher concentrations, the mean 2G5 density was unchanged (Fig. 5).

Comparison between DR antigen modulation and 2G5 RTA-IT potency enhancement after rIFN-γ treatment

As depicted in Figs 4 and 5, the pretreatment with rIFN-γ increased concurrently in a dose-dependent manner both the percentage of DR-positive cells and the DR antigen mean density as well as the killing rate by 2G5 RTA-IT (10^{-8} M). It is important to note that the rIFN-γ dose-related increase of ML-3 cell DR mean density did not correlate with the enhancement of immunotoxin potency (Fig. 4a and Fig. 5), 2G5 RTA-IT being fully active at 10^{-8} M (100% of cell kill) on DR-positive ML-3 cells which expressed a mean DR antigen density as low as 11 000. Furthermore, the immunotoxin-induced killing rate and the percentage of DR-positive ML-3 cells seemed to evolve as a

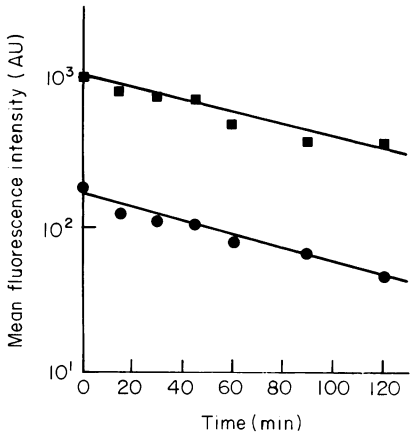

Fig. 7. Kinetics of 2G5 antigen modulation on ML-3 cells: ML-3 cells (●); and rIFN-γ-pretreated ML-3 cells (■). Mean fluorescence intensity was evaluated by flow cytometry and expressed in arbitrary units (AU) based on calibrated fluorescent microspheres. Mean of quadruplicate experiments; s.d. were less than 5%.

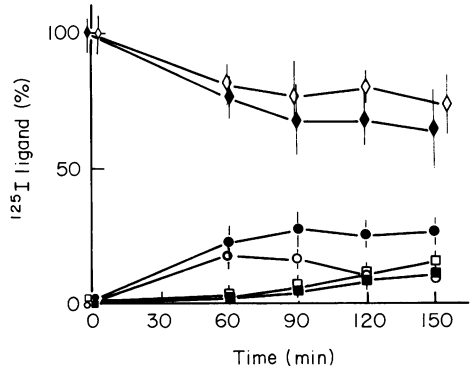

Fig. 8. Kinetics of 2G5 RTA-IT degradation and shedding on ML-3 cells pretreated (closed symbols) or not pretreated (open symbols) with rIFN-γ (500 U/ml). Cellular (diamonds); shedded (circles); and degraded (squares) ^{125}I-2G5 RTA-IT are expressed in percentage of the total radioactivity (100% of ^{125}I ligand).

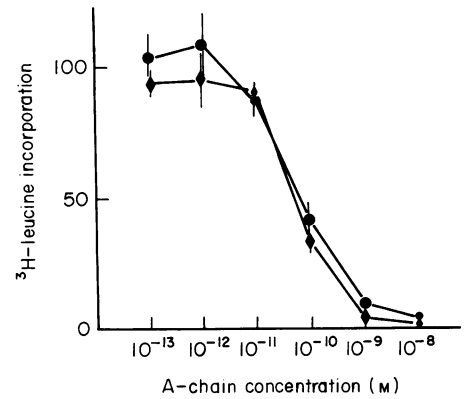

Fig. 9. 2G5 RTA-IT dose-dependent cytotoxicity on ML-3m$^+$ clone: ML-3 m$^+$ cells (●); and ML-3m$^+$ cells pretreated with rIFN-γ (500 U/ml) (◆). Percentage of ^{3}H-leucine incorporation ± s.d.

similar logarithmic function of rIFN-γ concentrations, both of them in the range 0–250 U/ml. Moreover, in the same range of rIFN-γ concentrations, the killing rate values and the percentage of DR-positive ML-3 cells were similar if not identical when the immunotoxin was used with NH_4Cl. These results suggest that rIFN-γ essentially acts by inducing DR expression on the DR-negative subpopulation when 2G5 RTA-IT was used at the optimal selected dose of 10^{-8} M. However, this dose was 100-fold higher than the saturating dose of 10^{-10} M; therefore, we investigated whether rIFN-γ could modify the potency of 2G5 RTA-IT used at lower concentrations on DR positive ML-3 cells.

Influence of rIFN-γ on 2G5 RTA-IT dose–effect cytotoxicity on HLA-DR-positive ML3 cells

As shown in Fig. 6, rIFN-γ notably modified in a dose-dependent manner immunotoxin dose–effect cytotoxicity on DR-positive ML-3 cells for immunotoxin concentrations lower than 10^{-9} M. In the presence of NH_4Cl (10^{-2} M), at the immunotoxin saturating dose of 10^{-10} M, the survival fraction of rIFN-γ-treated DR-positive ML-3 cells was much lower than that of their untreated counterpart (0% *versus* 55%). These differences suggested strongly that at the immunotoxin saturating dose, rIFN-γ increased intrinsic sensitivity of DR-expressing ML-3 cells. Several explanations could be argued, including a possible influence of rIFN-γ on the DR cellular density (10-fold higher for 500 U/ml) or rIFN-γ related modifications on internalization and degradation rates of 2G5 RTA-IT. In order to explore the latter hypothesis, we designed a study in which we compared 2G5 antigen modulation and 2G5 RTA-IT degradation kinetics on both rIFN-γ-treated and untreated ML-3 cells incubated with a saturating dose of 2G5 RTA-IT.

Influence of rIFN-γ on 2G5 RTA-IT entry and degradation

In these experiments, cells were incubated with an excess dose of 2G5 RTA-IT at 4°C in order to saturate fully cell membrane antigens; then cells were washed in cold medium to remove unbound immunotoxin, before warming them at 37°C. Modulation kinetics studies showed that after 2 h of RTA-IT treatment, 76% and 66% of cell membrane-bound 2G5 RTA-IT was lost for untreated and rIFN-γ-pretreated ML-3 cells, respectively. As depicted in Fig. 7, we did not find important modifications of 2G5 RTA-IT induced modulation kinetics between rIFN-γ-pretreated and untreated ML-3 cells. In both cases, the mean fluorescence intensity decreased exponentially as a function of incubation time. ^{125}I-radiolabelled immunotoxin studies showed that cell-associated (cellular), shedded (TCA-precipitable supernatant fraction) and degraded (TCA-soluble supernatant fraction) ^{125}I-2G5 RTA-IT evolved in a similar ratio in ML-3 cells irrespective of pretreatment with rIFN-γ (Fig. 8). Taken together, these findings showed that rIFN-γ did not modify internalization and degradation of 2G5 RTA-IT when used at saturating dose, in the initial phase of RTA-IT action.

Influence of rIFN-γ on ML-3m$^+$ subclone

ML-3m$^+$ subclone was derived from ML-3 parental cell line by limiting dilution and selected for its high 2G5 antigen expression. This subclone displayed a high sensitivity to 2G5 RTA-IT, with $IC_{50} = 6{\cdot}3 \times 10^{-11}$ M, which was not significantly enhanced by pre-incubation with rIFN-γ (500 U/ml during 48 h), as shown in Fig. 9.

Cytofluorometric analysis of ML-3m$^+$ revealed a high percentage of 2G5-positive cells (94%) with a 2G5 antigen mean density of 79 000 2G5 molecules/cell as measured by cytofluorometric quantitative analysis. The percentage of 2G5-positive ML-3 m$^+$ cells as well as 2G5 mean density was not significantly modified after 48 h following rIFN-γ treatment with concentrations up to 500 U/ml (data not shown).

DISCUSSION

Tumour antigen heterogeneity represents one of the major obstacles in immunotoxin therapy. The use of biological response modifiers that can modulate tumour antigen expression offers an attractive approach for improving the clinical potential of immunotoxins. Among them, interferon was found to be one of the most active agent either *in vitro* or *in vivo* for inducing or augmenting the expression of both several tumour-associated antigens (Giacomini *et al.*, 1984; Greiner *et al.*, 1984; Herlyn *et al.*, 1985; Taramelli *et al.*, 1986; Matsui *et al.*, 1988; Borden, 1988; Marth *et al.*, 1989; Ghosh *et al.*, 1989) and MHC antigens (Fellous *et al.*, 1979; Carrel, Schmidt-Kassen & Giuffre, 1985; Taramelli *et al.*, 1986; Piguet *et al.*, 1986; Rowlinson *et al.*, 1986; Amatruda *et al.*, 1987; Balkwill *et al.*, 1987; Testa *et al.*, 1988). The augmented expression of these tumour markers on the cell surface may help to improve the efficacy of immunotoxin against tumour cells that expressed a large heterogeneity of target cell antigen.

ML-3 cells are ideal for testing this hypothesis since they displayed a great heterogeneity in HLA class II antigen expression and were characterized as rIFN-γ-responsive cells in terms of differentiation and HLA class II expression enhancement (Amatruda *et al.*, 1987).

We found that pretreatment of ML-3 cells with rIFN-γ dramatically improved the cytotoxicity of an anti HLA-DR RTA-IT. This enhancement effect of rIFN-γ on immunotoxin potency could be explained by (i) the almost complete switch over of the DR negative ML-3 subpopulation to DR expressing cells; (ii) an increase of the cellular DR density on DR-expressing cells; or (iii) an intrinsic effect of rIFN-γ which could act independently from rIFN-γ-induced antigen modulation. The latter hypothesis seems unlikely, since pretreatment with rIFN-γ did not modify (i) the cytotoxicity of an irrevelant anti-CD8 RTA-IT or uncoupled ricin A-chain; (ii) 2G5 RTA-IT efficacy on ML-3m$^+$ subclone; or (iii) 2G5-RTA routing and degradation processes in ML-3 cells in the initial phase of RTA-IT action. Our findings suggest rather that the immunotoxin-enhancement effect of rIFN-γ is related to the interferon-induced dramatic changes of the DR expression level on ML-3 cells.

rIFN-γ increased concurrently in a dose-dependent manner both the percentage of DR positive ML-3 cells and the DR antigen density and improved the 2G5 RTA-IT dose–effect cytotoxicity. Such findings, therefore, would suggest that rIFN-γ acts by reducing the amount of DR-negative escaping cells and by increasing the individual cellular DR density of DR-expressing cells. However, our study gave some evidence that the respective influence of these two phenomena are not equally balanced.

In fact, the major influence of the target antigen switch-over was clearly demonstrated when the immunotoxin was used at the optimal dose of 10^{-8} M. In these conditions of treatment, the cytotoxicity of immunotoxin was exactly correlated with the percentage of DR positive cells which increased after rIFN-γ pretreatment in a dose-dependent manner. Moreover, when we addressed the question of immunotoxin potency on the DR-positive ML-3 cell subpopulation, we found that 2G5 RTA-IT used at 10^{-8} M was fully active (100% cell kill) on DR-positive ML-3 cells that expressed a mean DR antigen density as low as 11 000 molecules/cell. Therefore, our findings suggest that the cytotoxicity of a potent RTA-IT (such as anti HLA-DR RTA-IT) used at the optimal dose against heterogeneous cell population can be strictly related to the percentage of target antigen expressing cells, when the cellular antigenic density is above a minimal threshold.

However, the enhancement effect of rIFN-γ on immunotoxin dose–effect cytotoxicity on ML-3 cell line seems not to be exclusively related to the HLA-DR antigen induction under the influence of rIFN-γ. Indeed, when we analysed the dose–effect cytotoxicity on the DR-positive ML-3 cell subpopulation, we observed a dose-related enhancement effect of rIFN-γ on immunotoxin potency when used at concentrations lower than 10^{-8} M, a concentration 100-fold higher than the limiting membrane saturation dose. Such an effect, which was observed in a range of 0–500 U/ml rIFN-γ might be explained by the 10-fold increase of the DR cellular density on DR-expressing cells and subsequent higher membrane bound immunotoxin. Therefore, our study suggests that rIFN-γ enhances 2G5 RTA-IT dose–effect potency on ML-3 cells not only by inducing HLA-DR antigen on ML-3 cells but also by increasing the cellular DR antigen density on DR-positive cells, the respective influence of these two mechanisms being influenced by the immunotoxin dose.

Finally, this study shows that immunotoxin potency on a malignant cell population which displays heterogeneous target antigen expression, can be greatly improved by the use of rIFN-γ. Moreover, our study offers an attractive model for testing the potential of rIFN-γ in other immunotargetting approaches, when the heterogeneous target antigen distribution is considered as a limiting factor of immunoconjugates efficacy.

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