Properties of Immunotoxins against a Glycolipid Antigen Associated with Burkitt’s Lymphoma¹

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ABSTRACT

A monoclonal immunoglobulin M (IgM) antibody (38-13) which recognizes Burkitt’s lymphoma (BL) cells, by reacting with the neutral glycolipid Galα1 → 4-Galβ1 → 4-Glcβ1 → 1-ceramide, was recently characterized. This monoclonal IgM was coupled to either ricin A chain or gelonin. The two different immunotoxins obtained retained the apparent immunological specificity of 38-13 IgM, as shown by flow cytofluorometry analysis and complement-dependent cytotoxicity test. The BL Ramos cells and the apparently irrelevant Epstein-Barr virus-containing lymphoblastoid Priess cells were used as targets in in vitro assays of the cytotoxic inhibition of protein synthesis. Isolated ricin A chain, gelonin, and 38-13 IgM exhibited very low intrinsic cytotoxicity on both target cells. 38-13 ricin A chain and 38-13 gelonin conjugates exerted toxic effects on both target cells which were about 6000-fold and 3000-fold higher than uncoupled ricin A chain and gelonin, respectively. The toxicity of these conjugates almost reached that of intact ricin. On Ramos BL cells, the kinetics of action of the 38-13 ricin A chain conjugate was almost as fast as that of intact ricin, because 50% protein synthesis inhibition was reached after 3 hr. In contrast, the kinetics of action in the non-BL Priess was much slower (50% protein synthesis inhibition after 10 hr). An obviously irrelevant immunotoxin (anti-trinitrophenol IgM-ricin A chain) had no significant cytotoxic effect on BL Ramos and non-BL Priess cells. An excess of o-galactose (0.1 M) inhibited the cytotoxic effect of the two immunotoxins by measuring the inhibition of protein synthesis. Isolated ricin A chain, gelonin, and 38-13 IgM exhibited very low intrinsic cytotoxicity on both target cells. 38-13 ricin A chain and 38-13 gelonin conjugates exerted toxic effects on both target cells which were about 6000-fold and 3000-fold higher than uncoupled ricin A chain and gelonin, respectively. The toxicity of these conjugates almost reached that of intact ricin. On Ramos BL cells, the kinetics of action of the 38-13 ricin A chain conjugate was almost as fast as that of intact ricin, because 50% protein synthesis inhibition was reached after 3 hr. In contrast, the kinetics of action in the non-BL Priess was much slower (50% protein synthesis inhibition after 10 hr). An obviously irrelevant immunotoxin (anti-trinitrophenol IgM-ricin A chain) had no significant cytotoxic effect on BL Ramos and non-BL Priess cells. An excess of o-galactose (0.1 M) inhibited the cytotoxic effect of the two 38-13 immunotoxins, whereas it did not prevent the cytotoxic effect of the anti-trinitrophenol immunotoxin on the same trinitrophenol labeled target cells. These data suggest that the cytotoxic effect observed with 38-13 immunotoxins on non-BL Priess cells was mediated through their binding to a very low number of antigenic sites undetectable by conventional immunological methods. The main characteristics of 38-13 immunotoxins appear to be their fast kinetics of action and the very low number of antigenic sites required for the expression of their toxic effects. These properties could be related to the glycolipid nature of the reacting antigen. Such glycolipid antigens would represent valuable targets for therapeutic use of immunotoxins.

INTRODUCTION

Recent works have emphasized the potential therapeutic interest of ITs, which combine on the same molecule the specificity characteristic of antibodies with the cytotoxic efficiency of toxins. In several experimental models, it was clearly demonstrated that ITs could selectively kill target cells (reviewed in Ref. 24). Monoclonal antibodies have revived this approach by enabling toxins to be directed to well-characterized surface antigens (1, 5, 6, 8-11, 22, 23, 27). In humans, some tumor-associated antigens have been defined by monoclonal antibodies (2, 4, 5, 7, 19), and they appear as potential targets for the use of ITs in cancer therapy. We recently described a monoclonal IgM antibody, referred to as 38-13, which was raised against Daudi cells and reacted specifically with BL cells, regardless of their association with Epstein-Barr virus (25, 26). This monoclonal antibody appeared of particular interest for the immunotoxin approach, because it reacts with a glycolipid antigen (12), while all of the previously studied immunotoxins were directed against cell surface proteins. The structure of this glycolipid antigen [Galα(1 → 4)-Galβ(1 → 4)-Glcβ(1 → 1)-ceramide:ceramide trihexosyl] is related to the P blood group substance (15). The properties of 2 immunotoxins prepared with the 38-13 monoclonal antibody coupled with either RTA or GEL are reported here. It will be shown that some of these properties clearly differ from those of previously described immunotoxins.

MATERIALS AND METHODS

Cell Lines. The BL lines Ramos and Daudi were provided by Professor G. Klein (Stockholm, Sweden). The lymphoblastoid cell line Priess was kindly sent by Professor W. Bodmer (London, U. K.). All lines were cultivated in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum.

Monoclonal Antibodies. The production and characterization of the anti-BL 38-13 antibody has been described elsewhere (25). Immune ascites were grown in nude mice. IgM purification was performed by precipitation with 50% ammonium sulfate and filtration on a Sepharose 6B column in sodium phosphate buffer (10 mM pH 8.0; NaCl, 0.5 mM; NaN3, 0.02%). Hybridoma cells secreting the 13.6 IgM monoclonal antibody, directed against TNP, were kindly provided by Dr. J. Theze (Paris, France). 13.6 IgM was purified according to the same procedure as 38-13 IgM.

RTA and GEL. Ricin was extracted and purified from seeds of Ricinus communis according to the method of Lugnier and Dirheimer (13). A-and B-chain separation was performed according to the method of Jansen et al. (7). GEL was prepared from seeds of Gelonium multiform, obtained from M. C. Celo (Zweibrucken, Federal Republic of Germany) and purified as described by Stirpe et al. (20). The biological activity of the toxins was assessed by measuring the induced inhibition of protein synthesis in reticulocyte lysates (17).

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IT Preparation. RTA was coupled with IgM using the heterobifunctional cross-linker N-succinimidyl-3-(2-pyridyldithio)propionate (Phar- 
cacia Fine Chemicals), according to the procedure described previously (3). The number of RTA molecules coupled per IgM was determined by 
measuring the release of 2-thiopyridine (21). 38-13 IT and 38.6 IT contained about 10 RTA molecules per IgM.

GEL was coupled with IgM using N-succinimidyl 3-(2-pyridyldithio)propionate according to the method of Thorpe et al. (22). The obtained 
IT contained about 10 GEL molecules per IgM.

Cell Labeling with TNP. Ramos BL cells were TNP labeled with a 
solution of sodium trinitrobenzene sulfonate (10 mw in phosphate-buff-
ered saline). After 10 min, at 37° the reaction was stopped by addition 
of RPMI 1640 supplemented with 20% fetal calf serum.

Inhibition of Protein Synthesis in Cell Culture. Exponentially growing 
cells in culture were resuspended at 2 x 10⁶ cells/ml in leucine-depleted 
RPMI 1640 (Eurobio, Paris, France) supplemented with 10% fetal calf 
serum. Fifty µl of the culture medium containing various 
concentrations of IT were added. After various incubation times at 37°, 
0.5 µCi of [³H]leucine (New England Nuclear) was added in each culture 
well. After 90 min of incubation, cells were harvested, and the radioac-
tivity incorporated in the cellular proteins was determined in an LKB liquid 
scintillation counter. Results were expressed as a percentage of ³H 
uptake measured in untreated cells.

Controls of Immunological Specificity of Antibodies and ITs. The 
specificity of the antibodies and of the conjugates was tested by com-
fact-dependent cytotoxicity, measured by ⁵¹Cr release and by indi-
rect immunofluorescence assays, using fluorescein-conjugated goat anti-
rat IgM (Nordic). Labeled cells were examined either by fluorescence 
microscopy or by flow cytometry with a FACS IV cell sorter (Becton-
Dickinson).

RESULTS

Immunological Properties of 38-13 IT. In order to check that the 
chemical coupling of toxins did not modify the immunological 
specificity of the 38-13 antibody, the reactivity of both uncoupled 
and coupled 38-13 IgM was compared on BL and non-BL cells. It 
can be seen in Table 1 that the coupling of the toxin to 38-13 
did not affect its reactivity with Daudi and Ramos BL cells and 
did not confer any cytotoxicity against the apparently irrelevant 
Priess cells. Similarly, the binding of 38-13, 38-13-Gel, and 38-
13-RTA analyzed by flow cytometry appeared similarly on the 
Ramos BL cells, whereas no significant binding could be 
detected with any of these reagents on the non-BL Priess cells 
(Chart 1). It could be argued that, in the indirect immunofluores-
cence assay used here, it is not possible to distinguish between 

did not affect its reactivity with Daudi and Ramos BL cells and 

did not confer any cytotoxicity against the apparently irrelevant 

<table>
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<th>Target cells</th>
<th>2.5 µg/ml</th>
<th>0.1 µg/ml</th>
<th>0.005 µg/ml</th>
<th>2.5 µg/ml</th>
<th>0.1 µg/ml</th>
<th>0.005 µg/ml</th>
</tr>
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<td>Ramos</td>
<td>71</td>
<td>75</td>
<td>63</td>
<td>89</td>
<td>64</td>
<td>108</td>
</tr>
<tr>
<td>Daudi</td>
<td>109</td>
<td>69</td>
<td>66</td>
<td>77</td>
<td>80</td>
<td>96</td>
</tr>
<tr>
<td>Priess</td>
<td>0</td>
<td>0</td>
<td>0</td>
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Results are expressed as:

% of specific lysis = (Experimental ⁵¹Cr release - Maximal ⁵¹Cr release in the presence of complement) / Maximal ⁵¹Cr release in the presence of complement

Table 1

Inhibitory Effects of 38-13 IT on Protein Synthesis in Non-
BL Cells. The effects of 38-13-RTA and 38-13-GEL on the rate of 
protein synthesis appeared to be a relevant assay for analyzing 
of the cellular effect of IT. Typical effects of 38-13-RTA are 
shown in Chart 2. It can be seen that RTA and 38-13-IgM alone 
exhibited very low toxic effects. The RTA data are similar to 
previously published ones (7), indicating that our purification 
procedure resulted in A-chain preparations practically devoid of 
any B-chain contamination. On the other hand, the coupling of 
A-chain on 38-13 conferred a very large increase in the cytotoxic 
effect (about 6000-fold) which almost reached that of intact ricin. 
It was established previously that the antigen recognized by 38-
13 was the ceramide trihexoside glycolipid and that the terminal 
galactose was involved in the combining site, since free D-
galactose could inhibit the binding of the antibody on BL cells 
(12). Therefore, it was possible to check that the toxic effects of 
IT were mediated through their interaction with the antigenic 
sites by studying the inhibition of IT effects by D-galactose. Chart 
2 shows that, indeed, the presence of 0.1 M D-galactose in the 
medium was able to prevent the effect of IT, whereas the same 
dilution of D-galactose did not affect the cytotoxicity of 
RTA (Chart 5). Chart 3 shows the effects of 38-13-GEL on protein 
synthesis in Ramos cells. The results are similar to those 
obtained with 38-13-RTA with a clear gain in toxicity (about 
3000-fold) when GEL was coupled to 38-13 antibody. Here, 
again, 0.1 M D-galactose inhibited the effects of the 38-13-GEL, 
demonstrating that its action was mediated through its binding 
to specific sites.

Inhibitory Effects of 38-13 IT on Protein Synthesis in Non-
BL Cells. The effects of 38-13-RTA and 38-13-GEL on the rate of 
protein synthesis in the apparently irrelevant non-BL Priess 
cells are shown in Charts 3 and 4. Surprisingly, similar toxic 
effects of the 38-13-IT were observed on BL and non-BL cells.
ITs against Burkitt Glycolipid Antigen

Chart 2. Inhibition of protein rate synthesis measured in BL cells (Ramos) after 16 hr of incubation induced by intact ricin (•), RTA (○), 38-13 IgM (□), 38-13 IgM-RTA (▴), and 38-13 IgM-RTA in the presence of 0.1 M β-galactose (×).

Chart 3. Inhibition of protein rate synthesis measured after 16 hr of incubation induced by GEL (○) and 38-13-GEL (•) on Ramos cells and GEL (△) and 38-13-GEL (▴) on Priess cells.

Chart 4. Inhibition of protein rate synthesis measured in non-BL cells (Priess) after 16 hr of incubation, induced by intact ricin (•), RTA (○), 38-13 IgM (□), 38-13 IgM-RTA (▴), and 38-13 IgM-RTA in the presence of 0.1 M β-galactose (×).

Chart 5. Inhibition of protein rate synthesis measured after 16 hr of incubation, in Ramos-TNP (a), Ramos (b), and Priess (c) cells induced by intact ricin (•), RTA (○), 13-6 anti TNP IgM (×), and 13-6 anti TNP IgM-RTA (▴).

When the assays were performed after a 16-hr incubation. Again, the toxic effect of 38-13-IT on apparently irrelevant target cells appeared to be mediated through interaction with specific antigenic sites, since 0.1 M β-galactose could also prevent the effects of 38-13-RTA (Charts 2 and 4) and of 38-13-GEL (data not shown).

Effects of IgM anti-TNP-IT on BL and non-BL cells. Because of the observed toxic effects of 38-13 IT on non-BL cells, it appeared of importance to demonstrate that these cells were not binding any IgM in a nonspecific way that would allow the internalization of coupled toxin. An obvious irrelevant IgM was the monoclonal 13.6 IgM directed against the hapten TNP. An anti-TNP IgM-RTA conjugate was prepared by the same procedure as 38-13-RTA.

This anti TNP-IT was highly efficient against TNP-labeled Ramos cells (Chart 5a). In contrast, the same IT had no significant effect on unlabeled Ramos and Priess cells (Chart 5, b and c).

Furthermore, β-galactose, which was shown previously to inhibit the effects of both 38-13-IT, was unable to prevent the cytotoxicity of anti-TNP-RTA against TNP-labeled Ramos cells (Chart 6).

Kinetics of Action of 38-13-RTA. Chart 7 shows the compared kinetics of action of ricin and 38-13 RTA on BL Ramos and non-BL Priess cells. The observed kinetics of the ricin effects on the 2 target cells were identical. A 50% protein synthesis inhibition was achieved after about 2 hr. Such results are similar to those reported previously with other cell lines (7, 17). The observed kinetics of 38-13-RTA on Ramos cells was very fast. Indeed, its time of action was almost that of intact ricin. In contrast, the kinetics of action of 38-13-RTA was significantly slower on the non-BL Priess cells (50% protein synthesis inhibition reached after about 10 hr) than on the BL Ramos cells. These results differ strikingly from the kinetics reported for previously studied IT (50% protein synthesis inhibition reached after 3 to 30 hr according to Jansen et al. (7)).

DISCUSSION

In this paper, the properties of 2 immunotoxins prepared with the same monoclonal anti-BL antibody which react specifically with the neutral glycolipid Galα1 → 4Galβ1 → 4Glcβ1 → 1-ceramide are reported.

RTA and GEL were demonstrated previously to require cellular internalization through a cell binding carrier to exert cytotoxic activity (16, 17, 20). Both agents act by inactivating the ribosomes catalytically, and both induce protein synthesis inhibition in acellular systems at very low concentrations. Once internalized, one or very few molecules can be sufficient to kill a cell (17).

Our RTA preparation gave a 50% protein synthesis inhibition
in the studied tumor cells for concentrations of about 5 × 10^{-6} M, in agreement with the results of Jansen et al. (7). In addition, the low in vitro cytotoxicity of A-chain was not affected by o-galactose, whereas o-galactose does protect cells from the toxicity of intact ricin. These results show that our A-chain preparation was practically devoid of any B-chain contamination.

GEL is a single polypeptide chain devoid of any binding lectin-like properties and can be considered as a naturally occurring toxin A-chain (20). Therefore, its extraction and purification is much easier than that of RTA. In agreement with Stirpe et al. (20) and Thorpe et al. (22), we found that GEL elicited a very low cytotoxicity on human cells (Chart 3).

The coupling procedure apparently did not affect the antigen-binding property of the 38-13 monoclonal antibody conjugates. As shown in Table 1 and Chart 1, the immunological reactivity of the conjugate was identical to that of uncoupled 38-13 in complement-dependent cytotoxicity and FACS analysis. Indeed, with these traditional immunological assays, 38-13 IgM-RTA on Ramos cells (8) and Priess cells (9). The concentration of ricin or IT in the assay was 10^{-4} M.

Fast Kinetics of Toxic Effects. Jansen et al. (7) emphasized the slow kinetics of toxic action of most ITs. Fifty % inhibition of protein synthesis is observed after 13 hr with anti-Thy 1.2 IT on the murine WEHI-7 T leukemia cells (7), after 20 hr with the T101 IT on the human CEM T leukemia cells (7), and after 30 hr with the anti-P97 IT on the human SK-MEL 28 melanoma cells (4). In contrast, 50% protein synthesis is obtained after only 3 hr with the 38-13 IT on BL target cells.

Such behavior suggests that, as intact ricin, 38-13 IT follow a specific cellular entry pathway allowing fast internalization. Preliminary results of direct measurement of the rate of internalization of the 38-13 IgM after binding on BL cells showed that internalization required less than 5 min.4 In the prospect of in vivo utilization, the kinetics of IT action could be critical. Indeed, rapidly internalized IT would not have to be maintained at high serum levels for considerable lengths of time to achieve their therapeutic action.

Effects on Apparently Irrelevant Cell Lines. As already pointed out, the 2 38-13 ITs used here kept their apparent immunological anti-BL specificity when tested by usual immunological assays (including flow cytometric analysis). These IT were, nevertheless, clearly toxic on irrelevant Priess cells. However, as shown in Chart 7, the action kinetics of IT appeared slower for Priess than for the relevant Ramos BL cells.

On the one hand, it must be pointed out that the detection of antigens at the cell surface by usual immunological assays (complement-dependent cytotoxicity, immunofluorescence) requires a minimal number of target sites (at least 4000 in flow cytometric analysis) and a minimal stability of the antigen-antibody complexes at the cell surface. On the other hand, cell killing by ITs could require them only to bind to a very few number of specific antigenic sites, undetectable by traditional immunological methods but sufficient for driving the internalization of the toxin. A similar observation was made by Krockick et al. (8), who reported that murine BCL1 leukemic cells were killed by anti-6 IT, whereas no IgD could be detected on their surface by FACS analysis.

Our results show that the toxic action of 38-13 IT on the non-BL Priess cells is mediated to their binding to a low number of otherwise undetected antigenic sites, since o-galactose was able to compete with 38-13-IT and prevent their toxicity. The clearly irrelevant anti-TNP-IT was devoid of any toxic effect on the same target cells, whereas it was clearly active on TNP-labeled cells. Furthermore, o-galactose was unable to prevent the toxic effect of anti-TNP-IT on TNP-labeled target cells. These data show that nonspecific absorption of IgM-IT on the cell surface is unable to drive the internalization of the toxin.

The glycolipid nature of the BL-associated antigen could be responsible for the unusual properties of the 38-13 IT. An increasing number of human tumor-associated antigens which were defined by monoclonal antibody appear to be of a glycolipid nature (14, 15, 18). Such antigens might represent a type of structure that is widespread on tumoral cells. The detection of a very low number of antigenic sites by ITs directed towards such antigens might not be a disadvantage but could permit efficient and rapid killing of a wide variety of tumor cells.

Some properties of these 38-13 ITs must be emphasized, because they differ strikingly from those of previously described immunonjugates.

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**Chart 6.** Effect of o-galactose on the inhibition of protein rate synthesis measured as a function of the incubation times induced by ricin on Ramos cells (8) and Priess cells (9). 38-13 IgM-RTA on Ramos cells (8) and Priess cells (9). The concentration of ricin or IT in the assay was 10^{-4} M.

**Chart 7.** Inhibition of protein rate synthesis measured as a function of the incubation times induced by ricin on Ramos cells (8) and Priess cells (9). 38-13 IgM-RTA on Ramos cells (8) and Priess cells (9). The concentration of ricin or IT in the assay was 10^{-4} M.

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4 J. Wiel and P. Metaizeau, unpublished results.
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