Cancer Immunol Immunother (1993) 36: 346–350

Cancer Immunology Immunotherapy

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Short communication

In vitro and in vivo properties of an anti-CD5 – momordin immunotoxin on normal and neoplastic T lymphocytes

G. Porro[1], A. Bolognesi[2], P. Caretto[1], G. Gromo[1], P. Lento[1], G. Mistza[1], T. Sciumbata[1], F. Stirpe[2], D. Modena[1]

[1] Italfarmaco Research Centre, Via dei Lavoratori 54, I-20092 Cinisello B. (Mi), Italy
[2] Dipartimento di Patologia Sperimentale, Univ. degli Studi di Bologna, Via S. Giacomo 14, I-40126 Bologna, Italy

Received: 7 August 1992/Accepted: 30 November 1992

Abstract. An anti-CD5 monoclonal antibody (mAb) was linked to the plant toxin momordin, a type-1 ribosome-inactivating protein purified from *Momordica charantia.* The in vitro cytotoxicity of the immunotoxin was evaluated as the inhibition of protein and/or DNA synthesis on isolated peripheral blood mononuclear cells (PBMC) and on human T cell leukemia Jurkat. The potency of the immunotoxin on PBMC was very high ($IC_{50} = 1-10$ pM) and was not affected by blood components. The conjugate was also very efficient in the inhibition of the proliferative response in a mixed lymphocyte reaction ($IC_{50} = 10$ pM). Moreover, the in vitro performances of the immunotoxin compared favourably with those reported for other anti-CD5-based immunoconjugates containing ricin A chain. The in vivo activity of the immunotoxin was assessed in the model of *nu/nu* mice bearing Jurkat leukemia. A significant inhibition of the tumour development (80%, $P < 0.01$) in the animals treated with immunotoxin was observed. Taken together, the in vitro and in vivo results suggest that the anti-CD5–momordin conjugate may be useful for graft-versus-host disease therapy and potentially in the treatment of CD5-positive leukemias and lymphomas.

Key words: Immunotoxin – CD5 – Momordin – T lymphocyte – GVHD – Cytotoxicity

Introduction

The availability of mAb technology has made possible the development of antibody conjugates that can deliver a cytotoxic molecule to a defined cellular target in a highly specific manner. Several conjugates have been described as delivering drugs or toxins, of either bacterial [17] or plant origin [1], to neoplastic cells [37] or to T lymphocytes for prevention or therapy of graft-versus-host disease (GVHD) [6, 13].

Toxins are far superior to antimetabolites as cytotoxic agents, only one toxin molecule being sufficient to kill one cell [9]. The most frequently used plant toxin is the type-2 ribosome-inactivating protein (RIP), ricin. However, this toxin presents many problems due to the presence of the B subunit, a galactose-specific lectin that binds to virtually all cells, thus conferring very high aspecific toxicity to the immunotoxin [20]. This disadvantage can be overcome by the use of ricin A chain, either purified from the whole toxin extracted from castor beans [10] or produced by recombinant DNA technology [19]. An alternative approach is represented by the use of type-1 RIP, a class of single-chain ricin-A-chain-like toxins that only have the *N*-glycosidase activity necessary for protein inhibition. Type-1 RIP show several advantages over ricin and other type-2 RIP [32]: (a) low aspecific activity, (b) easier and safer preparation and handling, (c) high stability, (d) a wide spectrum of molecules that can be substituted during the therapy in patients developing anti-toxin antibodies, (e) a resultant immunotoxin that is sometimes more potent than those prepared with ricin A chain. Despite their good features, only a few reports can be found in the literature describing type-1-RIP-based immunotoxins and their applications in vitro [4, 7, 8, 18, 29, 30, 33] and in vivo [12].

Different mAb that recognize T lymphocytes surface molecules have been considered for immunotoxin preparations for bone marrow purging [35]. However, many questions have arisen about the efficacy of this approach in the prevention of GVDH. There is evidence that a complete T cell depletion increases the graft rejection rate [16] and it is still debated which types of T cell subpopulations are involved in the engraftment or graft failure phenomenon [5]. The use of a conjugate between anti-CD5 mAb and ricin A chain for in vivo therapy of steroid-resistant GVHD has been recently described [6]. CD5 is a 65-kDa membrane glycoprotein expressed on 90%–95% of human peripheral blood T lymphocytes, on 15% B lymphocytes, on most T-lymphocyte-derived tumours and on chronic B-lymphocytic leukemia [26].

Correspondence to: D. Modena

In the present paper we describe the in vitro and in vivo properties of an immunotoxin containing an anti-CD5 mAb chemically conjugated to the type-1 RIP momordin [2], purified from *Momordica charantia.* This immunotoxin has proved to be very active in lymphocyte killing both in vitro and in vivo and might be a good candidate for in vivo GVHD treatment.

Materials and methods

Monoclonal antibody

The anti-CD5 mAb (IgG1) was produced from the hybridoma cell line CRL 8000 [23] (American Type Culture Collection, Rockville, Md., USA) grown as ascites tumour in mice. The mAb was purified by affinity chromatography on Sepharose – protein-A (Pharmacia, Uppsala, Sweden) [11], followed by adsorption chromatography on hydroxyapatite (Bio-Rad, Hercules,. Calif., USA) [31]. The active fractions eluted from the column were pooled, concentrated through an Amicon YM30 membrane, filtered through a 0.2-μm Millipore filter and loaded on a Detoxigel column (Pierce, Beijerland, NL) according to the manufacturer's instructions, to remove the endotoxin content. The purity of the mAb was determined by sodium dodecyl sulphate/polyacrylamide gel electrophoresis [15].

Toxin

The type-1 RIP momordin was purified from the seeds of *M. charantia* as described by Barbieri et al. [3].

Immunotoxin

The anti-CD5 – momordin immunotoxin was prepared by linking the RIP to the antibody through an artificial disulphide bond introduced by 2-iminothiolane [34]. The in vitro activity of the conjugated momordin was tested on a rabbit reticulocyte lysate after reduction with 50 mM dithiothreitol as previously described [34]. The IC_{50} of the immunotoxin (concentration inhibiting 50% of protein synthesis) is expressed as the momordin content, the antibody: momordin ratio ranging from 1 : 1 to 1 : 4 in different preparations.

Cells

Peripheral blood mononuclear cells (PBMC) from healthy volunteers were separated by density gradient centrifugation on Ficoll/Hypaque (Pharmacia). The Jurkat cell line, derived from a human T cell leukemia, was mantained in RPMI-1640 medium (Biochrom, Berlin, FRG) containing 10% fetal calf serum (FCS) (Biochrom), and 50 μg/ml gentamicin at 37° C in a 5% CO_2 humidified atmosphere.

Detection of immunotoxin binding

Cell-surface binding of the immunotoxin to PBMC and Jurkat cells was assessed by flow cytofluorimetry on a fluorescence-activated cell sorter (FACScan; Becton Dickinson, Mountain View, Calif.) according to the manufacturer's instructions.

Cytotoxicity tests

a) PBMC. The cytotoxicity test was routinely performed as follows: 10^6 isolated PBMC were incubated with either immunotoxin, mAb or toxin 1 pM – 10 nM in a 5% CO_2 humidified atmosphere at 37° C for 1 h. After incubation PBMC were washed twice and seeded in microtiter plates (5×10^4 cells/well) in 0.2 ml RPMI-1640 medium supplemented with 15% FCS, 10 μg/ml phytohaemagglutinin (Wellcome, Crewe, UK) and 50 μg/ml gentamicin and cultured for 72 h. To evaluate whether longer exposure to the immunotoxin could increase its cytotoxic effect, PBMC were incubated as above in the presence of immunotoxin for 4 h or 72 h. To determine the DNA and protein synthesis, cultures were labelled either with 1 μCi/well [^{3}H]thymidine or [^{3}H]leucine respectively, 15 h before harvesting. The cells were transferred onto filter-paper disks by a cell harvester (ICN Biomedicals, Costa Mesa, Calif., USA) and the incorporated radioactivity measured by a liquid scintillation β counter (LKB, Uppsala, Sweden).

b) Whole peripheral blood. Either immunotoxin mAb or toxin was added to 1 ml heparinized whole peripheral blood immediately after it had been withdrawn, and the samples were incubated as described above. After 1 h the PBMC were separated from each sample by density gradient centrifugation. The isolated cells were washed, seeded and incubated for 72 h as above. The DNA synthesis was measured by [^{3}H]thymidine incorporation as described above.

c) Jurkat cell line. Jurkat cells were seeded in microtiter plates (2×10^4 cells/well) and incubated for different times (24, 48 and 72 h) with either immunotoxin, mAb or toxin as above. The protein synthesis was determined as described in paragraph (a).

Mixed lymphocyte reaction

PBMC were isolated from two unrelated histocompatibility-locus-antigen(HLA)-non-identical subjects (responder, R; stimulator, S). A sample of 5×10^4 R-PBMC was cocultured with 5×10^4 ^{137}Cs-irradiated (5000 Gy) S-PBMC in round-bottomed microtiter plates in the presence of either immunotoxin, mAb or toxin. After 7 days of incubation at 37° C in a 5% CO_2 humidified atmosphere, T lymphocyte proliferation was determined as DNA synthesis (see above).

In vivo immunotoxin treatment

Female *nu/nu* mice (background Swiss), 4 weeks old, were splenectomized and sublethally irradiated with ^{137}Cs (4.5 Gy) 3 days before transplantation. Mice were injected i. v. with 0.2 ml diluted anti-asialoGM1 antiserum 24 h after splenectomy and irradiation. Treated mice, hereafter referred as *nu/nu* SIA (splenectomized, irradiated, anti-asialoganglioside-treated) mice [25, 36], were challenged s.c. in the left inguinal region with 0.3 ml saline solution containing 10^7 Jurkat cells. The mice were divided into five groups 3 days after inoculation, treated with either immunotoxin (5 μg or 10 μg/mouse), mAb (10 μg/mouse), toxin (10 μg/mouse) or saline, and injected s.c. in the peritumoral area twice a week for nine injections. The tumour mass was measured twice a week and the tumour development was followed for 120 days.

Results and discussion

Conjugation of momordin to anti-CD5 mAb affected neither the toxin activity nor the mAb binding characteristics. This was demonstrated by the comparable activity of momordin on protein synthesis in a cell-free system before and after conjugation as well as by the percentage of CD5-positive cells recognized by the immunotoxin and native mAb (75% of PBMC and 99% of Jurkat cells) as determined by cytofluorimetric analysis (data not shown). The effect of the immunotoxin was evaluated on isolated PBMC cultured for 1 h in the presence of increasing con-

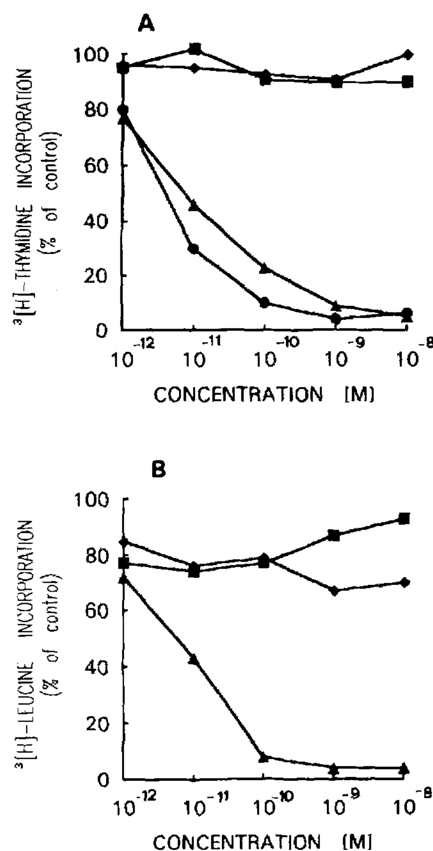

Fig. 1 A, B. Effect of the immunotoxin on DNA and protein syntheses of T lymphocytes. **A** Isolated peripheral blood mononuclear cells (PBMC) (▲) and heparinized whole peripheral blood (●) were treated with immunotoxin for 1 h. After incubation, PBMC from whole blood were separated and cultured in parallel to the preseparated sample in the presence of phytohaemagglutinin for 72 h. Control curves with toxin (◆) and mAb (■) were prepared using pre-isolated PBMC. The results are expressed as the percentage of [^{3}H]thymidine incorporation in the treated samples relative to the control. Data are expressed as means of six separate experiments for PBMC and three for whole blood, each performed in triplicate. SE never exceed 10%. **B** Isolated PBMC were treated with either Immunotoxin (▲), toxin (◆) or mAb (■) as described in Materials and methods. The results are epxressed as the percentage of [^{3}H]leucine incorporation in the treated samples relative to the control. Data are expressed as means of two separate experiments, each performed in triplicate

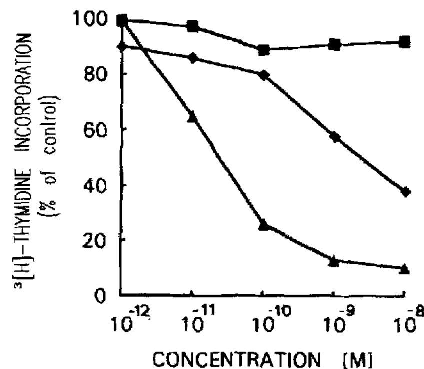

Fig. 2. Effect of immunotoxin in PBMC proliferation in mixed lymphocyte reaction. Responder and stimulator PBMC were cocultured in the presence of either immunotoxin (▲), toxin (◆) or mAb (■) for 7 days. The results are expressed as the percentage of [^{3}H]thymidine incorporation in the treated samples relative to the controls. Data are expressed as means of three separate experiments, each performed in triplicate

centrations, as described in Materials and methods. As shown in Fig. 1 A, B the immunotoxin inhibits both DNA and protein synthesis (IC_{50} = 7.5 pM and 6 pM, as momordin content, respectively) to a comparable extent. Longer treatment of PBMC with immunotoxin for up to 72 h did not increase the inhibition of [^{3}H]thymidine or [^{3}H]leucine incorporation (IC_{50} = 2 pM and 1 pM, as momordin content, for DNA and protein synthesis respectively), demonstrating that a short time is sufficient for the immunotoxin to exert its cytotoxic activity.

From the results reported above it appears that the in vitro activity of anti-CD5–momordin differs greatly from that reported for other anti-CD5-based immunoconjugates containing ricin A chain. These immunotoxins are active only upon longer incubation with cells and/or in the presence of enhancer compounds (NH_4Cl, chloroquine or other lysosomotropic compounds) [14, 22, 27] that have little or no effect on immunotoxins containing type-1 RIP [28]. This different behaviour might be explained by an easier uptake of the momordin-containing immunoconjugate and/or by differences in the intracellular routing of the different immunotoxins. A similar result is reported by Tazzari et al., who demonstrated that an anti-CD30 mAb conjugated to saporin performs better than the ricin A chain conjugate in a cytotoxicity assay [33].

In view of its possible in vivo use, we investigated the immunotoxin activity on T lymphocytes in freshly withdrawn human peripheral blood samples incubated with the conjugate for 1 h before density gradient separation and phytohaemagglutinin stimulation. As shown in Fig. 1 A, the pattern of DNA synthesis inhibition in unseparated cells was parallel to that obtained for separated PBMC (IC_{50} = 3 pM). This result is in keeping with preliminary stability data indicating that, after 2 h of incubation at 37°C in human plasma, 80% of the conjugate is still present in the intact form (HPLC analysis, unpublished results). Taken together these findings seem to indicate that the disulphide bond of mAb-momordin is not affected by blood cells and plasma factors. This stability is essential for the therapeutic efficacy of the conjugate.

It is noteworthy that in PBMC exposed for 1 h to the toxin or antibody alone, even at 10 nM concentration, no cytotoxic effect could be detected after 3 days of incubation (Fig. 1 A, B). A low level of toxicity was detectable only after 7 days of incubation in the presence of momordin (mixed lymphocyte reaction experiment, Fig. 2) probably because of aspecific endocytosis.

We choose the mixed lymphocyte reaction to evaluate the cytotoxic effect of the immunotoxin in an in vitro model of T cell proliferation closely related to the physiopathological activation. In this system the proliferation stimulus is mediated by non-self antigen recognition between T lymphocytes deriving from non-identical MHC donors. As shown in Fig. 2, following 7 days of incubation in the presence of the immunotoxin, the 50% inhibition of alloreactive T cell proliferation was achieved at an immunotoxin concentration of 20 pM. These results, taken together with those obtained in phytohaemagglutinin-

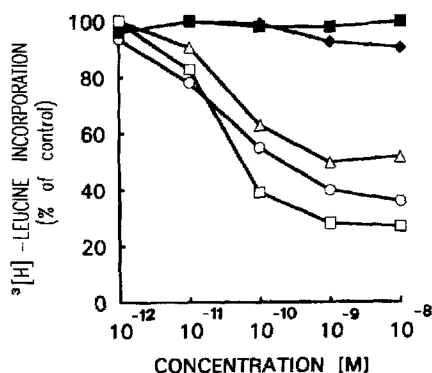

Fig. 3. Effect of incubation time on the inhibition of protein synthesis in Jurkat cells. Cells were cultured for different times: 24 h (△), 48 h (O) or 72 h (□), in the presence of immunotoxin as indicated in Materials and methods. Toxin (◆) and mAb (■) inhibition curves are referred to 72 h of incubation. The results are expressed as the percentage of [^{3}H]leucine incorporation in the treated samples relative to the control. Data are expressed as means of two separate experiments, each performed in triplicate

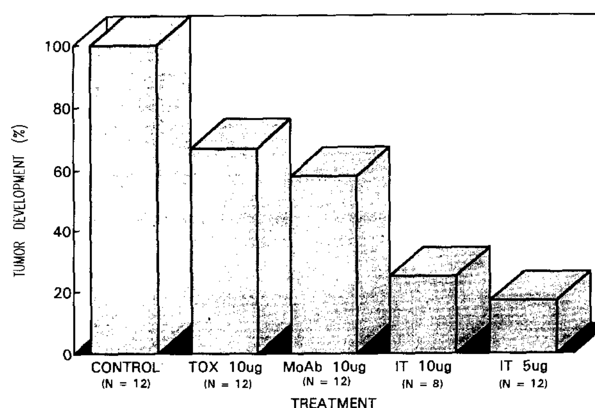

Fig. 4. Effect of immunotoxin on tumour development in *nu/nu* SIA mice. Samples of 10^7 Jurkat cells were injected into mice s.c. and, starting on day 3, the animals were treated twice a week for a total of nine injections with either immunotoxin (5 µg and 10 µg/mouse), toxin (10 µg) or mAb (10 µg). The results are expressed as the percentage of mice that developed the tumour. The number of animals for each group is indicated in the figure

stimulated lymphocytes, demonstrated that the immunotoxin is cytotoxic for activated T lymphocytes independently of the kind of proliferation stimulus. Moreover, the remarkable inhibition of the mixed lymphocyte reaction suggested that the conjugate could be a good candidate for GVHD therapy, at least comparable to anti-CD5-mAb-ricin-A-chain immunotoxins.

We investigated the in vivo activity in nude mice transplanted with the human T cell leukemia Jurkat. The susceptibility of this line to the immunotoxin was evaluated in vitro in a preliminary experiment as inhibition of [^{3}H]leucine incorporation into the protein. The assay was performed by incubating the cells in the presence of increasing immunotoxin concentrations for different lengths of time (24, 48 or 72 h) (see Fig. 3). The sensitivity of Jurkat cells to the immunotoxin was different from the one observed for PBMC, in contrast to data reported by other authors [21]. In fact, to reach the IC_{50} of 50 pM an incubation of 72 h was required, compared to the 1 h necessary for PBMC. Under the same conditions no cytotoxic effect of either mAb or toxin alone was observed at concentrations up to 10 nM. Considering that T lymphocytes and Jurkat cells show a similar CD5 surface-antigen density, the discrepancy in the cellular susceptibility could be due to a different rate of CD5 molecule internalization and/or to a different immunotoxin intracellular destination. For in vivo experiments, five groups of *nu/nu* SIA mice were injected s. c. with Jurkat cells and treated with either saline, mAb, toxin or immunotoxin as described in Materials and methods. The tumour was smaller in the animals treated with immunotoxin than in the other groups from the fourth week from the leukemia inoculum. At day 120 the tumour development was significantly reduced ($P < 0.01$) in the groups of animals treated with the immunotoxin, being 25% and 17% of the control with 10 µg and 5 µg respectively (Fig. 4); the difference between the two treatments is not statistically significant. The inhibition of the tumour growth (42%) observed in the group treated with mAb alone, may be explained by an antibody-dependent cellular cytotoxicity phenomenon mediated by the host natural killer cells and macrophages, which play an essential role in the host natural defence system [24]. In fact, SIA mice are not completely immunodeficient, the effect of anti-asialoganglioside serum and of irradiation being only transient.

Most of the murine models of human tumour development are restricted to the study of in situ tumours and do not simulate disseminated cancer growth; nevertheless, our in vivo results are very encouraging and suggest a potential use of the immunotoxin as an anticancer agent.

In conclusion the anti-CD5-mAb-momordin immunoconjugate may be a good candidate for T lymphocyte depletion in GVHD prevention/therapy. Further investigations are required to evaluate its efficacy as an anticancer drug.

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