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A dominant linear B-cell epitope of ricin A-chain is the target of a neutralizing antibody response in Hodgkin's lymphoma patients treated with an anti-CD25 immunotoxin

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SUMMARY

Hodgkin's lymphoma patients treated with an anti-CD25 Ricin toxin A-chain (RTA)-based Immunotoxin (RFT5.dgA) develop an immune response against the toxic moiety of the immunoconjugate. The anti-RTA antibody response of 15 patients showing different clinical features and receiving different total amounts of RFT5.dgA was therefore studied in detail, considering antibody titre, IgG and IgM content, average binding efficacy and ability to inhibit *in vitro* the cytotoxicity of a RTA-based Immunotoxin. No correlations were found between these parameters and the clinical features of the patients or the total amount of Immunotoxin administered. However, using a peptide scan approach we have identified a continuous epitope recognized by all patients studied, located within the stretch L161-I175 of the RTA primary sequence, close to a previously identified T-cell epitope. The ability of anti-L161-I175 antibodies to recognize folded RTA and to affect the biological activity of RTA by inhibiting RTA-IT cytotoxicity *in vitro* revealed that they may exert an important role in IT neutralization *in vivo*. Discovery of RTA immunodominant epitopes which are the target of anti-RTA immune response may lead to the development of immunomodulating strategies and to more successful treatment schedules.

Keywords human B lymphocytes ricin epitopes immunotoxins

INTRODUCTION

Cell-selective cytotoxic reagents (Immunotoxins, ITs) can be obtained by linkage of vehicle molecules (e.g. antibodies, ligands, growth factors) to potent enzymatic polypeptide toxins (e.g. ricin, Pseudomonas Exotoxin A) [1] and represent nowadays a powerful tool in the oncologist's armamentarium, particularly in the treatment of haematological malignancies [2].

Ricin is a member of the so-called A-B toxin family of potently toxic plant and bacterial proteins that are able to enter and kill mammalian cells [3]. It consists of a catalytically active polypeptide (the A-chain, or RTA), covalently linked via a single disulphide bond to a cell-binding polypeptide (the B-chain, or RTB) [3]. The isolated RTA is frequently used for the construction of anti-target cell ITs [4] and has been evaluated as the toxic component of ITs in the clinics [5,6]. Although RTA-ITs have yielded promising results in phase I-II trials, in most of the reported studies [5,7] the generation of neutralizing anti-IT antibodies limited their clinical impact [8–11]. Host immune response against the vehicle moiety of the ITs could be greatly reduced by using humanized antibodies [12] or cell-selective ligands of human origin (e.g. human Epidermal Growth Factor or transferrin) [13]. However, selective abrogation of the host immune response against the heterologous toxin RTA would require more complex approaches. With these regards, investigating the immunologic profile of RTA represents a crucial prerequisite.

Injection of RTA-ITs *in vivo* results in the production of antibodies belonging to the IgG class, indicating that RTA is a T-cell dependent antigen able to induce a secondary immune response [5]. Thus, identification of both T-cell dependent and B-cell dependent epitopes might open the way to epitope-targeted immunomodulating strategies. We have recently identified a dominant T-cell epitope of RTA recognized in the context of HLA-DRB1*03011 [14]. However, in spite of the critical role of the host antibody response observed in clinical trials using RTA-ITs, no information is presently available on B-cell epitopes of RTA.

RFT5.dgA consists of an anti-CD25 mAb covalently cross-linked to deglycosylated RTA [15] that showed moderate clinical effects in a group of patients refractory to conventional treatments [11,16]. However, the potential clinical effectiveness of such an IT was greatly reduced by the development of human

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anti-mouse antibodies (HAMA) and human anti-ricin antibodies (HARA) negatively affecting pharmacokinetics and pharmacodynamics of the injected IT [11,16]. We have therefore set out to investigating the HARA response in RFT5.dgA-treated patients with the goal of identifying possible target RTA epitopes to be considered in immunoprevention/immunosuppression schedules. To identify linear (continuous) epitopes we used overlapping 30-mer and 15-mer peptides spanning the entire sequence of RTA and assessed their reactivity with immunoaffinity purified anti-RTA antibodies from patients. Using this peptide-scan approach we have identified a dominant linear B-cell epitope recognized by all patients studied who developed neutralizing antibodies against it.

MATERIALS AND METHODS

Reagents

Goat anti-human IgG and IgM alkaline phosphatase-conjugated antisera used in ELISA were purchased from Sigma (Saint Louis, MO, USA). Recombinant Ricin Toxin A chain (rRTA) was expressed, purified and assayed for catalytic activity as described previously [17,18]. The ST.1-RTA IT [19] used in binding and cytotoxicity experiments was kindly supplied by Dr P. Casellas (Sanofi Recherche, Montpellier, France). Ribosome Inactivating Proteins type I (RIPs-I) purified from plants (dianthin, saporin-S6, saporin-L1, pokeweed antiviral protein-S, momordin and gelonin) [3] were provided by Prof F.Stirpe and Prof L.Barbieri (Dipartimento di Patologia Sperimentale, University of Bologna, Italy). Peptides (15-mer and 30-mer) based on RTA protein sequence were synthesized by an Applied Biosystem automated synthesizer on solid-phase [20]; their purity, assessed by HPLC and mass spectrometry, was found to be >90%.

Patients

We studied the sera of 15 Hodgkin's lymphoma patients. Clinical eligibility for submission to trial and RFT5.dgA IT treatment schedules were reported elsewhere [16]. Only patients who did not show evidence of HARA before treatment where considered. The RFT5.dgA IT [15] consists of a murine mAb IgG1 (RFT5), recognizing the alpha-chain of the IL-2 receptor (CD25) on the surface of activated T lymphocytes and covalently linked to deglycosylated RTA (dgA). In Table 1 are reported the main clinical features of the treated patients [16]. All patients considered in the present study gave written informed consent.

Assessment of human anti-RTA response

The quantification of IgG and IgM anti-RTA antibodies in the serum samples of the patients was carried out by ELISA. Wells of microtitre plates (Maxi Sorp Nunc, Denmark) were coated with purified rRTA (1 μg/well in 50 μl PBS) overnight at 4°C and saturated with 3% BSA for 1 h at room temperature. To determine the IgG titre, triplicates of serial dilutions of serum were incubated overnight at 4°C in the presence of 1% BSA. The ELISA was performed following standard procedures using an alkaline phosphatase conjugated goat anti-human IgG antibody (anti-human γ-chain, Sigma, code A-3312). Antibody titre was determined as the serum dilution yielding 50% of maximum signal (absorbance read at 405 nm). Data were expressed as reciprocal of the serum dilution corrected for the background signal (i.e. binding observed in the presence of preimmune sera). Preimmune sera showed no significant reactivity with either heat-denatured RTA (dRTA) or rRTA. An alkaline phosphatase conjugated anti-human IgM antibody (anti-human μ-chain, Sigma, code A-3437) was used to determine the relative IgM content of the sera, expressed as percent of the optical density signal obtained with anti-IgG.

Table 1. Main clinical features of patients treated with the Immunotoxin RFT5.dgA

Patient	Response to treatment†	Primary resistant
110	Partial remission	Yes
112	Partial remission	No
102	Minor response	No
117	Minor response	Yes
103	Stable disease	No
105	Stable disease	No
107	Stable disease	No
118	Stable disease	No
119	Stable disease	Yes
123	Stable disease	No
101	Progressive disease	No
108	Progressive disease	Yes
111	Progressive disease	No
114	Progressive disease	Yes
125	Progressive disease	Yes

†Treatment regimens and patients' features are described in detail in [16].

Anti-RTA antibodies from patients

HARA from each patient were purified by affinity chromatography on Sepharose CL-4B cross-linked RTA (Pharmacia, Sweden). After loading the serum, the column was washed with 9 volumes of phosphate buffer 5 mM (pH 7·5) containing 0·5 M NaCl. Bound antibodies were eluted using a solution containing 3·0 M NaSCN (pH 7·3), recovered and dialysed against PBS. The immunoglobulin content was estimated to be >95%, as evaluated by 8% SDS-PAGE under non reducing conditions.

The purified anti-RTA IgG antibodies were quantified by sandwich ELISA. Wells of microtitre plates were precoated with anti-human IgG (Sigma, code I-1886) and an alkaline phosphatase-conjugated anti-human IgG antibody was used for the detection of bound antibodies. Concentration of IgG antibodies purified from patients was derived from a standard IgG curve. To calculate the anti-RTA IgG concentration originally present in the patients' sera, the initial serum volume was considered. Concentration (mol/l) of antibodies required to obtain 50% binding to rRTA was then used to compare the binding ability of anti-RTA IgG from different patients.

Cross-inhibition of anti-RTA antibody binding

Samples of anti-RTA antibodies obtained from different patients (102, 111, 112, 117, 119) were biotinylated according to the manufacturer's instructions (Sigma). Briefly, antibodies were equilibrated in $NaHCO_3$ 0·1 M (pH 8·0) by dialysis and then incubated for 4 h at room temperature in the presence of a 20-fold molar excess of N-Hydroxy-Succinimidobiotin (NHS-Biotin, Sigma, code B-4531) dissolved in dimethylformamide (DMF, Pierce, Rockford, IL, USA). The excess reagent was removed by dialysis against PBS for 24 h. Non biotinylated antibodies from patient

107 were assayed for their ability to inhibit the binding to rRTA of biotinylated antibodies from other patients. Increasing amounts of antibodies purified from patient 107 (i.e. 10^{-11} M, 10^{-10} M, 10^{-9} M) were incubated for 2 h at 37°C with precoated rRTA (2 μg/well). Biotinylated antibodies (from either patients 102, 111, 112, 117 or 119) were then added and incubated for further 2 h. Bound biotinylated antibodies were detected by using 1 : 1000 dilution of alkaline phosphatase conjugated streptavidin (Amersham). The colourimetric reaction was monitored at 405 nm. Results were expressed as percent inhibition of binding to rRTA of the biotinylated antibodies in the presence of a 1-, 10- and 100-fold molar excess of 107 antibody.

Blockage of ricin and IT activity

To investigate the inhibitory effect of anti-RTA antibodies developed *in vivo* on the cytotoxic activity of ST.1-RTA and of ricin toxin, cytotoxicity experiments were performed *in vitro*. Jurkat cells (50 000/well) were plated and the samples tested in triplicates in 96 round bottom microtitre wells in leucine-free RPMI 1640 medium containing 2·5% FBS and 200 mM glutamine. Cytotoxicity of ST.1-RTA IT was potentiated by adding 50 nM monensin to the culture medium [21]. The assay was carried out under standard culture conditions (37°C, 5% CO_2) for 24 h in the presence of serial dilutions of anti-RTA antibodies (ranging from 10^{-9} M to 10^{-13} M) and a fixed amount of ST.1-RTA ($0{\cdot}5 \times 10^{-12}$ M). After 20 h, cells were pulsed for 4 h with 0·5 μCi of [^{14}C]-leucine (Amersham Life Science, Amersham, UK). When ricin was used ($0{\cdot}5 \times 10^{-12}$ M) the assay was instead performed for 6 h total comprehensive of 4 h of pulsing with[^{14}C]-leucine. The fixed IT or ricin concentration used corresponds approximately to the IC_{50} value (i.e. the concentration yielding 50% cytotoxicity), as evaluated in preliminary dose–response experiments. At the end of the assay cells were harvested and incorporated radioactivity was measured in a β-spectrometer. The results were expressed as percent of protein synthesis with respect to mock-treated control samples. The inhibitory potential of anti-RTA antibodies was determined as percent of protein synthesis rescue with respect to the positive control, in which the presence of the IT or ricin alone leads to maximum cytotoxicity (i.e. 50% cytotoxicity, see above).

Epitope mapping

Peptide scan. In order to identify linear (continuous) epitopes of RTA the protein sequence was subjected to epitope mapping [22,23] using a set of 30-mer overlapping peptides spanning the whole molecule. The reactivity of purified anti-RTA antibodies with solid-phase-coated peptides was detected by ELISA and was expressed as percent binding to whole dRTA. Further analysis was then carried out using a set of 15-mer overlapping peptides spanning the stretch L161-T190 (LPTLARSFII CIQMISEAARFQYIEGEMRT). Data obtained using the anti-RTA antibodies purified from the sera of 15 patients were compared. The median, the 10th, 25th, 75th and 90th percentile were considered to describe their statistical distribution.

Results obtained in peptide scan experiments were compared with the analysis of physicochemical parameters derived from the secondary and tertiary structure of RTA [24] to elucidate the structural properties of B-cell epitope(s) identified in this study. The amino acid sequence of RTA as deduced from the nucleotide sequence of its encoding gene [25,26] was therefore subjected to in silico analysis to predict hydrophobicity/hydrophilicity [27,28] and water accessibility [29]. These analyses were performed with bio-computing software programs from http://www.expasy.ch/cgi-bin/protscale.pl

Binding of anti-RTA antibodies to RIPs-I. RTA shares a high structural and sequence homology with toxins belonging to the family of RIPs-I [30]. To examine whether anti-RTA antibodies from patients' sera recognized domains of toxins belonging to the RIPs-I family, they were challenged with various RIPs-I (dianthin, saporin-S6, saporin-L1, pokeweed antiviral protein-S, momordin and gelonin) in an ELISA by using precoated RIPs-I (2 µg/well). Recognition of RIPs-I was expressed as percent binding to rRTA.

Competitive ELISA

In order to assess the ability of soluble linear peptides to displace the binding of antibodies to whole rRTA, fixed amounts of antibodies from three patients (105, 107, 111) were preincubated in the presence of increasing concentrations of competitor peptides (or PBS-BSA 1% as control reagent). After 5 h incubation at room temperature under rotation, antibodies were transferred to rRTA-coated plates and an ELISA was then performed following the protocol described above. The inhibitory effect of a RTA-derived peptide (L161-I175, LPTLARSFIICIQMI) was compared to the effect of another 15-mer RTA-peptide (S176-T190, SEAARFQYIEGEMRT) used as a negative control. Results were expressed as percent inhibition of antibody binding. The percent inhibition of binding represents the reduction in antibody binding measured in the presence of the competitor peptide, with respect to the maximum antibody binding to rRTA yielding 100% signal in ELISA.

Enrichment of anti-L161-I175 peptide antibodies by immunoaffinity

A pool of anti-RTA antibodies from 5 patients was enriched in antibodies recognizing the L161-I175 peptide by immunoaffinity. Antibodies were first loaded onto an immunoaffinity column prepared by cross-linking the L161-I175 peptide to a CNBr preactivated Sepharose (PRIMM, Milan, Italy). The column was then washed with approximately 10 volumes of 0·1 M Tris-HCl buffer containing 0·5 M NaCl (pH 8·0). Bound antibodies were eluted with Glycine-HCl 0·1 M buffer containing 0·5 M NaCl (pH 2·5) and immediately neutralized by dilution with 5 volumes of 1·0 M Tris-HCl (pH 8·0). Recovered antibodies were dialysed against PBS and quantified by measuring the absorbance at 280 nm. Equal amounts of total anti-RTA antibodies and of immunoaffinity purified antibodies were assayed by ELISA for binding to ST.1-RTA. To role out artefacts due to a possible partial unfolding of the rRTA directly coated onto plastic surfaces [31] the ST.1-RTA IT was anchored to microtitre wells precoated with an anti-mouse Ig antiserum recognizing its antibody moiety.

RESULTS AND DISCUSSION

Anti-RTA antibody response of patients treated with the Immunotoxin RFT5.dgA

The anti-RTA antibody (HARA) response of Hodgkin's lymphoma patients [11,16] (Table 1) was studied. Patients were treated at a dose corresponding to the Maximum Tolerated Dose (15 mg/m^2)/cycle for 2–4 cycles and blood samples were collected at the times indicated in Table 2. A homogeneous group of patients who underwent two treatment cycles and therefore received the same amount of RFT5.dgA was first considered. As

Table 2. Characterization of antibody response to RTA in Hodgkin's lymphoma patients treated with the Immunotoxin RFT5.dgA

Sample	Treatment cycle (day)*	Serum titre†	IgG content (μg/ml)‡	IgM content (% of IgG)§	Binding¶ (mol/lx10^{-12})
110	4 (46)	ND	1·7	11·4	ND
125	4 (46)	ND	1·6	22·1	ND
101	3 (31)	ND	ND	13·2	ND
112	3 (31)	ND	1·0	1·0	ND
117	3 (31)	ND	0·9	0·6	ND
119	2 (115)	1400	0·5	1·7	2·6
105	2 (65)	680	2·1	15·1	20
108	2 (65)	2000	0·4	2·1	1·4
102	2 (64)	3200	2·4	0·6	4·9
103	2 (55)	1100	ND	24·6	ND
123	2 (55)	<150	4·1	10·6	ND
111	2 (38)	640	5·9	10·5	62
118	2 (36)	1540	2·0	2·1	8·7
114	2 (35)	<80	0·8	11·6	ND
107	2 (34)	28200	10·2	10·0	2·4

*Values represent the number of treatment cycles (each cycle consisting of four bolus infusions over 7 days) with the Immunotoxin RTF5.dgA. An amount of 15 mg/m^2 IT per cycle, corresponding to the Maximum Tolerated Dose, was injected. Numbers in brackets correspond to the day of the treatment when the serum was collected. †Values represent the reciprocal of serum dilution required to obtain 50% maximum signal in an ELISA. ‡IgG content is calculated based on recovery of anti-RTA antibodies from immunoaffinity columns. §IgM content is expressed as a fraction of anti-RTA IgG antibodies. ¶Concentration of anti-RTA antibodies resulting in 50% binding expressed as mol/l.

illustrated in Table 2, the serum titre of anti-RTA IgG antibodies in this group of 10 patients was spread over a wide range (<1 : 80–1 : 28000). No correlation could be observed with either the day of serum collection or the clinical parameters reported in Table 1. In fact, patients' sera collected after the same time interval from the last IT administration showed different anti-RTA antibody titre, whereas patients sampled at different intervals showed very similar antibody titre (see for example patients 114, 107, and patients 119, 103). As also shown in Table 2, we found that the fraction of the IgM antibodies varied considerably in the same group of patients, from a minimum of 0·6% to a maximum of 24·6% of the total IgG content. The lack of correlation between IgG/IgM ratio and the treatment cycle is in agreement with findings by Byers *et al*. [8] who studied the antibody response to an RTA-IT in metastatic colon cancer patients. Likely, the patients studied by us could have also responded against the antibody portion of the Immunotoxin, and in principle both the antibody and the toxin moieties could be responsible for a carrier-effect response, providing helper epitopes for eliciting antibodies against the partner molecule. This aspect, however, was not investigated herein.

HARA were then purified by immunoaffinity on immobilized RTA and the amount of anti-RTA antibody in each patient's serum as well as the binding properties of the purified IgG were investigated. As summarized in Table 2, the HARA concentration, ranging from 0·5 μg/ml to 10·2 μg/ml, resulted to be variable from patient to patient regardless of the total amount of RFT5.dgA administered. Antibody concentration resulting in 50% RTA binding was expressed as mol/l and used to compare HARA in different patients' sera. We observed that, although antibody concentration yielding 50% binding to precoated rRTA varied from $1{\cdot}4 \times 10^{-12}$ mol/l to 62×10^{-12} mol/l (Table 2), only a few administration cycles of RFT5.dgA were sufficient to develop high-affinity anti-RTA antibodies.

To evaluate if anti-RTA antibodies raised in patients following RTA-IT treatment recognized common RTA domains we performed experiments of competition binding in ELISA, where binding to rRTA of biotinylated antibodies from various patients (102, 111, 112, 117, and 119) was displaced by an excess non biotinylated antibodies from patient 107. We found that purified anti-RTA IgG antibodies from patients 102, 111, 112, 117 and 119 were all displaced 46·5 – 99·5% by a 100-fold molar excess of anti-RTA antibodies from patient 107, suggesting that a considerable fraction of anti-RTA antibodies from different subjects can recognize common domains of the antigen in spite of their heterogeneous features.

Blockage of cytotoxic activity of ricin and ST.1-RTA

To evaluate if HARA elicited by RFT5.dgA IT were able to recognize RTA and interfere with its cytotoxicity when linked to a vehicle mAb or in its native molecular context (i.e. linked by a disulphide bridge to the ricin B subunit) [3], we performed cytotoxicity assays with Jurkat cells in the presence of increasing amounts of purified HARA and a fixed concentration of the anti-CD5 ST.1-RTA IT or of ricin. A different RTA-IT was used in the assays described below to avoid possible interference of antibodies directed against new epitopes which might have been created by the cross-linking of RTA to mouse Ig. These antibodies would partially recognize RTA and/or structures at/near the linkage site and could have been positively selected during the purification procedure on insolubilized RTA. As shown in Fig. 1, we found that human anti-RTA antibodies (from 8 different patients) were able to inhibit 50% cytotoxicity of ST.1-RTA at concentrations ranging between $1{\cdot}0 \times 10^{-12}$ M and $1{\cdot}5 \times 10^{-10}$ M, whereas the cytotoxicity of ricin was comparably inhibited only by adding higher antibody amounts, ranging between $1{\cdot}0 \times 10^{-9}$ M and $>3{\cdot}3 \times 10^{-9}$ M. In one instance (i.e. antibodies from patient 125) no inhibition of ricin cytotoxicity was observed. All the HARA samples showed therefore a neutralizing effect on the RTA-mediated cytotoxicity *in vitro*, both when the RTA is part of the heterodimeric ricin toxin and when it is linked to a mouse mAb,

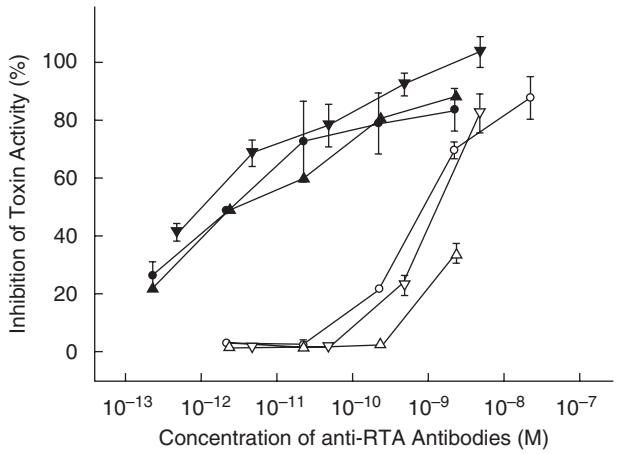

Fig. 1. Anti-RTA antibodies inhibit the cytotoxicity of a RTA-based Immunotoxin and of ricin. The effect of anti-RTA antibodies developed in RTA-IT treated patients was evaluated in a protein synthesis inhibition experiment. The inhibition of the cytotoxicity of $0{\cdot}5 \times 10^{-12}$ M ST.1-RTA IT (●, ▲, ▼) or of $0{\cdot}5 \times 10^{-12}$ M ricin (○, △, ▽) by increasing amounts of anti-RTA antibodies was considered. The percent of protein synthesis with respect to mock-treated control samples was calculated. The inhibitory potential of anti-RTA antibodies (y axis) was then determined as percent of protein synthesis rescue with respect to the control, in which the presence of the IT or ricin alone leads to maximum cytotoxicity. Data points represent the mean of triplicates ± SD. For the sake of clarity only data obtained with anti-RTA antibodies from patients 102 (▲, △), 107 (●, ○) and 111 (▼, ▽) are shown. Description of data obtained with all patients assayed is reported in the Results and Discussion section.

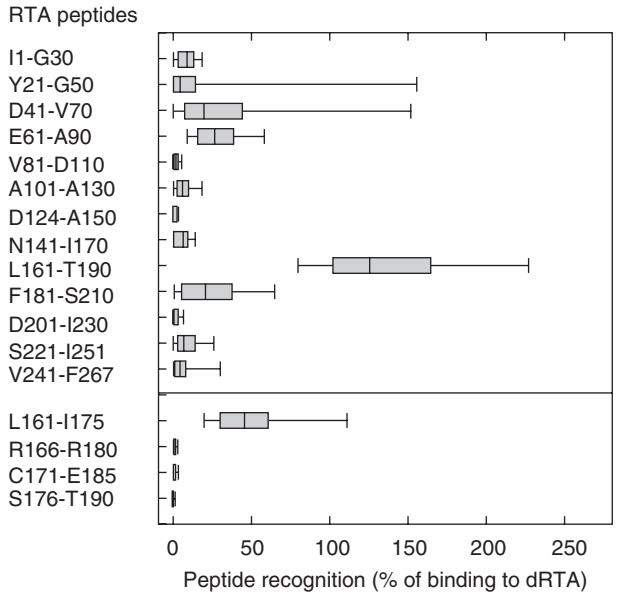

Fig. 2. Human anti-RTA antibodies recognize a common domain of RTA. Anti-RTA antibody response was dissected in RTA-IT treated patients by ELISA. Reactivity with discrete RTA domains was evaluated first using a panel of 30-mer peptides reproducing RTA primary sequence (a) and the response against the stretch L161-T190 was further investigated using 15-mer peptides (b). Results are expressed as percent binding to dRTA. Results of recognition patterns of 15 patients were subjected to statistical analysis and are represented as bars showing the 10th, 25th, 75th and 90th percentiles. The median is shown as a line within the bars.

similar to RFT5.dgA. The different extent of ricin *versus* ST.1-RTA inhibition could be attributed to a different conformation of RTA when it is linked to a carrier antibody and when it is bound to the RTB as a component of the ricin heterodimer. It must be noticed, however, that the crystallographic representation of RTA bound to RTB and of isolated recombinant RTA are identical, only minor conformational changes are due to interactions with the B chain [32]. Greater inhibition of IT *versus* ricin could therefore be also explained by an easier access of anti-RTA antibodies to antibody-linked RTA than to RTA bound to RTB.

Identification of a dominant continuous B epitope of RTA

Following this preliminary analysis of antigen recognition properties of HARA we proceeded to a peptide-scan approach [22,23] in order to narrow down the antibody binding regions of RTA.

Peptide scanning using 30-mer peptides allowed to identify firstly two RTA regions which were recognized by anti-RTA IgG from different patients. As shown in Fig. 2, the two regions were those reproduced by peptides Y21-G50, D41-V70 and E61-A90 and peptides L161-T190 and F181-S210. The antibody binding to peptides in ELISA was expressed as a fraction of binding to whole dRTA taken as a standard. Based on this criteria we found that purified anti-RTA antibodies from all patients were able to recognize the stretch L161-T190 with a higher efficiency than dRTA (Fig. 2), whereas the binding to other peptides is either undetectable (peptide D124-A150) or detectable only at very low levels (< 10% binding with respect to dRTA). No differences were observed between the homogeneous group of 10 patients that underwent 2 treatment cycles and those (5 patients) who underwent a higher number of cycles.

We then studied in greater detail the response to L161-T190 by evaluating the ability of anti-RTA antibodies to recognize shorter overlapping 15-mer peptides reproducing the L161-T190 sequence. As shown in Fig. 2b, all patients exclusively recognized the peptide L161-I175, indicating that a considerable antibody response to RTA is directed against this stretch of the molecule.

Unlike HARA, antibodies obtained from a rabbit hyperimmunized with dRTA did not react with peptide L161-T190 nor with peptide L161-I175 but recognized instead other RTA domains distributed over the entire RTA sequence. Moreover, rabbit antibodies blocked ricin and ST.1-RTA cytotoxicity with a 50–100-fold lower efficacy as compared to HARA (not shown). Taken together, these observations support our conclusion that anti-L161-I175 antibodies in humans are indeed involved in RTA neutralization.

To make sure that the interaction between anti-L161-I175 antibodies and RTA was specific and it involved a relatively high fraction of anti-RTA antibodies, we performed binding experiments to precoated rRTA in the presence of increasing amounts of a soluble peptide reproducing the stretch L161-I175. As illustrated in Fig. 3, preincubation of anti-RTA antibodies from patient 107 or patient 105 with increasing concentrations of the peptide L161-I175 prevented to a great extent the binding to rRTA (maximum values of percent inhibition of binding to rRTA were $26{\cdot}0 \pm 0{\cdot}8\%$ and $38{\cdot}3 \pm 3{\cdot}2\%$, respectively, in the presence of the highest peptide dose). A control RTA peptide (S176-T190) was unable to significantly interfere with the binding of HARA to rRTA (Fig. 3).

However, the observation that such a high amount of HARA in all patients' sera recognized the stretch L161-I175 was

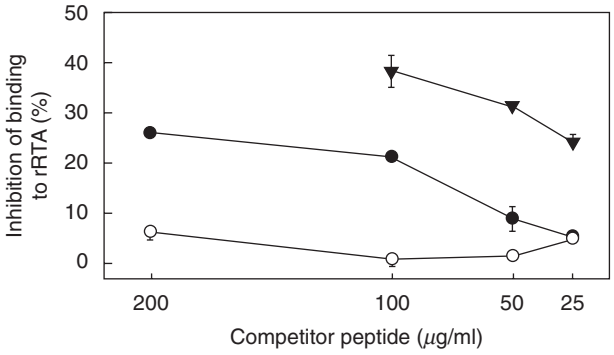

Fig. 3. The peptide L161-I175 inhibits the binding of anti-RTA antibodies to rRTA. The ability of peptide L161-I175 to displace binding of HARA to rRTA coated onto microtitre wells was evaluated by ELISA. HARA from patient 107 (●, ○) or from patient 105 (○, ▼) were preincubated with increasing concentrations of L161-I175 (●, ▼) or a control peptide S176-T190 which is not recognized by HARA (open circles). Curves obtained with the control peptide are superimposable and appear as one. Errors were calculated by pooling raw data from both curves. Results are expressed as percent inhibition of binding to rRTA ± SD. Values for the uninhibited (mock-treated) samples were considered 100% binding.

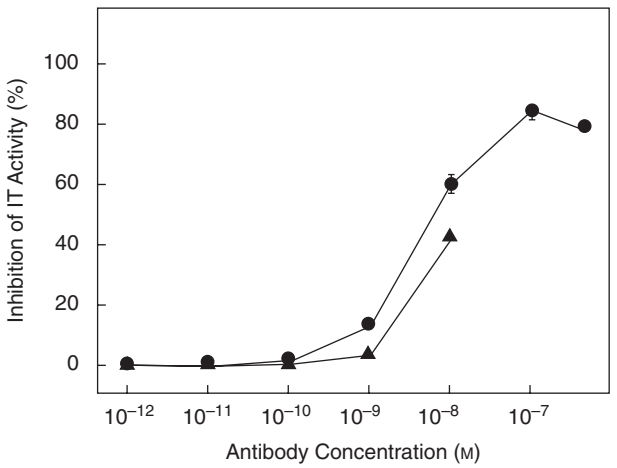

Fig. 4. Anti-L161-I175 antibodies neutralize the cytotoxic activity of a RTA-based Immunotoxin. The inhibitory potential of pooled immunoaffinity purified antibodies binding the peptide L161-I175 (▲) was evaluated in cytotoxicity assays in the presence of a fixed amount of ST.1-RTA IT corresponding to a concentration yielding >90% cytotoxicity and compared with the effect of the total anti-RTA antibodies (●).

somewhat unexpected. In fact, this segment is located close to the RTA core region, as determined by studies on the secondary and three-dimensional RTA structure [24]. Therefore it was not surprising that predictive analyses, taking in consideration several parameters [27–29] (i.e. protein hydrophilicity/hydrophobicity, water accessibility, etc.) did not identify the stretch L161-I175 as a likely B-cell epitope (data not shown and [33]). The epitope L161-I175 maps immediately upstream to a T-cell epitope previously identified by us [14] corresponding to the sequence I175-E185 which resides within the enzymatic site-cleft of RTA. It has been reported [34] that the induction of antibodies to a given sequence could in some cases depend on the existence of helper T cells specific for a stretch proximal to the antibody epitope. This so called 'T-B reciprocity' [35] could thus explain the high antigenicity of a limited RTA domain containing both a T-cell and a B-cell epitope and belonging to a predominantly hydrophobic RTA domain.

Role of anti-L161-I175 antibodies

Immunoaffinity purification of HARA binding the peptide L161-I175 allowed us to investigate if the linear epitope identified is recognized on native RTA, i.e. when RTA is in its properly folded functional conformation. To this end, the binding of antipeptide antibodies to RTA as a component of ST.1-RTA IT was first investigated. Purified anti-L161-I175 antibodies recognized ST.1-RTA with an efficacy of 53% with respect to the binding of total anti-RTA antibodies.

The inhibition of ST.1-RTA cytotoxicity by pooled anti-L161-I175 antibodies and by pooled total antibodies was then evaluated (Fig. 4). ST.1-RTA (used at concentrations inhibiting >90% protein synthesis of target cells) was blocked 40% by total anti-RTA antibodies at a concentration of $4{\cdot}0 \times 10^{-9}$ M. Anti-L161-I175 antibodies were also able to inhibit ST.1-RTA cytotoxicity, although with a slightly lower efficiency, yielding 40% of ST.1-RTA neutralization at a concentration of $1{\cdot}0 \times 10^{-8}$ M. It appears therefore that the common epitope residing within the stretch L161-I175 of RTA can elicit functionally neutralizing antibodies. Since RTA must be in a correctly folded conformation to exert enzymatic activity, it can be concluded that antibodies to L161-I175 peptide bind to an epitope on the native protein. The fact that the epitope L161-I175 is located close to the RTA active site might explain the considerable effect exerted by anti-L161-I175 antibodies. Because anti-L161-I175 antibodies showed a significant neutralizing effect *in vitro*, we cannot exclude that they may have a functional role of RTA-ITs blockade also *in vivo*. Functional blockade of administered RTA-ITs can thus be proposed as a possible mechanism of RTA-IT inactivation *in vivo* in addition to antibody-mediated clearance. The observation that ST.1-RTA cytotoxicity was more effectively inhibited by unfractionated HARA could be explained by the presence of additional antibodies directed against conformational epitopes which can be identified and investigated only by X-ray crystallography of antigen-antibody complexes.

Recognition of homologous RIPs

The heterodimeric toxin ricin is a member of the RIPs family which also includes a number of single chain RIPs-I, such as dianthin, momordin, saporin, Pokeweed Antiviral Protein (PAP) and gelonin [3]. The enzymatic subunit of ricin (RTA) and toxins belonging to the RIPs-I group are all of similar size, all carry out the same N-glycosidation reaction and show a high degree of sequence [36] and structural similarity [36,37]. The L161-I175 RTA stretch belongs to the region of highest sequence and structural homology between RTA and other RIPs [30]. Nevertheless, we observed no cross-reactivity of anti-RTA antibodies with all the RIPs assayed by us (binding of anti-RTA antibodies to native RIPs-I was lower than 10% with respect to native RTA).

It is therefore likely that sequence and conformational similarity do not lead to the induction of a cross-reactive immune response. In fact, RIPs assayed in the present work for cross-reactivity with anti-RTA antibodies are also not recognized by anti-RTA specific clonal T-cells [14] demonstrating that both T-cell and B-cell responses against toxins with similar structure and function are highly discriminatory.

CONCLUSIONS

The following conclusions also emerge from our investigation:

- patients responding to RTA develop antibodies against a major linear epitope located within a highly immunogenic stretch of the molecule and which could be involved in RTA inactivation *in vivo*; therefore, the whole immunogenic RTA region L161-E185 (this paper and [14]) could be the target of immunomodulating strategies aiming at a more general application of RTA-based ITs [4];
- the lack of T-cell and B-cell cross-reactivity of anti-RTA antibodies with homologous RIPs reinforces the suggestion [38] that different RIPs could be used in sequential IT treatments without eliciting a cross-reactive blocking immune response;
- knowledge on the immunologic profile of RTA might also be exploited in peptide-based vaccination strategies [39], counteracting the possible intoxicating effects of ricin, a potential tool in bioterrorism [40].To this purpose the presence of both T and B epitopes within a short stretch of the RTA sequence might be advantageous.

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