Proteolytic fragmentation of tetanus toxin by subcellular fractions of JY, a B lymphoblastoid cell line

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Proteolysis of ^{125}I -labelled tetanus toxin by subcellular fractions from an Epstein–Barr-virus-transformed B lymphoblastoid cell line, JY, was investigated. Fractions enriched in lysosomes and plasma membranes cleaved the toxin molecule at several sites, with a pH optimum of 5.5. N-Terminal sequence analysis of M_r -81000, -45000 and -35000 proteolytic fragments indicated cleavage of the Asp-460–Leu-461, Asp-872–Glu-873 and Ile-1013–Thr-1014 peptide bonds, all sites located within the heavy chain of the toxin molecule. Additional sites near the C-terminus of the heavy chain, giving rise to low- M_r peptides, were implicated. The toxin light chain was more resistant to proteolysis. A similar pattern of fragmentation was observed with tetanus toxin biosynthetically radiolabelled with ^{14}C -labelled amino acids, showing that the proteolysis was not an artifact caused by iodination. The proteolytic activity was inhibited by the serine proteinase inhibitor di-isopropyl phosphorofluoridate, thiol-blocking proteinase inhibitors *N*-ethylmaleimide and iodoacetamide, and by EDTA. These results represent a preliminary characterization of the processing *in vitro* of tetanus toxin by an antigen-presenting cell line.

INTRODUCTION

Most antigens must be processed intracellularly before they can be presented in association with major histocompatibility complex (MHC) molecules at the cell surface, for recognition by the antigen-specific receptor of T cells (Chain *et al.*, 1988; Townsend & Bodmer, 1989). This processing appears to involve limited proteolytic degradation of protein antigens, since lysosomotropic bases and some proteinase inhibitors block the presentation of the native protein, but not of the specific peptide epitope, to T cells (Ziegler & Unanue, 1981; Chesnut *et al.*, 1982; Buus & Werdelin, 1986; Puri & Factorovich, 1988). Mechanisms leading to the generation of antigenic fragments from the native protein are still poorly understood. The vaccinal antigen tetanus toxin is an ideal choice to investigate human antigen-processing pathways, because its gene has been cloned and sequenced (Eisel *et al.*, 1986; Fairweather & Lyness, 1986), enabling the expression in bacteria of tetanus-toxin fragments and the delineation of several T-cell epitopes (Demotz *et al.*, 1989a).

In the present paper, we describe an approach that uses the intracellular proteolysis *in vitro* of tetanus toxin by the human Epstein–Barr-virus-transformed B cell line JY, as an attempt to study the pattern of antigen processing and the proteinases involved.

MATERIALS AND METHODS

Materials

The proteinase inhibitors di-isopropyl phosphorofluoridate (DFP), leupeptin and pepstatin were purchased from Sigma, and E64 and antipain were from Boehringer.

Proteins

Tetanus toxin was generously given by Institut Mérieux, Lyon, France; it was further treated with 5 mM-DFP and purified by chromatography on a Sephacryl S300 HR column (Pharmacia). Tetanus toxin was radiolabelled with Na^{125}I (Amersham) by

using Iodogen (Pierce Chemicals)-coated tubes. ^{14}C -labelled tetanus toxin was prepared in the laboratory by growing *Clostridium tetani* (Massachusetts strain E88) in the presence of ^{14}C -labelled amino acids (CEA, Saclay, France).

SDS/PAGE

SDS/PAGE was performed by the method of Laemmli & Favre (1973). Reduction of samples was carried out in the presence of 1% (w/v) dithiothreitol for 5 min at 100 °C. The gels were dried and exposed to Fuji RX film at –70 °C. When ^{14}C -label was used, the gels were treated with Amplify (Amersham) before drying and exposed at –70 °C to Hyperfilm-MP from Amersham. Scans of autoradiographs were performed with a Shimadzu CS 9000 dual-wavelength flying-spot scanner.

N-Terminal amino acid sequence of proteins separated by SDS/PAGE was determined with a Applied Biosystem 470A sequencer after the transfer of protein bands from acrylamide gels to Immobilon membranes as described by Matsudaira (1987).

Cell culture

The human B lymphoblastoid cell line JY was a gift from Schering–Plough, Dardilly, France. Cells were grown in RPMI medium (Gibco) supplemented with 2 mM-glutamine, 5 µg of gentamicin/ml (Gibco), 50 µM-2-mercaptoethanol and containing 10% (v/v) heat-inactivated fetal-calf serum (IBF).

Cell fractionation

Subcellular fractionation of JY cells was performed as described by Darte & Beaufay (1983). Briefly, after being washed with 0.145 M-NaCl/10 mM- Na_2HPO_4 , pH 7.4, the cells were resuspended at 4 °C at a concentration of 4×10^7 – 5×10^7 /ml in homogenization buffer (pH 7.4) containing 0.25 M-sucrose, 1 mM-EGTA and 3 mM-imidazole. The cells were disrupted by 30 passes through a ball-bearing homogenizer (8.02 EMBL) to obtain more than 80% lysis, as monitored by phase-contrast microscopy, leaving nuclei intact. After centrifugation at 4 °C for 8 min at 1200 g, the nuclear pellet was discarded and the postnuclear supernatant was fractionated by either differential

Abbreviations used: DFP, di-isopropyl phosphorofluoridate; MHC, major histocompatibility complex.

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centrifugation or density equilibration in a linear gradient of sucrose.

Differential centrifugation was carried out at 4 °C in a JA 21 rotor and Beckman J2-21 M centrifuge. Fractions M, L and P were successively sedimented at 6600 *g* for 5 min, 25000 *g* for 10 min and 33000 *g* for 90 min and resuspended in the appropriate buffer. The final supernatant is denoted S fraction. According to the sedimentation profiles of reference enzymes (Darte & Beaufay, 1983), lysosomes and mitochondria are mainly found in fractions M and L, plasma membranes in fractions L and P, and microsomes in fractions P, and fraction S is the soluble cytosol.

For isopycnic centrifugation, sucrose gradients were made in 5 ml polyallomer tubes (Kontron) with an apparatus from Hoefer Scientific Instruments by mixing two solutions containing 27.6% (w/v) and 54.1% (w/v) sucrose in 1 mM-EDTA/5 mM-Tris/HCl, pH 7.4. After centrifugation for 160 min at 150000 *g* in a TST 54 rotor (Kontron), the gradient was recovered in 0.23 ml fractions by using a HBI Autodensity Flow IIC apparatus connected to a 200 PVP Isco collector. The density of each fraction was deduced from its refractive index measured with an Atago refractometer. The lysosomal marker acid β -*N*-acetylglucosaminidase was determined fluorimetrically (Leaback & Walker, 1961).

RESULTS

Proteolysis of tetanus toxin by subcellular fractions of JY cells

SDS/PAGE was used to analyse the proteolysis *in vitro* of 125 I-labelled tetanus toxin. Tetanus toxin is a two-chain protein in which the chains are linked by a disulphide bridge. In reduced conditions (Fig. 1, lane 5) the heavy chain migrates with an apparent M_r of 91000 and the light chain with an apparent M_r of 50000. The material that migrates with an apparent M_r of approx. 135000 corresponds to unprocessed protoxin.

Fig. 1 shows the electrophoretic pattern of 125 I-labelled tetanus toxin after incubation *in vitro* at acidic pH in the presence of subcellular fractions of decreasing sedimentation velocities (M, L and P), and of the soluble cytoplasm (S) from JY cells. Proteolysis of tetanus toxin by the M or L fraction is evident (Fig. 1, lanes 1 and 2): in reduced samples the intensity of the heavy-chain band decreased and corresponded to the appearance of a doublet with a major fragment of apparent M_r 81000, in addition to two fainter bands of apparent M_r 45000 and 35000 and peptides of lower M_r which migrated with the dye front. Degradation to low- M_r fragments increased with time of incubation (Fig. 2). Under the conditions used to assay proteolytic activity (hypo-osmotic buffer), most intracellular organelles would not remain intact, explaining the loss of structure-linked latency of the associated enzymes. Freezing and thawing of subcellular fractions M and L released part of the proteolytic activity in soluble form, suggestive of a loose association with the membrane of the proteinases involved. Micro-sequence analysis of the *N*-terminal portion of the proteolytic fragments confirmed that they belonged to the heavy chain. *N*-Termini of the 81000- M_r , 45000- M_r and 35000- M_r fragments resulted from the hydrolysis of the Asp-460-Leu-461, Asp-872-Glu-873 and Ile-1013-Thr-1014 bonds respectively, all sites localized in the heavy chain of the tetanus toxin (Eisel *et al.*, 1986). The light chain appeared to be more resistant to proteolysis.

We observed the same fragmentation pattern with other B-cell lines, Raji, Daudi, and the cloned lymphoblastoid cell line F43 C8, which bears membrane immunoglobulins specific for tetanus toxin. In contrast, a different fragmentation pattern was obtained when using similar fractions from U937, a monocytic cell line: the proteolysis was weaker than with JY; it generated a major fragment of M_r approx. 46000 resulting from the hydrolysis

of the Phe-854-Ser-855 bond located in the heavy chain, and minor fragments of approximate M_r 80000, 75000 and 67000 in addition to peptides of lower M_r migrating with the front.

Using the percentage of radioactivity lost from the intact heavy-chain band as a measure of proteolytic activity and taking

Fig. 1. Proteolysis of tetanus toxin by subcellular fractions of JY cells

Portions of each subcellular fraction corresponding to 10^7 cells were incubated for 3 h at 37 °C with 1 μ g of 125 I-labelled tetanus toxin in 10 mM-citrate buffer, pH 4.5, in a final volume of 50 μ l. The reduced samples were analysed by SDS/PAGE in a 7.5%-acrylamide gel. Lanes 1, 2, 3 and 4 correspond to tetanus toxin incubated respectively with M, L, P and S fractions, and lane 5 to tetanus toxin incubated alone in the same conditions.

Fig. 2. Time-dependence of the proteolysis of tetanus toxin by the L subcellular fraction from JY cells

Portions of L fraction corresponding to 4.4×10^6 cells were incubated at 37 °C with 1 μ g of 125 I-labelled tetanus toxin in a final volume of 50 μ l of 10 mM-acetate buffer, pH 5, for 15 min (lane 1), 30 min (lane 2), 1 h (lane 3) and 2 h (lane 4). Lane 5 corresponds to tetanus toxin incubated alone in the same conditions for 2 h. The reduced samples were analysed by SDS/PAGE in an 8.5%-acrylamide gel.

Fig. 3. Subcellular distribution in JY cells of proteolytic activity acting on tetanus toxin

The postnuclear supernatant obtained from 4×10^7 cells was fractionated by density equilibration in a sucrose gradient as indicated in the Materials and methods section. Each fraction was assayed for β -N-acetylglucosaminidase activity (thin line) and for proteolytic activity versus tetanus toxin (bold line). A $10 \mu\text{l}$ sample was incubated with $40 \mu\text{l}$ of 20 mM-acetate buffer, pH 5.2, containing $1 \mu\text{g}$ of ^{125}I -labelled tetanus toxin for 15 h at 37°C . The reduced samples were analysed by SDS/PAGE on an 8%-acrylamide gel. The percentage of radioactivity lost from the intact heavy chain of tetanus toxin was taken as a measure of the proteolytic activity. Results are presented as described by Darte & Beaufay (1983). Frequency is the fractional amount of activity in the gradient fraction divided by the density increment across that fraction. The dashed line indicates the demarcation between the sample layer and the preformed gradient.

Fig. 4. Proteolysis of ^{14}C -labelled tetanus toxin by the L fraction from JY cells

^{14}C -labelled tetanus toxin was incubated at 37°C in a final volume of $50 \mu\text{l}$ of 20 mM-acetate buffer, pH 5.2, with fraction L corresponding to 5×10^6 JY cells for 15 min (lane 1), 30 min (lane 2) and 3 h (lane 3). Lane 4 corresponds to toxin incubated alone in the same conditions for 3 h. Unreduced and reduced samples were analysed by SDS/PAGE in an 8%-acrylamide gel.

Fig. 5. Dependence of the proteolysis of tetanus toxin by the L fraction from JY cells on pH

Incubations were for 2 h at 37°C with $1 \mu\text{g}$ of ^{125}I -labelled tetanus toxin in a final volume of $50 \mu\text{l}$. The pH was varied by using different proportions of a mixture of 50 mM-acetic acid and 50 mM- Na_2HPO_4 . The reduced samples were analysed by SDS/PAGE in an 8%-acrylamide gel, and proteolytic activity was estimated as indicated in Fig. 3.

into account the protein concentration in the different fractions, the respective activities of fractions M, L, P and S were in the proportions 0.45:0.5:0.05:0. The L fraction was the most efficient and was used to elucidate some additional characteristics of the proteolysis.

When the postnuclear supernatant from JY cells was fractionated by isopycnic centrifugation in a sucrose gradient (Fig. 3), most of the proteolytic activity sedimented as a broad peak around 1.18, a distribution which is similar to that of the lysosomal marker acid β -N-acetylglucosaminidase. Smaller proportions of the activity were found in the sample layer, corresponding probably to soluble enzyme(s) released from disrupted lysosomal organelles, and at a slightly higher relative density (1.125), indicating a possible endosomal localization.

Proteolysis of ^{14}C -labelled tetanus toxin

Iodination of tetanus toxin has been shown to modify its conformation (Lazarovici *et al.*, 1984); in addition, this procedure labels only peripheral tyrosine residues, the localization of which in the molecule is unknown. For these reasons, biosynthetically ^{14}C -labelled tetanus toxin was used to check the pattern of fragmentation by the L fraction from JY cells (Fig. 4). Tetanus toxin is synthesized by *Clostridium tetani* as a single-chain protoxin (Fig. 4, lane 4). When incubated with fraction L, ^{14}C -labelled protoxin was partially processed into its two chains (Fig. 4, lane 1). Then the heavy-chain-derived 84000–81000- M_r doublet appeared, in conjunction with an increase in the radioactivity migrating with the electrophoretic front corresponding to low- M_r peptides (Fig. 4, lanes 2 and 3). No extra fragment was seen compared with the pattern of proteolysis of ^{125}I -labelled tetanus toxin.

Dependence of the proteolysis of tetanus toxin on pH

Fig. 5 illustrates the proteolytic activity in the L fraction from JY cells on varying the pH between 4.0 and 7.5. The activity is clearly dependent on the pH, with a maximum between pH 5.0 and 5.5, and becoming weak above pH 6.5.

Table 1. Effect of proteinase inhibitors on the proteolysis of tetanus toxin by the L fraction from JY cells

Tetanus-toxin proteolysis was measured in the same conditions in the absence or presence of the indicated inhibitor, and activity was estimated as in Fig. 3.

Inhibitor	Concn. (mM)	Percentage inhibition
Pepstatin A	7.3×10^{-2}	0
DFP	5	30
Leupeptin	1	0
Antipain	1	0
E64	2	0
N-Ethylmaleimide	2.2	60
Iodoacetamide	0.1	0
	1	33
	10	71
EDTA	10^{-2}	8
	5	> 80

Effect of inhibitors on the proteolysis of tetanus toxin

The effect of various proteinase inhibitors on the proteolysis of tetanus toxin by the L fraction from JY cells was investigated (Table 1). Pepstatin, an inhibitor of aspartate proteinases, did not inhibit the proteolysis. A partial inhibition was observed with DFP, an inhibitor of serine proteinases. The thiol-blocking reagents *N*-ethylmaleimide and iodoacetamide inhibited the proteolysis, whereas other thiol-blocking proteinase inhibitors, leupeptin, antipain and E64, did not. The inhibition by EDTA indicates that a metalloproteinase or a metal-activated proteinase may participate in the proteolysis.

DISCUSSION

We have shown that subcellular fractions from a B lymphoblastoid cell line are able to proteolyse tetanus toxin giving rise to discrete polypeptides of high M_r (81 000, 45 000 and 35 000) in addition to low- M_r products. The heavy chain of tetanus toxin is more sensitive to proteolysis than the light chain; this fact could reflect the overall conformation of this molecule, as it has been postulated that the heavy chain may protect the light chain (Lazarovici & Yavin, 1986; Ahnert-Hilger *et al.*, 1989). The major proteolytic fragment of M_r 81 000 corresponds to a shortened heavy chain (10 000 less than in the native molecule). Ten amino acids are removed from the *N*-terminus, i.e. from Asn-450 to Asp-460. These amino acids are localized in the nicking region of the toxin molecule, a short sequence *N*-terminal from the Cys-466 which is involved in the disulphide bond between the two chains (Weller *et al.*, 1988); this region is particularly sensitive to a wide range of proteinases. The removal of ten amino acids from the *N*-terminus of the heavy chain cannot account for the decrease in M_r reported above. Thus we postulate that the cleavage of a *C*-terminal fragment of the heavy chain may account for both the 81 000- M_r fragment and the generation of low- M_r products. The sensitivity to proteolysis of this part of the molecule has previously been observed (Weller *et al.*, 1988).

The requirement of an acidic pH for the proteolysis of tetanus toxin by subcellular fractions is in agreement with the subcellular localization (mainly lysosomal) of the proteinase(s) involved, and substantiates the observations made with intact cells that agents interfering with intracellular proton gradients block antigen presentation (Ziegler & Unanue, 1981; Chesnut *et al.*, 1982). Acid hydrolases are predominant in the subcellular

fractions M and L, which proteolyse tetanus toxin. L fraction is also enriched in plasma membranes and possibly endomembranes related to the plasma membrane (Darte & Beaufay, 1983). The broad partition of proteolytic activity among subcellular fractions M and L could reflect a dynamic equilibrium between plasma membrane and acidic vesicular compartments.

The implication of several proteinases, which could cleave the *N*- and *C*-termini of the heavy chain of tetanus toxin, is reflected by the pattern of inhibition by different proteinase inhibitors: EDTA, thiol-blocking reagents and, to a lesser degree, DFP inhibit proteolysis of tetanus toxin *in vitro*. Thiol (cysteine) and metalloproteinases, which function both at neutral and acidic pH, have been reported to be present in plasma membranes prepared from an antigen-presenting B-cell murine lymphoma (Chain *et al.*, 1989). Cathepsins, which belong to the papain superfamily, seem to be the predominant class of thiol proteinases in eukaryotes (Bond & Butler, 1987); many of them are localized mainly within lysosomal organelles. Mammalian cathepsin B, which functions at acidic pH, may act as a peptidyl dipeptidase cleaving peptides from the *C*-terminus; its role in the presentation of myoglobin by murine spleen cells to specific T-cell clones has been demonstrated (Takahashi *et al.*, 1989).

Processing of tetanus toxin by several human Epstein-Barr-virus-transformed B-cell lines has been assessed by their ability to present tetanus-toxin antigenic determinants to different T-cell clones (Demotz *et al.*, 1989a), and it has been suggested that the differences in antigen presentation observed with these B-cell lines were related to differential proteolytic activity (Demotz *et al.*, 1989b). The relationships between these results, obtained from experiments made with intact cells, and our observations made *in vitro* await clarification. It remains to be seen whether the tetanus-toxin fragments that we observed with the B-cell line used are the immunologically relevant ones that are generated *in situ* by an antigen-presenting cell.

Further insight into the processing of tetanus toxin would be gained by studying the changes induced in biosynthetically labelled tetanus toxin by intact cells and subcellular fractions, in conjunction with presentation to T cells of the recovered peptides by using glutaraldehyde-fixed antigen-presenting cells (Chain *et al.*, 1988). The present study represents a first step in a comparative analysis of the processing of tetanus toxin alone and complexed with the complement protein C3. This ligand can potentiate the presentation of tetanus toxin by enhancing its uptake by the antigen-presenting cell (Arvieux *et al.*, 1988), but may also influence qualitatively its processing in a manner similar to what has been shown for class II MHC molecules (Donermayer & Allen, 1989) and membrane immunoglobulins of antigen-specific B cells (Manca *et al.*, 1988).

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