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Granzyme A released upon stimulation of cytotoxic T lymphocytes activates the thrombin receptor on neuronal cells and astrocytes

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ABSTRACT **Granzymes are a family of serine proteases that are harbored in cytoplasmic granules of activated T lymphocytes and are released upon target cell interaction. Immediate and complete neurite retraction was induced in a mouse neuronal cell line when total extracts of granule proteins were added. This activity was isolated and identified as granzyme A. This protease not only induced neurite retraction at nanomolar concentrations but also reversed the stellation of astrocytes. Both effects were critically dependent on the esterolytic activity of granzyme A. As neurite retraction is known to be induced by thrombin, possible cleavage and activation of the thrombin receptor were investigated. A synthetic peptide spanning the N-terminal thrombin receptor activation sequence was cleaved by granzyme A at the authentic thrombin cleavage site Leu-Asp-Pro-Arg↓Ser. Antibodies to the thrombin receptor inhibited both thrombin and granzyme A-mediated neurite retraction. Thus, T-cell-released granzyme A induces cellular responses by activation of the thrombin receptor. As brain-infiltrating CD4⁺ lymphocytes are the effector cells in experimental allergic encephalomyelitis, granzyme A released in the brain may contribute to the etiology of autoimmune disorders in the nervous system.**

Cytolytic T lymphocytes (CTLs) are a major immunological means used to kill cells carrying non-self epitopes, such as virus-infected cells, tumor cells, or grafted tissues. CTLs specifically recognize their target cells via the T-cell receptor complex interacting with major histocompatibility complex-encoded proteins and its cognate antigen (for reviews, see refs. 1–5). This cell–cell interaction leads to dramatic changes in CTL morphology, resulting in the delivery of "lethal hit" proteins secreted from cytoplasmic granules. Subsequently, nuclear DNA of the target cell is fragmented and eventually the integrity of the plasma membrane is disrupted (6, 7).

Immature CTLs elaborate storage compartments (granules) upon appropriate stimulation (8). Their contents can be released in a regulated manner following specific interaction with the target cell (9). Biochemical analysis of the most abundant molecules stored in these secretory organelles has revealed the presence of perforin/cytolysin, a family of serine esterases (the granzymes), proteoglycans, calreticulin, and several lysosomal enzymes (10–12). Among the granule proteins, the only one with an unequivocal lytic character is perforin, a pore-forming protein with high structural and functional homology with the lytic complement proteins (13–15). Experiments using antisense oligonucleotides and perforin-knockout mice have revealed its crucial role in cytolysis (16, 60, 61).

Lymphocytes are involved in the etiology of some autoimmune neurological disorders (17, 18). In multiple sclerosis, for example, myelin destruction is believed to be mediated by these immune effector cells (19, 20). Experimental autoimmune encephalomyelitis (EAE), a rodent multiple sclerosis-like disease, can be caused by T lymphocytes reactive against myelin basic protein (MBP) (21). The encephalitogenic MBP-specific T lymphocytes are in most cases CD4⁺ and major histocompatibility complex class II-restricted (17). The pathogenicity in the central nervous system involves homing, extravasation, and induction of tissue damage.

Little is known about the molecular mechanism by which encephalitogenic T lymphocytes induce tissue destruction in the nervous system. Although perforin causes the lysis of target cells, it does not seem to be relevant in encephalitogenic clones, mainly because CD4⁺ lymphocytes are devoid of perforin and central nervous system neurons are highly refractory to perforin-mediated cytolysis (22). The role played by the serine proteases granzymes in the immune response is incompletely understood (23). The blood-borne serine protease thrombin has been reported to cause profound changes in the cytoarchitecture of neuronal and glial cells *in vitro* (24–29). This raised the question of whether the lymphocyte granule-associated proteases can induce effects similar to those seen with thrombin. Here we report that in a neuronal cell line and astrocytes, granzyme A causes profound morphological changes that are apparently mediated through cleavage-induced activation of the thrombin receptor.

MATERIALS AND METHODS

Materials. Granzymes and granzyme A were purified from granules of the B6.1 CTL line as described (30). α-Thrombin was from the same previously used preparation (25). Protease nexin 1 was purified from the conditioned medium of C6 glioma cells (31). The granzyme A inhibitor Ph-HNCONH-CiTEtOIC was a gift from J. Powers (Georgia Institute of Technology, Atlanta), and staurosporine was a gift from Thomas Meyer (CIBA, Basel). Other laboratory chemicals were of the highest grade commercially available.

Cell Culture. Mouse neuroblastoma NB2a cells cloned from the C1300 murine tumor cell line (32) were cultured as described (25).

Cultures of mixed rat glial cells were prepared essentially as described (28). Briefly, cortices from rat brains at postnatal day 0 or 1 were collected into DMEM, cleaned from the arachinoid meninge and blood vessels, and digested for 30 min at 37°C in an isotonic salt solution containing trypsin (0.5 mg/ml) and EDTA (0.16 mg/ml). The tissue was then triturated and the resulting suspension was passed through a large-mesh filter and spun in a benchtop centrifuge. The cell pellet was suspended in DMEM supplemented with 20% heat-inactivated fetal bovine serum and the material derived from one brain was plated in a 10-cm-diameter Falcon dish.

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Abbreviations: CTL, cytolytic T lymphocyte; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein.

The glial cells were grown under 5% CO_2 and 100% humidity at 37°C. After 24 and 48 hr the medium was changed to remove nonadhering cells and clumps.

Neurite Retraction Assay. NB2a cells were grown and differentiated as described (25). After experimental treatment and fixation, between 600 and 700 cells per dish were counted and the length of neurites which were equal to or longer than their cell diameter was measured.

Reversal of Astrocyte Stellation. Primary glial cells which were grown to confluency (≈5 days) as described above were dissociated, plated at a density of 2–4 × 10^5 per 35-mm dish, and grown in DMEM with 20% fetal bovine serum. When an appropriate density was attained (≈3 days), the cells were washed three times for 5–10 min with serum-free DMEM, and the medium was changed to DMEM containing 1 mM 8-bromo-cAMP (Sigma) and 50 μg of delipidated bovine serum albumin per ml. Morphological differentiation occurred under these conditions during a period of 3 hr. The great majority of the cells in these cultures (and those which responded to granzyme A) were considered astrocytes, since they were all glial fibrillary acidic protein-positive by immunocytochemical analysis.

Time-Lapse Video Microscopy. For this study, cells were grown on acid-washed 22-mm glass coverslips coated with poly(L-lysine) hydrobromide (molecular weight, 70,000–150,000; Sigma). Time-lapse video microscopy was carried out as described (25). The microscope's thermoregulated plate was set to 37°C and the temperature of medium in the well was 33 ± 0.5°C.

T-Lymphocyte-Conditioned Medium. The release of granule-associated molecules was induced from the CTL clone PbCSF12 (33). The lymphocytes (2 × 10^6 per ml of DMEM) were added to the wells of 96-well plates which had been coated with the anti-CD3-specific monoclonal antibody 1452C11 (15 μg/ml) (34). Degranulation was induced by 4 hr of incubation at 37°C in a 5% CO_2 atmosphere. Cells were spun down at 1000 rpm and 100 μl of the conditioned medium harvested from each well. The pool of 80 wells was concentrated 100-fold in a Centricon 100 (Amicon) and stored at −20°C. The concentrate was then diluted 100-fold into the assay medium in the presented experiment.

RESULTS AND DISCUSSION

Neurite Retraction by Lymphocyte Granule Proteins. Purified granule proteins of the CTL line B6.1 (30) were added to mouse neuroblastoma NB2a cells. Within seconds after the addition of granule extract, neurite retraction was observed (data not shown). The retraction involved swelling of the neurite and was followed by complete regression after a few minutes in the presence of the granule extract.

Although the procedure used for CTL granule isolation yields organelles of high purity, we could not exclude with certainty that the effect seen on neurites was due to a contaminating factor(s) not harbored in the granules. Since the specific release of granule-associated proteins can be triggered by the activation of the T-cell receptor signaling pathway (9), CTL clone PbCSF12 was incubated with immobilized anti-CD3 T-cell receptor complex antibodies during 4 hr. The conditioned medium containing the degranulated material was added to NB2a cells. Immediate neurite retraction was observed (Fig. 1). The medium conditioned by T cells not stimulated by anti-T-cell receptor antibodies showed no effect.

The Active Granule-Derived Agent Is Granzyme A. To characterize the granule-associated factor causing neurite retraction, granule proteins were separated by Mono S cation-exchange chromatography. Maximal neurite retraction activity (Fig. 2*A*; fractions 21–23) coincided with maximal granzyme A esterolytic activity (Fig. 2*C*) and granzymes A

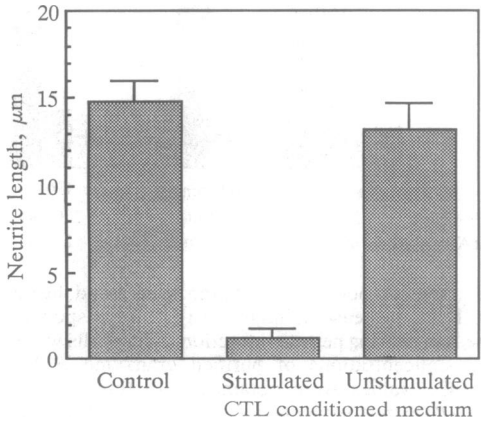

FIG. 1. Neurite retraction induced by a granule component of CTLs. Conditioned medium of CTLs (clone PbCSF12) induced to undergo exocytosis and to release their intragranular material (see *Materials and Methods*) was assayed for neurite retraction activity on NB2a cells. After an incubation of 20 min, the cells were fixed and the average neurite length was determined. The effect of the medium of unstimulated cells is shown for comparison. Results shown are averages and SDs of three determinations.

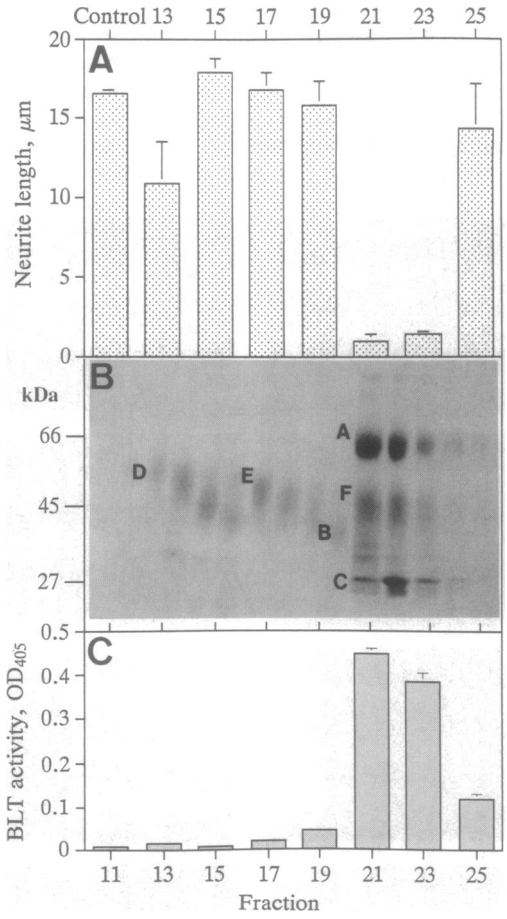

FIG. 2. Neurite retraction activity is coeluted with granzyme A activity. (*A*) Soluble granule proteins were applied to a Mono S cation-exchange column and the protein was eluted by an increasing salt gradient. Each second fraction, diluted 100-fold, was incubated for 15 min with NB2a neuroblastoma cells and the average neurite length was determined. Results shown are averages and SDs of three determinations. (*B*) The corresponding fractions were analyzed by SDS/PAGE (10%). Granzyme A is found in fractions 21–23. The letters adjacent to the bands (A–F) designate the different granzymes resolved by this analysis. (*C*) Esterolytic granzyme A activity was assayed for the above fractions using the substrate benzyloxycarbonyllysine thiobenzyl ester (30).

and C and protein content (Fig. 2*B*). This protease-induced neurite retraction activity was reminiscent of the response caused by thrombin (25, 26, 35). Granzyme A and thrombin have similar substrate specificities (36), and since no esterolytic activity has been demonstrated for granzymes C and F (30), granzyme A seemed the most likely candidate for the observed neurite-retracting activity. Indeed, when experiments were performed with highly purified granzyme A (37), rapid neurite retraction was observed (Fig. 3). After an initial swelling of the neurites, retraction proceeded to completion. The response also involved transient violent movement of the cell bodies and membrane blebbing as previously seen in response to thrombin (25). The blebbing seen in response to granzyme A are similar to those often seen on a target-cell interaction with CTL.

Astrocytes participate in several processes crucial to normal brain function, including the modulation of neuronal growth and activity (38). Cultured astrocytes acquire a stellate morphology when grown under serum-free conditions, an effect which is potentiated in the presence of membrane-permeant analogs of cAMP (39). Fig. 3 shows that 23 nM granzyme A reversed the stellate formation of such astrocytes. Virtually all processes had disassembled and the cells reverted within 20 min to an epithelial-like morphology.

The EC_{50} for neurite retraction was about 1 nM for granzyme A (Fig. 4*A*, a similar value was estimated for reversal of astrocyte stellation), a value which is larger than that of 1.7 or 50 pM observed with thrombin in the neurite retraction assay (25 or 24) or 0.5 pM in the reversal of astrocyte stellation assay (27). Granzyme A-induced morphological changes, like those caused by thrombin (25, 26), were completely blocked in the presence of the general protein kinase inhibitors staurosporine and H-7 (data not shown).

Effect of Granzyme A Is Dependent on Its Proteolytic Activity. The proteolytic activity of granzyme A was required to induce morphological changes because upon preincubation with the protease inhibitor Ph-HNCONH-CiTEtOIC (40), no neurite retraction was observed (Fig. 4*B*). This inhibitor at 67-fold molar excess did not attenuate the cellular response to thrombin, which was fully blocked by a 10-fold molar excess of protease nexin 1 (see also ref. 24). Protease nexin 1 only marginally inhibited the response to granzyme A (Fig. 4*B*) although a stronger inhibition could be expected in the presence of heparin (41). A similar sensitivity to the protease inhibitors was observed in the reversal of astrocyte stellation assay or when the forskolin-induced rise in cAMP accumulation in NB2a neuroblastoma cells (42) was inhibited by either of the proteases (data not shown). These results not only indicate that the granzyme A-induced metabolic and morphologic changes in neuronal cells are strictly dependent on its proteolytic activity but also provide further evidence

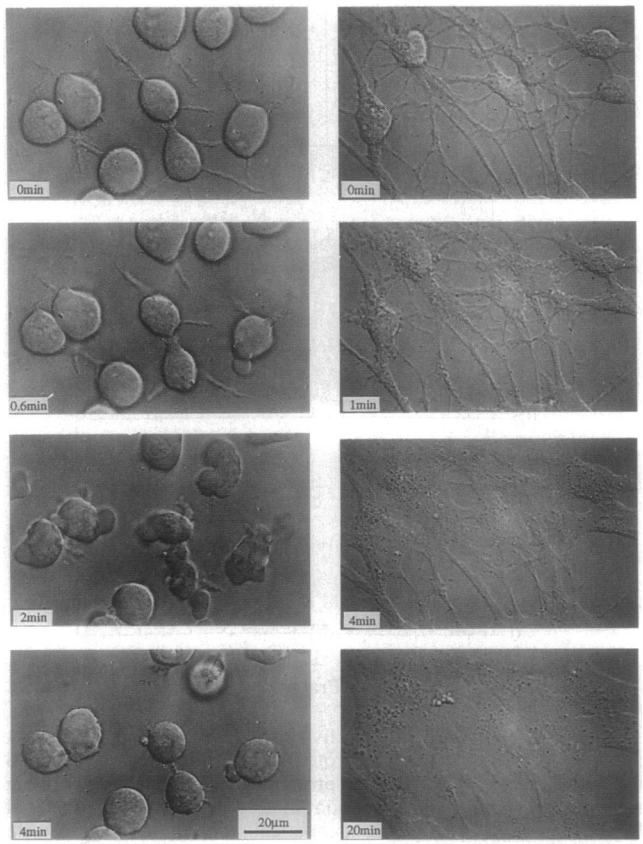

FIG. 3. Granzyme A-induced morphological changes in NB2a cells (*Left*) and astrocytes (*Right*) grown on poly(L-lysine)-coated glass coverslips. The morphological changes were recorded as described (25). Zero time designates the last recorded image before addition of 23 nM granzyme A purified from granules of the CTL cell line B6.1.

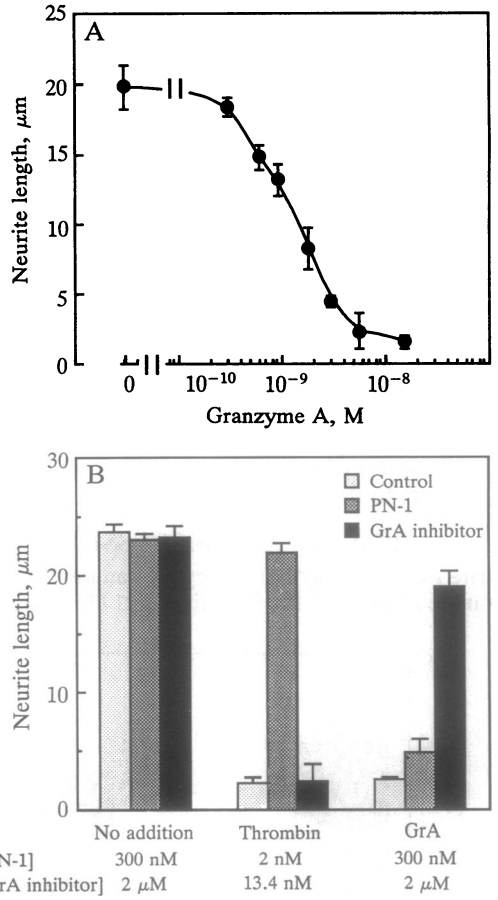

FIG. 4. Dose–response curve of granzyme A and the inhibition of its effect by a protease inhibitor. (*A*) Dose–response curve of Granzyme A in causing neurite retraction. NB2a cells were subjected to various concentrations of purified granzyme A for 15 min. Identical EC_{50} values were obtained with three preparations of granzyme A (two independent experiments). (*B*) Granzyme A (GrA)-induced neurite retraction is specific and dependent on the esterolytic activity of the protease. Granzyme A-induced neurite retraction was assayed in the presence and absence of protease inhibitors and compared with the activity of thrombin. Protease nexin 1 (PN-1) and Ph-HNCONH-CiTEtOIC (GrA inhibitor) at the indicated concentrations were incubated with thrombin (0.2 nM) or granzyme A (23 nM) for 30 min at 37°C before their addition to the cells. The results shown are averages and SDs of three determinations and are representative of three independent experiments.

that neurite retraction is specifically induced by granzyme A and not by a nonproteolytic contaminating factor.

Granzyme A Appears to Cleave and Activate the Thrombin Receptor. Since thrombin-mediated effects on neuronal cells are caused by the cleavage and subsequent activation of the thrombin receptor (25, 26), we examined whether granzyme A recognized the same receptor. Human thrombin receptor activation occurs through a unique mechanism whereby cleavage of the Arg^{41}-Ser^{42} bond within the extracellular domain has been proposed to occur through the generation of a new N-terminal sequence that functions as a tethered ligand (43, 44). The cleavage site within the thrombin receptor—i.e., Pro-Arg↓Ser—conforms to the substrate specificity of granzyme A which hydrolyzes synthetic substrates after positively charged amino acids with preference for arginine. Proline at the P2 position has been found to be the preferred amino acid (45, 46) and chloromethyl ketones terminating with Pro-Arg-CH_2Cl are potent inhibitors (47). A 32-mer peptide was synthesized which corresponded to amino acid residues 29–60 of the human receptor extracellular N-terminal region [Fig. 5 (44)]. Granzyme A or thrombin cleaved the

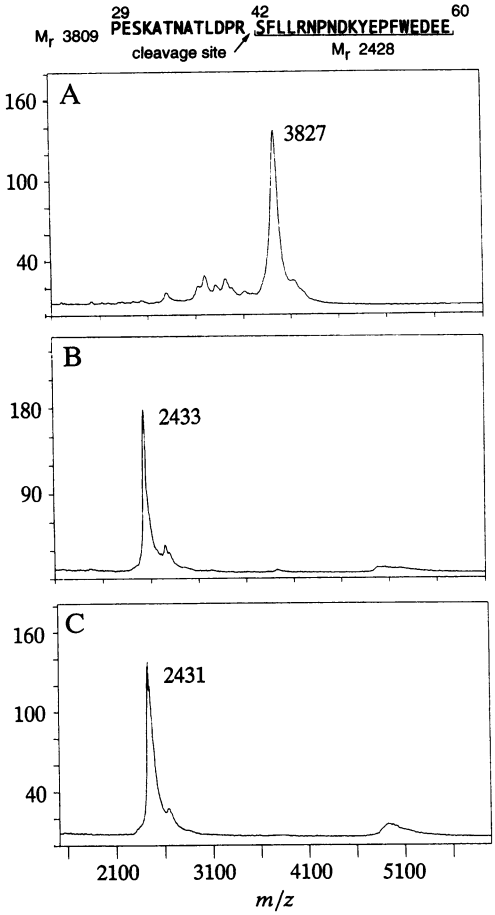

FIG. 5. Granzyme A cleaves a peptide covering part of the N-terminal sequence of the thrombin receptor. A peptide corresponding to residues 29–60 of the human thrombin receptor was synthesized according to the fluorenylmethoxycarbonyl strategy and purified by HPLC. The purity of the peptide was confirmed by matrix-assisted laser desorption mass spectroscopy (ref. 48; Linear Scientific, Reno, NV). Arrow points to the thrombin cleavage site. Theoretical molecular weights of the peptide and the C-terminal fragment, generated by thrombin, are indicated. Results of matrix-assisted laser desorption mass spectroscopic analysis of the intact peptide (*A*) and 10 mM peptide treated for 16 hr at 37°C with 1.8 μM thrombin (*B*) or 1.4 μM granzyme A (*C*) are shown. The units on the *y* axis are arbitrary, and *m/z* designates the mass/charge ratio. The N terminus of the cleaved peptide could not be detected, probably due to noise in the chromatogram covering the lower molecular weight range.

32-mer peptide at the same site as analyzed by mass spectroscopy. One of the fragments generated had a molecular mass of 2433 Da (thrombin) or 2431 Da (granzyme A) corresponding to the C-terminal peptide Ser^{42}–Glu^{60}, in accordance with the known thrombin cleavage site between Arg^{41} and Ser^{42} (Fig. 5). Furthermore, granzyme A- and thrombin-induced neurite retraction was completely blocked in the presence of an anti-thrombin receptor antibody that recognizes epitope(s) within Ser^{42}–Ser^{49} of the mouse receptor (Fig. 6). This antibody also completely blocked the reversal of astrocyte stellation that is caused by either thrombin or granzyme A (results not shown). Thus, granzyme A and thrombin appear to cleave the same or a highly homologous receptor with a similar activation sequence. The difference in the potencies of thrombin and granzyme A in their alteration of neuronal and glial cell morphology is most likely due to the absence in the latter of the extended basic substrate-binding surface, known as the thrombin anion-binding exosite. Structural alteration of this site was shown to cause a decrease in the potency of thrombin in several assays, including neurite retraction (25, 49).

The observation that granzyme A appears to activate the thrombin receptor in neuronal cells and astrocytes raised the question of whether this protease also could induce platelet aggregation, a major effect of thrombin in hemostasis. Interestingly, however, granzyme A (up to 230 nM) did not cause platelet aggregation but blocked that induced by thrombin (unpublished work). This could be, for example, due to differences between the receptor on platelets compared with that expressed by neuronal and glial cells. Although the molecular mechanism underlying the unresponsiveness of platelets to granzyme A remains to be elucidated, these observations do suggest that a receptor cleavage by the granule-derived protease in the nervous system and in other structures would not involve platelet aggregation.

Perspectives. There are multiple other effects of thrombin mediated by receptor activation (50). For instance, the protease is mitogenic for lymphocytes and causes endothelial cells to express adhesion proteins and synthesize growth factors. Receptor activation by granzyme A may also explain other heretofore unexplained effects of this lymphocyte protease. For example, spleen-derived B lymphocytes are

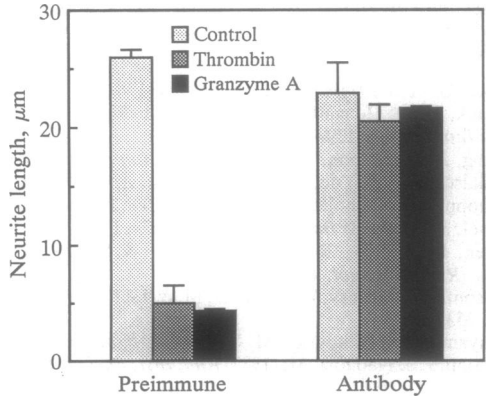

FIG. 6. Antibody raised against a thrombin receptor peptide blocks granzyme A-induced neurite retraction. A rabbit polyclonal antibody was raised against a 17-mer peptide of the mouse receptor (Ser^{42}–Asp^{58}) and affinity purified against a shorter peptide (Ser^{42}–Ser^{49}) of the mouse receptor. The 8-amino acid domain corresponds to and is identical to the sequence Ser^{46}–Ser^{53} of the rat receptor. The antibody or the protein A fraction of the preimmune serum was preincubated for 30 min with NB2a cells at a concentration of 54 μg of protein per ml. The cells were then subjected to 20 pM thrombin or 44 nM granzyme A for 15 min and fixed, and the average neurite length was determined. Results shown are averages of two determinations and are representative of two independent experiments.

induced to proliferate by granzyme A in the nanomolar range (51). In addition, some x-ray induced T-cell thymomas are poorly tumorigenic and secrete a growth factor which supports their own growth (52), and the mitogenic protein has been identified as granzyme A (53).

The EC_{50} value of about 1 nM granzyme A for receptor activation on NB2a cells and astrocytes, which is relatively high compared with that of thrombin, raised the question of whether this action of granzyme A is physiologically significant. The concentration of granzyme A in granules is estimated to be ≈3 mg/ml (0.1 mM monomer). If the proximity between a $CD4^+$ lymphocyte and its target upon interaction is considered, then a >10,000-fold dilution of granzyme A upon release into its microenvironment would still be sufficient for receptor cleavage. Accordingly, the relatively high EC_{50} value of granzyme A for receptor activation could be crucial for sparing noncognate bystander cells.

Brain infiltrating lymphocytes can transport granzyme A into the brain. For example, the encephalitogenic MBP-specific T lymphocytes in EAE are $CD4^+$ and granzyme A is found in activated $CD4^+$ and $CD8^+$ T lymphocytes (54–56). Granzyme A has also been reported to cleave MBP and may thus generate encephalitogenic peptides (57). It is therefore possible that excessive granzyme A release in the brain may exert deleterious effects on cells that express the thrombin receptor and that cause T-cell degranulation. Another possibility is that granzyme A-induced morphological changes in endothelial cells and astrocyte endfeet would increase the permeability of the blood–brain barrier, a characteristic of EAE and multiple sclerosis lesions. In addition, as thrombin is known to be mitogenic on astrocytes (27, 58), it would be important to determine whether granzyme A is involved in the gliosis featuring these pathologies. The use of inhibitors with broad specificity for serine and other proteases has been previously shown to attenuate the development of EAE (59). Due to the feasibility of designing granzyme A-specific inhibitors, our results point to the possible use of such molecules in therapeutic approaches leading to the treatment of autoimmune disorders in the nervous system.

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