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Production and characterization of monoclonal antibodies raised against recombinant human granzymes A and B and showing cross reactions with the natural proteins *

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The human serine proteases granzymes A and B are expressed in cytotoplasmic granules of activated cytotoxic T lymphocytes and natural killer cells. Recombinant granzyme A and granzyme B proteins were produced in bacteria, purified and then used to raise specific mouse monoclonal antibodies. Seven monoclonal antibodies (mAb) were raised against granzyme A, which all recognized the same or overlapping epitopes. They reacted specifically in an immunoblot of interleukin-2 (IL-2) stimulated PBMNC with a disulfide-linked homodimer of 43 kDa consisting of 28 kDa subunits. Seven mAb against granzyme B were obtained, which could be divided into two groups, each recognizing a different epitope. On an immunoblot, all mAb reacted with a monomer of 33 kDa protein. By immunohistochemistry, these mAb could be used to detect granzymes A and B expression in activated CTL and NK cells. The availability of these mAb may facilitate studies on the role of human cytotoxic cells in various immune reactions and may contribute to a better understanding of the role of granzymes A and B in the cytotoxic response in vivo.

Key words: Granzyme A; Granzyme B; Monoclonal antibody; Immunohistochemical staining

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Abbreviations: BSA, bovine serum albumin; CTL, cytotoxic T lymphocyte; FCS, fetal calf serum; HRP, horseradish peroxidase; IPTG, isopropyl β-D-thiogalactopyranoside; IL-2, interleukin-2; LAK, lymphokine activated killer; mAb, monoclonal antibody; M_r, relative mobility; NK, natural killer; PBS, phosphate-buffered saline, pH 7.4; PBMNC, peripheral blood mononuclear cells; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TC, target cell.

Introduction

Activated cytotoxic T lymphocytes (CTL) and natural killer (NK) cells are the effector cells in major histocompatibility complex (MHC)-restricted and in non-MHC-restricted cellular cytotoxicity, respectively. These cytotoxic reactions are important in the elimination of tumor cells and virus infected cells and for the process of allograft rejection. The cytoplasm of CTL and NK cells contains specialized, so-called cytotoxic granules, which are able to lyse susceptible targets. The major components of these cytotoxic

granules are the pore forming protein 'perforin' (Lichtenheld et al., 1988), proteoglycans and a unique family of highly homologous serine proteases, termed 'granzymes'.

In humans three serine proteases have been identified in the cytotoxic granules at the protein level: granzyme A, granzyme B and granzyme 3 (Krahenbuhl et al., 1988; Hameed et al., 1988). Full length cDNA has been cloned for granzymes A (Gershenfeld et al., 1988) and B (Trapani et al., 1988a; Caputo et al., 1988) but not for granzyme 3. Granzymes are synthesized as inactive precursor molecules containing a signal (pre-) peptide and a very short propeptide. In the cytotoxic granules granzyme A is present as a disulfide linked 50 kDa homodimer while granzyme B consists of a 29 kDa monomer. Both are stored as fully processed active enzymes, i.e., without the pre- and pro-peptide (Krahenbuhl et al., 1988).

In vitro studies have demonstrated that (1) granzyme A is secreted from CTL upon T cell receptor stimulation (Krahenbuhl et al., 1988); (2) lysis by CTL clones and isolated granules is inhibited by pretreatment with serine protease inhibitors (Hudig et al., 1991); and (3) the expression of granzyme A and perforin is correlated with the functional cytolytic potency in vitro (Garcia-Sanz et al., 1990). These findings suggest involvement of granzymes in the lytic process. Several investigators using in situ hybridisation, have provided evidence that in vivo expression of perforin and granzymes is found in inflammatory tissue in the mouse as well as in the human (reviewed by Griffiths and Mueller, 1991).

Studies on the role of granzymes in human disease, as markers for activated cytotoxic cells, would be facilitated by the availability of specific antibodies against these proteases. Here we report the production and characterization of mAb raised against recombinant human granzymes A and B, reacting with the natural proteins.

Materials and methods

Cloning of granzymes A and B cDNA

To obtain the cDNAs coding for the mature granzymes A and B (i.e., without the pre- and propeptide) respectively, specific primers were prepared based on the published cDNA sequence for granzyme A (Gershenfeld et al., 1988) and B (Trapani et al., 1988a), containing the appropriate restriction sites. Polymerase chain reaction (PCR) amplification was performed on first strand cDNA prepared from mRNA extracted from human peripheral blood mononuclear cells (PBMNC), cultured for 3 days at a concentration of 0.5×10^6 cells/ml in Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% (v/v) heat-inactivated fetal calf serum (FCS), streptamycin, penicillin, β-mercaptoethanol and 50 U of IL-2 (Cetus, Emeryville, CA) per ml. The amplified cDNA fragments were isolated, digested with the appropriate restriction enzymes and ligated into similar restriction sites in the vector PET 3b, such that its expression was under the control of the T7 RNA polymerase promotor. Plasmid DNA isolation, restriction enzyme digestion conditions and agarose gel electrophoresis were performed as described (Sambrook et al., 1989). The authenticity of the cloned cDNAs was confirmed by nucleotide sequence analysis (Sequenace kit, USB, Cleveland, OH).

Production and purification of recombinant granzymes A and B in E. coli

The expression plasmids PET 3b/granzyme A and PET 3b/granzyme B were used to transform *E. coli* BL 21(DE3) which expresses T7 RNA polymerase under control of the inducible lac UV 5 promotor. Single colonies of *E. coli*, transformed with either PET3b/granzyme A or PET3b/granzyme B, were used to inoculate 5 ml LC medium supplemented with ampicillin (100 μg/ml). The bacteria were grown at 37°C until the absorbance at 550 nm was 0.6. Then isopropyl β-D-thiogalactopyranoside (IPTG, Sigma) was added to a final concentration of 1 mM, and the *E. coli* were allowed to grow for another 2 h. In general, the pellet of 100 ml *E. coli* was resuspended in TE (10 mM Tris-HCl, pH 7.4, 10 mM EDTA), subjected to three freeze/thaw cycles and lysed by sonication. Recombinant proteins were pelleted by centrifugation, separated by SDS-PAGE (12.5%) and electroeluted from the gel using the Schleicher and Schüll protein elution apparatus. SDS was removed from the protein preparation as previously described (Konigs-

berg and Henderson, 1983). These preps were used for immunization (see further). After this SDS extraction procedure the purified proteins were insoluble. Therefore this material was sonicated for 30 s prior to use in coating of ELISA plates (at a concentration of 3 μg/ml in PBS).

Production and purification of mAb against granzymes A or B

BALB/c mice were immunized subcutaneously with 50 μg of purified recombinant granzyme A or granzyme B emulsified in complete Freund's adjuvant (CFA) followed by three subsequent injections of 50 μg of recombinant protein in incomplete Freund's adjuvant (IFA) at intervals of 2 weeks. 4 days after the last booster injection, spleen cells were isolated and fused with mouse myeloma Sp2/0-Ag14 cells under standard conditions. Resulting hybridomas were screened for anti-granzyme A or anti-granzyme B antibody production by an ELISA procedure using purified recombinant granzymes A and B and horseradish peroxidase-coupled goat anti-mouse IgG. Antibody-producing hybridomas were cloned by limiting dilution and cultured in bulk. All mAb were purified by protein G affinity chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden).

Immunoblotting

Recombinant granzymes A and B as well as cell lysates of IL-2 stimulated PBMNC were used in an immunoblot to test the specificity of the monoclonal antibodies obtained. The pellet of a bacterial suspension (200 μl) was lysed and the insoluble fraction, containing the recombinant proteins, was obtained by high speed centrifugation as described above. The cell lysates were obtained as follows: 40×10^6 PBMNC (stimulated for 7 days with 1000 U/ml IL-2) were resuspended in 1 ml of 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (w/v) NP40 (Sigma, St Louis, Mo), gently mixed and left for 30 min in an ice bath. The mixture was then centrifuged for 10 min at $200 \times g$ (4°C) and the supernatant was stored at −70°C until used. Briefly, SDS-PAGE separated recombinant proteins and lysate were transferred onto nitrocellulose sheets. Sheets were preincubated with PBS, 5% (w/v) non fat dry milk, 0.1% (w/v) Tween 20 for 30 min, and subsequently with the appropriate primary antibody diluted in the same buffer for 2 h. After washing three times with PBS, 0.1% (w/v) Tween 20 for 10 min, the blot was incubated with HRP-conjungated goat anti-mouse IgG for 2 h, washed as before and stained by addition of 4-chloro-1-naphthol (Sigma).

Immunofluorescence staining of single cells and conjugates

K562 cells and NK cell clone 76, generously provided by Dr. R.L.H. Bolhuis (Rotterdam, Netherlands), were cultured as described (Radošević et al., 1990; Bolhuis et al., 1984). NK-target cell conjugates were formed by mixing equal volumes of NK and K562 cells, each at a concentration of $1-2 \times 10^6$ cells/ml in RPMI 1640 supplemented with Hepes, 10% (v/v) FCS, 2 mM L-glutamine and antibiotics, followed by centrifugation for 5 min at 200 g, resuspension and incubation for another 20 min at 37°C. Aliquots of $1-2 \times 10^5$ cells were attached to poly-L-lysine (Sigma) coated coverslips and fixed with 4% (w/v) paraformaldehyde (10 min at room temperature), washed twice with PBS and permeabilized using methanol (2 min at room temperature). Coverslips were washed with PBS and cells were incubated with the appropriate mAb (50 μg/ml) in PBS-BSA followed by goat anti-mouse FITC, 1 to 50 diluted in PBS-BSA, each for 30 min at room temperature. After washing, the coverslips were sealed and examined with a confocal laser scanning microscope (CSLM) (Leica Lasertechnik, Heidelberg, Germany). The CSLM was equipped with an inverted fluorescence microscope (Fluovert, FU) and an argon-krypton laser.

Results

Expression of human recombinant granzymes A and B proteins

To obtain purified granzymes A and B for immunisation and screening purposes we decided to generate recombinant granzymes A and B antigen in a prokaryotic expression system. CDNA coding for the mature proteases (thus without the

pre- and propeptides) was generated by PCR, performed on first strand cDNA prepared from mRNA of IL-2 stimulated PBMNC (see materials and methods section). The nucleotide sequence of the mature granzyme A cDNA was completely identical to that published by Gershenfeld et al. (Gershenfeld et al., 1988). The nucleotide sequence of the granzyme B cDNA was the same as that published by Caputo et al. (Caputo et al., 1988). After the appropriate PCR products were cloned into the PET 3b expression vector and transfected into DE3 (BL21) bacteria, several clones were isolated for each construct, sequenced, and tested for recombinant protein production. Granzyme A and B recombinant protein production by the selected clones was analysed by SDS-PAGE. After induction of the bacteria containing the PET 3b/granzyme A or the PET 3b/granzyme B plasmid proteins were induced with M_r of approximately 26 and 29 respectively (data not shown). These recombinant proteins reacted specifically with polyclonal antisera raised against granzymes A and B N- and C-terminal peptides on immunoblot (data not shown), establishing their identity as granzymes A and B, respectively. Initial experiments indicated that both recombinant granzymes were produced in insoluble form, as so called 'inclusion bodies', and no soluble recombinant protein was detected. Efforts to solubilize and renature both recombinant proteins were not successful. Therefore, we purified each granzyme by preparative SDS-PAGE and obtained highly pure granzymes A and B recombinant proteins, with yields of approximately 1 mg of granzyme A and 2 mg of granzyme B from 100 ml bacterial cultures.

Characterization of mAb against granzyme A

From one fusion experiment, seven mAb were obtained, all of the IgG1 subclass, which were positive in the granzyme A ELISA. To determine their reactivity towards recombinant granzymes A and B the mAb were tested on immunoblot, as is shown for one mAb (GrA-8) in Fig. 1*A*. GrA-8 reacted only with recombinant granzyme A (lanes 1 and 2) and not with recombinant granzyme B (lanes 3 and 4). Granzyme A present in the cytotoxic granules has been reported to consist of a 50 kDa disulfide linked homodimer (Krahenbuhl et al., 1988). On an immunoblot of non-reduced LAK cell lysates, GrA-8 mAb appeared to bind to a protein band with a M_r of 43 kDa (Fig. 1*A*, lane 5). Under reducing conditions, the mAb reacted with a protein band with a M_r of 28 kDa. The other mAb against granzyme A all reacted in the same way as GrA-8 (not shown). Cross-blocking experiments, in which granzyme coated microtiter plates were incubated with saturating amounts of individual mAb, followed by an incubation with each of the biotinylated mAb, indi-

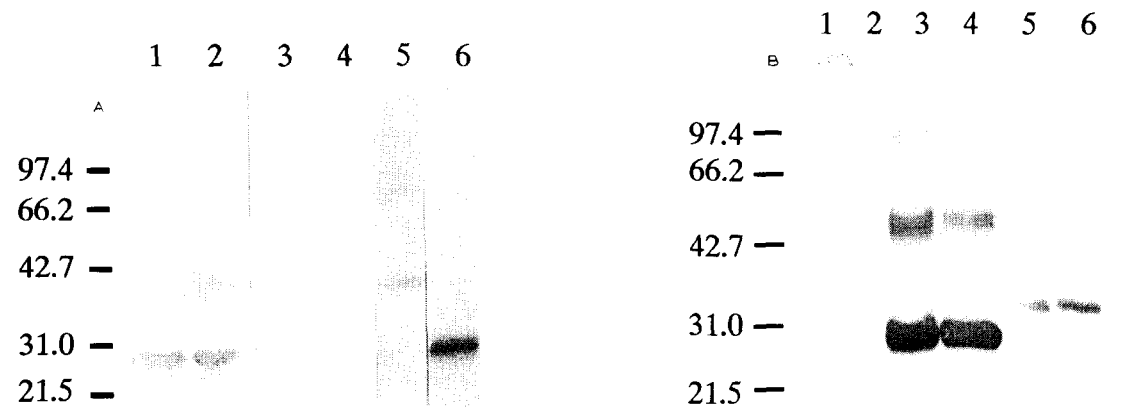

Fig. 1. Immunoblot analysis showing reactivity of mAb GrA-8 (*A*) and GrB-4 (*B*) with granzymes A and B respectively. Non-reduced (lane 1) and reduced (lane 2) recombinant granzyme A (200 ng of protein per lane), non-reduced (lane 3) and reduced (lane 4) recombinant granzyme B (200 ng of protein per lane) and non-reduced (lane 5) and reduced (lane 6) LAK cell lysates (150×10^5 cells) were electrophoresed on SDS gels and transferred onto nitrocellulose sheets. The blot was probed with mAb GrA-8 (*A* lanes 1–6) and with mAb GrB-4 (*B* lanes 1–6).

cated that all mAb against granzyme A competed for binding to recombinant granzyme A. Thus, the mAb were apparently all directed against the same or overlapping epitopes.

Characterization of mAb against granzyme B

From two fusion experiments, seven mAb against granzyme B were obtained, two of IgG2a and five of IgG1 subclass. The reactivity on immunoblot of one of these mAb, GrB-4, with recombinant granzymes A and B, respectively, is shown in Fig. 1*B*. This mAb only recognized recombinant granzyme B (lanes 3 and 4) and not recombinant granzyme A (lane 1 and 2). On a Western blot of LAK cell lysates, both under reducing and non-reducing conditions one protein band was detected by GrB-4 mAb (Fig. 1*B*, lanes 5 and 6) that migrated at approximatly 33 kDa.

In cross-blocking experiments, at least two groups of mAb against granzyme B were distinguished, each apparently recognizing a different epitope on granzyme B.

Immunofluorescent staining of NK-K562 cell conjugates with the mAb

The results thus far showed that the mAb recognized granzyme A and B proteins from IL-2 stimulated PBMNC on immunoblots. Another important application of the mAb against granzymes A and B is the detection of both proteases in activated CTL or NK cells by immunofluorescence or immunohistochemistry. Fig. 2 shows that in immunofluorescence staining the anti-granzyme mAb could be used to visualize the process of granule reorientation in NK cells toward the K562 target using confocal laser scanning. Clearly, a polar distribution was seen in the NK cell (left), whereas the cytotoxic granules in the other were distributed at random in the cytoplasm (right). With mAb GrB-4 similar patterns were observed (data not shown).

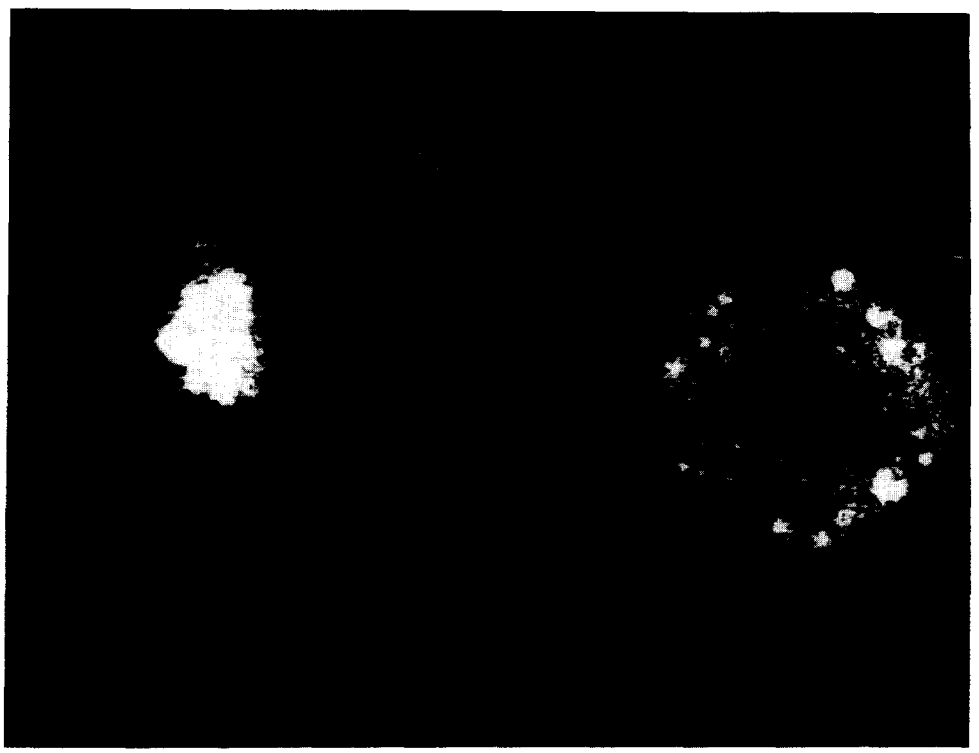

Fig. 2. Confocal laserscan of a NK-K562 cell conjugate stained with mAb GrA-8. Equal volumes of NK and K562 cells were incubated for 20 min at 37°C, attached to poly-L-lysine treated coverslips and fixed with 4% paraformaldehyde. Cytototoxic granules were visualized by incubating the fixed cells with GrA-8 followed by goat anti-mouse labeled with FITC.

Discussion

Studies on the role of granzymes in cytotoxic and other immune reactions would be facilitated by the availabilty of specific monoclonal and polyclonal antibodies. Although granzymes A and B proteins were purified in 1988 (Krahenbuhl et al., 1988), and full-length cDNA for both have been isolated, until now mAb against human granzymes have not been described. Here, we report the production of human recombinant granzymes A and B and the application of these recombinant proteins to raise a panel of specific mouse mAb directed against granzymes A or B. These antibodies all reacted with granzymes from activated CTL and NK cells.

Hameed et al. (1991) used a prokaryotic expression system to produce human granzyme B, against which exclusively specific polyclonal antibodies were raised. We used a similar expression system to produce sufficient amounts of recombinant granzymes A and B. The identity of both proteins produced by *E. coli* were confirmed by immunoblotting with polyclonal antibodies raised against C-terminal and N-terminal peptides of each granzyme. Although the recombinant proteins could not be solubilized, direct coating on ELISA plates of the sonicated protein aggregates provided an easy and successful method for the detection of mAb. On immunoblot the mAb not only recognized the recombinant but also the natural protein from LAK cells. The M_r of the natural granzymes A and B proteins detected by the mAb corresponded, in general, with those described (Krahenbuhl et al., 1988).

All seven mAb against granzyme A were of the IgG1 subclass and recognized the same or overlapping epitopes on the recombinant granzyme A protein. In contrast, at least two different types of mAb against granzyme B were obtained, each recognizing a different epitope on granzyme B.

As immunoblotting indicated that the mAb detected denatured natural proteins, we further studied the application of these mAb in immunohistochemistry. Both mAb against granzymes A and B bound to cytotoxic granules in formalin and in acetone fixed LAK cells (data not shown), suggesting that they can be used in double immunohistochemical staining techniques which may be valuable for determining the phenotype of cells expressing granzymes in vivo. We also demonstrated the application of the mAb in confocal laser scanning microscopy to monitor the process of granule reorientation in the cytotoxic cells.

In conclusion, we describe the production and characterization of mAb against human granzymes A and B. These mAb (hybridoma cell lines available on request) may facilitate studies on the role of human cytotoxic cells in various immune reactions and in this way may contribute to the better understanding of the role of granzymes A and B in the cytotoxic response in vivo.

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