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Abstract

Delta (Y), MB1 (X) and Z are the three catalytic β subunits located in the inner rings of the constitutive proteasome, an intracellular multicatalytic complex responsible for the generation of peptides presented by HLA class I antigens to T cells. When cells are incubated with IFN-γ, delta (Y), MB1 (X) and Z are replaced by LMP2, LMP7 and LMP10, respectively, leading to the expression of immunoproteasome which generates peptides with increased affinity for HLA class I antigens. The characterization of the expression of constitutive proteasome and immunoproteasome subunits in cells, normal tissues and malignant lesions has been hampered by the lack or limited availability of constitutive proteasome and immunoproteasome subunit-specific mAb which are suitable for immunohistochemical staining. To overcome this limitation, we generated human delta (Y), MB1 (X), Z, LMP2, LMP7 and LMP10-specific mAb secreting hybridomas from BALB/c mice immunized with peptides and recombinant fusion proteins. The mAb SY-5, SJJ-3, NB-1, SY-1, HB-2 and TO-7 were shown to be specific for delta (Y), MB1 (X) and Z, LMP2, LMP7 and LMP10, respectively, since they react specifically with the corresponding molecules when tested with a human B lymphoid LG2 cell lysate in Western blotting and with the peptide derived from each molecule in ELISA. The reactivity of the six mAb with the corresponding intracellular antigens resulted in intracellular staining when the mAb were tested with microwave-treated and saponin-permeabilized cells in indirect immunofluorescence and with formalin-fixed, paraffin-embedded tissue sections in

immunohistochemical reactions. These results suggest that the constitutive proteasome and immunoproteasome subunit-specific mAb we have developed are useful probes to characterize the expression of proteasome subunits in normal tissues and in pathological lesions.

Key words: delta (Y); immunohistochemistry; LMP2; LMP7; LMP10; MB1 (X); monoclonal antibody; proteasome subunit; Z

Introduction

Proteasomes are intracellular, multisubunit and multicatalytic complexes which are responsible for the degradation of proteins and generation of peptides presented by the major histocompatibility complex (MHC) class I antigens to T cells (1, 2). The 20S proteasome or constitutive proteasome is composed of twenty-eight subunits arranged in four stacked seven-membered rings, with the outer two rings containing seven non-catalytic α subunits and the inner rings containing ten β subunits in humans (3). Three of the β subunits, delta (Y), MB1 (X) and Z are catalytic (4, 5). When cells are incubated with interferon-γ (IFN-γ), delta (Y), MB1 (X) and Z subunits are replaced by low molecular weight protein (LMP)2, LMP7 and LMP10, respectively, leading to the replacement of constitutive proteasome with immunoproteasome (6, 7). Immunoproteasome generates a spectrum of antigenic peptides different from that produced by constitutive proteasome, since it generates peptides with increased affinity for HLA class I allospecificities and is not efficient in processing peptides derived from self-proteins (8, 9). As a result the specificity of the cytotoxic T lymphocyte (CTL) immune response elicited by constitutive proteasome and immunoproteasome is different. Studies with LMP2 and LMP7 knock-out mice suggest that defects in immunoproteasome subunits affect the CTL repertoire (10, 11).

Proteasomes are localized in both nucleus and cytoplasm in all types of eukaryotic cells (12). The constitutive proteasome subunits are evenly distributed throughout the

nucleus and the cytoplasm in rat liver (13). In contrast, immunoproteasomes are enriched around the endoplasmic reticulum (ER) in the cytoplasm, where they can provide efficient transport of peptides from the site of generation into the lumen of the ER in rat liver and in cultured human cells (14). LMP2 and LMP7 were observed in the nucleus, while delta (Y) was identified in both the nucleus and the cytoplasm in cultured human cells (15). Only scanty information is available about the localization of constitutive proteasome and immunoproteasome subunits in human cells and about their distribution in human tissues. The paucity of information we have described is due, at least in part, to the limited or lack of availability of mAb which detect constitutive proteasome and immunoproteasome subunits in tissues. Furthermore, in spite of their role in shaping the specificity of CTL immune response, the expression of constitutive proteasome and immunoproteasome subunits in dendritic cells under physiological and pathological conditions has been characterized only to a limited extent. Lastly, although defects in the antigen processing machinery in malignant cells have been documented, constitutive proteasome and immunoproteasome subunit expression has been analyzed in a limited number of malignant tumors and within each tumor in a limited number of lesions. Whether the phenotypes found in malignant lesions are normal or pathological cannot be determined in most of the cases because of lack of information about the phenotype of the corresponding normal tissues.

mAb represent the most reliable probes for this type of study, since conventional

antisera suffer from the potential interference of contaminating antibodies and antibodies purified by affinity chromatography from antisera suffer from the practical difficulties to produce a large amount of this type of reagents. To the best of our knowledge, mAb specific for only some of the human constitutive proteasome and immunoproteasome subunits have been developed. They include the anti-delta (Y) mAb MCP421, the anti-Z mAb MCP168 (16), the anti-LMP2 mAb VF101-39F7 and VF101-39G5 (17), the anti-LMP7 mAb VF103-5D5 and VF103-8C2 (17). Among them mAb VF101-39F7, VF101-39G5, VF103-5D5 and VF103-8C2, which had been developed by one of us, stained both frozen and formalin-fixed tissues. However, these mAb are not available anymore, since the corresponding hybridomas have been lost. Whether mAb MCP421 and MCP168 are suitable for intracellular and immunohistochemical staining is not known. To facilitate the analysis of constitutive proteasome and immunoproteasome subunit expression in normal tissues, and in pathological lesions we have developed and characterized mAb which react with these subunits in intracytoplasmic and immunohistochemical staining. The aim of this paper is to describe the immunization strategy and the characterization of the specificity and reactivity pattern of the panel of mAb we have developed.

Materials and Methods

Mice

Six to eight week old female BALB/c mice were bred and maintained in the Department of Laboratory Animal Resources at Roswell Park Cancer Institute (RPCI).

Cells and tissues

The mouse myeloma cell line P3-X63-Ag8.653, the human B lymphoid cell line LG2, the human LMP2 and LMP7 deficient lymphoblastoid cell line T2 (18) and the human melanoma cell line Colo 38 were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Bio-Whittaker, Walkersville, MD) in a 5% CO_2 atmosphere at 37°C.

A lymph node with no detectable pathological abnormalities was obtained from a patient who underwent surgery for therapeutic reasons. This lymph node was prepared and stored at -80°C in the Department of Pathology at RPCI. Esophageal mucosa with no detectable pathological abnormalities and primary esophageal carcinoma lesions were obtained from patients who underwent surgery at the Asahikawa Medical College Hospital, Asahikawa, Japan. Tissue samples were fixed with 20% buffered formalin, routinely processed, and embedded in paraffin.

Monoclonal and polyclonal antibodies and reagents

The anti-idiotypic mAb MK2-23 (IgG_1) used as an isotype negative control was developed as described (19). Peroxidase-conjugated goat anti-mouse IgG (Fcγ fragment specific)

antibodies were purchased from Jackson Immuno Research (West Grove, PA). R-phycoerythrin (RPE) and fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG antibodies were purchased from DAKO (Carpinteria, CA). Recombinant human IFN-γ was purchased from Roche Applied Science (Indianapolis, IN).

Insert Table 1 here

Synthetic peptides

Synthetic peptides corresponding to amino acid sequence stretches in human delta (Y), MB1 (X), Z, LMP2, LMP7, LMP10 and β_2-microglobulin (β_2-m) were purchased from Molecular Genetics Instrumentation Facility at the University of Georgia Research Service (Atlanta, GA). The amino acid sequences of the peptides utilized as immunogens and in binding assays are shown in Table 1. A cysteine residue was added at either the N-terminal or C-terminal to facilitate the subsequent conjugation of keyhole limpet hemocyanin (KLH) (Pierce, Rockford, IL) or of bovine serum albumin (BSA) (Sigma, St. Louis, MO) using the coupling agent maleimidobenzoyl-N-hydroxysuccinimide (Pierce).

Insert Table 2 here

Oligonucleotide primers and recombinant fusion proteins

The oligonucleotide primers used for synthesis of delta (Y), MB1 (X), Z, LMP2, LMP7 and LMP10 cDNAs were purchased from Integrated DNA Technologies Inc. (Coralville, IA) and show in Table 2. cDNA used to generate recombinant fusion proteins were cloned

using pBAD/TOPO® ThioFusion™ Expression system (Invitrogen, Carlsbad, CA) as described elsewhere (20). The recombinant fusion proteins were expressed and purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the manufacturer's instructions and used for immunization.

Generation of mAb secreting hybridomas

BALB/c mice were immunized with KLH-conjugated peptides (50-100 μg each) emulsified in Freund's complete adjuvant (Sigma) and an intraperitoneal injection of SDS-PAGE purified recombinant fusion protein (100-200 μl of gel suspension/injection) following the strategy previously described (20). Hybridization and subcloning were performed as described (21). Isotyping of mAb was performed utilizing IsoStrip (Roche Applied Science) following the manufacturer's protocol.

Binding assay with BSA-conjugated peptides

The enzyme-linked immunosorbent assay (ELISA) with peptides to determine peptide specific reactivity of sera from mice immunized with constitutive proteasome and immunoproteasome subunit-derived peptides and recombinant fusion proteins and of mAb was performed as described (22). Cell permeabilization, intracellular staining and flow cytometric analysis were performed as described elsewhere (23).

Immunochemical assay

Western blot analysis was performed as described (22).

Immunofluorescence analysis

Microscopic analysis of intracellularly stained cells was performed as described with minor modifications (14). Briefly, following fixation with -20°C absolute methanol for 10 min, cells were permeabilized by 30 min incubation with PBS containing 1% BSA and 0.1% saponin (Sigma) at 4°C. Then cells were sequentially incubated with an optimal amount of primary mAb and with FITC-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG antibodies. Cells were then analyzed under a fluorescence microscope (MC80 microscope camera; Carl Zeiss, Thornwood, NY).

Immunohistochemical staining of frozen and formalin-fixed tissue section

Immunohistochemical staining of frozen and formalin-fixed, paraffin-embedded tissue sections with mAb was performed utilizing the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) and the EnVision+ system (DAKO), respectively, as described elsewhere (20).

Results

Selection of amino acid sequence stretches of constitutive proteasome and immunoproteasome subunit to synthesize the peptides used as immunogens

Analysis of each constitutive proteasome and immunoproteasome subunit amino acid sequence with the computer-assisted program Protean™ (DNAStar, Inc., Madison, WI) identified immunogenic amino acid sequences in each subunit. This information in conjunction with the degree of homology with the amino acid sequence of the mouse counterparts was utilized to select the amino acid sequences to synthesize the peptides used as immunogens. The amino acid sequences selected were shown in Table 1. Each peptide was conjugated to KLH and administered with the corresponding recombinant protein to a group of at least 5 BALB/c mice. Mice were boosted up to 5 times every two weeks and the immune response was monitored by testing sera with the immunizing peptide in ELISA and/or with a human B lymphoid cell line LG2 lysate in Western blotting.

Generation of constitutive proteasome and immunoproteasome subunit-specific mAb

More than 80% of the immunized mice developed antibodies reacting with the immunizing peptide in ELISA following at least two immunizations. More than 20% of them developed antibodies reacting with the proteasome subunit when tested with a human B lymphoid cell line LG2 lysate in Western blotting. When the titer of the antibodies with the immunizing peptide was at least 1: 1,000 in ELISA and/or when testing of immune serum with a LG2 cell lysate showed a strong reactivity with a component with the

expected size in Western blotting, mice were rested for one month. In the 3-5 day following an intraperitoneal booster with the soluble peptides, the immunized mice were sacrificed. Splenocytes were harvested, hybridized with murine myeloma cells P3-X63-Ag8.653 and seeded in 96 well plates. Colonies developed in at least 30% of the seeded wells. Screening in ELISA with the immunizing peptide of the supernatant from each individual hybridoma generated from the fusions resulted in the identification of one clone (mAb SY-5) for delta (Y), one clone (mAb SY-1) for LMP2, and one clone (mAb TO-7) for LMP10. Screening with a LG2 cell lysate in Western blotting of a pool of supernatants from 10-15 wells of hybridomas resulted in the identification of one clone (mAb SJJ-3) for MB1 (X), one clone (mAb NB-1) for Z and one clone (mAb HB-2) for LMP7. Isotyping of mAb showed that mAb SY-5 is an IgG_{2b}/κ and all the other mAb are IgG_1/κ.

Insert Table 3 here

Characterization of constitutive proteasome and immunoproteasome subunit-specific mAb

Characterization of proteasome subunit-specific mAb is shown in Table 3. All proteasome subunit-specific mAb except mAb SJJ-3 displayed a dose dependent reactivity with the immunizing peptide in ELISA (Fig. 1). This reactivity is specific since all of the mAb did not react with a peptide derived from human β_2-m and a peptide derived from an amino acid sequence stretch of each protein different from that used to synthesize the peptides

used as immunogens. The specificity of the mAb was corroborated by their reactivity pattern with a lymphoid LG2 cell lysate in Western blotting (Fig. 2). The mAb SY-5, SJJ-3, NB-1, SY-1, HB-2 and TO-7 reacted with a 25 kDa component corresponding to delta (Y), a 23 kDa component corresponding to MB1 (X) and a 29 kDa component corresponding to Z, a 21 kDa component corresponding to LMP2, a 25 kDa component corresponding to LMP7, and a 29 kDa component corresponding to LMP10, respectively. The specificity of mAb SY-1 and HB-2 was also supported by their lack of reactivity in Western blotting with the lysates of the LMP2 and LMP7 deficient cell line T2 and of the mouse myeloma cell line P3-X63-Ag8.653. The specificity of mAb SY-5, NB-1 and TO-7 was corroborated by their lack of reactivity with a mouse myeloma cell line P3-X63-Ag8.653 lysate in Western blotting. mAb SJJ-3 cross-reacted with a 23kDa component, when tested with a mouse myeloma cell line P3-X63-Ag8.653 lysate in Western blotting.

Insert Fig. 1 and 2 here

Flow cytometric analysis of cells stained with constitutive proteasome and immunoproteasome subunit-specific mAb

Fig. 3 shows representative examples of intracellular staining of cells by mAb which recognize constitutive proteasome and immunoproteasome subunits. The staining intensity obtained with the six mAb was enhanced by microwave treatment of cells. The staining is specific since mAb SY-1 and HB-2 did not stain the human LMP2 and LMP7

deficient cell line T2 and mAb SY-5, NB-1, SY-1, HB-2 and TO-7 did not stain the mouse myeloma cell line P3-X63-Ag8.653. Only mAb SJJ-3 stained weakly the mouse myeloma cells P3-X63-Ag8.653, probably because of a weak crossreactivity with mouse MB1 (X). Flow cytometric analysis showed that the staining intensity of the human B lymphoid cells LG2 with mAb SY-1, HB-2 and TO-7 was upregulated following incubation with IFN-γ, while that with mAb SY-5, SJJ-3 and NB-1 was downregulated slightly.

Insert Fig. 3 here

Immunofluorescence staining of cells with constitutive proteasome and immunoproteasome subunit-specific mAb

Fig. 4 shows representative examples of the cellular distribution of the staining when cells were stained with constitutive proteasome and immunoproteasome subunit-specific mAb. The staining was distributed both in nucleus and in cytoplasm when cells were stained with delta (Y)-specific mAb SY-5, MB1 (X)-specific mAb SJJ-3 and Z-specific mAb NB-1 (panels A, B and C, respectively). On the other hand, the staining was strong in the cytoplasm and weak in the nucleus, when cells were stained with LMP2-specific mAb SY-1, LMP7-specific mAb HB-2 and LMP10-specific mAb TO-7 (panels D, E and F, respectively).

Insert Fig. 4 here

Immunohistochemical staining of tissue sections with constitutive proteasome and immunoproteasome subunit-specific mAb

Only mAb HB-2 stained frozen tissue sections weakly. A representative example is shown in Fig. 5, which presents the weak staining of a frozen tissue section of a lymph node. All six mAb stained formalin fixed paraffin embedded tissue sections. Representative examples of the staining patterns with the six mAb are also shown in Fig. 5. The delta (Y)-specific mAb SY-5, the MB1 (X)-specific mAb SJJ-3 and the Z-specific mAb NB-1 stained basal layer and lymphoid cells in normal mucosa. Furthermore, while mAb SY-5 and NB-1 stained an esophageal carcinoma lesion strongly, no staining of this carcinoma lesion was detected with mAb SJJ-3. This pattern may reflect the selective loss of MB1 (X) or of the determinant recognized by mAb SJJ-3 in the lesion analyzed. The LMP2-specific mAb SY-1, the LMP7-specific mAb HB-2 and the LMP10-specific mAb TO-7 stained basal layer and lymphoid cells in normal mucosa weakly and the esophageal carcinoma lesion tested, strongly.

Insert Fig. 5 here

Discussion

Several lines of evidence indicate that immunization of BALB/c mice with immunogenic peptides identified in constitutive proteasome and immunoproteasome subunit amino acid sequences and with recombinant fusion proteins has generated the mAb SY-5, SJJ-3, NB-1, SY-1, HB-2 and TO-7 which are specific for delta (Y), MB1 (X), Z, LMP2, LMP7 and LMP10, respectively. First, the six mAb react with moieties with the appropriate size when tested in Western blotting with a lysate of human B lymphoid cells LG2 which express all the constitutive proteasome and immunoproteasome subunits. Second, the six mAb stained human B lymphoid cells LG2 intracellularly and the intensity of staining of lymphoid cells was modulated by IFN-γ. Third, with the exception of mAb SJJ-3, all the mAb reacted with the immunizing peptide in a binding assay. The reactivity of the mAb in the three assays are specific, since the anti-LMP2 mAb SY-1 and the anti-LMP7 mAb HB-2 do not react with any components when tested in Western blotting with a lysate of the human T2 cells which do not express these immunoproteasome subunits and of the mouse myeloma cells P3-X63-AG8.653. The latter two cell lines were also not stained intracellularly by the two mAb. Similar results were obtained with the anti-delta (Y) mAb SY-5, the anti-Z mAb NB-1 and the anti-LMP10 mAb TO-7. On the other hand, the anti-MB1 (X) mAb SJJ-3 reacted weakly with the mouse counterparts when tested with a lysate of mouse myeloma cells P3-X63-AG8.653. The latter finding is likely to reflect the high degree of homology of the amino acid sequence of human and mouse MB1 (X).

Since delta (Y), MB1 (X) and Z displayed a 61%, 69% and 58% amino acid sequence homology with LMP2, LMP7 and LMP10 subunits, respectively (5, 24), one might wonder why the mAb we have generated do not crossreact with the other constitutive proteasome and immunoproteasome subunits. This finding is likely to reflect for at least five of the six developed mAb specificity for a region of the constitutive proteasome and immunoproteasome subunits corresponding to the immunizing peptides. The amino acid sequence of each of them is unique of each constitutive proteasome and immunoproteasome subunit. With the exception of mAb SJJ-3, all of the mAb reacted with the immunizing peptides although the immunogen contained also a large part of the protein. These findings indicate that the administration of synthetic peptides corresponding to up 15 residues of the constitutive proteasome and immunoproteasome subunit amino acid sequence focuses the immunized mouse immune response to determinant(s) expressed by immunizing peptides.

In the present study, we demonstrated the localization of constitutive proteasome and immunoproteasome subunits in human cultured cells stained with the six mAb. The anti-delta (Y) mAb SY-5, the anti-MB1 (X) mAb SJJ-3 and the anti-Z mAb NB-1 stained both cytoplasm and nucleus in human melanoma Colo 38 cells. In contrast, the anti-LMP2 mAb SY-1, the anti-LMP7 mAb HB-2 and the anti-LMP10 mAb TO-7 stained the cytoplasm strongly. The staining in the cytoplasm appeared to be associated with the ER. These data are in agreement with the report previously described about the

localization of delta (Y), LMP2 and LMP7 in human cultured cells (15).

In immunohistochemical staining, the six mAb stained formalin-fixed, paraffin-embedded tissue sections. However, the six mAb with the exception of anti-LMP7 mAb HB-2 did not stain frozen tissue sections. This finding is not so surprising, since the selection of the immunogens used was determined by our emphasis on the development of mAb which react with proteasome subunits in formalin-fixed, paraffin-embedded tissue sections. We immunized with both recombinant proteins purified by SDS-PAGE and synthetic peptides selected from each proteasome subunit for their likely immunogenicity. These immunogens were selected, since they are likely to elicit antibodies reacting with determinants expressed on denatured proteins and therefore likely to be expressed in formalin fixed, paraffin embedded tissue sections. Fixation of tissues in formalin is likely to denature proteins and to change their antigenic profile.

The six mAb we have developed are expected to facilitate the characterization of constitutive proteasome and immunoproteasome subunit expression in normal tissues and in pathological lesions and the analysis of the localization of the subunits in human cells.

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Figure legends

Fig. 1. Reactivity of constitutive proteasome and immunoproteasome subunit-specific mAb with immunizing peptides in ELISA. Delta (Y)-specific mAb SY-5 (panel A), MB1 (X)-specific mAb SJJ-3 (panel B), Z-specific mAb NB-1 (panel C), LMP2-specific mAb SY-1 (panel D), LMP7-specific mAb HB-2 (panel E) and LMP10-specific mAb TO-7 (panel F) were added to an ELISA plate coated with BSA-conjugated immunizing peptides (●), with BSA-conjugated peptides derived from β_2m (87-99) (○) and with BSA-conjugated peptides derived from a region of each proteasome subunit different from that corresponding to the immunizing peptide (▼). Binding of the mAb to the peptide was detected using peroxidase-conjugated goat anti-mouse IgG (Fc fragment-specific) antibodies. The color reaction was developed by the addition of TMB substrate and stopped by the addition of 1N H_2SO_4. Results are expected as absorbance at 450 nm.

Fig. 2. Western blotting analysis of the specificity of constitutive proteasome and immunoproteasome subunit-specific mAb. Lysates of a human lymphoid cell line LG2 (lanes 1, 3, 5, 7, 10 and 13), a mouse myeloma cell line P3-X63-AG8.653 (lanes 2, 4, 6, 8, 11 and 14) and a human LMP2 and LMP7 deficient cell line T2 (lanes 9 and 12) were separated on 12% polyacrylamide gels under denaturing conditions. After a transfer procedure, membranes were incubated with an appropriate amount of delta (Y)-specific mAb SY-5 (lanes 1 and 2), MB1 (X)-specific mAb SJJ-3 (lanes 3 and 4), Z-specific mAb

NB-1 (lanes 5 and 6), LMP2-specific mAb SY-1 (lanes 7 to 9), LMP7-specific mAb HB-2 (lanes 10 to 12) and LMP10-specific mAb TO-7 (lanes 13 and 14). Membranes were then incubated with peroxidase-conjugated goat anti-mouse IgG (Fc fragment-specific) antibodies. After incubation with ECL Western blotting detection reagents, membranes were exposed to Hyperfilm ECL.

Fig. 3. Flow cytometric analysis of cells stained with constitutive proteasome and immunoproteasome subunit-specific mAb. Cultured human B lymphoid cell line LG2 (panels A, C, E, G, J and M) were incubated with IFN-γ (250U/ml) or without IFN-γ for 48h at 37°C. Mouse myeloma cells P3-X63-AG8.653 (panels B, D, F, H, K and N) and human LMP2 and LMP7 deficient cells T2 (panels I and L) were used as controls. Cells were fixed with 2% paraformaldehyde, treated with microwave and permeabilized with saponin. Cells were incubated with an appropriate amount of delta (Y)-specific mAb SY-5 (panels A and B), MB1 (X)-specific mAb SJJ-3 (panel C and D), Z-specific mAb NB-1 (panel E and F), LMP2-specific mAb SY-1 (panels G to I), LMP7-specific mAb HB-2 (panels J to L) and LMP10-specific mAb TO-7 (panels M and N). Following an additional incubation with RPE-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG antibodies, cells were analyzed by FACScan. The unbroken line represents fluorescence intensity of cells incubated with proteasome subunit-specific mAb. The line with solid fill represents fluorescence intensity of cells incubated with anti-idiotypic mAb MK2-23 used

as an isotype control. The dotted line represents fluorescence intensity of IFN-γ treated LG2 cells incubated with mAb.

Fig. 4. Immunofluorescence staining of Colo 38 cells with constitutive proteasome and immunoproteasome subunit-specific mAb. Cultured human melanoma cells Colo 38 were fixed with absolute methanol followed by permeabilization with saponin. Cells were then incubated with an appropriate amount of delta (Y)-specific mAb SY-5 (panel A), MB1 (X)-specific mAb SJJ-3 (panel B), Z-specific mAb NB-1 (panel C), LMP2-specific mAb SY-1 (panel D), LMP7-specific mAb HB-2 (panel E) and LMP10-specific mAb TO-7 (panel F). Cells were incubated with FITC-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG antibodies and then analyzed under a fluorescence microscope.

Fig. 5. Immunohistochemical staining of frozen and formalin-fixed, paraffin-embedded tissue sections with constitutive proteasome and immunoproteasome subunit-specific mAb. Frozen tissue sections of a lymph node (panels A to C and J to L) and formalin-fixed, paraffin-embedded tissue sections of normal esophageal mucosa (panels D to F and M to O) and esophageal carcinoma lesion (panels G to I and P to R) were stained with delta (Y)-specific mAb SY-5 (panels A, D and G), MB1 (X)-specific mAb SJJ-3 (panels B, E and H), Z-specific mAb NB-1 (panels C, F and I), LMP2-specific mAb SY-1 (panels J, M and P), LMP7-specific mAb HB-2 (panels K, N and

Q) and LMP10-specific mAb TO-7 (panels L, O and R). Tissue sections were then incubated with Vectastain ABC or EnVision+ reagent. Staining was visualized using the liquid DAB substrate-chromogen system (×200).

Table 1. Synthetic peptides used as immunogens to induce anti-proteasome subunit mAb

mAb	Specificity	Molecular size	Synthetic peptide[a]	Residue number[b]
SY-4	delta (Y)	25 kDa	FTPDWESREVSTGTT(C)	22-36
SY-5			(C)IIAGWDPQEGGQGY	133-146
SJJ-3	MB1 (X)	23 kDa	(C)YDLEVEQAYDLARR	149-162
NB-1	Z	29 kDa	(C)KNKLDFLRPYTVPNKK	223-238
SY-1	LMP2	21 kDa	GVDHRVILGNELPKFYDE(C)	202-219
SY-3	LMP7	25 kDa	GAPRGQRPESALPVA(C)	8-22
HB-2			(C)KVESTDVSDLLHQYREANQ	258-276
TO-6	LMP10	29 kDa	(C)TGAKLLRTLSSPTEPVK	220-236
TO-7				

a: Amino acid sequence of the proteasome subunits

b: Length of each synthetic peptide of the proteasome subunits

Table 2. Oligonucleotides used as primers for synthesis of cDNA encoding recombinant fusion proteins

mAb	Specificity	Sense primer	Antisense primer	Recombinant protein[a]
SY-4 SY-5	delta (Y)	5'-atggcggctaccttactagctgct-3'	5'-ggcgggtggtaaagtggcaacggcgaa-3'	F.L.[b] 1-239
SJJ-3	MB1 (X)	5'-gacggtgaagaaggtgatag-3'	5'-ttgactgcacctcctgagta-3'	P.L.[c] 94-179
NB-1	Z	5'-atggcggctgtgtcggtgta-3'	5' ggaagtgtccattgtttggactg-3'	F.L. 1-277
SY-1	LMP2	5'-atgctgcgggcgggagaagtcc-3'	5'-ctcatcatagaattttggcag-3'	F.L. 1-219
SY-3 HB-2	LMP7	5'-gcgctactagatgtatgc-3'	5'-gcgttgattggcttcccggtactg-3'	F.L. 1-276
TO-6 TO-7	LMP10	5'-atgctgaagccagccctggagccccga-3'	5'-ctccacctccatagcctgcacagtt-3'	F.L. 1-273

a: Length of each recombinant protein and correspondence to the amino acid sequence of the proteasome subunits

b: Full length

c: Partial length

Table 3. Characterization of constitutive proteasome and immunoproteasome subunit-specific mAb

mAb	Specificity	Screening method	ELISA	Western blot/ Flow cytometry			Immunohistochemistry	
				LG2	T2	AG8.653	Frozen	Formalin-fixed
SY-5	delta (Y)	peptide ELISA	+	+	n.a.[a]	−	−	+
SJJ-3	MB1 (X)	Western blot	−	+	n.a.	+	−	+
NB-1	Z	Western blot	+	+	n.a.	−	−	+
SY-1	LMP2	peptide ELISA	+	+	−	−	−	+
HB-2	LMP7	Western blot	+	+	−	−	+	+
TO-7	LMP10	peptide ELISA	+	+	n.a.	−	−	+

+: Reaction　−: No reaction

a: Not applicable

Fig. 1

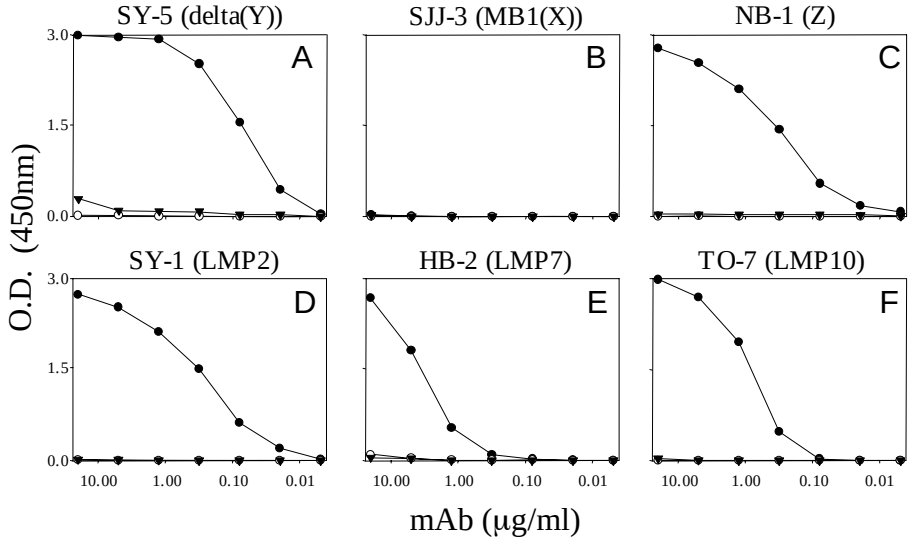

SY-5 (delta(Y))
SJJ-3 (MB1(X))
NB-1 (Z)
SY-1 (LMP2)
HB-2 (LMP7)
TO-7 (LMP10)
A
B
C
D
E
F
O.D. (450nm)
3.0
1.5
0.0
10.00
1.00
0.10
0.01
mAb (μg/ml)

Fig. 2

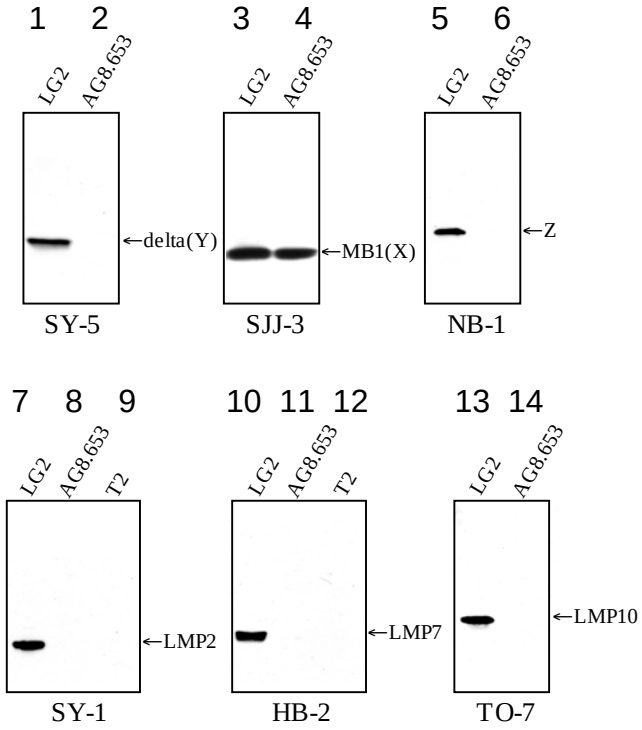

1 2
LG2
AG8.653
←delta(Y)
SY-5
3 4
LG2
AG8.653
←MB1(X)
SJJ-3
5 6
LG2
AG8.653
←Z
NB-1
7 8 9
LG2
AG8.653
T2
←LMP2
SY-1
10 11 12
LG2
AG8.653
T2
←LMP7
HB-2
13 14
LG2
AG8.653
←LMP10
TO-7

Fig. 3

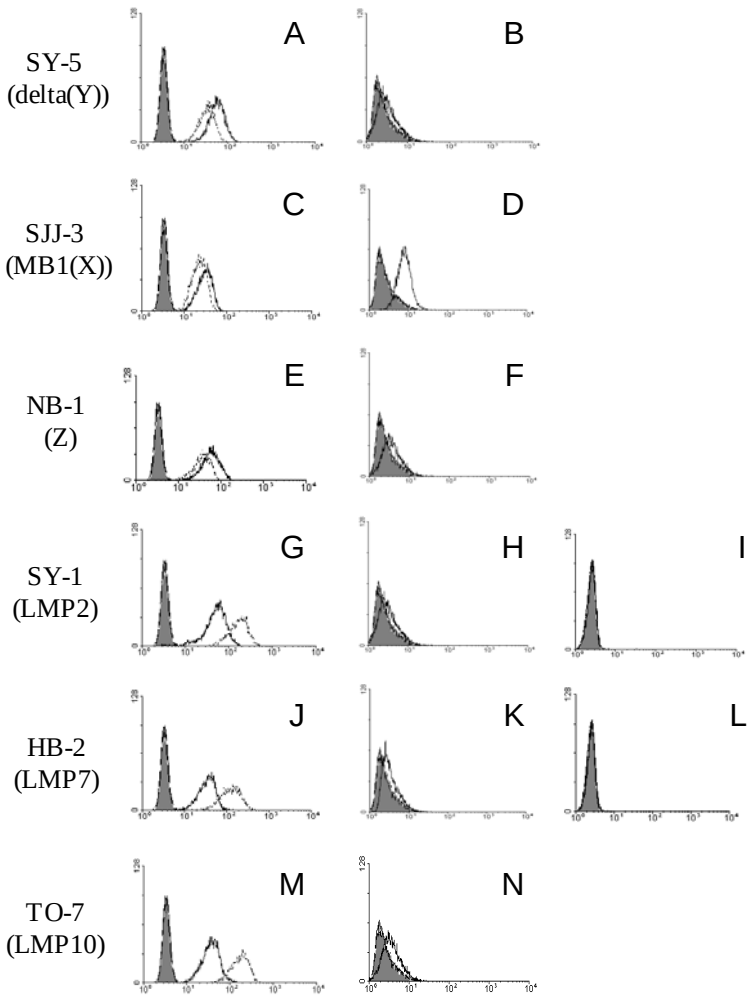

SY-5
(delta(Y))
A
B
SJJ-3
(MB1(X))
C
D
NB-1
(Z)
E
F
SY-1
(LMP2)
G
H
I
HB-2
(LMP7)
J
K
L
TO-7
(LMP10)
M
N
Fluorescence intensity

Fig. 4

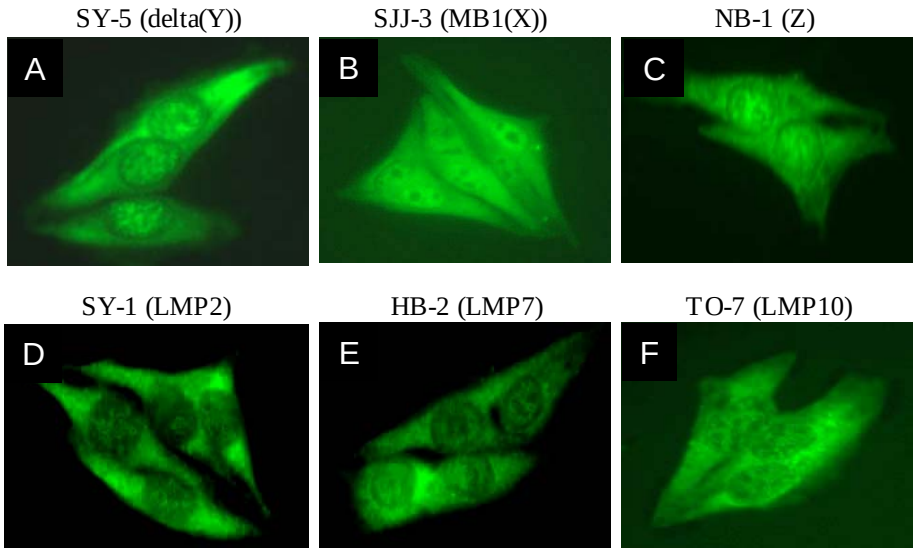
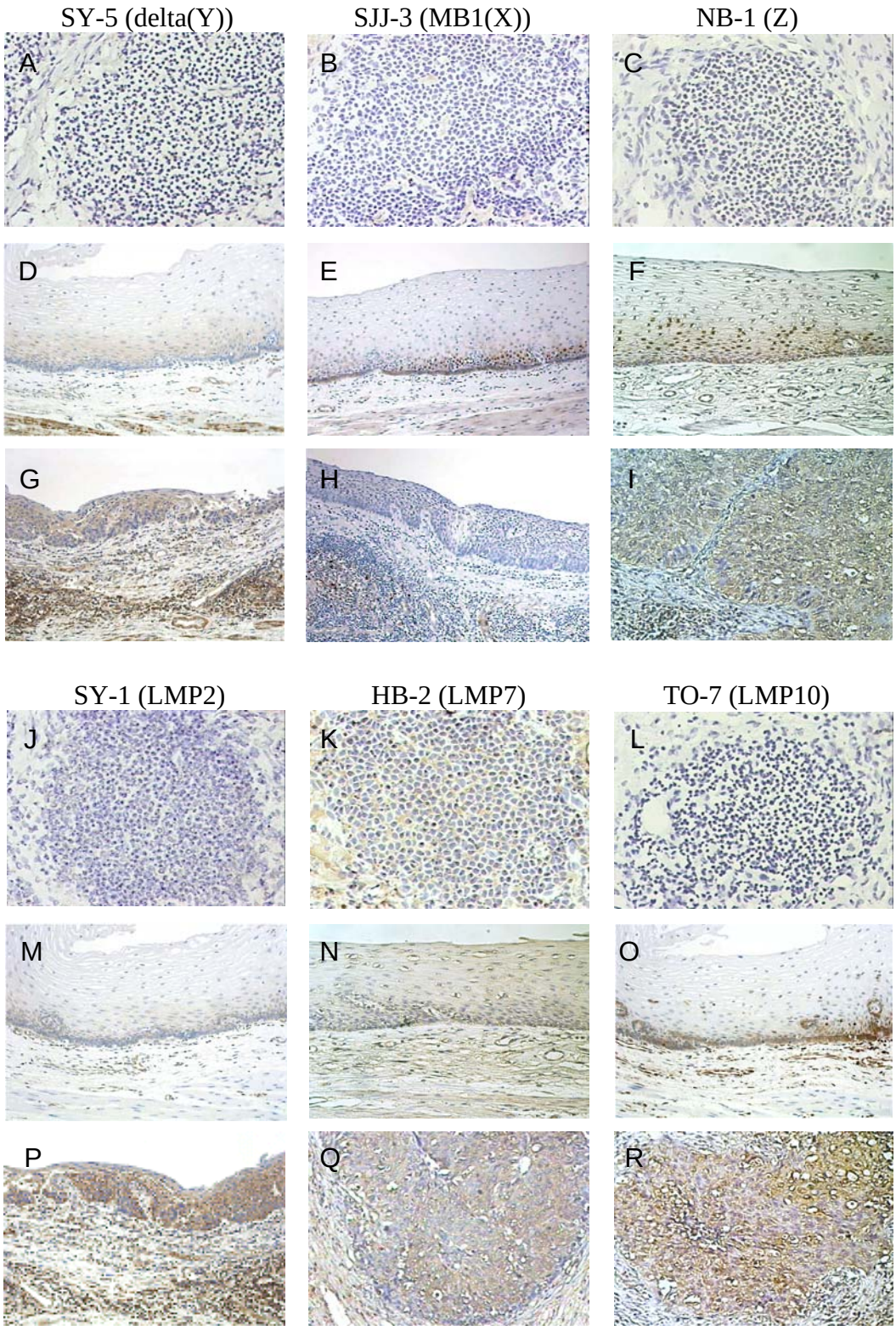


Fig. 5


SY-5 (delta(Y))
SJJ-3 (MB1(X))
NB-1 (Z)
A
B
C
D
E
F
G
H
I
SY-1 (LMP2)
HB-2 (LMP7)
TO-7 (LMP10)
J
K
L
M
N
O
P
Q
R