Gamma-interferon causes a selective induction of the lysosomal proteases, cathepsins B and L, in macrophages

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Abstract Previous studies have indicated that acid-optimal cysteine proteinase(s) in the endosomal-lysosomal compartments, cathepsins, play a critical role in the proteolytic processing of endocytosed proteins to generate the antigenic peptides presented to the immune system on major histocompatibility complex (MHC) class II molecules. The presentation of these peptides and the expression of MHC class II molecules by macrophages and lymphocytes are stimulated by γ-interferon (γ-IFN). We found that treatment of human U-937 monocytes with γ-IFN increased the activities and the content of the two major lysosomal cysteine proteinases, cathepsins B and L. Assays of protease activity, enzyme-linked immunosorbant assays (ELISA) and immunoblotting showed that this cytokine increased the amount of cathepsin B 5-fold and cathepsin L 3-fold in the lysosomal fraction. By contrast, the aspartic proteinase, cathepsin D, in this fraction was not significantly altered by γ-IFN treatment. An induction of cathepsins B and L was also observed in mouse macrophages, but not in HeLa cells. These results suggest coordinate regulation in monocytes of the expression of cathepsins B and L and MHC class II molecules. Presumably, this induction of cysteine proteases contributes to the enhancement of antigen presentation by γ-IFN.

Key words: Antigen-presenting cell; Cathepsin B, L, D; MHC class II; Macrophage; γ-Interferon

1. Introduction

Antigen-presenting cells (APCs) display on their surface membranes peptides derived from endocytosed proteins in complexes with class II major hystocompatibility complex (MHC) molecules [1,2]. This step is a critical early event in eliciting antibody production and inflammatory responses. Antigen presentation on class II molecules (in contrast to the presentation of intracellular antigens on MHC class I molecules) is restricted to certain cells, especially monocytes and macrophages. In these cells, the presented peptides are generated from the endocytosed proteins by limited proteolytic digestion in the endosomal or lysosomal compartments. Before binding the antigenic peptides, the newly synthesized MHC class II molecules form a complex in the endoplasmic reticulum with the invariant chain (Ii), which stabilizes the MHC molecules and facilitates their transport into the acidic endosomal-lysosomal compartments. There, the inhibitory invariant chain undergoes proteolytic inactivation, which exposes the binding site on the MHC molecules [3]. The proteolytic events that generate the antigenic peptides and inactivate the invariant chains in these compartments depend on the decreased pH, the reducing environment [4], and the activities of the various lysosomal proteinases, known as cathepsins. However, the precise roles of different endosomal-lysosomal proteinases in antigen presentation and the mechanisms that regulate the activities of these proteases and prevent the complete digestion of the antigenic peptides are still unclear.

Among the most prominent lysosomal proteases are cathepsin D, an aspartic proteinase which has its optimum activity in the very acidic range (pH 3.5–4.5), and the cysteine proteases, cathepsin B and cathepsin L, which degrade proteins optimally in the pH range from 4.5–6.5. There is a growing series of observations indicating a critical role of these cysteine proteases in class II presentation. Takahashi et al. [5] and Diment [6], using inhibitors, demonstrated in vivo that cysteine proteinases were clearly important for the proteolytic processing of bovine albumin by APCs, even though the aspartic proteinase, cathepsin D, was able to degrade this antigen in vitro into fragments that could be recognized by Th1 lymphocytes. Recently, Towatari et al. [7], also using selective inhibitors, showed that a cysteine proteinase, in particular cathepsin B, is critical in the processing of recombinant hepatitis B antigenic subtype (HBsAG) by APCs. Other studies [8] provided evidence that the processing of different polypeptides may require digestion by distinct proteases to reveal antigenic epitopes. Using protease inhibitors to modulate the ability of splenic APC and B cell hybridoma to present chicken ovalbumin (OVA), Vidard et al. [9,10] demonstrated that the endosomal-lysosomal proteases have the capacity to both generate and destroy immunogenic peptides, and that different epitopes on the same protein may be processed in distinct fashions by different APCs. In fact, in epithelial cells, cathepsin E had been shown to play a critical role in the elicitation of IgA responses [11].

MHC class II antigen presentation is under the control of the interferons (IFNs), a family of antiviral immune modulatory proteins that regulate the expression of a large number of cellular genes [12]. All three types of IFNs induce MHC class I expression, but only γ-IFN also stimulates class II presentation [1,3,13,14]. It was demonstrated recently [15] that γ-IFN acts by inducing the transactivator gene, CIITA, which directly

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Abbreviations: AMC, 7-amino-4-methylcoumarin; γ-IFN, gamma-interferon; Z-Ala-Arg-Arg-AMC, (Z-ARR-AMC) benzoylcarbonyl-alaninyl-arginyl-arginyl-7-amino-4-methylcoumarin; Z-Phe-Arg-AMC (Z-FR-AMC), benzyloxycarbonyl-phenylalanine-arginyl-7-amino-4-methylcoumarin.

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controls both the constitutive and inducible MHC class II expression. In stimulating class II presentation, γ-IFN may also enhance expression of other genes in the APC in addition to MHC class II molecules. The stimulation by γ-IFN of MHC class I presentation involves the increased expression of class I molecules, peptide transporters on the endoplasmic reticulum, and the LMP2 and LMP7 proteins which function as subunits of the 20 S and 26 S proteasome [16,17]. The incorporation of these γ-IFN-induced subunits in the proteasome, enhances certain peptidase activities and suppresses others [13,15–17]. As a result, the degradation of cytosolic proteins by the ubiquitin-proteasome pathway should yield more of the types of oligopeptides that were transported selectively into the ER [18,19] and that bind preferentially to class I molecules [16,17].

The present studies were undertaken to test the possibility that the stimulation of MHC class II presentation by γ-IFN may also involve an alteration in the proteolytic capacity of the lysosomal-endosomal compartment, analogous to the changes in the proteases that generate peptides for MHC class I presentation. We have tested whether, in the class II system, γ-IFN treatment may change the content of lysosomal proteases and thus may alter the pattern of protein digestion of endocytosed proteins. This study demonstrates for the first time that exposure to γ-IFN causes the selective enhancement of the expression and activities of cathepsins B and L in human and mouse monocyte lines, which could have important consequences for MHC class II antigen presentation.

2. Materials and methods

2.1. Cell cultures and γ-IFN treatment

U-937 (human hystiocytic lymphoma, ATCC CRL 1593) cells (0.15×10^6/ml) were grown in RPMI160 medium containing 10% fetal calf serum (FCS) and antibiotics at 37°C for 72 h with and without 1,000 U/ml human recombinant γ-IFN, which was kindly provided by Biogen Inc., Cambridge, MA. This concentration of γ-IFN was found to cause maximal stimulation of MHC class I antigen presentation and maximal alteration in proteasomal peptidase activities [16]. A3.1A.7 is an immortalized mouse macrophage cell line [20]. HeLa cells were obtained from American Type Culture Collection (Rockville, MD) and grown in DME medium in the presence of 10% fetal calf serum.

2.2. Subcellular fractionation

Macrophages were collected by centrifugation for 5 min at $700 \times g$, washed twice and resuspended in the homogenization buffer, containing 50 mM Tris, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 2 MM ATP, 250 mM sucrose at pH 7.4. Cell suspensions were homogenized using a Dounce homogenizer (Wheaton), followed by vortexing for 3 min with glass beads. The crude extracts were obtained by centrifugation at $10,000 \times g$ for 20 min and a further centrifugation at $100,000 \times g$ for 1 h to obtain the microsomal pellet and the soluble (cytosolic) fraction, which was the source of proteasome fraction used in previous studies [16]. The 'low speed pellets' from the crude extracts (sedimenting at $10,000 \times g$), which contained nuclear and lysosomal particles, and the microsomal pellets (the 'high speed pellets' sedimenting at $100,000 \times g$) were extracted with 50 mM Tris buffer, containing 0.5% Triton X-100 and 1 mM EDTA. They were homogenized with 5 passages in a Potter-Elvehjem (Teflon) homogenizer, and centrifuged in an Ultracentrifuge (Beckman) at $100,000 \times g$ for 30 min to obtain the clear supernatants used for the biochemical analyses.

Alternatively, in many experiments, the monocytes were disrupted by nitrogen cavitation and homogenized with the Dounce homogenizer. Cell debris and nuclear pellets were separated by a centrifugation at $1,500 \times g$ for 10 min. The supernatant was centrifuged at $12,000 \times g$ for 20 min to obtain the lysosome-endosome-enriched fraction (the '12K pellet'), which was extracted in 50 mM Tris buffer, containing 0.5% Triton X-100 and used for analysis. Further centrifugation of the supernatant at $100,000 \times g$ min for 60 min resulted in the microsomal pellets and the soluble supernatant (cystosolic fraction), which was also studied in parallel.

2.3. Enzyme activity assays

Cathepsin D was determined by the Anson procedure for determination of aspartic proteases, using hemoglobin as a substrate, as modified by Turk et al. [16]. Cathepsin B activity assay [18] was modified as described in details previously [19]. Briefly, 20 μl of the sample were added to 280 μl of the activation buffer containing 400 mM phosphate buffer, pH 6.0, containing 4 mM EDTA and 1.2 mM dithiothreitol and pre-incubated for 5 min at 37°C, before the substrate, 100 μl Z-Ala-Arg-Arg-7-AMC (Z-ARR-AMC) (Bachem, PA, USA), was added. After 150 min incubation, the reaction was stopped with cold 1 mM iodoacetic acid. The linearity of Cat B activity for 240 min at 37°C in crude homogenates has been established (unpublished data). Blanks were prepared without the addition of the enzyme. Samples and controls, containing 1 mM L-epoxy succinylleucyllamido-(4-guanidine)-butane (E-64) were carried out in duplicate. Fluorescence was read at an excitation wavelength of 370 nm and an emission wavelenght of 460 nm in an SLM Aminco S2 Luminescence Spectrophotometer and standardized against AMC. Cathepsin L activity was assayed after the samples were pre-incubated for 3 h at 37°C in 34 mM acetate buffer, pH 4.2, containing 2 mM dithiothreitol and 1 mM EDTA. Each enzyme activity assay was performed in triplicate.

Cathepsin L activity was measured using Z-Phe-Arg-AMC (Z-FR-AMC) as the substrate in the presence and absence of the selective inhibitor, Z-Phe-Phe-CHN_2 (Enzyme System Products), as described previously [21]. After preincubation at 37°C in acetate buffer at pH 4.2 to activate Cat L precursor, as described in detail by Mason et al. [22], 100 μl of 0.34 mM acetate buffer, pH 5.5, containing 1 mM EDTA and 1.3 mM DTT, were added, followed by 100 μl of 2 mM inhibitor solution to a final volume of 400 μl. Control samples received 100 μl of water instead of the inhibitor, and the reaction was started by adding 100 μl of the substrate solution to each sample. The reaction was stopped after 150 min incubation at 37°C by 500 μl of 1 mM iodoacetate. Cathepsin L enzyme units were calculated as described above for cathepsin B. The difference in fluorescence between the samples and the controls represented cathepsin L activity. These activities are expressed in EU/g protein, where 1 EU represents the amount of enzyme releasing 1 μmol of 7-AMC per min. Protein concentration was determined by the Bradford method (Bio-Rad, USA) using bovine serum albumin as the standard [23].

2.4. Enzyme–protein immunochemical determination

Cathepsin D was measured in the extracts by the competitive enzyme immunosorbent-linked assay (ELISA) obtained from Triton Diagnostics (CA, USA). The assay recognizes all forms of the enzyme (total enzyme mass) using the combination of monoclonal anti-human cathepsin D antibody and rabbit polyclonal antibody, both specific for cathepsin D. Cathepsins B and L protein content were determined by a double-sandwich ELISA (BioAss, Diessen, Germany) using immunospecific polyclonal rabbit and sheep anti-human cathepsin B antibodies. According to the protocol (BioAss), both immunoassays detected all free enzyme forms (precursor and active forms), as well as their complexes with the endogenous inhibitors.

2.5. SDS-PAGE and immunoblotting

Slab-gel electrophoresis was carried out in 10% polyacrylamide gels in the presence of SDS followed by blotting on nitrocellulose membranes, as described by Towbin et al. [24]. For immunoblotting, proteins separated by SDS-PAGE were transferred electrophoretically to nitrocellulose membranes, using a BioRad Trans-Blot apparatus. The blots were incubated with the polyclonal primary antibodies and with goat secondary antibodies, conjugated with horseradish peroxidase (Pierce, USA) and detected using a silver-gold enhancement kit (Amersham, USA), as recommended by the manufacturer. Primary polyclonal rabbit antibodies for cathepsin B, generously given by Dr. Joza Babnik (Jozef Stefan Institute, Ljubljana, Slovenia), were used in a 1:250 dilution. Immunospecific polyclonal sheep antibodies, raised against human brain cathepsin D, kindly provided by Dr. Ralph Nixon (McLean Hospital-Harvard Medical School, MA, USA), were used in a 1:400 dilution.

3. Results

3.1. Cathepsin activities in monocytes and HeLa cells

To define the lysosomal-endosomal compartment, we measured the specific activities of the lysosomal proteinases, cathepsins B and L, in all subcellular fractions from the human macrophage line, U937, and the mouse monocyte line, A3.1A.7. These two lysosomal markers were found to be greatly enriched in the 10–12,000 × *g* pellets: Cathepsin B activity in various preparations of this fraction was 9- to 45-fold higher than in microsomal pellets, and at least 500-fold higher than in the 100,000 × *g* supernatant (Table 1). Cathepsin L activity was enriched 2- to 5-fold in various preparations of the 12,000 × *g* pellet over the 100,000 × *g* pellets and at least 200-fold over the soluble supernatant. Therefore, in subsequent experiments, the rapidly sedimenting pellets were used to measure proteases in the lysosomal compartment.

Human monocytes were harvested after treatment with or without γ-IFN for 3 days, and the activities of cathepsins B, L and D were measured in the 10,000 × *g* or 12,000 × *g* pellets. Large increases were observed in the activities of both cysteine proteases in the cell lines grown in the presence of human γ-IFN, compared to control cells grown in parallel without the cytokine (Table 2). The mean increases in cathepsin B were 4.5-fold in the 10,000 × *g* pellets and 6.2-fold in the 12,000 × *g* pellets. Similarly, the mean activity of cathepsin L increased 4.6-fold in both pellets. By contrast, no significant changes were observed in cathepsin D activity, although the content of this enzyme was at the lower limit of sensitivity of the hemoglobin-degradation method (data not shown).

To test if these findings on the human macrophage line were also found in all human cell lines, HeLa cells were treated with γ-IFN in a similar fashion. In these cells, γ-IFN is known to induce a number of genes [16,17] and to stimulate MHC class I antigen presentation [13,17]. No significant change in cathepsin B and L activities was observed in HeLa cells exposed to γ-IFN under conditions where the cytokine did induce MHC class I molecules and the MHC encoded proteasome subunits, LMP1 and LMP2 [16]. We, therefore, tested if this response might be restricted to monocytes, which do play a prominent role in class II presentation. Table 2 shows that treatment of mouse macrophages with murine γ-IFN for several days enhanced the activities of both cathepsin B and L approximately 2-fold. These changes appeared smaller than those seen with U937 cells. However, the human cytokine was used at concentrations determined previously to cause maximal changes in U937 cells, while the most effective concentrations of the murine γ-IFN have not been determined.

Table 1
The activities of cathepsins B and L in subcellular fractions of U937, a human macrophage cell line

Subcellular fraction	Activity (EU/g)	
	Cathepsin B	Cathepsin L
Endosomal-lysosomal (12,000 × *g* pellet)	450	150
Microsomal (100,000 × *g* pellet)	10	37
Soluble supernatant	0.63	0.6

A representative experiment is shown with the extracts of different subcellular fractions, obtained from human monocytes (U937), as described in section 2. Specific enzyme activities were measured as described previously [21,22] and in section 2.

Table 2
Effects of γ-interferon treatment on cathepsin B and L activities in the endosomal-lysosomal fraction of human (U937), mouse (A3.1A1.7) and HeLa cells

Preparation	Activity (EU/g; mean ± S.D.)	
	Cathepsin B	Cathepsin L
U937 cells		
12,000 × *g* pellets		
Control	720 ± 10	746 ± 34
+ γ-IFN	4507 ± 3	1803 ± 26
Mean ratio (n = 3)	6.2	2.4
10,000 × *g* pellets		
Control	240 ± 129	20.2 ± 5.6
+ γ-IFN	802 ± 140	92.5 ± 35
Mean ratio (n = 7)	4.5	4.6
A3.1A1.7		
12,000 × *g* pellets		
Control	427 ± 50	4666 ± 593
+ γ-IFN	1006 ± 150	10762 ± 1007
Mean ratio (n = 3)	2.2	2.1
HeLa cells		
12,000 × *g* pellets		
Control	1467 ± 190	1263 ± 590
+ γ-IFN	1382 ± 642	1287 ± 204
Mean ratio (n = 3)	1.1	0.9

Cathepsin activities were determined in endosomal-microsomal fractions, defined as either the 12,000 × *g* or as 10,000 × *g* pellets (in certain initial experiments on U937 cells), obtained from (n) independent preparations. In each, the cells were cultured with or without human γ-IFN for 3 days. Mean ratios were obtained in (n) pairs of control and treated cells, which were cultured and prepared in parallel. The extracts were prepared as described in section 2, and specific enzyme activities of cathepsins B and L, using fluorigenic substrates, were measured as described [22,23].

3.2. Cathepsin content in human monocytes

γ-IFN may increase the activities of the cysteine proteinases in extracts either by increasing the amount of these enzymes or by activation of these proteases or by decreasing the level of the endogenous inhibitors, cystatins. To investigate whether γ-IFN treatment increased the amounts of cathepsins D, B and L, we measured the content of all three proteases immunochemically by ELISAs (Table 3). In human macrophages, cathepsin D was present in the highest concentration, followed by cathepsins L and B. Only the concentration of the cysteine proteinases were affected by γ-IFN treatment. The protein content of these cathepsins was not determined in the mouse monocytes, because the ELISAs are designed to measure only the proteases of human origin. These changes resembled the increase in enzyme activities described above.

This effect of γ-IFN on cathepsin B content in macrophages was confirmed using immunoblotting (Fig. 1). The lysosomal fraction from U937 cells treated with and without γ-IFN were normalized for protein concentration and applied to SDS-PAGE. A marked increase in the cathepsin B band was observed; in human macrophages, its heavy chain appeared as a darker, double bank with an M_r of 25,000 and 27,000, as was also noted in other cell lines [25]. In accord with the assays of enzyme activities, immunoblotting with anti-human cathepsin D antibodies showed no difference between the γ-IFN-treated

and control samples in the intensity of the major protein band, which corresponds to the M_r 31,000 heavy chain of the mature cathepsin D form. Together, these findings indicate that γ-IFN enhances cathepsin B and L activities by stimulating the production of these proteins.

4. Discussion

The present studies have demonstrated for the first time a selective regulation of the protease content of lysosomes by the cytokine, γ-IFN. This agent caused a large increase in cathepsin B and a moderate increase in cathepsin L in macrophages, while the other abundant lysosomal protease, cathepsin D, did not change. It is of particular interest that these findings were restricted to macrophages, which play a major role in antigen presentation on MHC class II molecules, and were not seen in HeLa cells, which do not present on class II molecules. This selective response is also noteworthy because of the special role of macrophages in phagocytosis of microbial pathogens and antigen–antibody complexes and in the killing of neoplastic cells. It seems likely that this increase in the content of cathepsin B and L in macrophages by γ-IFN is important in enhancing the proteolytic processing of the endocytosed antigens for presentation to the immune system or in the inactivation of the inhibitor Ii chains. Possibly, this increase in protease content may also enhance the macrophage's ability to digest the endocytosed materials and the tumoricidal activity of tumor-infiltrating macrophages. For example, the marked anti-neoplastic effects of γ-IFN, e.g. in regression of local recurrences of breast carcinoma [26], appears to be due in part to the activation of tumor-infiltrating macrophages, which show a marked increase in expression of MHC class I and class II.

The proposed role of cysteine proteinases in antigen processing [5–8] is supported by recent observations that cathepsin B can produce peptide fragments bearing T-cell epitopes from recombinant human growth factor [27] and from IgG2a in vitro [28]. Most direct evidence for the involvement of cathepsin B in the humoral response to vaccination with hepatitis-B type (HBsAg) was recently provided by Matsunaga et al. [7,29], who showed that selective inhibition of cathepsin B can block this process, while inhibition of cathepsin L or cathepsin H could not. Our finding that this protease is induced when class II presentation is accelerated and when γ-IFN production rises are thus of particular interest.

The selective induction of cathepsins B and L, together with MHC class II proteins, suggests that γ-IFN regulates the proteolytic activity necessary for the partial digestion of endocytosed proteins to the types of peptides that can be presented on class II. An increase in protease content could either enhance

Table 3
Effects of γ-Interferon treatment on the content of cathepsins D, B, L proteins in lysosomal-endosomal fraction of human U937 macrophages

	Activity (ng/mg)		
Preparation	Cathepsin D	Cathepsin B	Cathepsin L
Control	5340 ± 1700	188 ± 25	25 ± 20
γ-IFN	6185 ± 1250	440 ± 240	110 ± 83
Mean ratio (n = 3)	1.1	2.4	4.4

Cathepsins concentrations were measured by ELISAs, as described in section 2 and expressed per mg of total protein in the endosomal-lysosomal preparation. Mean values of three measurements of cathepsins in controls and γ-IFN-treated cell lines are presented. Mean ratio was obtained in pairs of control and treated cells, which were cultured simultaneously.

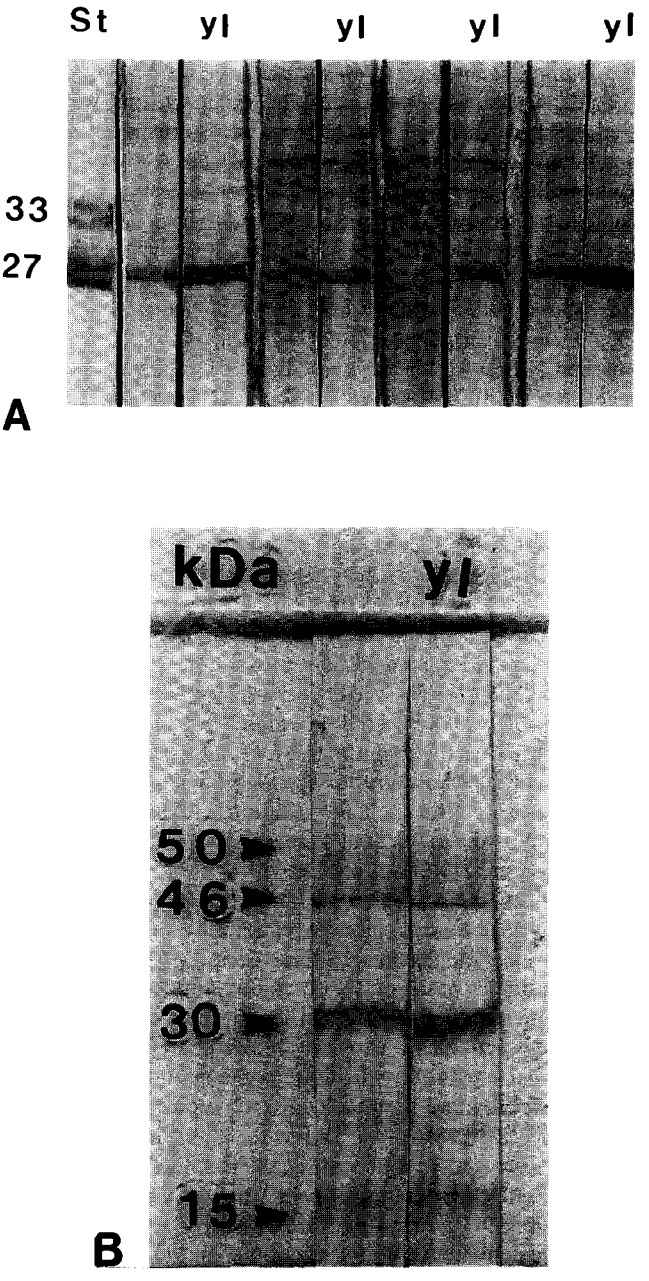

Fig. 1. Immunoblotting of cathepsins D and B in endosomal-lysosomal fraction of human monocyte U 937 cell line. Immunoblotting was carried out according to Towbin [25] and as described in details in section 2. (A) Immunostaining with polyclonal antibodies, raised against human cathepsin B, resulted in double band of cathepsin B heavy chain at M_r 25,000–27,000 in endosomal-lysosomal pellets (10,000 × g). First lane represents standard human cathepsin B (Medor, Hersching, Germany). Lanes represent four matched pairs grown without or with γ-IFN. (B) Immunostaining with polyclonal antibodies, raised against human cathepsin D, stained most intensively the heavy chain of mature Cat D at M_r 31,000. The intensity of the staining did not differ in five matched pairs of 10,000 pellets prepared from treated and control samples of human macrophages. A typical matched pair is shown.

or reduce production of an antigenic peptide depending on the polypeptide, the sequences of the antigenic epitope, and the selectivity of cathepsins B and L. Recently, a domain in the invariant chain of the class II molecules was reported to share an extensive sequence homology with the cystatins, the endogenous inhibitors of cysteine proteases [30]. Interestingly, the invariant chain Ii strongly inhibited cathepsins H and L, but not B [7,30]. Moreover, a fragment of the Ii (p 41 Ii) has been found associated with cathepsin L and thus may block its activity in lysosomes in vivo [31]. It is conceivable that cathepsin L specifically binds this fragment of Ii after its cleavage and release from the class II complexes. This hypothesis may also account for the observations by McCoy et al. [32], who found that an excess of cathepsin L precursor, added to cell culture media and internalized together with the protein antigen, inhibited antigen presentation. Presumably, this inhibition was due to excessive proteolysis not counterbalanced by the endogenous Ii fragments (and/or cystatins), and resulting in complete cleavage of antigen epitopes.

γ-IFN also stimulates the presentation of cytosolic antigens on the MHC class I pathway, in part due to a modification of proteasome activity, which results in the generation of more peptides that end with hydrophobic (and basic) residues. Such peptides are more readily transported into the ER [33,34] and selectively bind to surface MHC class I molecules [4,16,17,35]. The present observations suggests that γ-IFN may act in an analogous way in the MHC class II pathway to alter the enzymatic content and proteolytic cleavage patterns of lysosomes. Interestingly, Matsunaga et al. [29] have noted structural similarities between the active endoproteolytic site on cathepsin B and the peptide-binding region of MHC class II molecules. Thus, this protease may be particularly important in determining the carboxyl-terminus of the presented peptide. By inducing cathepsin B, γ-IFN may favor production of those types of peptides that bind preferentially to MHC class II. Very recently, Nadler et al. [36] have reported that the pattern of peptides generated by monocytes from endocytosed albumin does differ significantly after treatment with γ-IFN. It remains to be established whether this modulation of the way proteins are processed is of importance for immunological responses and whether it is due to the induction of cathepsins B and L.

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