CMLS, Cell. Mol. Life Sci. 59 (2002) 1503–1512
1420-682X/02/091503-10

CMLS Cellular and Molecular Life Sciences

Review

Immunomodulatory properties of cystatins

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Received 11 April 2002; accepted 18 April 2002

Abstract. Cystatins are natural tight-binding reversible inhibitors of cysteine proteases. Because these cysteine proteases exist in all living organisms and because they are involved in various biological and pathological processes, the control of these protease functions by cystatins is of cardinal importance. Cystatins are found in mammals but cystatin-like molecules are also present in mammals and parasites. In the immune system, cystatins modulate cathepsin activities and antigen presentation. They also induce tumor necrosis factor α and interleukin 10 synthesis, and they stimulate nitric oxide production by interferon γ-activated murine macrophages. In turn, nitric oxide has inhibitory activity on cysteine proteases, especially those from parasitic protozoa. Cystatins isolated from parasitic nematodes also have immunomodulatory activities that are distinguishable from those induced by lipopolysacharide-like molecules from endosymbiotic bacteria. On the whole, cystatins and cystatin-like molecules belong to a new category of immunomodulatory molecules. Doubtless increasing data will improve our knowledge of this property, leading to practical applications in immunotherapy.

Key words. Cystatins; cytokines; NO; antigen presentation; parasitic protozoa; nematodes.

Introduction

The cystatin superfamily

Cystatins are natural tight-binding, reversible inhibitors of cysteine proteases. Because these cysteine proteases exist in all living organisms and because they are involved in various biological and pathological processes, including protein catabolism, antigen processing, inflammation, dystrophy and metastasis, control of their enzymatic function by cystatins is of cardinal importance [1–5].

The cystatin superfamily contains three families. The stefins (family 1, or cystatins A and B, or class I or type 1; approximative MW 11 kDa) are cytosolic and have a single domain, with no disulfide bonds nor carbohydrates. Human stefin B (or cystatin B) and bovine stefins are representative of this family [1, 6–7]. Due to the presence of one region having homology with the other two families, the stefin family probably represents the archetype of the cystatin molecules, and a common evolutionary origin for stefins, cystatins and kininogens has been proposed [8].

The cystatins (family 2, or cystatins C, E and S, or class II or type 2; approximative MW 13 kDa) are secreted and found in most biological fluids. However, experimental evidence shows that cystatin C can also be intracellular [9]. Cystatins have a single domain with two disulfide bonds [10] and no carbohydrates. To date, chicken cystatin (CC) (or chicken egg-white cystatin) is one of the best-known members of this family [11].

The kininogens (family 3, or class III or type 3) are found in blood plasma [12–13]. They are made of several cys-

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tatin-like domains (D) and contain a bradykinin moiety. Rat T kininogen is a member of this family. Kininogens of low molecular weight have a heavy chain with four domains (D1, D2, D3 and D4, which contains the bradykinin moiety) and a light chain with the D5 domain and carboxy terminal. Kininogens of high molecular weight have the same structure as kininogens of low molecular weight except that the light chain has two domains (D5 and D6).

The reactive site binds and blocks the protease active site by a noncovalent link. A highly conserved sequence between residues 53 and 57 [the QVVAG (Glu-Val-Val-Ala-Gly) region] is located in a β-hairpin loop, between the amino-terminal segment G-A (Gly-Ala) and the second hairpin loop PW (Pro Trp). In some cystatins, only the QxVxG or the QxxxG motifs are conserved [4, 11, 14].

New cystatins and molecules with similar functions

Many cystatins have been recently described and may be included in a first group of "*sensu stricto* cystatins" on the basis of their inhibitory effect on cysteine proteases, for example new cystatins with yet unknown functions such as leukocystatin (or cystatin F) and cystatin E (or cystatin M) [15–18]. Similarly, cystatins from the free living nematode *Caenorhabditis elegans* and from the gut of a parasitic nematode, *Haemonchus contortus,* have been described. The *H. contortus* cystatin is probably involved in the control of a cathepsin L-like cysteine protease [19–20]. Other molecules have been identified as having cystatin-like domains and cystatin-like activity. For example, cathepsin F, the invariant chain (Ii), quail cystatin, equistatin from sea anemone and a complementary DNA (cDNA) from eggs of *Schistosoma mansoni* (a parasitic trematode) featuring cystatin sequences from family 1 have all been reported [21–28].

Kininogens contain three cystatin-like domains (see above). The D1 domain of the kininogens is a member of a second group of cystatin-like molecules having structural homology with *sensu stricto* cystatins, but is devoid of any inhibitory function on cysteine proteases.

A third group involves other cystatin-like molecules with an inhibitory function of cysteine proteases but acting through a completely different mechanism. For instance, chagasin is a cysteine protease inhibitor without any homology with cystatins. It has been found in *Trypanosoma cruzi*, a parasitic protozoa affecting humans in Latin America. Cruzipain (or cruzain or GP57/51) is a papain-like cysteine protease localized at the parasite membrane and involved in host cell invasion. It has been shown recently that chagasin inhibits cruzipain [29–30]. Surprisingly, BbCl is a cruzipain inhibitor isolated from *Bauhinia bouhinoides* seeds without any related structure with cystatins [31].

Clearly, cystatins and cystatin-like molecules constitute a large and growing field of interest for biochemists, immunologists and parasitologists. The diversity of these inhibitors is related to the diversity of cysteine proteases. Many reviews deal with various aspects of cystatins [1–6, 32–34]. This review focuses on the immunomodulatory properties of cystatins.

Cathepsins, cystatins and antigen presentation

Antigen-presenting cells

Present in most tissues and organs, dendritic cells (DCs) act as sentinels of the immune system and are potent antigen-presenting cells (APCs). They capture and process antigens to prime rare naive T lymphocytes. For this, acidic proteases (cathepsins; [35]) degrade the Ii associated with the α and β chains of major histocompatibility (MHC) class II heterodimers within endosomal and lysosomal compartments. Antigenic peptides are then linked to MHC class II molecules and transferred to the membrane. At the same time, DCs migrate towards T-lymphocyte-rich areas of lymph nodes and spleen. They undergo a process of maturation: (i) they lose their capacity to internalize antigens, (ii) they increase the expression of MHC class II and costimulatory molecules (e.g. CD80, CD86 and CD40) and (iii) they present peptide and MHC class II complexes at their membrane [36, 37]. Once in the lymphoid organs, they interact with antigen-specific T lymphocytes and initiate the adaptive immune response. Macrophages and B lymphocytes are also well known APCs but they only activate T lymphocytes previously primed by DCs [38–41].

Ii plays an important role in intracellular trafficking and peptide loading of MHC class II molecules. However, to reach efficient antigen presentation, Ii has to be progressively degraded within endosomes, in smaller fragments from Ii to Lip 23, Lip 10 and finally in a 3-kDa peptide called CLIP (class II-associated invariant chain peptide). Ii degradation is performed by various cathepsins depending on the APC types [reviewed in 35, 42].

Cystatin C

Cystatin C (γ-trace [43]) is found in humans, rats and mice. Secreted by mononuclear phagocytes, it is involved in the modulation of chemotaxis, phagocytosis and respiratory burst [13, 44–47]. In addition, cystatin C is a potent inhibitor of cathepsins S and L. High amounts of cystatin C are detected in class II-positive lysosomes of both immature DCs and Langerhans cells (immature DCs in the epidermis). In contrast, upon lipopolysaccharide (LPS)-induced maturation, (i) cathepsin S level remains unchanged in DCs but (ii) cystatin C concentration is sharply reduced, (iii) cystatin C is found mainly in perin-

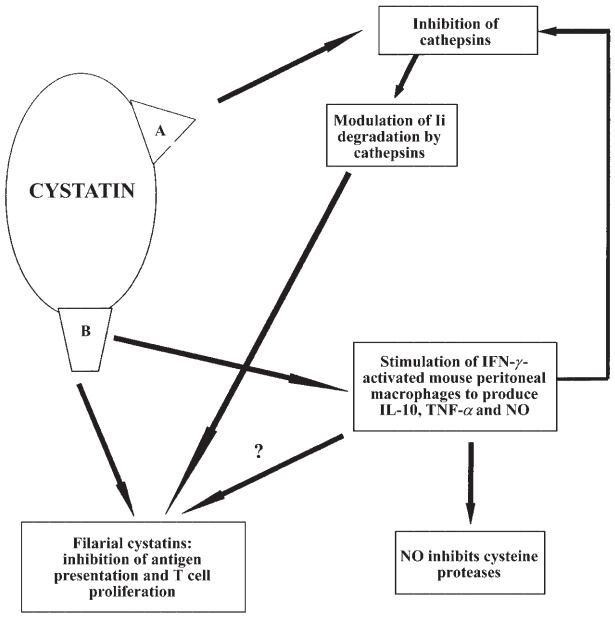

Figure 1. Schematic representation of immunomodulatory properties of cystatines. (*A*) Inhibitor site of papain, papain-like and cysteine proteases. (*B*) Putative site for induction of cytokines and NO by IFN-γ-activated macrophages. ?, existence of a specific receptor.

uclear cytoplasm and (iv) most of class II molecules are seen at the surface of the cells and no more in the lysosomes. These data indicate that the maturation process of DCs leads to a reduced level of cystatin C that favors the proteasic activity of cathepsin L. This is confirmed by experiments showing that in a murine A20 B cell line transfected with cDNA coding for mouse cystatin C there is an accumulation of Ii-p10 and a redistribution of class II into lysosomes. Thus, cystatin C is an intracellular cystatin since it is likely that it leaves the Golgi apparatus and directly reaches the endocytic pathway. The process of DCs maturation modifies the intracellular distribution of cystatin C, downregulates cystatin C levels and, consequently, the cystatin C to cathepsin S ratio and the biological activity of cathepsin S. On the whole, the stoechiometric relation between cystatin C and cathepsin S strongly suggests an intracellular control of Ii degradation and antigen-presentation pathway (fig. 1) [9].

Leukocystatin or cystatin F

Leukocystatin (also called cystatin F) is a cystatin recently discovered and mainly found in human natural killer (NK) cells, in DCs derived from activated $CD1a^+$ and $CD14^+$ stem cells, and in murine T cells. As shown by immunoassays, blood contains low levels of this cystatin (0.9 ng/ml). The recombinant protein obtained in *Escherichia coli*, or in a baculovirus expression system, inhibits papain (the archetype of cysteine proteases) and also cathepsin L, but with lower affinity than other cystatins. To date, the target of this cystatin is not known. It is suggested that this target might be a yet unidentified lysosomal protease, or even a protease from a different family. Given its inhibitory activity on papain and cathepsin L and its presence in human DCs, one could speculate that cystatin F could play a role in antigen presentation [16–17].

Cystatin-mediated modulation of cytokines and nitric oxide

Th1/Th2 immune responses

Type 1 immune response is characterized by immune cellular response and type 1 cytokines produced by $CD4^+$ Th1 lymphocytes. Interaction between CD40 (expressed on DCs) and CD40L (or CD154; expressed on activated T lymphocytes) induces interleukin (IL)-12 production, which in turn stimulates interferon γ (IFN-γ) production together with other cytokines such as IL-2. This is a protective response, particularly against intracellular pathogens through the production of nitric oxide (NO, see below) [48]. On the other hand, production of type 2 cytokines (IL-4, IL-5, IL-9, IL-10, IL-13) is a cardinal feature of the immune response mediated by $CD4^+$ Th2 lymphocytes together with antibody production [49].

Among others, three cytokines are largely involved in anti-infectious immune response through their modulation of NO production. Among other important properties, IFN-γ is so far the only cytokine able to induce on its own the synthesis of NO synthase-2 (NOS-2) and the subsequent synthesis of NO by mouse peritoneal macrophages (MPMs). Tumor necrosis factor α (TNF-α) is a proinflammatory pleitropic cytokine produced by several cell types (T and B cells, mastocytes and NK cells), including macrophages, in response to a large array of stimuli (infectious microorganisms, LPSs). IFN-γ-induced TNF-α is a prerequisite for in vitro production of NO released by MPMs. IL-10 is an activating as well as deactivating cytokine produced by several cell types (T cells, certain B cells and monocytes), including macrophages. It can enhance but also inhibits NO synthesis by IFN-γ-activated MPMs depending on whether it is added before or after IFN-γ (or LPS)-mediated activation of macrophages.

NO

NO is important in various physiological processes (vasodilatation, smooth muscle regulation, neurotransmission) and particularly in the immune response. It is involved in several inflammatory diseases and is cytotoxic or cytostatic in a wide range of infections [50–52]. NO is synthesized from L-arginine by NOS. Three isoforms of NOS have been identified; they differ in their tissue

distributions and regulation. Neuronal NOS (nNOS or NOS-1) and endothelial NOS (eNOS or NOS-3) types are constitutively present in neurons and endothelial cells, respectively, and are calcium dependent [52]. The third NOS isoform is an inducible enzyme (iNOS or NOS-2) synthesized in response to specific signals by cells such as hepatocytes, fibroblasts and macrophages and is calcium independent [53]. Its synthesis is induced mainly by IFN-γ and leads to the rapid production of large amounts of NO. In addition, other cytokines such as TNF-α cooperate synergistically with IFN-γ to significantly increase production of NO [54], whereas IL-10 modulates IFN-γ-induced production of NO [55–59].

Immunomodulatory properties of cystatins

In preliminary experiments performed to determine whether natural inhibitors of cysteine proteases are able to block cruzipain and subsequent host cell invasion, we observed that IFN-γ-activated MPMs produce large amounts of NO when exposed to representative members of the three cystatin families (human stefin B, CC and T kininogen) (fig. 1). This is not the case with other cysteine protease inhibitors such as E64 nor with inhibitors of aspartyl and serine proteases. Similarly, aprotinin (clinically used as a trypsin-like serine protease inhibitor) is also without effect. This is in line with reports showing that aprotinin is a competitive inhibitor of NOS-1 and NOS-2 [60, 61]. Upon complexation with saturating amounts of reduced-alkylated papain, cystatins still remained active in increasing NO production, suggesting that the inhibitory site is not involved in this mechanism and thus that another biologically active site is likely present on cystatins [62]. The fact that CC does not inhibit cruzipain is now better understandable since we know that cruzipain is only inhibited by chagasin, which is devoid of any cystatin domain [63].

Enhanced NO production by CC/IFN-γ-activated MPMs correlates with increased production of TNF-α, IL-10 and NOS-2. The addition of recombinant TNF-α alone or in combination with recombinant IL-10 mimics the effect of CC, while the addition of neutralizing anti-TNF-α antibodies sharply reduce NO production by CC/IFN-γ-activated MPM [64]. A putative site present on CC probably interacts with yet unknown receptors expressed on macrophages and triggers production of cytokines which, in turn, augment NO synthesis by IFN-γ-activated macrophages.

These experiments, performed in vitro, are now confirmed by recent in vitro and in vivo experiments [65]. *Leishmania donovani* is a parasitic protozoa transmitted by sand flies and infecting macrophages. It is the etiological agent of visceral leishmaniasis (kala-azar; [66]) in humans. The promastigotes (the vector form) invade macrophages. They transform into amastigotes (the intracellular form of multiplication), which survive and multiply inside parasitophorous vacuoles of host cells. Treatment of *L. donovani*-infected MPMs with a combination of CC/IFN-γ induces increased production of NO. These data not only confirm previous results [62, 64] but also indicate that CC/IFN-γ stimulation can overcome the inhibition of NO synthesis driven by *L. donovani* parasites in IFN-γ-activated MPMs. They also extend previous results to an in vivo model of infection. Treatment of *L. donovani*-infected mice with a combination of CC and IFN-γ induces reduced splenomegaly together with a lowered spleen parasite burden. This correlates with an increased production of NO. Moreover, the type 2 immune response is switched towards a type 1 response as shown by the increased production of IL-12 versus decreased production of IL-4. On the whole, CC/IFN-γ treatment clinically cures highly susceptible BALB/c mice through enhanced NO production and a type 2/type 1 switched immune response [65].

NO is an inhibitor of parasitic cysteine proteases

CC/IFN-γ-mediated production of NO has a protective effect on leishmanial infection both in vitro and in vivo [65]. In addition to a direct antiparasite action of NO (see above), this protective effect could also be related to inhibition of leishmanial cysteine proteases. Indeed, it has been shown that NO donors inhibit *L. infantum* cysteine protease [67]. Cysteine proteases produced by *Leishmania* seem to play a major role in intracellular survival and could constitute parasite virulence factors [68, 69]. NO-mediated inhibition of cysteine proteases is reminiscent of reports showing that *Leishmania mexicana* mutants lacking cysteine protease genes induce an attenuated infection in mice and a reduced rate of macrophage infection probably because these enzymes are necessary to their promastigote/amastigote transformation and to their survival into the macrophage parasitophorous vacuole [70, 71]. Furthermore, these parasites deficient in cysteine proteases induce a shift from a Th2- towards a protective Th1-immune response when compared with control mice infected with wild-type *L. mexicana* [72]. This is in line with a Th2/Th1 shift observed with CC/IFN-γ-treated and *L. donovani*-infected mice [65].

Interestingly, other parasitic protozoa also possess cysteine proteases (i.e. *T. cruzi*: cruzipain; *Plasmodium falciparum*: falcipain), and NO donors inhibit these proteases, that is by S-nitrosylation of the Cys 25 catalytic residue of cruzipain [73–75]. It would be interesting to study CC (and other cystatins) in its capacity to stimulate NO production and to look at a possible protective effect in experimental models of *T. cruzi* and *Plasmodium* infections.

T. cruzi (South America) and *Trypanosoma brucei* (Af-rica) are quite different not only by their geographic

distribution but also in their life cycle. However, a common ancestor likely exists for south american and african trypanosomes, *T. cruzi* and *T. brucei* respectively [76, 77], and the existence of chagasin suggests that related cysteine protease inhibitor(s) likely exist(s) also in african trypanosomes such as *T. brucei* [78]. In addition, congopain has also been described for *Trypanosoma congolense* [79]. On the whole, these data unveil an interesting new field of research for comparative studies.

Reversible inactivation of calpain isoforms by NO has also been described [80] and must be added to the other enzymes inhibited by NO. Indeed, NO-mediated toxicity for intracellular parasites depends on its rapid diffusion into infected cells, and it acts by inhibiting some of their key enzymes. Oxidoreductases (mitochondrial electron transport system), aconitase (a citric acid cycle enzyme) and enzymes essential for DNA synthesis contain catalytically active Fe–S groups which are inactivated by NO as a result of formation of nitrosyl-iron-sulfur complexes [50].

Immunomodulatory properties of filarial cystatins

Filariae are arthropod-borne worms (Phylum Nematoda) which are parasites of the lymphatic, subcutaneous and cutaneous tissues of humans. The adult females produce larvae called microfilariae which are found in the peripheral blood or in the skin and cutaneous tissues. Interestingly, cystatins have recently been isolated and characterized from several filariae. They interfere with antigen presentation, cellular proliferation, cytokine synthesis and NO production (fig. 1).

Bm-CPI-2 of *Brugia malayi*

B. malayi is a mosquito-borne filaria which inhabits the lymphatic vessels of humans and is found in Asia and India. Two genes coding for new members of the cystatin family, Bm-CPI-1 and Bm-CPI-2 (CPI-2), have recently been identified. In particular, CPI-2 of *B. malayi* is expressed and secreted in parasite stages that mature in the mammalian host and therefore has the potential to interfere with host immune responses. It blocks the hydrolysis of synthetic fluorogenic substrates preferentially cleaved by cathepsin S, cathepsins B and L, and asparaginyl endopeptidase (AEP**)**. It also blocks the in vitro processing of the tetanus toxin C fragment by purified B cell lysosome fractions or purified pig kidney AEP. CPI-2 also inhibits the presentation of selected T cell epitopes from tetanus toxin by Epstein-Barr virus (EBV)-transformed B cell lines. These data suggest involvement of asparagine-specific cysteine endopeptidases rather than lysosomal cathepsins in antigen processing. Filarial cystatins are likely involved in the downregulation of antigen presentation and thus participate in escape mechanisms evolved by parasitic worms to survive in their host [81]; for review, see [34].

Cystatin of *Acanthocheilonema viteae*

A. viteae is a tick-transmitted rodent filarial parasite producing a cystatin C-like molecule (17-kDa antigen, Av17) that has been isolated and characterized. Av17 is released in vitro by all parasite stages dwelling in the mammalian host [82]. Culture supernatants of *A. viteae* female worms inhibit proliferation of murine splenic cells activated with concanavalin A, which to a high proportion is due to the cystatin molecule. Its recombinant form, expressed in *E. coli*, downregulates T cell proliferation induced by concanavalin A and thymocytes treated with anti-CD3 antibodies. Similar results were obtained with a T cell clone and its corresponding T cell hybridoma, both specific for a T cell epitope of sperm whale myoglobin, indicating that Av17 downregulates nonspecific and antigen-specific T cell proliferation.

Av17 has potent activity in cytokine production. Indeed, the recombinant cystatin enhances the production of IL-10 by naive and concanavalin-treated splenic cells, while IL-4 production is decreased and no effect is observed on IL-2 and IFN-γ production. Treatment of MPMs from BALB/c or C3H/HeJ mice with a combination of *A. viteae* cystatin/IFN-γ triggers the production of NO [83]. C3H/HeJ mice are low responders to LPS because of mutations in *Tlr4* gene [84]. Polymyxin (an antibiotic that neutralizes LPS activity) also fails to inhibit the upregulated NO production in the presence of Av17. These data indicate that the cystatin-mediated NO production is not due to LPS contamination.

Onchocystatin of *Onchocerca volvulus*

O. volvulus is a subcutaneous parasitic filaria which is the etiological agent of river blindness, transmitted by bites of the black fly (*Simulium damnosum*) and affecting humans in Africa and Latin America. Filarial cystatin of *O. volvulus* (onchocystatin) is closely related to cystatins (family 2) and fragment of kininogens (family 3). Clearly identified during the molting of L3 to L4, it is suggested that onchocystatin may modulate cysteine protease during this process [85]. However, onchocystatin may also play a role in the reduced cellular immune response and the persistence of the parasite in its host for many years. A recent report shows that recombinant onchocystatin strongly inhibits cathepsins L and S, whereas the activity of human cathepsin B is less efficiently inhibited. Onchocystatin interferes with the proliferation of human peripheral blood mononuclear cells (PBMCs) upon stimulation by phytohemagglutinin, or by anti-CD3 antibodies

or by PPD. The cellular hyporeactivity induced by onchocystatin coincides with an upregulation of IL-10 production and a reduced expression of HLA-DR and CD86 by human PBMCs and monocytes. The addition of neutralizing anti-IL-10 antibodies restores the expression of HLA-DR and CD86. Depletion of monocytes from the PBMC completely reverses the inhibitory effects on cellular proliferation induced by onchocystatin, suggesting that monocytes could be the target cells of immunomodulation [86]. As is the case for Av17 or *A. viteae* cystatin, (i) onchocystatin inhibits nonspecific and antigen-specific proliferation of T lymphocytes and (ii) onchocystatin/IFN-γ-treated MPMs from BALB/c and C3H/HeJ mice also have enhanced production of NO, even in the presence of polymixin B, confirming that NO production is not triggered by LPS contaminants [83, 87]. In addition, onchocystatin has a possible role in the evasion of immune responses of its simuliid vector as it blocks immunocompetent proteases in the vector, which facilitates establishment of the parasites [88].

Cystatin of *Litomosoides sigmodontis*

L. sigmodontis is a filarial nematode which infects cotton rat. It is a suitable experimental murine model of filariasis, as a complete life cycle is obtained in immunocompetent mice. BALB/c mice are more susceptible to infection than C57BL/6 mice but with a disparate outcome, as in the case of the natural infection [86]. In addition, similarly to human filarial infections, there is immunosuppression, a long persistence of female worms releasing microfilariae and the type 1 immune response is switched towards type 2.

Cystatin from *L. sigmodontis* has been detected in several developmental stages of the worm and also in the supernatants of in vitro cultured worms. It has overall similarities with *O. volvulus*, *A. viteae* and *B. malayi* cystatins of 71, 80 and 83%, and identities of 57, 64 and 66%, respectively. When recombinant *L. sigmodontis* cystatin is introduced into the peritoneal cavity of mice via a microosmotic pump, TNF-α is upregulated, and antigen-specific proliferative response of spleen cells to microfilariae is diminished. NO production measured in vivo is reduced [89]. Previous experiments from the same group show that microfilariae of *L. sigmodontis* (but not adult worms) are susceptible to NO from a chemical NO donor (*S*-nitroso-*N*-penicillinamin), and NO production is triggered by microfilarial-activated macrophages from C57/BL/6 mice but less in macrophages from BALB/c mice. In contrast, neither pharmaceutical inhibition of NO synthesis nor genetic deletion of the NOS-2 gene abrogate resistance. So there is a protective effect of NO in vitro and ex vivo but not in vivo [86, 90].

Cytokines and NO production induced by endosymbiotic bacteria in filariae

IL-10 and NO production by cystatin of *A. viteae* and TNF-α, IL-10 and NO production by onchocystatin of *O. volvulus* are not due to LPS since this also occurs with macrophages from C3H/HeJ mice in the presence of polymyxine B, and recombinant control proteins – which contain higher levels of LPS than the recombinant filarial proteins – do not induce similar effects [83, 87]. This is an important point. Indeed, TNF-α, IL-1-β and NO production by macrophages is also triggered by LPS-like molecules from endosymbiotic *Wolbachia* bacteria living in several filariae, for example *B. malayi*. In some reports of experiments with *A. viteae*, which lacks endosymbionts, no production of TNF-α, IL-1β and NO is determined [91, 92]. In contrast, another study shows that microfilariae-primed cells restimulated with an extract of *A. viteae* produce elevated levels of NO in response to *A. viteae* antigen [93].

Enhanced production of TNF-α and IL-10 by human monocytes is also observed in the presence of *O. volvulus* antigen extract due to LPS-like molecules released from endosymbiotic *Wolbachia* bacteria [94, 95]. The implication of a LPS-like molecule derived from *Wolbachia* bacteria is confirmed by the fact that cytokine and NO production does not occur with macrophages from C3H/HeJ mice, in the presence of *B. malayi* or *O. volvulus*.

Nippocystatin of *Nippostrongylus brasiliensis*

N. brasiliensis is a small (3–6 mm) reddish nematoda, a common parasite of the small intestine of wild rats and quite distinct from filarial nematodes. Nippocystatin has been isolated from the excretory and secretory products of the worm and is present in L3 and adult stages. It presents a high percentage of homology with other cystatins of nematoda. Recombinant nippocystatin also inhibits cathepsin L and cathepsin B.

Proliferation of splenic cells from ovalbumin (OVA)-immunized and nippocystatin-treated mice is inhibited in the presence of OVA, but not concanavalin A. Furthermore, the processing of OVA by lysosomes is inhibited in vitro in the presence of nippocystatin. Mice immunized with recombinant nippocystatin become more resistant to infection by infective larvae. On the whole these data indicate that nippocystatin is involved in the escape mechanism evolved by this worm in front of the immune system [96, 97].

Conclusions

Cystatins are cysteine protease inhibitors that exert several immunomodulatory functions (fig. 1). They control degradation of Ii by cathepsins and modulate antigen presentation. A putative site interacting probably with yet

unknown receptors stimulates production of cytokines which, in turn, augment NO synthesis by IFN-γ-activated macrophages. NO inhibits many enzymes, in particular cruzipain, and participates in the control of *T. cruzi* infection. A similar process is probably at work in leishmanial infection, where CC induces the Th2 towards a Th1 switch. Several filarial cystatins inhibit antigen presentation as well as antigen-specific and nonspecific lymphoproliferation, contributing to parasite survival in the host. The recent discovery of the immunomodulatory effects of cystatins warrants further investigation to better understand which mechanisms are involved in these process.

To approach the problem of the cystatin domain involved in the increased synthesis of NO synthase in IFN-γ-activated mouse macrophages, we have made a sequence analysis of all cystatins, which were shown to share that property (table 1). Only one consensus sequence was found which could correspond to such a domain because it is not involved in protease inhibition and because it is solvent and thus ligand accessible in the cystatins whose three-dimensional structures were resolved. As can be seen in figure 2, the domain is characterized by an acidic

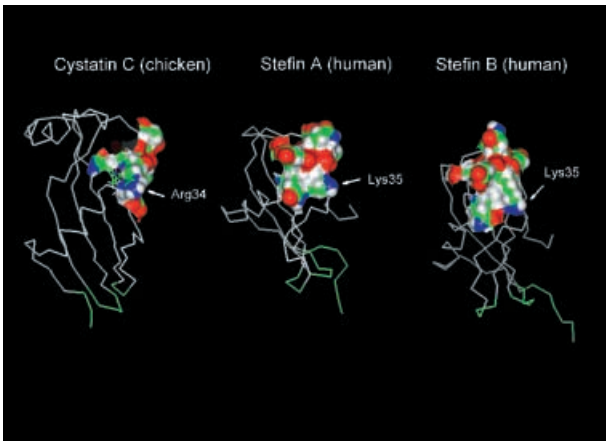

Figure 2. The trace structure of chicken cystatin (PDB 1CEW), human stefin A (PDB 1GD3) and human stefin B (PDB 1STF) are shown with the Connolly surface of the domain corresponding to amino acids 31–37 (atoms accessible to a solvent molecule of 1.4 Å as calculated by the Insight II software of Accelrys). Atoms are colored as usual (H, white; C, green; N, blue and O, red). The domains involved in cysteine protease inhibition are colored in green.

Table 1. Clustal analysis of the sequences of cystatins increasing NO synthase activity.

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                                                                    ↓
chicken       -MAGARGCVVLLAAALMLVGAVLGSEDRS-----------------------RLLGAPV
human         -MAGPLRAPLLLLAILAVALAVSPAAGSSPGKPP-----------------RLVGGPM
acantho       MMLSIKEDGLLVVLLLSFGVTTVLVRCEEP---ANMESEVQAP---------NLLGGWQ
litomosoides  -MMSVKGVLLVPFLSLFG---VVLVNCLGH---GNMESEAR-----------VVGGWQ
onchocerca    -MLTIKDGTLLIHLLLFSVVALVQLQGAKS---ARAKNPSKMESKTGENQDRPVLLGGWE
stefA         ------------------------------------------------------MIPGGLS
stefB         ------------------------------------------------------MMCGAPS
                                                                     : *.

chicken       PVDENDEGLQRALQFAMAEYNRASNDKYSSRVVRVISAKRQLVSGIKYILQVEIGRTTCP
human         DASVEEEGVRRALDFAVGEYNKASNDMYHSRALQVVRARKQIVAGVNYFLDVELGRTTCT
acantho       ERNPEEKEIQDLLPKVLIKLNQLSNVEYHLMPIKLLKVSSQVVAGLRYKMEIQVAQSECK
litomosoides  ERSPDDNEIQEMLPSILTKVNQQSNDAYHLMPIKVLKVSSQVVAGMKYKMEIQVARSDCK
onchocerca    DRDPKDEEILELLPSILMKVNEQSNDEYHLMPIKLLKVSSQVVAGVKYKMDVQVARSQCK
stefA         EAKPATPEIQEIVDKVKPQLEEKTNETYG--KLEAVQYKTQVVAGTNYYIKVRAG-----
stefB         ATQPATAETQHIADQVRSQLEEKENKKFP--VFKAVSFKSQVVAGTNYFIKVHVG-----
                .              : :.  *  :    .. :    *:*:* .* :.:. .

chicken       KSSGDLQSCEFHDEPEMAKYTTCTFVVYSIPWLNQIKLLESKCQ------
human         KTQPNLDNCPFHDQPHLKRKAFCSFQIYAVPWQGTMTLSKSTCQDA----
acantho       KSSGEEVNLKTCKRLEGHPDQIITLEAWEKSWENFLQVKILEKKEVLSSV
litomosoides  KSSNEKIDLKTCKKLEGHPDQIITLEVWEKAWEDFLQVNILETKLLS---
onchocerca    KSSNEKVDLTKCKKLEGHPEKVMTLEVWEKPWENFMRVEILGTKEV----
stefA         ---DNKYMHLKVFKSLPGQNEDLVLTGYQVDKNKDDELTGF---------
stefB         ---DEDFVHLRVFQSLPHENKPLTLSNYQTNKAKHDELTYF---------
                 :       .         :  :        :
```

Arrow shows start of matured cystatins.
Amino acids in green: interaction sites with cysteine proteases.
Amino acids in red: putative interaction site needed to increase NO synthase synthesis.

environment in which a basic residue is included. The parasite cystatins all have a basic residue at the N-terminal position of this domain (table 1). Although sequence or structural similarity is not a proof of function, it can help to set up site-directed mutagenesis studies which could confirm or disprove the hypothesis.

Acknowledgements. We acknowledge the constant support of Prof. Dr Michel Goldman, Laboratoire d'Immunologie Experimentale, ULB, Brussels, Belgium. The technical assistance of Vincent Vercruysse and the secretarial help of Iolanda Mazza were much appreciated. This work was supported by a grant from Centre de Recherche Interuniversitaire en Vaccinologie (CRIV).

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