Ym1 Is a Neutrophil Granule Protein That Crystallizes in p47phox-deficient Mice*

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Crystals were discovered within the aged lung and at sites of chronic inflammation in a mouse model of chronic granulomatous disease. Following re-crystallization at neutral pH, the crystals were identified as the chitinase-like protein Ym1, expressed in organs of the lymphoreticular system, the lung, and distal stomach. Ym1 was shown to be a neutrophil granule protein and to have weak β-N-acetylglucosaminidase activity, indicating that it might contribute to the digestion of glycosaminoglycans. Crystal formation is likely to be a function of excess neutrophil turnover at sites of inflammation in the chronic granulomatous disease mouse. Failure to remove subcutaneous Ym1 crystals injected into knockout mice indicates that a failure of digestion may also contribute to crystallization.

Chronic granulomatous disease (CGD)† is a rare genetic disease that presents with recurrent life-threatening bacterial and fungal infections. The commonest autosomal recessive cause of CGD, affecting 25% of patients (1), is caused by defects in the p47phox subunit of the NADPH oxidase. We have created a mouse model of CGD by disrupting this subunit in order to increase the understanding of the biochemistry and cell biology of this disease. During histological review of our p47phox knock-out (p47phox−/−) mouse, we discovered crystals in the lung of aged mice, which was not described in the report of the original p47phox−/− mouse in 1995 (2).

Recently Ym1, a member of a family of mammalian proteins (Table I) that shares sequences with lower organism chitinases, has been described as crystallizing in the lungs of three immunodeficient mice as follows: the moth-eaten mouse, the CD40L-deficient mouse, and a transgenic mouse with lung-specific human tumor necrosis factor receptor expression (3). Increased expression of Ym2, a Ym1 splice variant (4), has been found in a murine model of asthma, believed to be macrophage-derived and dependent on CD4+ T cells (5). Gastric hyalinosis in CYP1A2-null B6, 129 mice has recently been identified as Ym2 (6), and in the same study, anti-Ym1 immunoreactivity demonstrated to pulmonary macrophage crystals. Unidentified pulmonary crystalline deposits have been described in other transgenic, immune-deficient mice (7) and in certain strains of immune-competent mice, particularly on a 129Sv or C57BL/6 background (8), especially during experimental chronic pulmonary infection (9). These were previously thought to be Chacot-Leyden-like crystals, but it now seems likely that they are composed of Ym1 protein.

Ym1 mRNA is expressed in the spleen and lung and a highly homologous gene, ym2, in the thymus, stomach, and kidney (4). The distribution of Ym1 protein within the cells of these and other tissues has not been determined, although it has been assumed to be a macrophage protein because it was first discovered in a peritoneal macrophage cDNA library (10). Recently, enhanced macrophage ym1 gene expression, mediated by a homologue of macrophage migration inhibitory factor, has been found associated with Th2 cytokine inducing helminth infection (11). The function in vivo is unknown, although recently the same researchers (12) have both deduced the protein structure, which contains a saccharide-binding site, and shown that Ym1 functions as a lectin, binding multivalent hexosamines and heparin (10). Ym1 may have weak chitinase activity, thus cleaving several moieties of β-1,4-linked N-acetyl glucosamine (GlcNAc) (3), and therefore may have a defensive role. Finally, the protein has been shown to be chemotactic for eosinophils (13), and high concentrations are associated with eosinophil recruitment in vivo (11).

In this study, we demonstrate that p47phox−/− murine pulmonary crystals are indeed Ym1 and that the protein is stored in neutrophil granules. In addition, we show that pure Ym1 can function as a β-N-acetylhexosaminidase and not a chitinase. We show that Ym1 crystals easily form in vitro and speculate that excessive Ym1 release during acute inflammation causes crystal formation in vivo.

EXPERIMENTAL PROCEDURES

Generation of CGD Mouse—Compounds were purchased from Sigma unless stated otherwise. The p47phox gene was isolated from a P1 mouse ES cell library (Strain 129Sv; Genome Systems, St Louis, MO) and partially characterized. A neomycin resistance gene was inserted into the third exon of the p47phox gene as an XhoI/SalI fragment after conversion of a BspMI site in exon 3 into a SalI site by linker ligation. A PvuI site 5′ of exon 3 was converted into XhoI by linker ligation to provide a unique restriction site for linearization of the targeting vector before transfection. The HSV-Tk gene was attached to the 3′ end of the region of homology to permit negative selection of clones with random integration of the targeting vector. Gene targeting in mouse embryonic stem cells was done as described previously (14). p47phox−/− mice were established on the 129 background by mating transmitting chimeras with 129Sv females. Mice were maintained in conventional housing.
facilities. All mice received water and standard mouse chow (SDS-3 expanded, Lillico, UK) ad libitum. Acute colitis with portal pyemia, cholangitis, and liver abscess formation was induced in a cohort of mice using oral administration of dextran sodium sulfate (DSS) (15).

**Purification and Identification of Lung Crystals**—Lungs from 12-month-old p47<sup>phox</sup><sup>−/−</sup> mice were gently Dounce-homogenized and incubated at 37 °C for 90 min with collagenase (1 mg/ml in water; 5 mg/lung), DNase (30 μg/ml in water), and protease inhibitors (1 μg/ml leupeptin; 1 μg/ml pepstatin A; 1 μg/ml tosyl-lysine chloromethyl ketone; 0.5 mM phenylmethylsulfonyl fluoride). The digest was layered on top of Lymphoprep (Nycomed, Oslo, Norway) and centrifuged (100,000 × g, 4 °C, 30 min) 3 times. Enriched crystals were dissolved in 10 mM CHES, pH 9.5 (250 μl/μg), for 1 h and then 100 mM MES, pH 6, added to the supernatant for 18 h (1:3 v/v; 4 °C; resultant, pH 6.06) to re-crystallize the protein. This was washed 3 times in water and further purified by re-crystallization. Protein size and purity was assessed by staining with Coomassie Brilliant Blue.

**Ym1 Tissue Distribution**—Formalin-fixed tissues for light microscopy were paraffin-embedded and stained with hematoxylin/eosin or Wright/Giemsa. Lung samples for electron microscopy were retrieved from formalin-fixed paraffin-embedded p47<sup>phox</sup><sup>−/−</sup>—/− tissue using a combined toluene/osmium retrieval method. Silver to gold ultrathin sections were cut on a Reichart-Jung Ultracut II (Leica Microsystems, UK) and double-stained with Reynold’s lead citrate and uranyl acetate. Sections were examined and photographed on a Jeol 1200 EX transmission electron microscope (JEOL Ltd.). For immunohistochemistry, rabbit polyclonal sera reactive to Ym1 (20) was affinity-purified on a HiTrap affinity column (Amersham Biosciences) (21). 3-μm sections from paraffin-embedded tissues were dried overnight at 60 °C, taken to absolute alcohol, blocked for 10 min with 0.5% hydrogen peroxide in methanol, and rinsed thoroughly in water. They were digested for 10 min in a 0.1% solution of chymotrypsin containing 0.5% hydrogen peroxide in methanol, and rinsed thoroughly in water. They were digested for 10 min in a 0.1% solution of chymotrypsin containing 0.1% calcium chloride (pH 7.8, 37 °C) and rinsed in water and then in 0.05% Tween 20 in Tris-buffered saline (pH 7.4). Endogenous biotin activity was blocked (Avidin/Biotin Blocking Kit, Vector Laboratories); the sections were incubated for 10 min in 1/10 normal swine serum (Dako Ltd., Ely, UK) and then incubated for 60 min with the primary antibody (1/5000 anti-Ym1 or rabbit polyclonal anti-thyroglobulin as a nonspecific control). They were developed using a biotinylated 1/200 swine anti-rabbit secondary antibody (Dako) and a streptavidin-biotin-peroxidase complex (Dako) and visualized with DAB (Kem-on-Tec, Copenhagen, Denmark). For immunoblotting, mouse organs from two p47<sup>phox</sup><sup>−/−</sup>—/− and two wild-type mice aged 3 weeks, 6 weeks, 5 months, and 10 months

![Figure 1](image-url)
Ym1 protein at different stages of purification. Lane 1, homogenate; lane 2, Immunoblots of 2 evaluation. anti-rabbit immunoglobulin (Amersham Biosciences), detected using were probed with anti-Ym1 (1/1000 dilution of nanogold goat anti rabbit Fab’ conjugate (Nanoprobes, particle size 1.5 nm) in PBS containing 0.5% bovine serum albumin, and 0.01% sodium azide. Neutrophils were similarly obtained 18 h after thioglycolate administration. The cells were filtered through 60 μm of gauze (Millipore Corp., Bedford, MA) and stained with the primary antibody GR1-FITC (PharMingen) specific for neutrophils (23) and F4/80-FITC (Serotec) specific for macrophages (24). Neutrophils and macrophages were sorted at 4 °C using an Epics Elite cell sorter, orifice size 75 μm (Coulter, UK; Expo 300 software, Applied Cyto Systems, UK). T cells were obtained by gently grinding the thymus between the roughened glass of 2 microscope slides, and the cells were washed free with buffer. 20 μg of cellular protein (equivalent to 25,000 neutrophils) was analyzed by immunoblotting for Ym1 and neutrophil elastase (25) and for Ym1 by immunocytochemistry of cytospin smears (Cytospin 2, Shandon, UK).

Subcellular Fractionation of Wild-type Murine Neutrophils—Thioglycollate-induced peritoneal neutrophils were obtained from eight wild-type mice. 2 μl of di-isopropyl fluorophosphate (1 mM in ethanol) was added to the cell pellet, followed by 2.5 ml of break buffer (10 mM PIPES, pH 7.1; 100 mM KCl; 3 mM NaCl; 3.5 mM MgCl2; protease inhibitors). The cells were sonicated (Soniprep 150, Sanyo, Japan) on ice for 3 cycles of 5 s, centrifuged (10,000 × g, 4 °C, 10 min), the post-nuclear supernatant layered onto a 10 ml of continuous sucrose gradient (15–55% w/w in break buffer containing 1 mM EDTA, pH 7.4; 5 units/ml heparin; and protease inhibitors), and centrifuged in a Beckman TLS55 head (150,000 × g, 4 °C, 1 h). The gradient was collected in 40 fractions by aspiration from the superior surface. Sucrose concentration was determined by refractometry. Fractions were analyzed for Ym1 and lactoferrin by immunoblotting and for myeloperoxidase (MPO) activity (26).

Immunogold Electron Microscopy of Peritoneal Neutrophils—Wild-type thioglycollate-induced peritoneal neutrophils, fixed in 2% paraformaldehyde containing 0.5% glutaraldehyde in PBS, were dehydrated through a graded series of alcohol, followed by two changes of propylene oxide, and embedded with araldite CY212 resin. Silver to gold ultrathin sections were cut with a diamond knife, collected onto 200-mesh carbon-coated nickel grids, incubated with 1% periodic acid for 10 min, washed with distilled water, incubated with 50 mM glycine for 5 min, washed in PBS, and incubated for 5 min in PBS containing 0.5% bovine serum albumin, 0.1% Triton X-100, 0.1% lysine, and 0.1% sodium azide. After overnight incubation in a 1:2000 dilution of anti-Ym1, the cells were washed with PBS and incubated for 1 h with a 1/100 dilution of nanogold goat anti rabbit Fab’ conjugate (Nanoprobes, particle size 1.5 nm) in PBS containing 2% normal goat serum. After washing for 30 min in PBS, the sections were post-fixed with 1% glutaraldehyde for 10 min and washed for a further 15 min. The detection of gold particles was enhanced with HQsilver (Nanoprobes). Equal parts of initiator and moderator were mixed before adding activator and mixing thoroughly. The mixture was put on ice under safe lights, and the sections were

peritoneal cavity of 3-month-old wild-type mice. Macrophages were retrieved 5 days later by peritoneal lavage with ice-cold phosphate-buffered saline (PBS) containing heparin (5 units/ml), 0.5% bovine serum albumin, and 0.01% sodium azide. Neutrophils were similarly obtained 18 h after thioglycolate administration. The cells were filtered through 60 μm of gauze (Millipore Corp., Bedford, MA) and stained with the primary antibody GR1-FITC (PharMingen) specific for neutrophils (23) and F4/80-FITC (Serotec) specific for macrophages (24). Neutrophils and macrophages were sorted at 4 °C using an Epics Elite cell sorter, orifice size 75 μm (Coulter, UK; Expo 300 software, Applied Cyto Systems, UK). T cells were obtained by gently grinding the thymus between the roughened glass of 2 microscope slides, and the cells were washed free with buffer. 20 μg of cellular protein (equivalent to 25,000 neutrophils) was analyzed by immunoblotting for Ym1 and neutrophil elastase (25) and for Ym1 by immunocytochemistry of cytospin smears (Cytospin 2, Shandon, UK).

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Ym1 in Murine Neutrophil Granules

**Fig. 4. Ym1 expression in the CGD mouse.** A, anti-Ym1 (1 μg/ml) immunoblot of wild-type mouse organs demonstrating the major sites of expression; a, Ym1 (1 μg); b, venous blood; c, colon; d, ileum; e, duodenum; f, gastric antrum; g, proximal stomach; h, bone marrow; i, lung; j, brain; k, heart; l, thymus; m, kidney; n, testis; o, liver; p, spleen. 50 μg of protein was loaded in each lane. B, immunohistochemistry for Ym1 confirmed the major tissues of protein expression in the p47phox−/− mouse as spleen (Sp) showing scattered positive cells in the red pulp, bone marrow (Bm) showing staining of the granulocytic cell lineage, non-specialized type gastric mucosa in the gastric antrum (Sa) showing both parietal and chief cell staining, and lung (L) showing staining of crystals and macrophages. Immunoreactivity within an inflamed bile duct (Bd) and skin abscess (Sa) is also shown.

**RESULTS**

**Generation of p47phox-deficient Mice**—The p47phox gene was disrupted in mouse embryonic stem cells by insertion of a neomycin resistance gene into the third exon of the p47phox gene (Fig. 1). Two targeted embryonic stem cell clones (9.3, K1) were obtained and used to generate chimeric mice. Chimeras transmitting the mutation were used to establish mutant mouse lines on the 129 genetic background. This mutation neither affects neutrophil development nor recruitment but leads to complete inactivation of the neutrophil respiratory burst in response to stimulation with phorbol myristate acetate or Candida albicans as shown previously (25).

**Crystal Location and Identification**—Spontaneous pulmonary crystal formation was noted in the majority of p47phox−/− mouse lungs but never in wild-type lungs (n = 25/33 p47phox−/−, 5.5 ± 2.6 months (mean ± S.D.); n = 0/33 wild-type, 5.6 ± 1.9 months). Crystals were seen in mice aged greater than 2 months, with density increasing with age (R² = 0.39). The distribution was patchy, greatest in basal and peripheral foci. Crystals were mainly extracellular, multifaceted, ~10–100 μm in length, and often associated with giant cells and macrophages. In addition, they were frequently noted in chronically inflamed p47phox−/− bile ducts following oral DSS and in spontaneous skin abscesses (Fig. 2). In two wild-type mice they were seen in DSS-induced, severely inflamed bile ducts. Ym1 crystals were resistant to degradation in p47phox−/− mice, because copious crystals persisted in the p47phox−/− mice 10 days after subcutaneous Ym1 injection, associated with a giant cell response, whereas none were seen in wild-type mice. No Ym1 crystals were seen at sites injected with uric acid, suggesting that the findings were not due to neo-crystal formation in response to the introduction of skin bacteria at the time of injection.

We discovered that Ym1 crystals entered solution at alkaline (and acid) pH but were insoluble at pH 7.0 (Fig. 3). This allowed the purification of ~2.5 mg of protein from each aged p47phox−/− mouse (Fig. 3). Mass fingerprinting identified the major band at 43 kDa as mouse secretory protein Ym1 and lower molecular weight bands as Ym1 cleavage products. This finding pre-dated and therefore was independent of the report by
Guo et al. (3), identifying pulmonary crystals in other mice strains as Ym1. By using Mascot, the probability-based Mowse score with 10/13 peptide matches was 105 ($p_{H11021}/100.05$). Edman degradation confirmed the identity.

**Ym1 Tissue Distribution**—Western blot analysis showed that protein expression was greatest in the lung, spleen, bone marrow, and gastric antrum of normal and p47phox/−/− mice. The results were confirmed by immunohistochemistry (Fig. 4). Smaller amounts of Ym1 were seen in the liver, kidney, brain, heart, proximal duodenum, and submandibular gland and in one mutant mouse in the thymus. There was similar Ym1 expression (with a gradual increase with mouse age) in p47phox/−/− and wild-type mice, except in the aged p47phox/−/− lung, where very high levels were observed consistent with the development of crystals (at 42 weeks p47phox/−/− 20.6 ± 1.0 ng of Ym1/µg of lung protein; wild-type 0.9 ± 0.2 ng/µg). Gastric antrum immunoreactivity occurred at a different molecular weight to the other tissues (45 and 42 kDa) and probably represents antibody cross-reactivity with Ym2, recently shown to cause gastric hyalinosis in CYP1A2-null B6, 129 mice (6).

The finding of Ym1 immunoreactivity within the gastrointestinal fluid suggests the protein is secreted into the stomach lumen, where it may aid digestion (stomach 0.8 ± 0.9 ng of Ym1/µg of protein; ileum 3.8 ± 2.1; colon 5.9 ± 4.1).

**Ym1 Is a Neutrophil Granule Protein**—Before this study, the main cell of origin of Ym1 was presumed to be the macrophage, and the subcellular location was not known. We demonstrated here that Ym1 was neutrophil-derived (2.5 ng/µg neutrophil protein) and present equally in both peritoneal and bone marrow-derived neutrophils. Ym1 was also present within purified peritoneal macrophages but at a much lower concentration (0.2 ng/µg) (Fig. 5). Ym1 was not detected in T or B cells (data not shown). Subcellular fractionation by sucrose density centrifugation showed that Ym1 colocalizes with neutrophil granule proteins. This was confirmed by immunogold electron microscopy of murine neutrophils, which demonstrated very clearly a granular distribution. The peaks of Ym1, lactoferrin, and MPO occurred at sucrose densities of 37–39%, but Ym1 and MPO were also present in denser fractions (43–45%) (Fig. 6). This suggests Ym1 may be present in the azurophilic granules as it coincided with the azurophilic granule protein MPO rather than with the specific granule protein, lactoferrin (28).

**Ym1 in Murine Neutrophil Granules**

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**Fig. 5.** Ym1 is preferentially expressed in bone marrow (Bm-n) and peritoneal (Per-n) neutrophils compared with peritoneal macrophages (Per-m). A, Coomassie Brilliant Blue (BDH)-stained gel (top) demonstrates equal protein loading of immunoblots with anti-Ym1 (middle); anti-elastase (bottom) confirms correct sample assignment; N/D, not done. B, cytospin preparations of bone marrow (Bm-n) and peritoneal (Per-n) neutrophils, peritoneal macrophages (Per-m), and T-lymphocytes (T-lym) stained with anti-Ym1. Occasional Ym1 staining in the thymus extract was within macrophages.

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**Fig. 6.** Ym1 is a neutrophil granule protein. A, aliquots from a sucrose density gradient (*) separation of murine neutrophils probed with anti-Ym1 and anti-lactoferrin and assayed for MPO. Ym1 (●) and MPO (●) are in similar proportions in the same subcellular fractions of the neutrophil. Lactoferrin (●) is not seen in the denser fractions. B, electron micrograph of a neutrophil sectioned through its center, showing its nucleus (top, ×16,000; print magnification ×4,000). The cytoplasmic granules are electron dense and heavily labeled with silver-enhanced gold particles, adherent to anti-Ym1 antibody; there is no labeling elsewhere in the cytoplasm (bottom, ×40,000; print magnification ×110,000).
assessed using sensitive assays based on the release of free MU. There was maximal cleavage of the GlcNAc₂ analogue, MU-(GlcNAc)₂, at pH 4 and 4.5, but no significant cleavage of MU-(GlcNAc)₃ or MU-(GlcNAc)₄ (Fig. 7). This defines Ym1 as having β-N-acetylhexosaminidase and not chitinase activity (29). We confirmed the finding (3) that cleavage was specific to the β-D-linkage of GlcNAc because Ym1 did not cleave MU-N-acetyl-α-D-glucosamine (data not shown). The Vₘₐₓ and Kₘ values were 0.023 μmol/min/mg Ym1 and 120.8 μM, respectively (30). The Kₘ is similar to known values for chitinases found in S. marcescens (31), but the Vₘₐₓ is several orders of magnitude less. Ym1 has a small amount of autofluorescence, equivalent to a maximum of 0.07 μmol/mg protein, which represents a part of the small amount of net fluorescence emitted with MU-(GlcNAc)₂ and MU-(GlcNAc)₃. Chitinase from S. marcescens cleaved MU-(GlcNAc)₂, MU-(GlcNAc)₃, and MU-(GlcNAc)₄ equally with an activity ~1000-fold more than Ym1 (data not shown).

**DISCUSSION**

Lungs from aged p47phox⁻/⁻ mice showed patchy consolidation with a heavy intra-alveolar chronic inflammatory cell infiltrate composed predominantly of macrophages together with numerous crystals, which we identified as the chitinase-like protein Ym1. We also demonstrated that similar crystals within the inflamed biliary tree were Ym1. The protein was confirmed as Ym1 and not the very similar protein ECF-L (13) because the mass of the tryptic peptide 102–117 was 1722.82 Da, corresponding to proline at position 106 and not serine as in ECF-L. It is also not derived from the highly homologous gene ym2 (4), because the mass of the tryptic peptide 220–231 was 1343.70 Da, which corresponds to aspartate at position 220 and not asparagine as in Ym2.

Ym1 has been assumed to be macrophage-derived (5) because it was sequenced from an activated peritoneal macrophage cDNA library (10), and microscopically Ym1 crystals are associated with macrophages (3). However, we have shown Ym1 to be mainly a neutrophil granule protein. What is the explanation for this disparity? Neutrophils have a circulation half-life of about 6–8 h and are the predominant cell type during the first 24 h of an inflammatory stimulus (32). They apoptose and are phagocytosed and digested by macrophages, which have a life span of weeks to months (33), and can very rapidly degrade apoptotic neutrophils (up to 1 neutrophil/macrophage/30 min) (34). Extracellular non-degradable crystalline Ym1, or Ym1 contained within apoptotic neutrophils, will appear to be mainly associated with macrophages. This is compatible with the low concentration of Ym1 in thioglycolate-induced peritoneal macrophages, where the majority of cells will have been recently synthesized from bone marrow promonocytes and blood monocytes (35) and therefore will not have had the opportunity to phagocytose many neutrophils. It is noteworthy that another member of this family of proteins, YKL-40, has been found in the specific granules of human neutrophils (36) and that chitinase activity in human leukocytes has been shown to be mainly from granulocytes (37). It is possible that Ym1 is expressed also by eosinophils because the experimental induction of murine macrophage crystalline inclusions has been described after the intraperitoneal injection of human eosinophil-rich granulocytes (38), and both alveolar macrophage crystals in C57/BL6 mice (9) and Ym2 expression during experimental allergy (5) increase with peripheral eosinophilia.

Ym1 was able to cleave the GlcNAc₂ analogue, MU-(GlcNAc)₂, but not MU-(GlcNAc)₃ or MU-(GlcNAc)₄. Therefore, it can be classified as a β-N-acetylglucosaminidase rather than a chitinase (40) which is compatible with the mutated chitinase active site of Ym1 (41). The minimal activity with MU-(GlcNAc)₂ and MU-(GlcNAc)₃ shows that Ym1 cannot cleave N-acetylhexosamine monomers sequentially from the same molecule of MU-(GlcNAc)₂ or MU-(GlcNAc)₃, as has been described for chitinase A and B of S. marcescens (31). Contrary to our findings, Guo et al (3) recently suggested that Ym1 also could cleave MU-(GlcNAc)₂,₃. However, it is possible that their preparation contained cellular debris including chitinases of a similar molecular weight as Ym1 (see Table I) or enzymes capable of sequential β-N-acetylglucosaminidase or exo-chitinase activity, allowing Ym1 to release 4MU from MU-(GlcNAc)₁. Such problems in distinguishing between exo- and endo-chitinase activity have been described (42). We used purified Ym1, and therefore the enzyme kinetics are specific for Ym1. It is noteworthy that the enzyme activity had the same pH dependence as shown recently for Ym1 binding to certain (oligo)saccharides with a free amine group (10), although binding and cleavage sites of the protein may be distinct. After invasion by chitin containing microorganisms, chitin and other foreign antigens must be digested to prevent a granulomatous tissue response. Chitin can be degraded with a binary enzyme system using a combination of a chitinase and an β-N-acetylglucosaminidase (31). The weak activity demonstrated here may underestimate in vivo kinetics because functionality may require synergy with other proteins, shown previously for chitinase (43).

What can be deduced about protein crystallization from our findings? First, crystals may form due to a high local concen-

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**FIG. 7. Ym1 is a weak β-N-acetylhexosaminidase.** Cleavage of the substrate MU-(GlcNAc)₂,₃ at pH 2.5 (●), 3.0 (■), 3.5 (△), 4.0 (○), 4.5 (×), 5.0 (●), and 5.5 (○) by pure Ym1 in solution to release MU demonstrates only β-N-acetylhexosaminidase activity, maximal at pH 4 and 4.5.
tration of Ym1 secondary to neutrophil degranulation during repeated episodes of inflammation. This is analogous to the deposition of α1-antitrypsin variants within the liver, where polymerization of the protein is enhanced during episodes of inflammation (44), and to Charcot-Leyden crystal formation, where lyso phospholipase, a granule protein comprising 10% of an eosinophil’s protein (45), crystallizes during asthmatic inflammatory reactions. In support of this, Ym1 crystals increased with age and were found within the lung, a major portal of antigen entry, in peripheral and basal sites, suggesting a response to inhaled matter; and increased Ym1 expression and Ym1 crystals occurred at extrapulmonary sites of inflammation. Second, a mildly acidic pH milieu within the CGD portal of antigen entry, in peripheral and basal sites, suggests a mildly acidic pH milieu within the CGD phagolysosome of the human CGD neutrophil has been shown to be normally acidic (6 min post-phagocytosis, pH 6.1 cf. pH 7.4 in control subjects (47)). Assuming similar pH changes occur in the murine CGD phagolysosome, the concentration of Ym1 protein may exceed the crystallization threshold due to excessive degranulation. There is 1 ng of Ym1 in 10,000 murine neutrophils; the volume of granules in one human neutrophil is 52.5 fl (48); assuming human and murine neutrophils have a similar granule volume, the granule concentration of Ym1 protein may exceed the crystallization threshold when the concentration exceeds 0.4 μg/ml. Third, crystalline Ym1 may be resistant to degradation within the CGD macrophage due to absence of functional NADPH oxidase. This is likely because skin injection experiments showed that only wild-type mice were able to clear Ym1 crystals from their subcutis. In contrast to the CYP1A2-null B6, 129 mouse, no protein crystals were seen within the stomach. This may be due to the extreme acidic environment and/or a lower Ym2 concentration in the p47phox−/− mouse.

It is interesting that a protein that may play a role in the digestion of bacteria and fungi is found as crystal deposits in the CGD mouse, which is susceptible to repeated bacterial and fungal infections. Could a similar phenomenon occur in humans? There are no reports of similar crystals in human patients with CGD. After comprehensive searches against protein, nucleotide, and expressed sequence tag data bases using the Basic Local Alignment Search Tool (National Center for Biotechnology Information (www.ncbi.nlm.nih.gov)), no known human homologue for Ym1 was found. Although there is 61% protein sequence homology to a human lung gene, TSA1902 (50), the human gene has a different murine homologue called acidic mammalian chitinase, recently reported by Boot et al. (51). Acidic mammalian chitinase is similar to Ym1/Ym2 because both proteins are expressed predominantly by gastric epithelium with maximum activity in an acidic milieu. Ym1/Ym2 may persist in the mouse to protect against mouse-specific microorganisms in the lung or stomach, because mice have evolved in habitats that contain a high concentration of chitin-containing fungal spores. The function of Ym1 is likely to be digestive rather than fungicidal, because neither Ym1 nor anti-Ym1 has anti-C. albicans activity in vitro at an acidic or normal pH (data not shown).

The function of Ym1 is still unclear but by defining the cellular location as the neutrophil granule and gastric epithelium, it is plausible to suggest that it has a major role in digestion during acute inflammation. Although knockout mice may help determine the function of Ym1, redundancy within this family of proteins may mean that a specific deficiency can be tolerated without a phenotypic change. This is known to be the case with human chitotriosidase deficiency (52). It will be important to study chitinase/β-N-acetylgalactosaminidase activity in the human CGD macrophage and neutrophil because it is possible that similar proteins to Ym1 are sequestered, which could enhance the inflammatory reaction seen in these patients.

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### Table I

| Name                        | Mass  | Cellular origin          |
|-----------------------------|-------|--------------------------|
| Human YKL-39<sup>a</sup>    | 43,039| Articular chondrocyte (53)|
| Human HCgp-39 (YKL-40)<sup>b</sup> | 42,613| Macrophage (54); neutrophil (36); articular chondrocyte (55)|
| Human oviductal glycoprotein<sup>c</sup> | 75,421| NK                       |
| Human chitotriosidase<sup>d</sup> | 51,681| Monocyte (39)            |
| Human TSA1902-1<sup>e</sup> | 40,082| NK                       |
| Mouse AMCase<sup>f</sup>    | 50,000| NK                       |
| Mouse ECP-1<sup>f</sup>     | 44,448| NK                       |
| Mouse Ym1<sup>f</sup>       | 44,528| Neutrophil; gastric epithelia (6) |
| Mouse BRF39<sup>g</sup>     | 43,001| NK                       |
| Mouse oviductin<sup>h</sup>| 78,807| NK                       |

<sup>a</sup> NCBI protein database accession number AAC50597.
<sup>b</sup> NCBI protein database accession number AAA16074.
<sup>c</sup> NCBI protein database accession number AAC86946.1.
<sup>d</sup> NCBI protein database accession number AAC50246.1.
<sup>e</sup> NCBI protein database accession number BAA69801.1.
<sup>f</sup> NCBI protein database accession number AAG60018.
<sup>g</sup> NCBI protein database accession number BAA13458.2.
<sup>h</sup> NCBI protein database accession number AAB62394.2.
<sup>i</sup> NCBI protein database accession number CA63603.1.
<sup>j</sup> NCBI protein database accession number Q62010.
