A Macrophage Protein, Ym1, Transiently Expressed during Inflammation Is a Novel Mammalian Lectin*

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Nan-Chi A. Chang‡‡‡, Shuen-Iu Hung‡, Kuo-Yuan Hwa‡, Ikunoshin Kato**, Ju-Eng Chen‡, Cheng-Hsiu Liu‡, and Alice Chiang‡‡‡

From the *Institute of Microbiology and Immunology, ‡Institute of Neuroscience, and ‡‡Center for Neuroscience, School of Life Science, National Yang-Ming University, Taipei, Taiwan 112, Republic of China, the *Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan 115, Republic of China, and **Biomedical Group, Takara Shuzo Co., Ltd., Otsu, Shiga 520-21, Japan

Oral infections of mice with Trichinella spiralis induce activation of peritoneal exudate cells to transiently express and secrete a crystallizable protein Ym1. Purification of Ym1 to homogeneity was achieved. It is a single chain polypeptide (45 kDa) with a strong tendency to crystallize at its isoelectric point (pI 5.7). Coexpression of Ym1 with Mac-1 and scavenger receptor pinpoints macrophages as its main producer. Protein microsequencing provide information required for full-length cDNA cloning from libraries constructed from activated peritoneal exudate cells. A single open reading frame of 388 amino acids with a leader peptide (21 residues) typical of secretory protein was deduced and later deposited in GenBank™ (accession number M94584) in 1992. By means of surface plasmon resonance analyses, Ym1 has been shown to exhibit binding specificity to saccharides with a free amine group, such as GalN, GlcN, or GlcN polymers, but it failed to bind to other saccharides. The interaction is pH-dependent but Ca\(^{2+}\) and Mg\(^{2+}\) ion-independent. The binding avidity of Ym1 to GlcN oligosaccharides was enhanced by more than 1000-fold due to the clustering effect. Specific binding of Ym1 to heparin suggests that heparin/heparan sulfate may be its physiological ligand in vivo during inflammation and/or tissue remodeling. Although it shares ~30% homology with microbial chitinases, no chitinase activity was found associated with Ym1. Genomic Southern blot analyses suggest that Ym1 may represent a member of a novel lectin gene family.

Macrophages exhibit a myriad of critical functions in host defense mechanism. The differentiation, activation, and effector functions of macrophages have intrigued intensive studies (1–3). Macrophages are responsible for antigen presentation and destruction against microbes and neoplastic cells. In some instances, they serve as nonspecific scavenger cells; in others, they may be subjected to modulation by selective cytokines for enhanced competence to destroy facultative and obligate intracellular parasites (4, 5). Furthermore, macrophages are also producers of a wide variety of membrane and secretory proteins pivotal for the development of tolerance, cell-cell recognition, and cell-mediated cytotoxicity (6–8). As the list of known functions of macrophages continues to grow, studies concerning the development of competence to execute particular functions of this class of highly versatile cells have brought consensus that macrophage activation is enormously complex. It can be regulated by multiple signals, inductive or suppressive, and by the sites of their residency and the biochemical changes of the local milieu (9, 10).

In order to elicit emigrant peritoneal exudate cells (PEC)\(^1\) as a source of activated macrophages functionally distinct from that induced by thioglycollate, Sephadex G-50, or Corynebacterium parvum, we have adopted the paradigm of natural parasitic infection using Trichinella spiralis, a systemic migration and muscle-penetrating parasite (11, 12). We have previously noted that marked cellular changes took place inside the peritoneal cavity of T. spiralis-infected mice. Cellular accumulation peaks at around day 15 postinfection (13). During this time, infective newborn larvae will migrate via the peritoneal cavity of the host en route to their final destination in the skeletal muscle cells (14).

The biochemical changes within the peritoneal cavity were also monitored during the entire infection course. A novel protein transiently expressed by the lavaged PEC was identified (13). The inducible expression of this protein and the profound cellular changes paralleling its appearance suggested to us that it might bear functional significance to the development of either host defense against or tolerance to this nematode infection. We have designated this protein as Ym1 and subsequently proceeded toward its purification and molecular characterization (i.e. cloning, sequencing, and expression). We report the following in the present study: (i) Ym1 is synthesized and secreted by activated macrophages during inflammation elicited by parasitic infections; (ii) Ym1 is purified, cloned, and characterized at both the protein and molecular levels; (iii) Ym1 is a novel mammalian lectin exhibiting a pH-dependent, specific affinity toward GlcN oligomers and heparin, which

\( ^1\) The abbreviations used are: PEC, peritoneal exudate cell(s); PEF, peritoneal exudate fluid; PCCS, PEC culture supernatant; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; HSPGs, heparin/heparan sulfate proteoglycan(s); ECM, extracellular matrix; BACH, 6-(biotinyl)-aminocaproylhydrazide; kb, base pairs; kb, kilobase pair(s).
Ym1, a Lectin Secreted by Macrophages

Amino Acid Composition and Protein Microsequencing—Purified and crystallized Ym1 was subjected to amino acid composition analyses using a D400 amino acid analyzer (Durrum Co.). Amino acid sequences of the NH2-terminal fragment and the CNBr fragments of Ym1 were determined by protein microsequencing on an Applied Biosystems 1470 gatoh protein sequencer (MS 299).

Library Screening and DNA Sequencing—Total RNA of PEC collected from 50 mice orally infected with T. spiralis for 15 days was prepared by the guanidinium-CaCl2 centrifugation method (25). Poly(A)+ mRNA purified by an oligo(dT) column were primed by both oligo(dT) and random hexamers and custom constructed into two cDNA libraries (PEC-1 and PECCS). PEC-1 cDNA library was packaged into a λgt11 vector, whereas PECC-2 cDNA library was packed into a λZAP-II vector. In order to prepare Ym1-specific probes by polymerase chain reaction (PCR) (26), a sense primer was designed based on the sequence of the NH2-terminus (P1, 5′-ATGTTG/TCC/TCT/TAT/CTGACC/A/CTTG/GCG/AA/GA-3′). Antisense primers were designed based on sequences derived from CNBr fragment 2 (P2, 5′-TTT/CCT/CCTCC/CTGA/GTTA/ICGC/AG/C/TCT/TCT-3′) and 3 (P3, 5′-TGC/TCC/TCT/C/TCC/AA/GA/CGC/GCAT-3′), respectively. PEC-1 cDNA library was screened by PCR using primer pairs as designed. PEC-2 cDNA library and a mouse lung cDNA library (Stratagene, λZAP-II vector) were also screened using partial clones of Ym1; PEC21 and PEC15 were obtained from PEC-1 library as probes. Full-length cDNA clones (AC-11 and AC-17) were sequenced, and the sequence was later identified and purified from both libraries, respectively. Nucleotide sequence was determined according to the dideoxy chain termination method of Sanger et al. (27). Mouse genomic library 129SvS in λFIXII (Stratagene) was screened using the same probes in search of genomic clones of Ym1. A total of 107 phage clones were screened, and 15 clones were amplified, purified, and sequenced.

Southern Blot Hybridization—Genomic DNA from testes of 129Sv mice was prepared as described (28). Aliquots of DNA (30 μg) were digested with selected restriction enzymes as specified, separated on 0.8% agarose gel, and transferred onto Immobilon-N membrane (Millipore) before hybridization. Exon probes (Ep17-14 and Ep4-6) were amplified from AC-11, and intron probe (Ip1-2) was amplified from a 3-kb EcoRI fragment of genomic DNA subcloned in pBluescript-II SK (Stratagene) by PCR, respectively. Probe labeling, prehybridization, and hybridization were conducted as described (28). Primer pairs used as follows: for Ep17-14, Ep17 (5′-TGGAGGACCATGAGGAGC-3′) and Ep14 (5′-GCCCTAACTGATGAGGCTC-3′); for Ep4-6, Ep4 (5′-CTTCTCTTGGTCTTGGG-3′) and Ep3 (5′-AGACCTGAGGCTCT-3′); for Ip1-2, Ip1 (5′-GGTACATTACCTACACTCCC-3′) and Ip2 (5′-CTTATTACACACTACC-3′).

Chitase Assays—Three different methods were used to examine if purified, native Ym1 or recombinant Ym1 has chitinase activity. The first method is based on an agar plate enzyme assay (29). Briefly, 0.02% glacial chitin or chitin glosan in an agar plate were used as substrate. The second gel overlay method was carried out as described by Trudel and Asselin (30). Samples were resolved first by 10% basic native PAGE and hybridized with a chitinase-specific probe, GlcNAc oligomers, GlcNAcα1,6. The initial substrates and products were separated on a PLAPAK type N column (Takara; 4.6 × 250 mm) and detected by a fluorescent detector (excitation wavelength, 315 nm; emission wavelength, 380 nm) on a high performance liquid chromatography (HPLC) system (31).

Carbohydrate Binding Specificity Determined by Surface Plasmon Resonance Technology—BLAcore X (Amersham Pharmacia Biotech) was used to screen for carbohydrate ligands that interact with Ym1. The Biocore sensor chips CM5 and SA (streptavidin covalently immobilized on a CM5 chip) and chemical activation reagents (amine coupling kit) were obtained from Amersham Pharmacia Biotech. BHS-EP buffer (10 mM HEPES with 150 mM NaCl, 3 mM EDTA, and 0.005% (v/v) surfactant P20) was used as a continuous running buffer over the sensor surface in the BLAcore experiments. Monosaccharides, various oligosaccharides, and 6-(biotinyl)-aminocaproylhydrazide (BACH) were from Sigma or Merck. N-acetyllactosamine was from Calbiochem. Chitobiose (di-GlcN), chitotriose (tri-GlcN), and chitotetraose (tetra-GlcN) were from Seikagaku.

In order to test the binding specificity of monosaccharides and GlcN oligomers, CMS purified Ym1 was immobilized onto the sensor chip CM5 surface via reaction with primary amines as suggested by the manufacturer. Ym1 (100 μg/ml) in a 10 mM sodium acetate buffer (pH 4.25) or buffer control was introduced separately onto the activated surface of flow cells number 1 and 2, respectively. Excess N-hydroxysuccinimide ester groups were blocked by 1 M ethanolamine hydrochloride, pH 8.5, for 7 min. Monosaccharides and GlcN oligomers in HBS buffer were

May be structurally related to its natural ligand; and (iv) Ym1 shares significant sequence homology with several mammalian chitinase-like proteins of unknown function reported recently. However, none exhibits any chitinase activity, whereas our genomic Southern blot analyses provide evidence supporting the notion that Ym1 and these proteins may belong to a multigene family. The putative functional role of Ym1 in vivo is discussed. In addition, the x-ray structure of Ym1, the first of the gene family, is reported in the accompanying article (79).

MATERIALS AND METHODS

Induction of Ym1 Expression— Infective larvae of T. spiralis were prepared from infected Sprague-Dawley rats as described (15). Female ICR mice were orally infected with 250 (primary infection) and 500 (secondary infection) T. spiralis larvae. Groups of 3–6 mice were sacrificed at selected intervals postinfection. PEC of both control and infected mice were recovered by lavaging each with 5 ml of ice-cold phosphate-buffered saline (20 mM phosphate buffer, pH 7.4, 0.85% NaCl). Total cell number in the lavaged fluid was enumerated and determined using a hemocytometer. To purify activated macrophages, PEC (5 × 107 cells/75 mm2) were plated out in serum-free Dulbecco’s modified Eagle’s medium supplemented with gentamycin (10 μg/ml), incubated in 5% CO2 at 37 °C for 2–3 h, and washed three times with serum-free medium to remove nonadherent cells. The percentage of attached cells capable of phagocytizing zymosan particles estimated was routinely over 95% after differential Giemsa staining. Both cell-free peritoneal exudate fluid (PEF) and cell culture supernatant (PECCS) collected daily were used as sources for Ym1 purification.

Purification of Ym1 by Preparative Polyacrylamide Gel Electrophoresis (PAGE)— For a typical run of purification, 50 ICR mice were orally infected with 250 T. spiralis larvae. By day 15, all animals were sacrificed to collect PEF and PECCS. Protein concentrations were determined by Coomassie Blue assay reagent (Pierce) before subjecting to preparative basic PAGE isolation according to Davis (16). On the average, the amount of total proteins obtained from PEF was 60 mg, and that from PECCS 16 mg. However, Gel discs containing Ym1 were subsequently sequenced with 20, 40, and 10% sucrose solution before filling up the tube with 0.4% etha
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injected across the surface at a flow rate of 5–30 μl/min, and the real
time binding curves were observed. To stop the reaction, HBS buffer,
ph 7.4, was introduced onto the sensor chip to start the dissociation.
The bulk effect of refractive index changes was subtracted from in-line
reference flow cell to yield true binding response.

For calculation of $K_D$ and competition analyses, biotinylated tetra-
GlcN was immobilized onto a streptavidin chip (SA chip). The biotin-
ylation of tetra-GlcN with BACH was performed as described (32).
Briefly, GlcN oligomers (200 nmol) in 100 μl of water were mixed with
100 μl of a 400-nmol solution of BACH in 30% acetonitrile at 95 °C for
2 h. The product and initial substrates were analyzed by TLC. Approx-
imately 40 nmol of GlcN oligomers was applied on HP-TLC Silica Gel 60
aluminum plates (Merck), developed in n-propyl alcohol, water, 32%
ammonia (55:20:25, v/v/v). Biotinyl GlcN oligosaccharides, unlabeled
GlcN oligosaccharides, and BACH were visualized by spraying
α-naphthol/H2SO4 onto a reference lane, and the desired product was
identified and scraped from the plate. The purified biotinylated GlcN
oligomers were extracted with n-propyl alcohol, water, 32% ammonia
(55:20:25, v/v/v) twice. The purity of extracted biotinylated GlcN oli-
gomers was confirmed by HP-TLC and the molecular weight of purified
(55/20/25, v/v/v) twice. The purity of extracted biotinylated GlcN oli-
gomers was confirmed by HP-TLC and the molecular weight of purified
biotinylated tetra-GlcN was identified by FAB-mass spectrometry at a
m/z 1038 (MNa+ ) and m/z 1016 (MH+ ).

The biotinylated tetra-GlcN (1 pmol) was reconstituted in HBS-EP
buffer and immobilized onto a SA chip. Biotinyl tetra-GlcN (109 res-
one units) was captured in flow cell number 2. Flow cell number 1 on
the same sensor chip was used as a reference surface. Kinetic data were
computed using the BIA evaluation software 3.0 (Pharmacia Biosensor
AB). The plot of subtracted responses versus the analyte concentrations
was used to derive the dissociation constant ($K_D$) in the Ym1-immo-
bilized system. The $K_D$ value of the tetra-GlcN immobilized assay system
was calculated using the simultaneous $k_1/K_2$ fitting.

Heparin/Heparan Sulfate Binding Assay—Various carbohydrates or
alumina/streptavidin-conjugated agarose beads were obtained from
Sigma and extensively washed with HBS-EP buffer. The biotinylated
di-GlcN was immobilized onto streptavidin-conjugated agarose beads.
The pH of T. spiralis-elicited PECs containing Ym1 was first adjusted to
4.75 with 50 mM sodium acetate buffer. To 100 μl of beads, 200 μl of
conditioned medium was added. After a 2-h incubation with gentle
mixing at ambient temperature, the beads were then extensively
washed with HBS-EP buffer, pH 4.75, to remove the nonspecific bound
proteins. Proteins bound on the carbohydrate-conjugated beads were
eluted in HBS-EP buffer, pH 7.4, and the presence of Ym1 was revealed
by Western blot.

RESULTS

Cellular and Biochemical Changes in the Peritoneal Cavity of
T. spiralis-infected Mice—Total cell counts in 5 ml of PEF
recovered from the lavages were plotted against time after
infection. A significant increase in the number of PEC at day 9
after primary infection was noted as compared with control
cells. The cell number peaked at around day 15 (4.2 × 107
cells/mouse) and subsided rapidly thereafter. The second infec-
tion of 500 T. spiralis larvae given at day 42 after primary
infection elicited a much faster and enhanced accumulation of
PEF (Fig. 1A). The cell counts peaked at around day 15 and day 12 after
the infections, respectively. The number of cells infiltrated to the peritoneal
cavity after the secondary infections is ~2-fold higher. B, the protein
profile of PEF was resolved on basic PAGE as a function of time postpri-
mary infection. The arrow denotes the appearance of Ym1 at day 15.

Purification of Ym1—To purify the transiently expressed
Ym1, conditioned medium of cultured PEC (PECCS) of infected
mice was harvested, and a total of 16 mg of proteins were
subjected to preparative basic PAGE. Proteins in unstained gel
slices with a $R_m$ of 0.77 were pooled, and ~7.4 mg of proteins
were subjected to IEF-PAGE in a broad pH range (pH 3.5–
10). The gel bands corresponding to pH 5.7 were again sliced
out and pooled, and 2.8 mg of proteins were subjected to IEF-
PAGE in a narrow pH gradient (pH 5–7) (Fig. 2A). After the
narrow IEF-PAGE, Ym1 actually is visible in gel as crystals
without staining (Fig. 2B). Under a phase-contrast microscope,

Ym1 appears as cross- or scissors-like crystals (Fig. 2C). These
crystals may be eluted from the gel discs with 0.01 M sodium
bicarbonate buffer (pH 9.0) and recrystallized out after pro-
longed dialysis against deionized distilled water; a total of 2.3
mg of Ym1 protein may be obtained. The crystals then acquire
a square sheet-like configuration and may “grow” into larger
four-ridged pyramids (Fig. 2D). The purity of the protein band
focused at pH 5.7 was further verified by 10% SDS-PAGE.

![FIG. 1. Cellular and biochemical changes in peritoneal cavity of T. spiralis-infected mice. PEC and PEF were harvested from T. spiralis infected mice at 3-day intervals. A, the total number of PEC in 5 ml of lavaged fluid of each time point was enumerated and plotted as a function of time postinfection: primary at day 0 and secondary at day 42. The cell counts peaked at around day 15 and day 12 after the infections, respectively. The number of cells infiltrated to the peritoneal cavity after the secondary infections is ~2-fold higher. B, the protein profile of PEF was resolved on basic PAGE as a function of time postprimary infection. The arrow denotes the appearance of Ym1 at day 15.](http://www.jbc.org/Downloaded from)

![FIG. 2. Purification and crystallization of Ym1. A, Coomassie Blue staining pattern of Ym1 revealed through a series of gel electrophoresis. Lane 1, the bracket denotes the position of Ym1 after PECCS was resolved on basic PAGE. Lanes 2 and 3, the arrows denote Ym1 after broad range (pH 3.5–10) and narrow range (pH 5–7) IEF-PAGE, respectively. B, Ym1 crystals in sandy appearance may be visualized in gel discs collected after isoelectric focusing gel electrophoresis. C, cross- or scissors-like Ym1 crystals in gel visualized under a stereomicroscope. D, after extensive dialysis, the purified Ym1 formed crystals in shapes such as a square sheet (arrow) or pyramid. E, the purity of Ym1 was evaluated (lane 6), and the molecular mass (arrow) was determined (lane 4) in SDS-PAGE.](http://www.jbc.org/Downloaded from)
Results indicate that Ym1 has been purified to homogeneity with an estimated molecular mass of 45 kDa (Fig. 2E). The final yield of purified and crystallized Ym1 after dialysis was about 15% of the starting materials.

Polyclonal Antibodies against Purified and Crystallized Ym1—After primary immunization, rabbits were subjected to at least two additional booster injections. The specificity of antibodies was determined by Western blot of PECCS (Fig. 3A). An indirect immunofluorescence test was used to identify the cell type in PEC responsible for Ym1 synthesis. To verify whether the Ym1-positive leukocytes are adherent cells, activated PEC were cultured with daily medium change for 3 days to deplete nonadherent cells. Essentially all adherent cells are Ym1-positive (Fig. 3B, a and c), suggesting that activated peritoneal macrophages are cells responsible for Ym1 expression. The notion was further supported by co-localizing macrophage marker proteins Mac-1 (Fig. 3B, b) and scavenger receptor (Fig. 3B, d).

Molecular Cloning and Sequence Characterization—Amino acid composition analyses of Ym1 indicate the presence of ~34% hydrophobic residues and relatively high content of glutamic acid (9.1%) and aspartic acid (11.4%). The data are consistent with the fact that Ym1 has a tendency to crystallize despite having an acidic pi of 5.7. Protein microsequencing data of the NH2-terminus and three peptide fragments of Ym1 derived from CNBr cleavage revealed a total of 100 residues. Pairs of primers were designed based on amino acid sequences derived from NH2-terminus and two CNBr fragments of purified Ym1. Two DNA fragments, Y1 (437 bp) and Y2 (605 bp) were initially amplified from PEC-1 cDNA library (λgt11) by PCR. DNA sequence data obtained indicated that Y1 and Y2 are overlapping clones and contain partial sequences identical to that derived from protein microsequencing of Ym1 (Fig. 4). After screening the PEC-1 cDNA library with Y1 and Y2, Ym1-positive clones were identified, purified, subcloned, and sequenced. Two clones (PEC21 (1035 bp) and PEC15 (1475 bp)) overlapping by as many as 970 bp, constitute a single open reading frame of 398 amino acids. The same sequence was enclosed in a single cDNA clone (AC-11) after rescreening a second PEC cDNA library (PEC-2, AZAP-II) (Fig. 4). In addition, a typical eukaryotic ribosome binding motif (33) adjacent the putative initiator codon is present. The first 21 amino acids also fit well as the predicted secretory signal peptide with a cleavage site at residue Y, which is the NH2-terminal residue of the purified Ym1. Sequences of all peptide fragments obtained from protein microsequencing analyses are present in the deduced open reading frame of Ym1 (Fig. 4). The calculated pi value (5.4), molecular weight (44,456), and amino acid composition using PC Gene (IntelleGenetics) correlate well with the empirical results obtained from the purified Ym1. The DNA and the deduced protein sequence of Ym1 were submitted to GenBank™ in 1992 and assigned with accession number M94584.

Homology search in the protein sequence data bank (GenBank™/EBI Data Bank) revealed that partial homology (11–36%) exists between Ym1 and members of the chitinase protein family. Chitinase activity of Ym1 was examined using a conventional agar plate assay, gel overlay assay, and the more sensitive pyridylamination-HPLC method as described. No chitinolytic activity was found associated with Ym1 either in the purified form obtained from activated macrophages or in the recombinant form expressed by the baculoviral expression system. Significant homology (Fig. 5) has also been noticed between Ym1 and several “chitinase-like” proteins reported recently, namely human HC-gp39 (46% identity) (34), human chitotriosidase (46%) (35), porcine gp38k (45%) (36), and Drosophila DS-47 (25%) (37). With the exception of chitotriosidase, it was intriguing to note that all of these proteins, just as Ym1, do not exhibit chitinase activity.

Ym1 Presents as a Member of a Multiple Gene Family—Genomic Southern blot analyses were carried out using probes generated from different parts of the coding region in order to determine whether Ym1 is encoded by a single copy gene and whether it contains introns. Genomic DNA was subjected to restriction enzyme digestion before hybridizing at high stringency to probe Ep17-14. Multiple bands of different sizes were detected after digestion with EcoRI (9.0, 5.3, and 3.2 kb), HindIII (4.6, 4.2, and 3.4 kb), XbaI (8.0, 6.5, 3.2, and 2.0 kb), SacI (16 and 6.5 kb), and NcoI (15 and 2.0 kb), respectively (Fig. 6B). Data suggest the possible existence of introns, pseudogenes, and/or other members of a gene family. Detailed sequence studies of Ym1 phage clones purified from a mouse (129SvJ) genomic library revealed the consensus splice donor and accep-
3.2 Genomic Southern blot analyses thereby suggest that Ym1
peptide one band in each lane, while the exon probe hybridized fairly
cate that the intron probe (Ip1–2) hybridized strongly to only

tor sites, defining at least 11 introns and 11 exons (Fig. 6A) in
the coding region toward the 3'-untranslated region. Reevaluation of the genomic Southern blot of Ym1 using intron-specific
probe Ip1–2 suggests that Ym1 is a single copy gene (Fig. 6C).

Same pairs of exon and intron probes were employed in murine chromosome mapping studies using the interspecific backcross analyses (in collaboration with Dr. N. G. Copeland, NCI-Frederick Cancer Research and Development Center). Results indicate that the intron probe (Ip1–2) hybridized strongly to only one band in each lane, while the exon probe hybridized fairly well to five bands in each lane at relative low stringency. All of the bands hybridized with both probes co-segregated and mapped to the middle region of mouse chromosome 3. It thus appears that the two probes are recognizing a family of closely
related genes and that these genes are linked on chromosome 3.2

Genomic Southern blot analyses thereby suggest that Ym1 may represent a member of a gene family.

Screen for Saccharides Interacting with Ym1—Although Ym1 is not a chitinase, significant sequence homology to chitinase would suggest that Ym1 and other members of the “chitinase-like” proteins may interact with saccharides sharing structural features similar to chitin. We have therefore screened for carbohydrate ligands that will interact with Ym1 bound on a CM5 sensor chip, using surface plasmon resonance.

As shown in Fig. 7, among various monosaccharides tested, GlcN and GalN exhibited specific binding to Ym1, whereas other monosaccharides tested did not. Data obtained from oligosaccharides further indicated that those with free amine groups are more potent ligands for Ym1 as compared with other monosaccharides tested. Data obtained from oligosaccharides further indicated that those with free amine groups such as GlcN and GalN are capable of blocking the binding response, while similar monosaccharides did not.

To evaluate the clustering effect, GlcN oligomers at concentra-

Fig. 4. Full-length cDNA sequence and the deduced amino acid sequence of Ym1. A single PEC cDNA clone (1526 bp) encoding the full-length Ym1 was identified and sequenced. The open reading frame consists of a protein of 398 amino acids with a typical Kozak sequence

FIG. 4. Full-length cDNA sequence and the deduced amino acid sequence of Ym1. A single PEC cDNA clone (1526 bp) encoding the full-length Ym1 was identified and sequenced. The open reading frame consists of a protein of 398 amino acids with a typical Kozak sequence

2 Chromosomal mapping by N. G. Copeland and N. C. Chang, unpublished results.
tions as specified were incubated with Ym1 prior to injection onto a tetra-GlcN-bound chip. Results obtained from the competition analyses indicate that all GlcN saccharides tested were effective competitors; however, the potency increases as a function of valences (Fig. 8D). The IC$_{50}$ was determined to be 100 mM for GlcN, 500 mM for di- and tri-GlcN, and 100 mM for tetra-GlcN, respectively. The fact that tetra-GlcN is 1000-fold more effective than GlcN monomer suggests that Ym1 is a mammalian lectin that exhibits specific binding toward multivalent hexosamines, and a clustering effect was observed in the interaction between Ym1 and GlcN oligomers.

**DISCUSSION**

Macrophages are perhaps one of the most versatile cell types in the body, participating in a vast array of biological processes from fighting infections to tissue remodeling and wound healing. The diversity of its functional repertoire strongly suggests that its differentiation and activation may be subjected to the profound influence of environmental changes (39–42). We have adopted a paradigm of natural parasitic infection using *T. spiralis* in order to further explore the roles of activated macrophages during the course of a complex immune response evoked by the host against parasites. Cellular infiltration in the peritoneal cavity peaked around day 15 and day 12 after the primary and secondary infection, respectively (Fig. 1A). The shorter time course required for cell accumulation after the secondary infection reflects a typical memory response. Transient appearance of Ym1 paralleling the rise and rapid fall of the PEC numbers during the course of inflammation was observed. The peak of Ym1 expression occurs at a stage during infection right after the intestinal expulsion of the adult worms and at the beginning of an intense systemic migration of newborn larvae throughout the body of the host (13). The peritoneal cavity at this time would have experienced a traumatic process that calls for tissue repair. The transient appearance of Ym1 (Fig. 1B) would suggest that it may be involved in the establishment of an inflammatory management control in the peritoneal cavity. To establish its identity as an immune mediator and its cellular origin, purification and molecular characteriza-
secretion has also been studied by metabolic labeling techniques. Cultured macrophages were first starved in methionine-free Dulbecco’s modified Eagle’s medium for 1 h, pulsed with [35S]methionine for 10 min, and chased from 2 to 20 h in medium containing 0.1 mM nonradioactive L-methionine. The secretion was synthesized and thereby labeled Ym1 was monitored as a function of time by radioimmunoprecipitation. The results indicated a half-maximal release time of 4–6 h for Ym1. Upon the addition of the Ca2+ ionophore A23187, 10 nM was sufficient to evoke a much enhanced secretion of newly synthesized Ym1 presumably due to Ca2+ influx (data not shown).

The developmental and spatial expression pattern of Ym1 in normal or infected mice has been examined by Western blot and immunocytochemical analyses. Constitutive, basal expression of Ym1 is restricted to bone marrow, spleen, and lung, whereas transient or inducible expression may be detected in embryonic liver and at additional sites (e.g. peritoneal cavity and brain), where inflammation or tissue injury was introduced. These results are consistent with the fact that fetal liver and bone marrow are the original sources of myeloid lineage cells, which will eventually develop into tissue macrophages (46, 47). In our experience, *Ascaris suum* was also able to induce the production of Ym1 in the peritoneal cavity to a comparable level. However, agents such as thioglycolate, Sephadex G-50, and *C. parvum* could elicit the accumulation of active peritoneal macrophages 3–4 days after peritoneal injections to a much lesser extent. None of these agents would induce the expression of Ym1 in quantity that permitted its purification (data not shown).

To delineate the function of Ym1, we proceeded toward its gene cloning. Molecular cloning of Ym1 cDNA was achieved by PCR employing biochemical data obtained from microsequencing of the purified and crystallized protein. Amino acid sequence deduced from the cDNA revealed the existence of a typical Kozak motif, and a signal peptide. In addition, sequences corresponding to the NH2-terminal fragment of Ym1 (secretory form) and that of three CNBr cleavage peptides are also present (Fig. 4). The calculated pl of 5.4 and molecular mass of 45 kDa are also consistent with the biochemical features of the purified Ym1 determined empirically. Furthermore, cDNA-directed synthesis and secretion of recombinant Ym1 in both mammalian and baculoviral expression systems were validated using specific antibodies in Western blot analyses (data not shown). We have thereby concluded that the cDNA clones on hand do contain the complete sequence information for encoding the secretory Ym1, a protein transiently expressed by activated macrophages during the course of inflammation against parasitic infections. We were the first to submit the Ym1 cDNA and protein sequences to GenBankTM. An accession number of M94584 was assigned to Ym1 in 1992. Recently, Owhashi et al. (48) reported the cloning of a cDNA (submitted in 1996, GenBankTM accession number D87757) that encodes a protein with a sequence identical to Ym1. The protein was accredited with a function as eosinophil chemotactic cytokine (ECF-L) (48). We are conservative about this functional assignment for the following reasons. (i) Chemotactic activity was one of the initial activities we examined when purified and crystallized Ym1 was available. No chemotactic activities were ever found associated with purified Ym1 in either in vitro chemotaxis assays or in vivo assessment by intradermal injections on the back of mice. (ii) Ym1 is expressed by cells of myeloid lineage such as activated macrophages.

3 S. I. Hung, A. C. Chang, I. Kato, and N. C. Chang, submitted for publication.
phages and myeloid progenitors in bone marrow. It has never been detected in the lymphoid system, e.g. thymus and lymph node. In addition, selective up-regulation of Ym1 has been reported in a committed myeloid progenitor cell line EPRO but not in a multipotent cell line EML capable of differentiating into lymphoid cells (49). (iii) In their own report, Owhashi et al. (48) stated that the calculated isoelectric point (pl) derived from cDNA-deduced protein is 5.3, whereas pl of the chemotactic activity was determined empirically to be 3.6. A difference of 1.7 in pH units would strongly imply that the amino acid composition of the protein with the chemotactic activity is different from that of the protein whose cDNA they have cloned. (iv) In their assays, the chemotactic activity expressed by the postinfection splenocytes (or CD8+ T-cells) was dependent on the inclusion in the assay of parasitic extracts (i.e. 5–15 μg/ml Toxocara canis (50) or 100 μg/ml Mesocyclostoides corti larvae (48)); both are mixtures of unknown compositions and activities.

Molecular cloning of the Ym1 gene from a mouse genomic library (129 Sv/J) was pursued in parallel for the establishment of knock-out mice. Genomic organization of the Ym1 gene revealed the existence of at least 11 exons (Fig. 6A). The complex pattern derived from the exon probe and the rather simple pattern derived from the intron probe (Fig. 6B) suggest the possible existence of pseudogenes or a family of closely related genes. Using the same pairs of probes, murine chromosome mapping studies revealed that the intron probe (Ip1–2) hybridized strongly to only one band in each lane, while the exon probe hybridized well to five bands in each lane at relatively low stringency. In addition, bands hybridized with both probes co-segregated and mapped to the middle region of mouse chromosome 3. Multiple genes hybridized by exon probes are not pseudogenes but more likely different members of a closely related gene family. Employing our Ym1 cDNA sequence as probes, a separate study reported recently by Jin et al. (51) has reached the same conclusion.

Approximately 30% homology was revealed between Ym1 and many members of the microbial chitinase gene family (52, 53). However, no chitinolytic activity was detected in either purified or recombinant Ym1 using three different assay methods as stated under “Materials and Methods.” The notion that Ym1 is a chitinase was further supported by the fact that the two conserved acidic residues key to chitinase activity (i.e. Asp200 and Glu204) of Bacillus enzyme) are Asn and Gln in Ym1, respectively. Site-directed mutagenesis studies of Watanabe et al. (54) have clearly demonstrated that mutations from Asp200 to Asn200 or from Glu204 to Gln204 will both severely impair the enzyme activity. In addition to microbial chitinases, significant homology was also found between Ym1 and several “chitinase-like” proteins reported recently (i.e. human cartilage HC-gp39, human macrophage chitotriosidase, porcine smooth muscle gp38k, and Drosophila DS-47) (Fig. 5). Human HC-gp39 was first reported as a secretory glycoprotein found in the inflamed joint of arthritis patients only (34). The cell type responsible for HC-gp39 production was subsequently verified as the differentiating macrophages (55, 56). Human macrophage chitotriosidase is a chitinolytic enzyme found markedly elevated in plasma of Gaucher disease patients (57, 58). Porcine gp38k is a secretory glycoprotein synthesized by vascular smooth muscle cells during differentiation from monolayer to nodular form (59). Drosophila DS-47 is synthesized and secreted by macrophage-like hemocytes and cells in the fat body, which is equivalent to mammalian liver (37). Several intriguing points have emerged after comparing features unique to Ym1 and these proteins. (i) Just like Ym1, all share sequence similarity with chitinase, but none exhibits the respective enzymatic activity, with the exception of chitotriosidase. However, it is possible that their substrates may share structural features similar to chitin. (ii) All are secretory proteins, suggesting that their sites of action are most likely to be extracellular. (iii) All are inducible; peak expression is found during inflammation, development/differentiation, tissue remodeling, and/or wound healing. (iv) Despite all similarities, tissue-specific expression patterns of these proteins would argue against their being species variants of the same protein. It is conceivable that this group of proteins of “unknown” functions (except chitotriosidase) may be members of the Ym1 gene family, especially when one considers that HC-gp39 and chitotriosidase were mapped to the same chromosome (chromosome 1) where the human homologue of Ym1 and its gene members would be located (34, 51, 60).

Parallel studies of Ym1 using x-ray crystallography (79) revealed that Ym1 has a βα TIM barrel structure shared by many glycolytic enzymes and proteins that bind and transport metabolites (61). This further supports the notion that Ym1 may interact with its natural ligand bearing carbohydrate moiety. The binding properties of Ym1 to various saccharides were evaluated by a biosensor based on surface plasmon resonance (BIacore) (62, 63). Data indicate that Ym1 binds not to chitin (oligomer of GlcNAc) but to saccharides with a free amine group, such as GlcN, and its oligomers (Fig. 7). To increase the sensitivity of detection, biotinylated tetra-GlcN was immobilized onto the sensor chip for all subsequent binding studies (64). The optimum pH for the binding is in the range of pH 4.5–5.0 (Fig. 8A), which would suggest that the electrostatic force between Ym1 and GlcN saccharides is important, since the binding was significantly affected by pH. The affinity between Ym1 and tetra-GlcN was determined to be about 223 nm.

In addition, the clustering effect, a characteristic feature in carbohydrate-lectin interaction (65, 66), was revealed in the competition analyses, in which inhibition of binding by tetra-
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Ym1 (2.8 μM) was examined. Soluble oligosaccharides in buffer with pH as specified was injected and allowed to interact with the tetra-GlcN-coated surface. The responses were measured across a broad pH range from 4.0–9.0. Optimal binding was observed at a narrow pH range from 4.5 to 5.0. B, the relative binding affinity of GlcN oligosaccharides to immobilized Ym1 on a CM5 chip at pH 4.75 was examined. GlcN oligomers (0.5 mM) were separately injected over the Ym1-immobilized surface, and the sensorgrams were monitored at a flow rate of 30 μl/min for 180 s. C, binding kinetics of Ym1 to immobilized tetra-GlcN at pH 4.75 were examined. The reaction is concentration-dependent, and the slower kinetics in this binding paradigm allowed the estimation of the Kd. D, competition assays with GlcN saccharides. The binding of Ym1 (1.4 μM) to immobilized tetra-GlcN at pH 4.75 was competed by co-injection of various GlcN saccharides at specified concentrations. ●, GlcN monomer; ○, di-GlcN; ×, tri-GlcN; ■, tetra-GlcN. The ability of GlcN saccharides to compete for Ym1 was plotted as percentage inhibition.

GlcN is 1000-fold more effective than that by monomers of GlcN (Fig. 8D). Although numbers of macrophage-derived lectins have been identified and reported with binding specificity to mannose, mannose 6-phosphate, or Gal/GalNAc, respectively (67–69), Ym1, a secretory mediator produced by activated macrophages during inflammatory response, is a novel lectin with a GlcN binding specificity. To identify other ligands of Ym1, its ability to interact with heparin was examined. Specific binding of Ym1 to heparin in addition to GlcN dimers was revealed (Fig. 9). Although it is not clear how Ym1 interacts with heparin and if Ym1 interacts with heparan sulfate, we postulated that Ym1 might interact with heparin/heparan sulfate proteoglycans (HSPGs) via GlcN, as selectins (70). Further analysis should be performed to address this issue.

HSPGs have been recognized as ubiquitous ligands on cell surface and in extracellular matrix (ECM) (71). Enormous structural heterogeneity can be generated through specific HSPG chain modifications during their biosynthesis, as well as from the diverse nature of their core proteins. The GlcN residues in HSPGs may be N-sulfated, N-acetylated, or N-unsubstituted. Proteins anchored on glycosaminoglycan side chains of HSPGs may serve a variety of functional purposes, from simple immobilization or protection against degradation to modulation of distinct biological activities (72, 73). Induced expression of HSPGs (i.e. syndecan-2) on the surface of activated human macrophages has been reported (74). The functional significance of this transient and selective expression of HSPGs was elucidated as to deliver the sequestered growth factors (e.g. fibroblast growth factor, vascular endothelial growth factor, epidermal growth factor) also produced by inflammatory macrophages, to their appropriate receptors on fibroblasts or endothelial cells for signaling new tissue growth during the repair processes (74). Syndecan-1 has been identified as the major HSPG on murine macrophage cell surface (75). Whether secretory Ym1 would be sequestered by binding to syndecan-1 and delivered by its producing macrophages to its site of action warrants further study. Alternatively, inflammatory responses elicited by tissue injury or infections require the emigration of subsets of leukocytes from circulation to the inflamed loci. Selective recognition between extravasated leukocytes, endothelial cells, and the local ECM adjacent to the inflammatory tissues all involve lectin-carbohydrate binding (76, 77). Evidence supports the possibility that the nature of a particular inflammatory stimulus determines the extravasation of certain immune cell subtypes and the compositional changes of ECM. The immune mediators secreted by the leukocytes would act in concert with ECM to either promote or diminish the inflammatory responses (42). Reports have established that the N-unsubstituted GlcN residues are enriched in native heparan sulfate species capable of binding to P- and L-selectins and thus potentially involved in regulating leukocyte traffic (70, 72, 78). Based on the observations in the present study, secretory Ym1 is transiently expressed in the peritoneal cavity after T. spiralis infection. Its inducible expression is not at the initial stage of infection but rather at a stage (day 15–17) when the infiltrating leukocytes are “leaving” the inflammatory peritoneal cavity, as evidenced by the parallel rapid fall of total cell counts (Fig. 1A). With a binding specificity similar to that of the homing receptors (selectins) for leukocytes, it is tempting to propose that a pulse of Ym1 secretion at the inflammatory foci was competing for binding sites on local ECM occupied by the infiltrating leukocytes. Diminished local inflammatory reactions may subsequently initiate the reestablishment of homeostasis. Structure features of Ym1 responsible for GlcN binding are reported in the accompanying x-ray crystallography study (79), which will provide definitive insights.
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for future design of agents that may be important for prevention and/or treatment of inflammation.

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A Macrophage Protein, Ym1, Transiently Expressed during Inflammation Is a Novel Mammalian Lectin
Nan-Chi A. Chang, Shuen-Iu Hung, Kuo-Yuan Hwa, Ikunoshin Kato, Ju-Eng Chen, Cheng-Hsiun Liu and Alice Chien Chang

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