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Identification of an Inflammation-inducible Serum Protein Recognized by Anti-disialic Acid Antibodies as Carbonic Anhydrase II

Zenta Yasukawa[1,2], Chihiro Sato[2,3,*] and Ken Kitajima[1,2,3]

[1]Laboratory of Animal Cell Function, Bioscience and Biotechnology Center; [2]Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences; and [3]Institute for Advanced Research, Nagoya University, Nagoya 464-8601, Japan

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Acute-phase proteins are an important marker of inflammation and sometimes have a role in the general defense response towards tissue injury. In the present study, we identified a 32-kDa protein that was immunoreactive with monoclonal antibody 2-4B (mAb.2-4B), which is specific to di/oligoNeu5Gc structures, and that behaved as an acute-phase protein following stimulation with either turpentine oil or lipopolysaccharides. The 32-kDa protein was identified as carbonic anhydrase II (CA-II), based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analyses of the purified protein. Mouse and human CA-II was immunoreactive and immunoprecipitated with mAb.2-4B, but contained no sialic acid. In addition to mAb.2-4B, the mAb. S2-566 an antibody specific for diNeu5Ac-containing glycans, recognized the CA-II, whereas an anti-oligo/polysialic acid antibody did not. These results indicate that a part of the CA-II protein structure mimics the disialic acid structure recognized by the monoclonal antibodies. This is the first report that CA-II circulates in the serum following inflammation.

Key words: acute-phase protein, carbonic anhydrase II, disialic acid, inflammation, serum.

Abbreviations: diSia, disialic acid; DMB, 1,2-diamino-4,5-methylenedioxybenzene; PBS, phosphate-buffered saline; PBS-T, PBS containing 0.05% Tween 20; PVDF, polyvinylidene difluoride; CA-II, carbonic anhydrase II.

After injury, a host organism attempts to prevent ongoing tissue damage by isolating and destroying the infective organism, and activating repair processes. This cumulative homeostatic process is known as inflammation (*1*, *2*). Many physical and chemical factors, such as hot/cold stimuli, wounds, lipopolysaccharides (LPS), turpentine oil and tetrachloromethane, induce inflammation and acute-phase responses (*3*). The acute-phase response is induced by cytokines acting as messengers between the local site of injury and the hepatocytes synthesizing the acute-phase proteins (*1*, *2*, *4*). A major systemic event that occurs in mice following the induction of inflammation is a marked change in the levels of certain serum proteins. The concentrations of these serum proteins are considered to be sensitive indicators, useful for diagnostic and prognostic assessments of inflammation and infection (*5*).

Although most acute-phase proteins are glycoproteins, little attention has been paid to the alterations of the glycotopes on glycoproteins during inflammation, except for concanavalin A (ConA)-reactive *N*-glycans (*6*). Structural changes of the *N*-linked glycans on some serum glycoproteins, such as α_1-acid glycoprotein, α_1-esterase and α_1-protease inhibitor, during inflammation were studied using crossed affinity immunoelectrophoresis with ConA, demonstrating that the ConA glycotope on serum glycoproteins changes from ConA strongly bound forms to ConA weakly bound forms in mouse (*6*). Previously, we focussed on changes in the expression of α2,3-, α2,6- and α2,8-linked sialic acid glycotopes on serum glycoproteins, especially the expression of acute-phase glycoproteins, under inflammatory conditions using lectins and a monoclonal antibody 2-4B (mAb.2-4B) that recognizes a disialic acid structure [α2,8-linked *N*-glycolylneuraminic acid (Neu5Gc) dimer]. In mouse serum, four components of the 30, 32, 70 and 120 kDa were shown to be immunostained with the mAb.2-4B. Recently, we identified three of the four components as immunoglobulin, vitronectin and plasminogen and disalic acid residues were clearly identified by chemical analyses (*7*). Although these three components did not increase after inflammation, the 32-kDa component was found to behave as an acute-phase protein (*8*). In the present study, we tried to identify the acute-phase protein reactive with mAb.2-4B and demonstrated that the 32-kDa-gp was carbonic anhydrase II.

Carbonic anhydrase (CA) catalyses the reversible hydration of carbon dioxide (*9–11*), and function in a wide variety of biologic activities, such as acid–base balance, bone resorption, calcification, etc (*12–15*). Among a large number of CA family members, CA-II is

*To whom correspondence should be addressed. Tel: +81-52-789-4297, Fax: +81-52-789-5228, E-mail: chi@agr.nagoya-u.ac.jp

described as a non-secretory, cytoplasmic resident protein that cooperates with integral plasma membrane transport proteins that regulate bicarbonate and proton passage in most organs (*15–18*). However, it is also reported that CA-II is secreted from the coagulating gland (*19*). In addition, CA-II is present in the blood vessel epithelial cells in some cancer patients (*20*). Furthermore, anti-CA-II antibodies are present in serum from patients with chronic viral hepatitis (*21*) or systemic sclerosis (*22*). Therefore, it is known that CA-II is present not only in the cytoplasm, but also in the serum by an unknown mechanism.

In the present study, we identified serum CA-II as an acute-phase protein by MALDI-TOF MS analysis, although there was no sialylation on CA-II in serum.

MATERIALS AND METHODS

Materials—Sephacryl S-100, Protein G Sepharose and enhanced chemiluminescence reagents were purchased from Amersham Biosciences (Piscataway, NJ, USA). DEAE–Toyopearl 650M and *p*-aminomethylbenzenesulfonamide-agarose resins were purchased from Tosoh (Tokyo, Japan) and Sigma (St Louis, MO, USA), respectively. Polyvinylidene difluoride (PVDF) membrane (Immobilon P) was a product of Millipore (Bedford, MA, USA). Prestained molecular markers were purchased from Bio-Rad (Hercules, CA, USA) or Daiichi Pure Chemicals Co., Ltd (Tokyo, Japan) or Sigma. Anti-carbonic anhydrase II antibody was purchased from Rockland (Gilbertsville, PA). Peroxidase-conjugated rat anti-mouse IgM was purchased from Zymed Laboratories Inc. (San Francisco, CA, USA). Mouse monoclonal IgM antibody 2-4B, which recognizes Neu5Gcα2→(8Neu5Gcα2→)$_{n-1}$, $n \geq 2$ was prepared as described previously (*23*). Mouse monoclonal IgM antibody S2-566, which recognizes Neu5Acα2→8Neu5Acα2→3Gal, mouse monoclonal IgM antibody 12E3, which recognizes (8Neu5Acα2→)$_n$, $n \geq 5$, mouse monoclonal IgG antibody AC1, which recognize (Neu5Gc)n, $n = 2$–4, was gifted from Dr Koichi Furukawa (Nagoya University, School of Medicine, Nagoya Japan), Dr Tastunori Seki (Juntendo University School of Medicinem Tokyo Japan) and Dr Keiko Nohara (Environmental Health Sciences Division, National Institute for Environmental Studies, Tsukuba, Japan), respectively, and were prepared as described previously (*23*). Peroxidase-conjugated rabbit anti-goat IgG, goat antiserum to human haptoglobin β-chain and rat anti-mouse IgM were purchased from Cappel (West Chester, PA, USA). 1,2-Diamino-4,5-methylenedioxybenzene (DMB) was purchased from Dojindo (Kumamoto, Japan). Male, 8-week-old ddY mice were obtained from Japan SLC Co. (Hamamatsu, Japan). Turpentine oil was kindly provided by Dr Hiroaki Oda (Nagoya University, Nagoya, Japan). Lipopolysaccharide was purchased from Sigma.

Experimental Inflammation—Injection of turpentine oil subcutaneously into ddY mice to induce inflammation and preparation of sera were performed as described previously (*8*). In case of inducing inflammation with lipopolysaccharide, mice were injected with 100 μl of 1 mg/ml of lipopolysaccharide diluted in 0.9% sterile saline into peritoneal cavity. The present study was approved by the Animal Experimental Committee of the Graduate School of Bioagricultural Sciences, Nagoya University.

Purification of the 32-kDa Component from Mouse Serum—Inflamed mice sera (20 ml) was mixed with 20 ml of saturated $(NH_4)_2SO_4$ (final concentration, 50%), stirred at 4°C for 3 h and centrifuged at 10,000 *g* for 15 min. The supernatant was mixed with 26.6 ml of saturated $(NH_4)_2SO_4$ (final concentration, 70%), stirred at 4°C for 12 h and centrifuged at 10,000 *g* for 15 min. The pellet was dissolved in 50 mM Tris–HCl (pH 8.0) containing 50 mM NaCl and applied to Sephacryl S-100 chromatography [1.3 × 80.5 cm, equilibrated with 50 mM Tris–HCl (pH 8.0) containing 50 mM NaCl]. The elution profile was monitored by absorbance at 280 nm and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Coomassie Brilliant Blue (CBB) staining and western blotting using mAb.2-4B. The fractions containing the 32-kDa component were pooled, applied to DEAE–Toyopearl 650 M chromatography [1.0 × 24 cm, equilibrated with 50 mM Tris-HCl (pH 8.0) containing 50 mM NaCl] and eluted in a step-wise manner with 0.05, 0.1, 0.15, 0.3 and 1.0 M NaCl containing 50 mM Tris–HCl (pH 8.0). The 0.05 M NaCl eluted fractions containing the 32-kDa component were pooled, concentrated and re-applied to Sephacryl S-100 chromatography [1.3 × 80.5 cm, equilibrated with 20 mM Tris–HCl (pH 9.25) containing 10 mM NaCl]. The fractions containing the 32-kDa component were re-applied to DEAE–Toyopearl 650 M column [1.0 × 24 cm, equilibrated with 20 mM Tris–HCl (pH 9.25) containing 10 mM NaCl], and eluted in a step-wise manner with 0.01, 0.02, 0.04, 0.05, 0.1 and 1.0 M NaCl containing 20 mM Tris–HCl (pH 9.25). The elution profile was monitored as described earlier.

SDS–Page and Western Blotting—Samples were dissolved in Laemmli buffer with 5% mercaptoethanol and boiled at 100°C for 3 min. The samples were electrophoresed on 10 or 15% polyacrylamide gel and visualized by CBB staining or silver staining. The glycoproteins separated were electroblotted onto PVDF membranes using a semi-dry blotting apparatus. The membrane was blocked with 10 mM sodium phosphate buffer (pH 7.2) and 0.15 M NaCl with 0.05% Tween 20 containing 1% bovine serum albumin at 25°C for 1 h. The membrane was incubated with a primary antibody, mAb.2-4B (0.50 μg/ml), S2-566 (1.0 μg/ml), 12E3 (0.36 μg/ml) or anti-haptoglobulin antibody (5.0 μg/ml) at 4°C for 16 h. For secondary antibody, peroxidase-conjugated anti-mouse IgM (1/5000 dilution) or anti-goat antibody (1/5000 dilution) was used. The colour development was carried out as described (*24*).

ELISA Analysis—ELISA was carried out as described (*24*).

2-Dimensional Gel Electrophoresis—Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was performed with a PROTEAN IEF Cell (Bio-Rad) using 7 cm pH 3–10 ReadyStrip IPG Strip (Bio-Rad). Protein samples were resuspended in rehydration buffer (9.8 M urea, 4% CHAPS and 100 mM DTT) with the ratio of 1: 3. Isoelectric focussing was performed at 250 V for 15 min, 250 to 4000 V linear ramp for 2 h and 4000 V

for 5 h at 20°C. After the gel strips were equilibrated for 10 min in 6 M urea, 2% SDS, 0.375 M Tris–HCl (pH 8.8), 20% glycerol and 130 mM DTT, and then re-equilibrated for 10 min in the same buffer containing 135 mM iodeacetamide in place of DTT. The proteins were separated by SDS–PAGE.

In Gel Digestion and MALDI-TOF MS—In gel trypsin digestion of CBB stained bands and MALDI-TOF MS analyses were performed as described (*7*, 8).

Affinity Purification of Carbonic Anhydrase II—Inflamed mice serum (0.8 ml) was resuspended with 4.2 ml of 10 mM HEPES (pH 7.5) and applied to *p*-aminomethylbenzenesulfonamide agarose. The column was washed with 10 mM HEPES (pH 7.5) and eluted in a step-wise manner with 0.01, 0.05, 0.1 and 1 M NaCl in 10 mM HEPES (pH 7.5).

Fluorescent C_7/C_9 Analysis, and Mild Acid Hydrolysis-DMB Derivatization Followed by Anion-Exchange Chromatography—Fluorescent C_7/C_9 analyses were performed as earlier (*25*). Mild acid hydrolysis-DMB derivatization followed by anion-exchange chromatography was conducted as described previously (*26*).

Tryptic Digestion of CA-II—Bovine CA-II (2 μg) in 0.1 M NH_4HCO_3 was heat-denatured at 100°C for 3 min, and was added 0.1 μg of trypsin and digested at 37°C for 22 h.

Immunoprecipitation of CA-II—Human CA-II (9 μg) in PBS or inflamed mouse serum (12.5 μl) that had been pretreated with protein G-Sepharose were subjected to immunoprecipitation using protein G-Sepharose coupled with mAb.2-4B via goat anti-mouse IgM antibodies (Sigma). Immunoprecipitates were subjected to the SDS–PAGE/western blot analysis with anti-CA-II antibody as described earlier.

RESULTS

In a previous study (*8*), we reported that four components in mouse serum were immunoreactive with mAb.2-4B and that the expression of a 32-kDa component was upregulated after turpentine oil injection. To confirm that the 32-kDa component is an acute-phase protein, we used two different reagents, turpentine oil and LPS, to induce inflammation. Under normal conditions, the 32-kDa component was slightly or not at all reactive with mAb.2-4B in mouse serum depending on individual mice (Fig. 1a, IB: 2-4B, day 0). Turpentine oil injection induced an increase in the amount of the 32-kDa component (Fig. 1a, IB: 2-4B, day 2 and 4) and the intensity of mAb.2-4B immunostaining increased until day 2 to reach a 3.7-fold higher level compared to that at day 0 (Fig. 1c). Stimulation with LPS also induced an increase in the amount of the mAb.2-4B-immunoreactive 32 kDa component (Fig. 1b, IB: 2-4B) and the intensity of mAb.2-4B immunostaining increased until day 2 to reach a 9.6-fold higher level compared to that at day 0, and decreased at day 5 (Fig. 1c). The induction of inflammation by injection of turpentine oil or LPS was confirmed by an increase of haptoglobin, which is a known acute-phase protein, detected with anti-haptoglobin antibodies (Fig. 1a and b). The mAb.2-4B-reactive component that was detected at 30 kDa (Fig.1a and b) was already identified as a light chain of immunoglobulins (*7*), and its expression was independent of the inflammation stimuli. These results indicate that the 32-kDa component is an acute-phase protein.

Purification of the 32-kDa Component—The 32-kDa component was salted-out with 70% $(NH_4)_2SO_4$ using supernatant derived from 50%-saturated ammonium sulphate solution of inflamed mouse serum. The precipitates were separated by Sephacryl S-100 gel filtration chromatography. The elution was monitored by absorbance at 280 nm (Fig. 2a) and by SDS–PAGE followed by CBB staining and western blotting using mAb.2-4B (data not shown). Fractions 44 to 61 were pooled and further separated by DEAE–Toyopearl 650 M anion exchange chromatography and eluted in a step-wise manner. The elution was monitored by absorbance at 280 nm (Fig. 2b) and by SDS–PAGE followed by CBB staining and western blotting (data not shown). The 32-kDa component was detected in fractions eluted with 50 mM NaCl. For further purification of the 32-kDa component, the fractions eluted with 50 mM NaCl were pooled and purified with Sephacryl S-100 gel filtration chromatography. The elution was monitored as described earlier (Fig. 2c, top). The 32-kDa component was detected in fractions 39 to 43 by SDS–PAGE, followed by CBB staining (Fig. 2c, middle) and western blotting with mAb.2-4B (Fig. 2c, bottom). Fractions 39 to 41 were pooled and further purified by DEAE–Toyopearl 650 M anion exchange chromatography and eluted in a step-wise manner with NaCl (data not shown). The 32-kDa component was detected in fractions eluted with 40 and 50 mM NaCl (Fig. 2d, lanes c and d), by SDS–PAGE, followed by CBB staining (Fig. 2d, left) and western blotting (Fig. 2d, right).

MALDI-TOF MS Analyses of the 32-kDa Component—The 32-kDa component was further separated by two-dimensional gel electrophoresis using fractions eluted with 40 and 50 mM NaCl (Fig. 3a). Spots of the 32-kDa component that appeared at pI 7–7.4 were excised and digested with trypsin. The obtained peptides were analysed by MALDI-TOF MS analyses (Fig. 3b). Based on the peptide mass fingerprints obtained by MALDI-TOF MS followed by database searches, the observed peaks from the 32-kDa component were considered to come from the CA-II (Fig. 3c and Table 1). Of 12 peptide sequences, 5 were confirmed by tandem mass spectrometry (MS/MS) analyses (Fig. 3d–h). The observed sequences accounted for 63% of the CA-II (**Q7TPE1**).

Affinity Purification of CA-II Using p-aminomethylbenzenesulfonamide-agarose—MALDI-TOF MS strongly suggested that the 32-kDa component, which was specifically immunostained with mAb.2-4B, was CA-II (Fig. 3 and Table 1). To confirm this result, we purified CA-II from inflamed mouse serum using a conventional method. CA-II can be purified using a *p*-aminomethylbenzenesulfonamide coupled column (*27*). Thus, we applied inflamed mouse serum to this column, and eluted CA-II with KSCN. Western blot analysis using mAb.2-4B as a primary antibody indicated that the fraction eluted with 0.4 M KSCN contained the 32-kDa

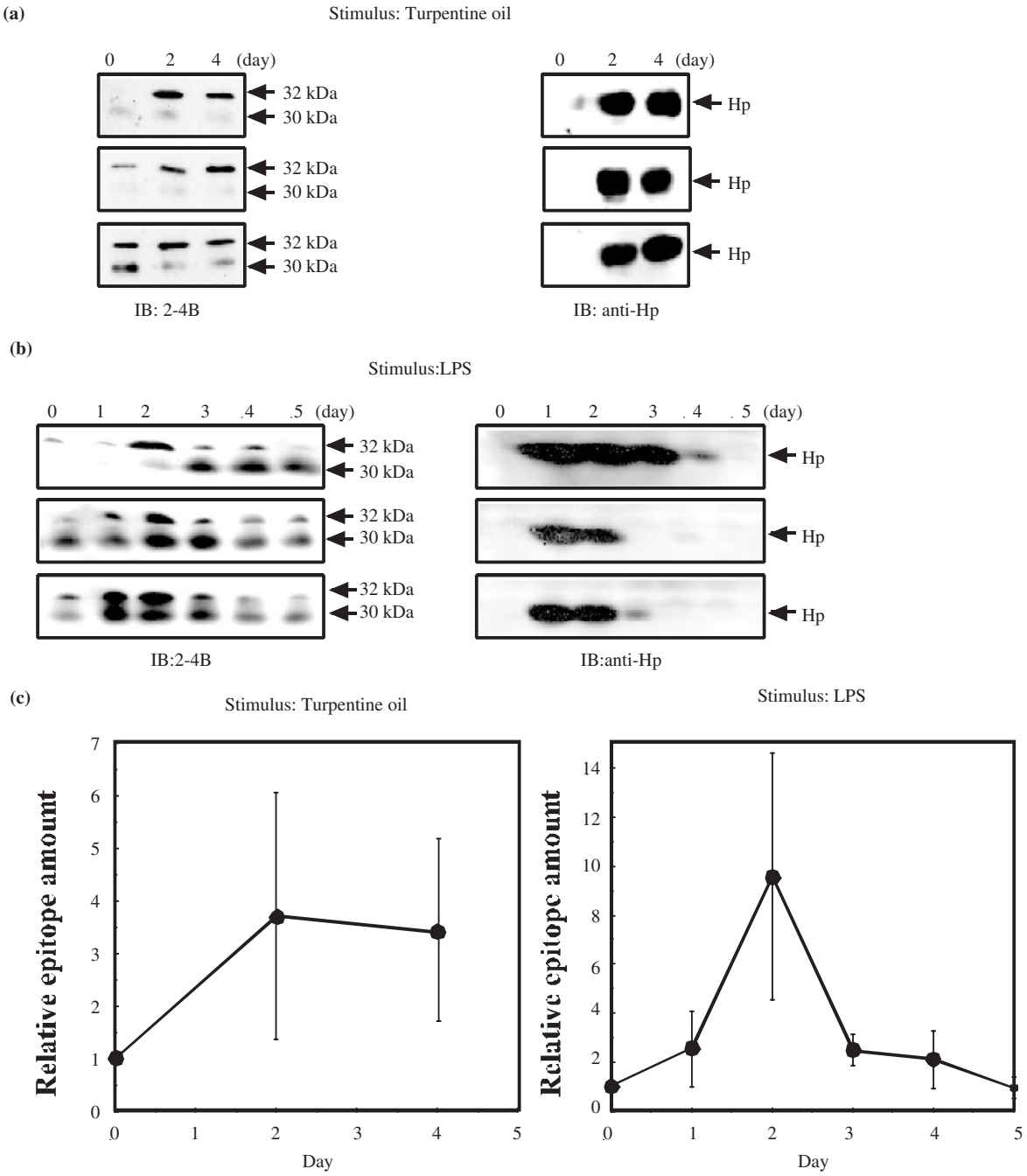


Fig. 1. **Upregulation of the 32-kDa component under and turpentine oil- and lipopolysaccharide (LPS)-induced inflammation.** Three mice were subcutaneously injected with turpentine oil (a) or intraperitoneally injected with LPS (b) at day 0. Before (day 0) and 1–5 days after the injection (days 1–5), blood was collected and serum was prepared. Serum (0.5 μl; 15 μg as protein) was subjected to SDS–PAGE/electroblotting on PVDF membrane, and the membrane was immunostained using mAb.2-4B, which is specific to the Neu5Gcα2→8(Neu5Gcα2→)$_{n-1}$, ($n \geq 2$) structure, as a primary antibody (IB: 2-4B). Immunoblot was also performed with anti-haptoglobin polyclonal antibodies (IB: anti-Hp) to verify inflammation. (c) Relative mAb.2-4B-epitope amount. The values at each day represent relative intensity of mAb.2-4B-immunostained bands. The average values at day 0 are set equal to 1.0. The standard deviations are indicated by the bar ($n = 3$).

component (Fig. 4a, IB: 2-4B, lane g). To further demonstrate that the mAb.2-4B-immunostained band at 32-kDa component is CA-II, we analysed the mouse serum before and after inflammation using both mAb.2-4B and anti-CA-II antibody. As shown in Fig. 4b, the 32-kDa component was immunostained not only with mAb.2-4B, but also with anti-CA-II antibody. These results shown in Figs. 3 and 4 indicate that the 32-kDa component was CA-II. In Fig.4b, we used two antibodies, anti-CA-II antibody (polyclonal IgG) and mAb.2-4B (monoclonal IgM). The amount of mAb.2-4B epitope per molecule is smaller than that of polyclonal

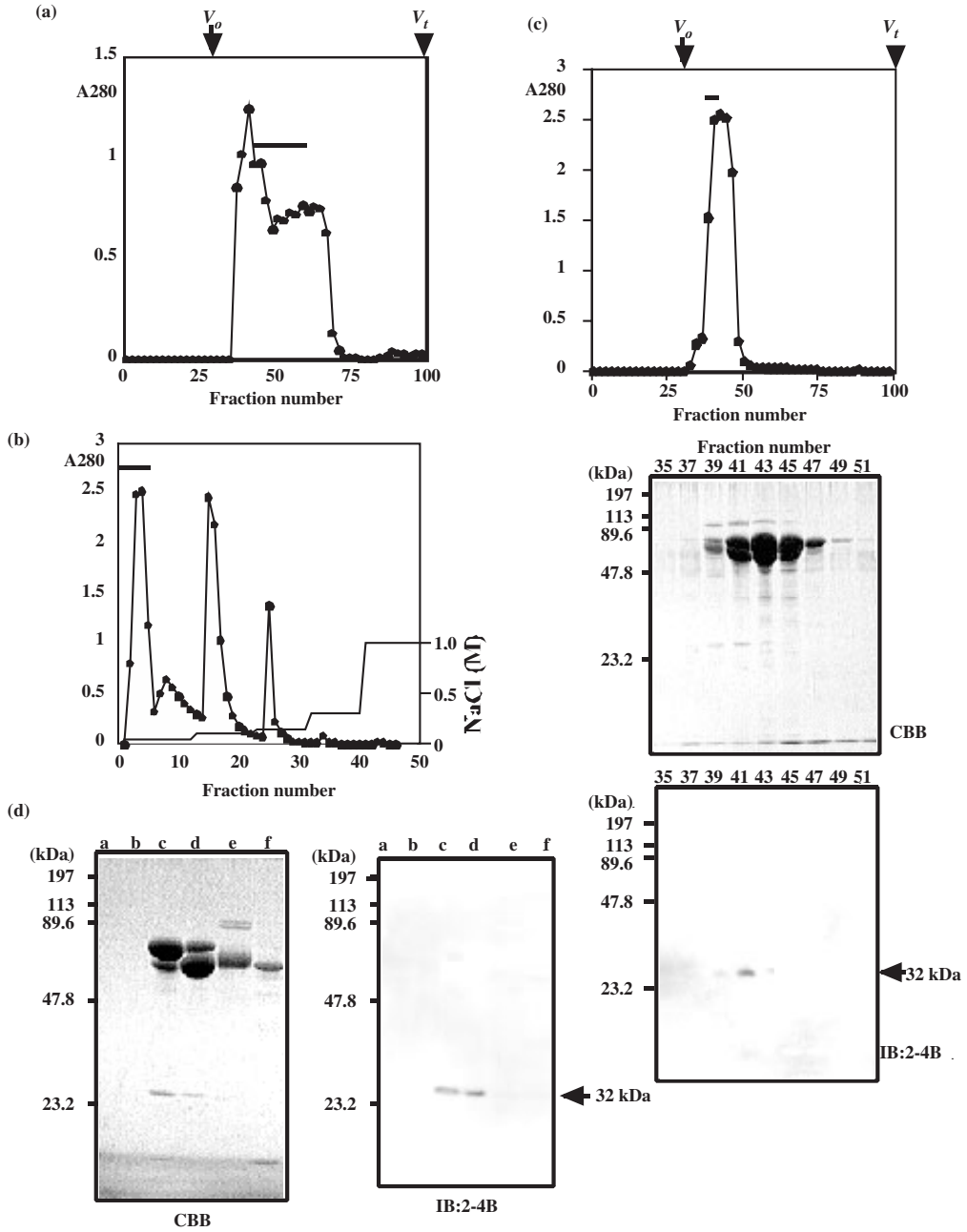


Fig. 2. **Purification of the 32-kDa component from inflamed mouse serum.** (a) Sephacryl S-100 chromatography of 50–70% ammonium sulphate precipitates of inflamed mouse serum. Mice were intraperitoneally injected with LPS and 2 days after the injection blood was collected and serum was prepared. The serum was subjected to ammonium sulphate precipitation as described under section 'Materials and Methods'. The 50–70% ammonium sulphate precipitate was applied to the column (1.3 × 80.5 cm) and eluted with 0.05 M NaCl in 50 mM Tris–HCl (pH 8.0). The elution profile was monitored by the absorbance at 280 nm. V_o, void volume, V_t, total volume. Major 32-kDa component-containing fractions (fractions 44–61), indicated by the bar, were pooled. (b) DEAE–Toyopearl 650 M chromatography of the major 32-kDa component-containing fraction in (a). The fraction was applied to a DEAE–Toyopearl 650 M column (Cl^- form; 1.0 × 24 cm), and eluted with discontinuous gradients of NaCl in 0.05 M Tris–HCl (pH 8.0) as described under section 'Materials and Methods'. The NaCl concentration is indicated by the line without symbol. The 32-kDa component-containing fractions (fractions 1–8 at 0.05 M NaCl) were pooled as indicated by the bar. (c) Sephacryl S-100 chromatography of the pooled fraction in (d). The column (1.3 × 80.5 cm^2) was eluted with 0.01 M NaCl in 20 mM Tris–HCl (pH 9.25). The elution profile was monitored by the absorbance at 280 nm. The major 32-kDa component-containing fractions (fractions 39–41) indicated by the bar, were pooled. The fractions indicated were analysed by SDS–PAGE/CBB staining (CBB) and western blotting with mAb.2-4B (IB: 2-4B). Positions of the molecular mass markers are indicated on the left. (d) SDS–PAGE/CBB staining (CBB) and western blotting with mAb.2-4B (IB: 2-4B) of the fractions eluted from the re-chromatography of the DEAE–Toyopearl 650 M chromatography. Aliquots (100 μl) of fractions were concentrated with Microcon YM-10 and loaded on 10% polyacrylamide gels. The 0.01 M NaCl eluate (lane a), 0.02 M NaCl eluate (lane b), 0.04 M NaCl eluate (lane c), 0.05 M NaCl eluate (lane d), 0.1 M NaCl eluate (lane e) and 1.0 M NaCl eluate (lane f) were analysed. Positions of the molecular mass markers are indicated on the left.

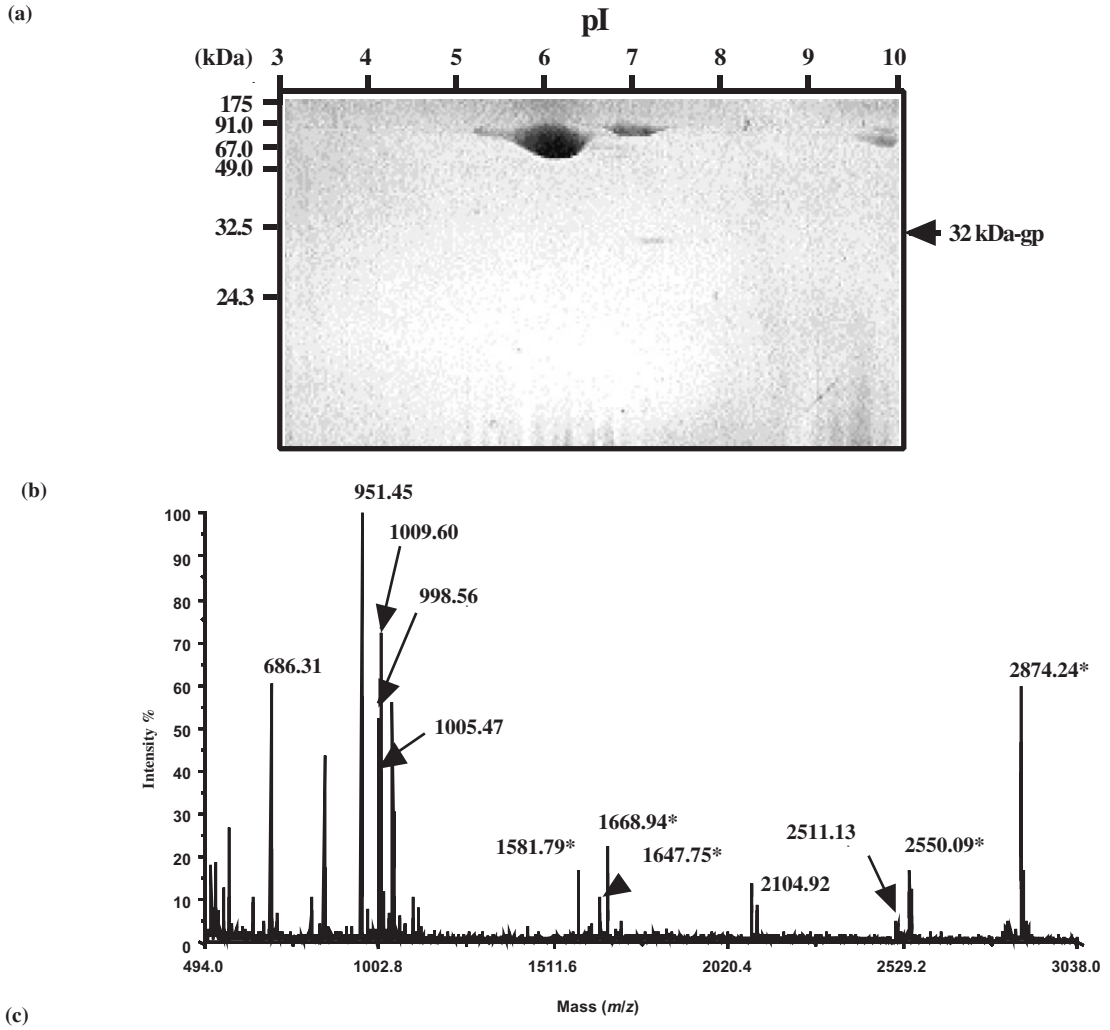
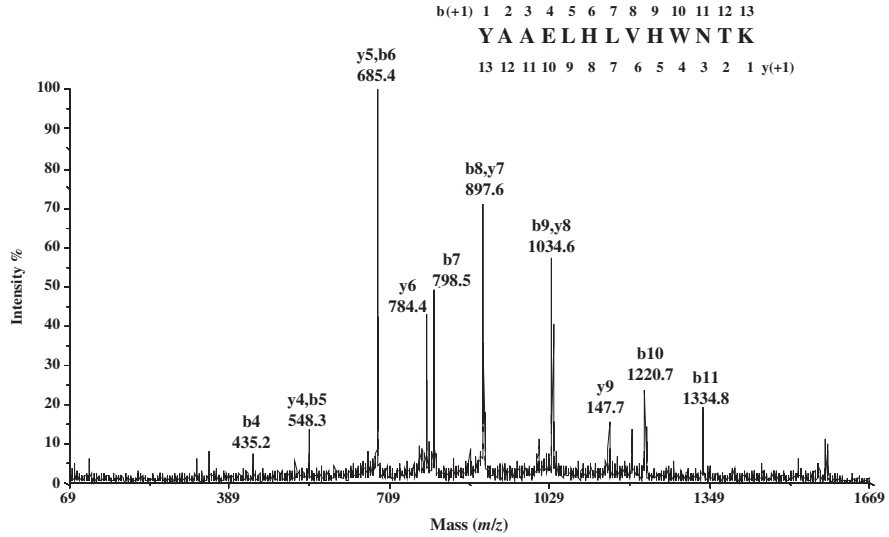


Fig. 3. **MALDI-TOF MS and MS/MS analyses of a tryptic digest of the 32-kDa component.** (a) Two-dimensional gel electrophoresis patterns of the 0.05 M NaCl-eluate of DEAE–Toyopearl 650 M chromatography. The eluate (90 μg as BSA) was concentrated by Microcon YM-10 and subjected to two-dimensional PAGE as described under section 'Materials and Methods'. The gel was stained with CBB. (b) MS spectrum (reflectron mode) of a tryptic digest of the reduced and alkylated 32-kDa component. The 32-kDa component spot observed in (a) was excised, digested in gel by trypsin and analysed by MALDI-TOF MS as described under section 'Materials and Methods'. Asterisks indicate the peptides that were further validated by MS/MS. (c) Amino acid sequence of CA-II (Accession number Q7TPE1). Observed peptides are underlined in bold. (d)–(h) Mass spectrometric fragmentation (MS/MS) spectra of $MH^+ = 1581.79$ (d), 1647.75 (e), 1668.94 (f), 2550.09 (g) and 2874.24 (h). Characteristic b- and y-type ions that were used for sequence tagging of the peptide are indicated. The determined sequence was matched to CA-II (c).

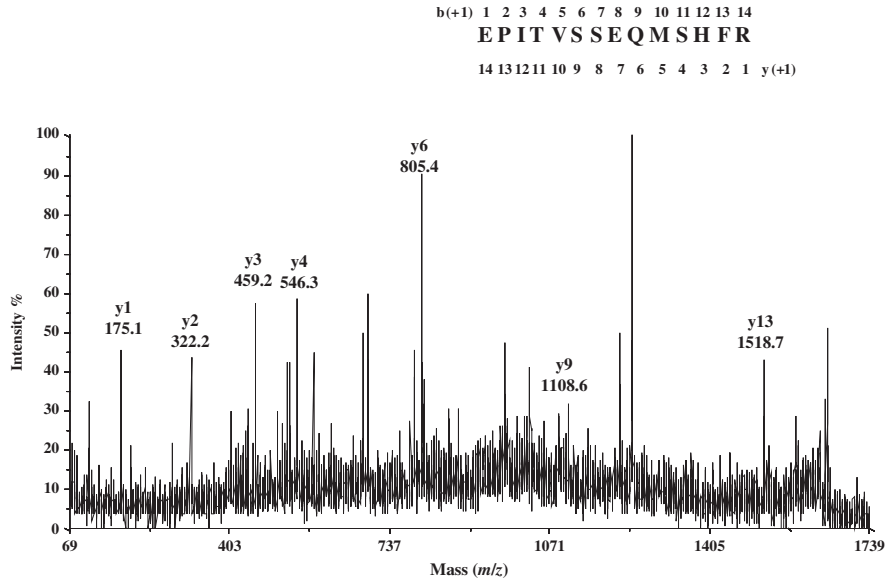
anti-CA-II epitope. In addition, IgM often shows lower affinity than IgG. Taken together, the difference of the intensity of the immunostaining between mAb.2-4B and anti-CA-II (Fig. 4b) might come from the properties of the antibodies we used (monoclonal *vs* polyclonal and IgM *vs* IgG).

Chemical Analyses of Sialic Acids—Neither disialic acid nor sialic acid was detected by fluorometric C_7/C_9

(d) 1581.79



(e) 1647.75



(f) 1668.94

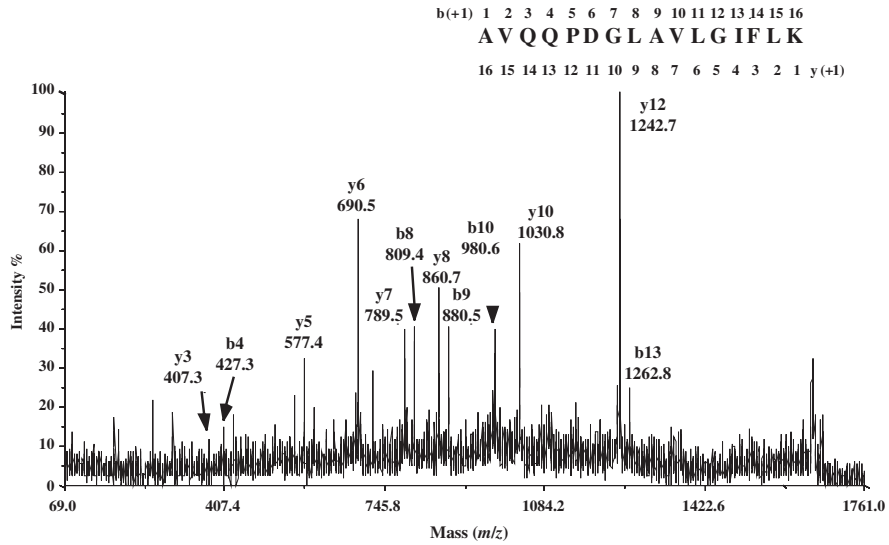


Fig. 3. Continued.

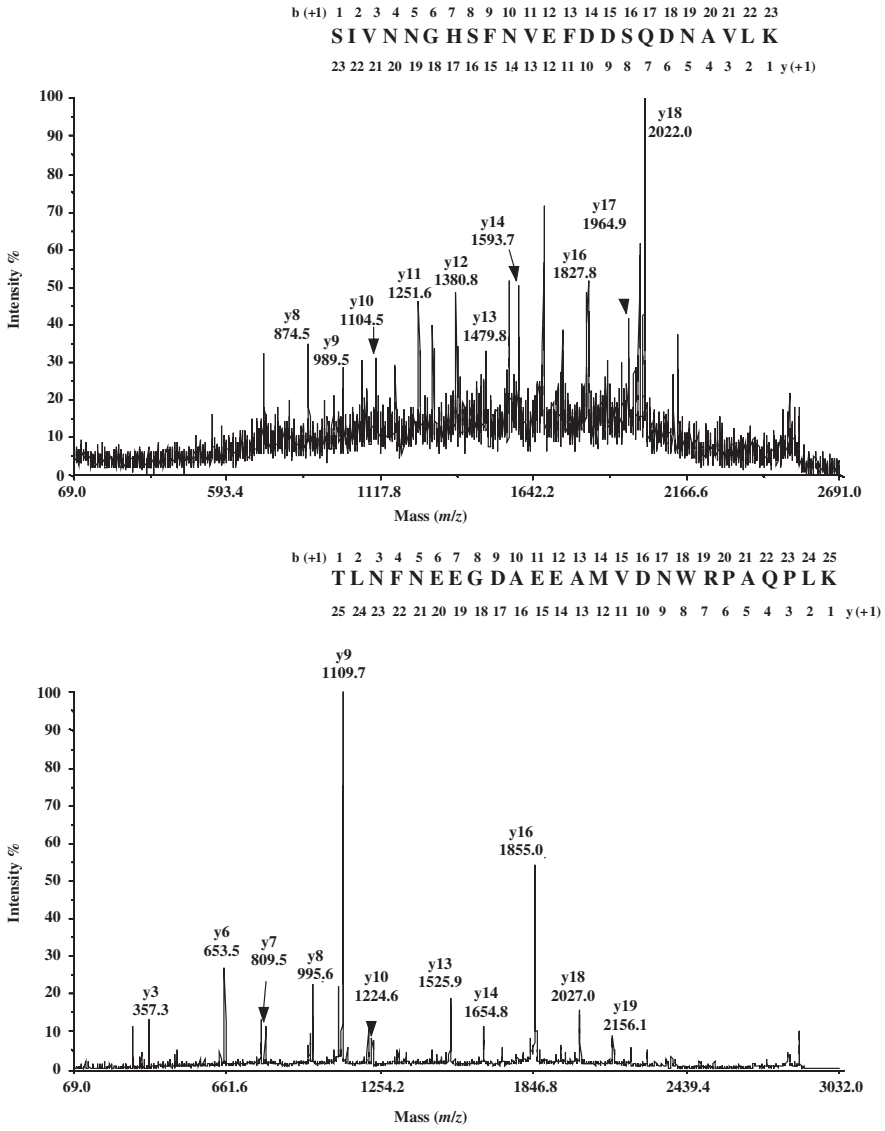


Fig. 3. Continued.

Table 1. **Peptide fragments obtained by the MALDI/TOF MS analysis of the 32 kDa-gp.**

Peptide sequence	Miscleavage	MH^+ obs.	Mr theor.	Delta (Da)
HNGPENWHKDFPIANGDR (10–27)	1	2104.92	2102.97	0.94
DFPIANGDR (19–27)	0	1005.47	1003.47	0.99
SIVNNGHSFNVEFDDSQDNAVLK (58–80)	0	2550.09	2548.18	0.90
GGPLSDSYR (81–89)	0	951.45	950.45	0.00
LIQFHFHWGSSDGQGSEHTVNK (90–111)	0	2511.13	2510.17	−0.05
YAAELHLVHWNTK (114–126)	0	1581.79	1580.81	−0.02
YGDFGK (127–132)	0	686.31	685.31	−0.00
AVQQPDGLAVLGIFLK (133–148)	0	1668.94	1667.96	−0.03
IGPASQGLQK (149–158)	0	998.56	997.56	0.00
VLEALHSIK (159–167)	0	1009.60	1008.60	0.00
EPITVSSEQMSHFR (213–226)	0	1647.75	1646.77	−0.02
TLNFNEEGDAEEAMVDNWRPAQPLK (227–251)	0	2874.24	2873.33	−0.10

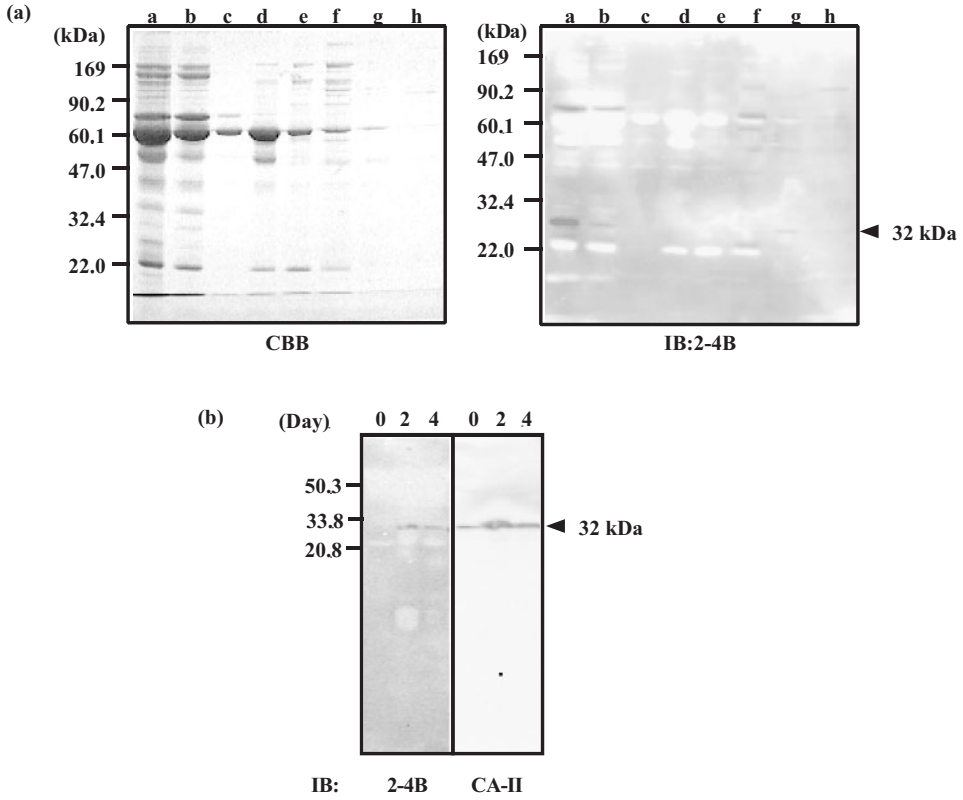


Fig. 4. **Affinity purification of CA-II.** (a) CA-II was affinity-purified using *p*-aminomethylbenzenesulfonamide agarose as described under section 'Materials and Methods' and subjected to SDS–PAGE/CBB staining (CBB) or western blotting using mAb.2-4B (IB: 2-4B). Mouse serum (lane a), the 10 mM HEPES (pH 7.5) eluate (lane b), 0.01 M NaCl in 10 mM HEPES (pH 7.5) eluate (lane c), 0.05 M NaCl in 10 mM HEPES (pH 7.5) eluate (lane d), 0.1 M NaCl in 10 mM HEPES (pH 7.5) eluate (lane e), 1 M NaCl in 10 mM HEPES (pH 7.5) eluate (lane f), 0.4 M KSCN in 10 mM HEPES (pH 7.5) eluate (lane g) and 3 M KSCN in 10 mM HEPES (pH 7.5) eluate (lane h) were analysed. Positions of molecular mass markers are indicated on the left. (b) Mouse was subcutaneously injected with turpentine oil at day 0. Before (day 0) and 2 and 4 days after the injection, blood was collected and serum was prepared. Serum (0.5 μl; 15 μg as protein) was subjected to SDS–PAGE/electroblotting on PVDF membrane, and the membrane was immunostained using mAb.2-4B (IB: 2-4B) and anti-carbonic anhydrase II antibody (IB: CA-II).

analysis or mild acid hydrolysis anion exchange chromatography analysis (data not shown).

Western Blot Analyses of CA Derived From Mouse, Human and Bovine—CA-II is immunoreactive with mAb.2-4B, which is immunospecific for the di/oligoNeu5Gc structure. Di/oligosialic acid, however, was not observed. To examine the differences in CA immunostaining between species, we analysed mouse and human erythrocytes, which are a rich source of CA-II (*27*) by western blotting using mAb.2-4B. We also analysed purified bovine CA-II. Mouse, human and bovine CA were immunostained with mAb.2-4B (Fig. 5a). Because humans have negligible Neu5Gc residues in their tissues due to the absence of CMP-Neu5Ac hydroxylase activity (*28*), and because mAb.2-4B does not react with di/oligoNeu5Ac, the mAb.2-4B immunostaining was not due to the presence of sialyloligomers, but rather from the protein portion of CA-II. To examine the immunoreactivity of anti-sialyloligomer antibodies towards CA-II, we used another disialic acid-recognizing monoclonal IgM, S2-566 (epitope: Neu5Acα2,8Neu5Acα2,3Gal) and a polysialic acid-recognizing monoclonal IgM antibody, 12E3 (epitope: oligo/polymer of α2,8-linked Neu5Ac structure). Although mAb.S2-566 clearly immunostained mouse and human CA, 12E3 did not (Fig. 5b). A monoclonal IgG antibody AC1 recognizing the α2,8-linked Neu5Gc dimer was also shown to react mouse CA-II (Suppl. 1). Thus, it might be a general feature that anti-disialic acid antibodies recognize CA-II. We also tested the reactivity of mAb.2-4B, S2-566 and 12E3 towards CA-II by ELISA. These anti-disialic acid antibodies (mAb.2-4B, S2-566) but not polysialic acid antibody (12E3) were reactive with CA-II immobilized on ELISA plate (Fig. 5c). To further investigate the ability of mAb.2-4B to capture the CA-II, we immunoprecipitated the CA-II using mAb.2-4B antibody from purified human CA-II and inflamed mouse sera. As shown in Fig. 5d, human intact CA-II under physiological conditions was specifically immunoprecipitated with mAb.2-4B antibody (lane 2). mAb.2-4B could capture the CA-II from inflamed mouse serum as well (Fig. 5d, lanes 3 and 4). Finally we further examined if mAb.2-4B recognized the tryptic peptides of CA-II by western blotting, and no peptides were immunostained (Suppl. 2) although all the peptides cleaved with trypsin were not guaranteed to be immobilized on the PVDF membrane.

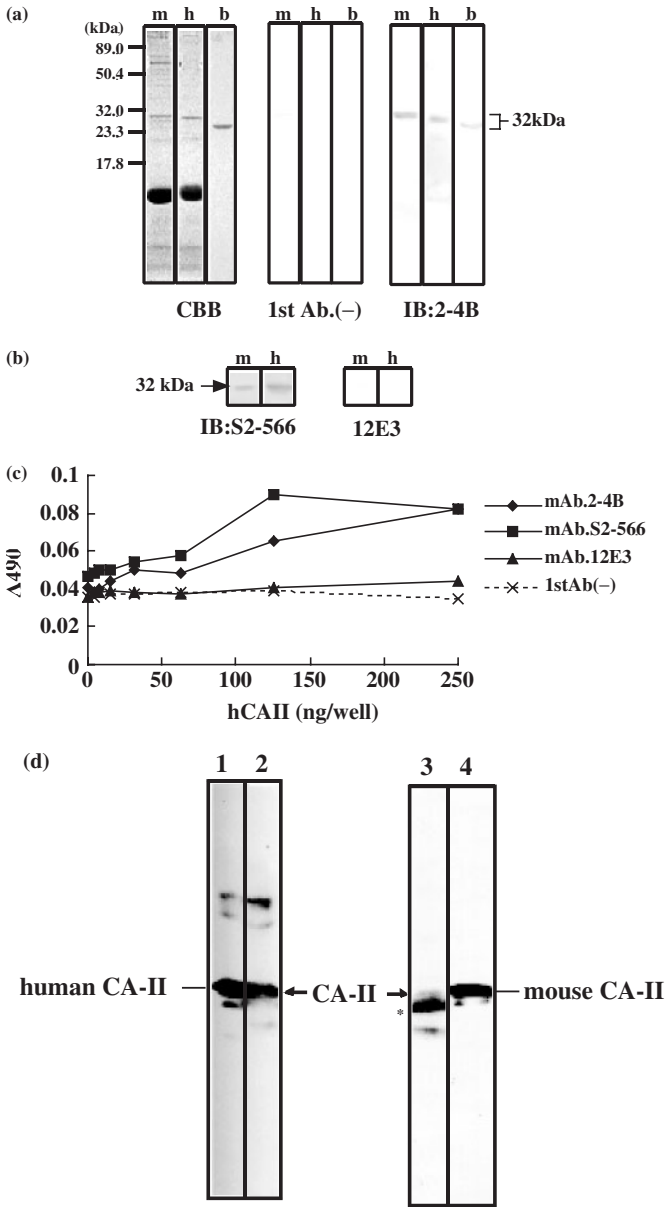


Fig. 5. **Western blot and ELISA analyses of CA from mouse and human hemolysates and bovine CA-II.** (a) Hemolysates (10 μg) from mouse (lane m), human (lane h) and purified CA-II (0.5 μg) from bovine hemolysate (lane b) were subjected to SDS–PAGE/CBB staining (CBB) and western blotting with mAb.2-4B (IB: 2-4B). No immunostaining was observed in the control blots without the primary antibody [1st Ab. (-)]. Positions of molecular mass markers are indicated on the left. (b) Hemolysates (10 μg) from mouse (lane m) and human (lane h) were subjected to SDS–PAGE and western blotting with mAb.S2-566 (IB: S2-566), which is specific to the Neu5Acα2→8Neu5Acα2→3Gal structure, or mAb.12E3 (IB: 12E3), which is specific to the Neu5Acα2→8(Neu5Acα2→)$_{n-1}$ ($n \geq 5$) structure. (c) ELISA analysis of CA-II. 96-wells were immobilized with CA-II and blocked with 1% BSA. Then primary antibodies (2-4B and S2-566) were incubated at 4°C for overnight. The absorbance at 490 nm indicates the amount of antibody bound to CA-II. (d) Immunoprecipitation of CA-II. Human CA-II (lane 1) and mouse serum (lane 4) were subjected to the SDS–PAGE and immunoblotted with anti-CA-II antibody. Immunoprecipitates from 9 μg of human purified CA-II (lane 2) and 12.5 μl of inflamed mouse serum (lane 3) with mAb. 2-4B were subjected to the SDS–PAGE and blotted on the PVDF membrane. The membrane was then immunodetected with anti-human CA-II antibodies. Arrows in lane 2 and 4 indicate the immunoprecipitated CA-II. * indicates the non-specific binding of mouse immunoglobulin light chains.

DISCUSSION

In a previous study (*8*), we demonstrated that four components of mouse serum were immunoreactive with mAb.2-4B, which specifically recognizes the (Neu5Gc)*n*, $n \geq 2$ structure, and that the 32-kDa component was remarkably upregulated by turpentine oil injection (*8*). Recently, we identified three of these disialic acid-containing components as immunoglobulin, vitronectin and plasminogen (*7*), whose amounts in serum did not increase after inflammation. In the present study, we clearly identified the mAb-2-4B-positive 32-kDa component to be CA-II based on MALDI-TOF MS analyses and specific antibodies. Using two reagents to induce inflammation via separate but overlapping inflammatory cytokines, we further demonstrated that CA-II is an acute-phase protein. Turpentine oil, which induces inflammation via the production of both interleukin (IL)-1 and IL-6 (*29*), upregulated the expression of CA-II at day 2 in serum and the increased level of CA-II was maintained until day 4 (Fig. 1c, left). In contrast, inflammation stimulated by LPS, which produces IL-1 and tumour necrosis factor-α (*30*, *31*), induced an upregulation of CA-II that peaked at day 2 and was dramatically decreased at day 4 (Fig. 1c, right). The appearance and disappearance of CA-II was the same as that of haptoglobin, which is a typical acute-phase protein (*3*, *5*).

In this study, we showed the presence of CA-II in serum not only under normal conditions, but also in inflammatory conditions. Wilhelm *et al*. (*19*) previously reported that CA-II is secreted from the coagulating gland. However, we observed the CA-II as an acute-phase protein in serum derived from both male and female mice (data not shown). Because the coagulating gland is a male organ, CA-II might not come exclusively from this tissue. Another possibility is that the CA-II in serum is a result of haemolysis. It is interesting that haptoglobin, which is a typical acute-phase protein, is involved in recycling of haemoglobin from hemolyzed red blood cells in the serum (*4*).

CA-II secreted from the coagulating gland is modified by carbohydrates, although the modification is not an *N*-linked or conventional mucin-type glycosylation (*19*). The presence of sialic acids in the CA-II, however, has not been described (*19*). Consistent with this, we did not detect sialic acids in mouse CA-II purified from inflamed mouse serum, although the presence of other carbohydrates was not examined. Mouse CA-II, however, was detected with mAb.2-4B, which specifically recognizes the di/oligoNeu5Gc structure. Human and bovine CA were also detected with mAb.2-4B. Although humans contain negligible amounts of Neu5Gc, CA from humans was immunostained with mAb.2-4B (Fig. 5a) and chemical analyses also demonstrated the absence of sialic acid in human CA (data not shown). In mouse serum, three other components were detected by

mAb.2-4B and were identified as immunoglobulin kappa chain, vitronectin and plasminogen (*7*). In these cases, however, the disialic acid epitope was confirmed by chemical analyses (*7*). The fact that CA-II is immunostained with mAb.2-4B, but contains no sialic acid, suggests that CA-II might have a very similar structure to that of the mAb.2-4B epitope. Because mAb.2-4B recognizes di/oligosialic acid, we analysed mouse and human CA using another disialic acid-recognizing monoclonal antibody and an oligo/polysialic acid-recognizing monoclonal antibody. CA was not immunostained with anti-oligo/polysialic acid antibody (12E3) (Fig. 5b). In contrast, CA was immunostained with anti-disialic acid antibody (S2-566), which is similar to mAb.2-4B (Fig. 5b). The anti-disialic acid antibodies, but not anti-oligo/polysialic acid antibodies, can also recognize CA-II immobilized on ELISA plate (Fig. 5c). We also demonstrate that mAb.2-4B can capture the intact human CA-II in physiological state and mouse CA-II in the inflamed serum (Fig. 5d). It has been reported that the peptides that mimic the α2,8-disialic acid and polysialic-acid epitopes have been established using a phage-display method and the epitopes were a specific amino acid sequence (*32*, *33*). We could not observe these specific amino acid sequences. However, CA-II contains amino acid sequences containing carboxyl groups, such as clusters of aspartic acid and glutamic acid (Fig. 6, 71–75, 233–248) and these sequences might mimic the disialic-acid epitope (which has a di-carboxyl group). We could not detect reactivity of mAb.2-4B toward the tryptic peptides of CA-II (Suppl. 2), either although it is uncertain if all the tryptic peptides were analysed. Further analyses will be necessary to determine the CA-II structure mimicking the disialic-acid epitope.

Natural antibodies against disialic-acid-containing gangliosides are present in the serum and are sometimes involved in diseases such as neuropathy (*34*). The antigens of these antibodies are considered to be some bacterial LPS or lipooligosaccharides, which mimic disialic-acid-containing gangliosides and these antibodies are involved in the neuropathy due to the binding to the epitope, disialic-acid-containing gangliosides resident in the neural tissues (*34–36*). Some of these antibodies, conversely, might arise from some specific region of acute-phase proteins, such as CA-II, because mAb.2-4B and mAb.S2-566, which were developed using the disialic-acid epitope-containing glycoconjugates cross-react with CA-II. It is noteworthy that anti-carbohydrate antibodies produced by conventional methods are mostly immunoglobulin M, like mAb.2-4B and mAb.S2-566, while the natural antibodies towards carbohydrates are sometimes immunoglobulin G (*37*).

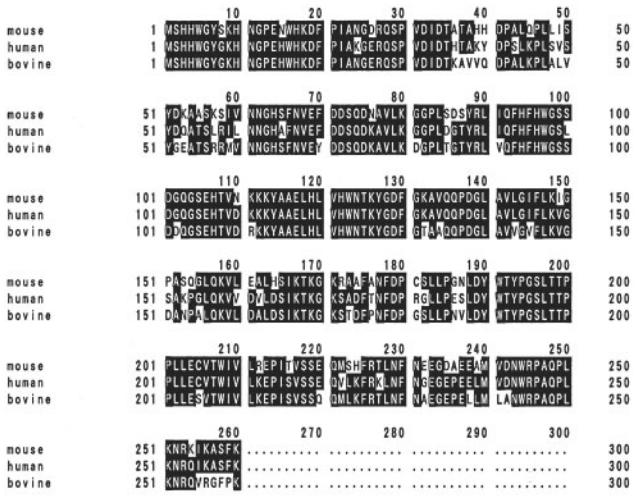


Fig. 6. **Comparison of the amino acid sequences of mouse, human and bovine CA-II.** The black boxes show conserved residues. Accession numbers are **Q7TPE1** for the mouse CA-II; **NP_000058** for human CA-II; and **NP_848667** for bovine CA-II.

Some people also have autoantibodies against CA-II in normal conditions (*21*) and P/NCr and DBA/1J mouse strains contain anti-CA-II autoantibodies after injection with phosphate-buffered saline (*43*), although the reason of the presence of autoantibodies remains unclear. As shown in Figs. 1 and 4b, significant amounts of CA-II are observed in mouse serum under normal conditions and thus might cause the autoantibodies against CA-II in normal conditions. In serum of patients, the presence of anti-CA–II antibodies has been reported in systemic lupus erythematous (*38*), primary Sjögren's syndrome (*38*), progressive systemic sclerosis (*38*), dermatomyositis (*38*), autoimmune-related pancreatitis (*39*), type 1 diabetes (*40*) and ulcerative colitis (*41*). The mechanisms of the anti-CA-II antibody acquisition and the pathology induced by the anti-CA-II antibody are not known. In this study, we observed the increase of serum CA-II in inflammatory conditions, and this might cause the production of anti-CA-II antibodies in serum of patients as described earlier. Natural antibodies towards CA-II might react not only with CA-II, but also with the disialic acid structure that occurs ubiquitously in various tissues (*42*). Furthermore, mice immunized with CA-II tend to suffer from sialoadenitis (*43*). Although the cause of disease is not known, it might be due to the cross-reaction of the anti-CA-II with disialic acid in the sialaden, a rich source of sialic acid (*44*).

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