

ORIGINAL ARTICLE

Application of an enzyme-labeled antigen method for visualizing plasma cells producing antibodies against Strep A, a carbohydrate antigen of *Streptococcus pyogenes*, in recurrent tonsillitis

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ABSTRACT

Streptococcus pyogenes is the main causative pathogen of recurrent tonsillitis. Histologically, lesions of recurrent tonsillitis contain numerous plasma cells. Strep A is an antigenic carbohydrate molecule on the cell wall of *S. pyogenes*. As expected, plasma cells in subjects with recurrent tonsillitis secrete antibodies against Strep A. The enzyme-labeled antigen method is a novel histochemical technique that visualizes specific antibody-producing cells in tissue sections by employing a biotin-labeled antigen as a probe. The purpose of the present study was to visualize plasma cells producing antibodies reactive with Strep A in recurrent tonsillitis. Firstly, the lymph nodes of rats immunized with boiled *S. pyogenes* were paraformaldehyde-fixed and specific plasma cells localized in frozen sections with biotinylated Strep A. Secondly, an enzyme-labeled antigen method was used on human tonsil surgically removed from 12 patients with recurrent tonsillitis. *S. pyogenes* genomes were PCR-detected in all 12 specimens. The *emm* genotypes belonged to *emm12* in nine specimens and *emm1* in three. Plasma cells producing anti-Strep A antibodies were demonstrated in prefixed frozen sections of rat lymph nodes, 8/12 human specimens from patients with recurrent tonsillitis but not in two control tonsils. In human tonsils, Strep A-reactive plasma cells were observed within the reticular squamous mucosa and just below the mucosa, and the specific antibodies belonged to either IgA or IgG classes. Our technique is effective in visualizing immunocytes producing specific antibodies against the bacterial carbohydrate antigen, and is thus a novel histochemical tool for analyzing immune reactions in infectious disorders.

Key words enzyme-labeled antigen method, recurrent tonsillitis, Strep A, *Streptococcus pyogenes*.

The Group A β -hemolytic *Streptococcus*, *S. pyogenes*, is a gram-positive non-motile pathogen capable of causing a wide variety of human diseases, including acute and recurrent tonsillitis, an infection of the palatine tonsil (1–4). *S. pyogenes* comprises 20–40% of the bacteria that cause tonsillitis (5). The first event in streptococcal acute tonsillitis is bacterial attach-

ment to the surface epithelium (6), followed by colonization that induces an inflammatory response (7). Clinical manifestations of acute phase tonsillitis include sore throat, fever and cervical lymphadenopathy (8).

The classification of *Streptococci* depends upon the serologic reactivity of cell wall polysaccharide antigens,

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List of Abbreviations: CDC, Center for Disease Control and Prevention; DAB, diaminobenzidine; DAPI, 4',6-diamidino-2'-phenylindole; TBS, tris-buffered saline.

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as originally described by Lancefield (9). Rapid diagnostic kits employing immunochromatography for detecting *S. pyogenes* are available from commercial sources (10). The test strips are coated with antibodies specific to a carbohydrate antigen of the cell wall of *S. pyogenes*, named Strep A (11). Strep A, or the group A capsular polysaccharide (molecular weight: 20 kDa), is a polymer of *N*-acetylglucosamine and rhamnose (12). A distinct line develops on a strip when a throat swab specimen is colonized by Strep A-positive *S. pyogenes*.

In chronic tonsillitis accompanied by multiple recurrences of acute-type lesions, namely recurrent tonsillitis, numerous plasma cells are observed as a component of the inflammatory cells (13). In the human tonsil, plasma cells are characteristically distributed within the vascularized reticular squamous mucosa (14). Because they are secreted within the lesion, the antibodies seen locally must be involved in the pathogenesis of tonsillitis. Plasma cells in recurrent tonsillitis are therefore strongly expected to locally produce antibodies against Strep A.

The enzyme-labeled antigen method is a histochemical technique that visualizes specific antibody-producing cells in tissue sections by using labeled antigens (15). With this novel histochemical approach, we have succeeded in identifying specific antibodies against *Porphyromonas gingivalis* in gingival radicular cyst and periodontitis (16, 17), and autoantibodies in rheumatoid synovitis (18). So far, we have used biotinylated protein antigens as probes. The aim of the present study was to localize plasma cells producing specific antibodies in prefixed frozen sections by employing the biotinylated carbohydrate antigen, Strep A. The target lesions to be studied included rat lymph nodes immunized with boiled *S. pyogenes* and 12 surgically removed human specimens from subjects with recurrent tonsillitis.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats aged 5 weeks and weighing 150 g (Chubu Kagaku Shizai, Nagoya, Japan) were housed in the animal laboratory of Fujita Health University, Toyoake, Japan, with a 12 hr light/dark cycle (light on at 08:00) and access to food and water *ad libitum*. The animal experiments were conducted as described previously (15, 16) and the procedures were approved by the institutional Animal Care and Use Committee of Fujita Health University, Toyoake (acknowledgment number M2152).

Immunization

Three rats were immunized with boiled *S. pyogenes* (strain: J17A4, serotype: M6, inoculum size: 4×10^6 CFU per rat, supplied by Eiken Chemical, Nogi, Tochigi, Japan) emulsified with Freund's incomplete adjuvant (Difco Laboratories; Detroit, MI, USA). The footpads of all four legs of the animals were each injected three times, the second and third injections being administered 1 and 5 weeks after the initial challenge. Three rats immunized with an emulsion of saline and the adjuvant served as control "non-immune" animals.

Animal tissue sampling

Two weeks after the third injection, the rats were killed by inhalation of diethyl ether and their popliteal and axillary lymph nodes sampled bilaterally. The tissues were immersion-fixed in 4% paraformaldehyde in 10 mM PBS, pH 7.4, at 4°C for 4 hr. After rinsing in 10% sucrose-containing PBS at 4°C overnight, they were soaked in 15 and 20% sucrose-containing cold PBS for 4 hr each. The tissues were then embedded in an embedding medium (Tissue Mount; Chiba Medical, Saitama, Japan), quickly frozen in dry ice-acetone, and sectioned on a cryostat at 3 µm thickness. The frozen sections mounted on 3-aminopropyltriethoxysilane-coated glass slides were dried for 30 min with a drier at room temperature, and stored at -30°C until staining.

Patients and surgical specimens

Palatine tonsils were surgically removed from 12 patients with recurrent tonsillitis (Table 1). The patients were aged from 2 to 26 years (mean, 10.6; median 6 years). The male to female ratio was 11:1. The frequency of recurrence of tonsillitis was categorized as low (once in 2 months), moderate (once a month), and high (twice or more a month). Six cases were in the high frequency of recurrence category. Microbial cultures were performed in three patients and *S. pyogenes* was detected in all three of them. Two negative control tonsils surgically removed from one patient with sleep apnea syndrome and one with IgA nephropathy, both with no symptoms of recurrent tonsillitis, were similarly analyzed to serve as control cases.

The surgical specimens of the tonsils were divided in two, one portion being processed for prefixed frozen sections in the same way as in the rat experiment and the other for DNA extraction. The tissues were washed and homogenized in PBS, pH 7.2, in a proportion of one part of tissue (wet weight) to ten parts of PBS. The homogenates were centrifuged at 15,000 rpm for 5 min twice and the supernatant stored at -80°C prior to total

Table 1. Summary of clinical data and analyzed results in 12 cases of recurrent tonsillitis

Case	Age/sex	Frequency of recurrence	<i>S. pyogenes</i> detection				Area of section (mm ²)	Number of positive cells/section		Percentage of positive cells Anti-Strep A/CD38 (%)
			Microbial culture	PCR		<i>S. pyogenes</i> <i>emm</i> type		Anti-Strep A	CD38	
				V-ATPase	speB					
1	4M	High	+	+	+	12	22	11	843	1.30
2	4M	High	NE	+	+	12	55	21	1032	2.00
3	7M	High	+	+	+	12	34	13	786	1.70
4	20M	High	NE	+	+	12	16	23	1218	1.90
5	20F	High	NE	+	+	1	29	12	1154	1.00
6	26M	High	+	+	+	12	30	5	1066	0.50
7	4M	Moderate	NE	+	+	1	17	5	1011	0.50
8	18M	Moderate	NE	+	+	12	16	4	939	0.40
9	5M	Moderate	NE	+	+	1	12	0	113	0
10	12M	Moderate	NE	+	+	12	39	0	1097	0
11	2M	Low	NE	+	+	12	24	0	463	0
12	5M	Low	NE	+	+	12	18	0	775	0
13	7M	0	NE	-	-	-	10	0	41	0
14	25F	0	NE	-	-	-	13	0	67	0

Cases 13 and 14 are negative controls (Case 13, sleep apnea syndrome; Case 14, IgA nephropathy). Frequency of recurrence: high, twice or more a month; moderate, once a month; low, once every two months. NE, not examined; speB, streptococcal pyrogenic exotoxin B; V-ATPase, vacuolar-type Na⁺ ATPase. + indicates positivity according to *S. pyogenes* culture or PCR analysis.

genomic DNA extraction. Total genomic DNA was extracted using a NucleoSpin Tissue kit (Takara Bio, Otsu, Japan), according to the manufacturer's instructions.

The use of human materials was approved by the Ethical Review Board for Clinical and Epidemiological Investigations at Fujita Health University, Toyoake (approval number: 12-084). Written informed consent was obtained from each patient or the parents of the pediatric patients.

Polymerase chain reaction amplification for *S. pyogenes*-specific DNA

Polymerase chain reaction was performed in a 25 µL volume containing 0.4 µM primers, 200 µM each of the deoxynucleoside triphosphates, 2.5 µL of 10× Ex-Taq buffer (Takara Bio), 0.625 units of Ex-Taq DNA polymerase (Takara Bio), and 1 µL of total genomic DNA extract from the tissue. Thermocycling conditions were as follows: 94°C for 10 min, followed by 45 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 7 min. The primer sequences specific for the gene of V-type Na⁺ ATPase subunit B of *S. pyogenes* were prepared according to a previous report (19), giving a PCR product of 121 bp.

Polymerase chain reaction analysis of streptococcal pyrogenic exotoxin B was also performed, as previously described (20), giving a PCR product of 257 bp. PCR amplicons were visualized on a 2% agarose gel after staining with 0.5 µg/mL ethidium bromide in 40 mM tris-acetate ethylenediamine tetraacetic acid buffer, pH 8.3, and photographed under ultraviolet illumination. Special care was taken to avoid specimen contamination.

M protein gene (*emm*) typing of *S. pyogenes*

The *emm* gene encoding the M protein, a major virulence (anti-phagocytic) factor on the cell wall of *S. pyogenes* (21), was genotyped according to the protocol of the CDC (22), except for extended thermocycling to 45 cycles. The PCR-amplified products, around 1300 bp-long, were purified by a MinElute spin column (Qiagen, Hilden, Germany). FASMAC Company (Atsugi, Japan) sequenced the 5'-end (820 bp-long) of the PCR-amplified products using a BigDye terminator cycle sequencing ready-reaction kit (Perkin-Elmer Applied Biosystems, Tokyo, Japan). The first 160 bases of the 5'-end of the *emm* gene sequences were compared with those in the CDC database of the *S. pyogenes emm* gene sequence (23). An *emm* genotype showing over 95%

homology with a CDC reference strain was identified as that particular *emm* genotype.

Enzyme-labeled antigen method using biotinylated Strep A

After a brief dip in running water and endogenous peroxidase inactivation using methanol containing 0.3% hydrogen peroxide at room temperature for 30 min, paraformaldehyde-prefixed frozen sections were treated with 50 mM TBS, pH 7.6, containing 5 µg/mL proteinase K at room temperature for 15 min. A PBS rinse was interposed between every step thereafter. Biotinylated Strep A solution (supplied by Eiken Chemical) at a concentration of 50 µg/mL of PBS was incubated overnight at room temperature, followed by incubation with HRP-labeled streptavidin (prediluted, Nichirei Bioscience, Tokyo, Japan) at room temperature for 1 hr. The reaction products were visualized in 50 mM Tris-HCl buffer, pH 7.6, containing 20 mg/dL DAB and 0.006% hydrogen peroxide. Finally, the nuclei were lightly counterstained with Mayer's hematoxylin.

Direct immunoperoxidase demonstration of total plasma cells using HRP-labeled polyvalent antibodies against rat IgG, IgA, and IgM

Prefixed frozen sections of the rat lymph nodes were rinsed in tap water for 5 min to quench endogenous peroxidase activity. After a brief dip in tap water, the sections were treated with TBS containing 5 µg/mL proteinase K at room temperature for 15 min. A PBS rinse was interposed between every step thereafter. A cocktail of HRP-conjugated goat anti-rat IgG, IgA and IgM polyvalent antibodies (MP Biomedicals; Solon, OH, USA), diluted at 1:1,000, was incubated at room temperature for 1 hr, after which DAB coloring reaction and brief hematoxylin counterstaining were performed. By examining consecutive sections, the percentage of Strep A-reactive antibody-producing cells (identified by the enzyme-labeled antigen method) of all immunoglobulin-positive plasma cells was evaluated.

Absorption experiment for the enzyme-labeled antigen method

An excess amount of unlabeled Strep A (Eiken Chemical) was utilized to abolish specific staining with the enzyme-labeled antigen method. Briefly, 10-times concentrated unlabeled Strep A was added to the

biotinylated Strep A solution and the mixture used for staining.

Double immunofluorescence using biotinylated Strep A and anti-CD79a monoclonal antibody

CD79a is a pan-B-cell (including plasma cells) marker (24). After a brief dip in running water, frozen sections of the human tonsil were treated with TBS containing 5 µg/mL proteinase K at room temperature for 15 min. The sections were incubated with a mixture of biotinylated Strep A (50 µg/mL) and anti-human CD79a monoclonal antibody at a 1:200 dilution (clone: JCB117, Dako, Carpinteria, CA, USA) overnight at room temperature, followed by incubation with a mixture of Alexa Fluor 568 (red)-labeled streptavidin (diluted 1:300; Molecular Probes, Tokyo, Japan) and Alexa Fluor 488 (green)-labeled goat anti-mouse IgG (diluted 1:300; Molecular Probes) at room temperature for 1 hr. Finally, the sections were mounted in a hydrophilic mounting medium (Prolong Gold Antifade Reagent with DAPI, Molecular Probes). The nuclei were stained blue with DAPI.

Immunostaining for CD38, pan-cytokeratin, collagen type IV and CD20

To demonstrate the topographic orientation of plasma cells producing antibodies against Strep A within the tonsils, immunoperoxidase staining was performed using monoclonal antibodies against CD38 (for detecting plasma cells), pan-cytokeratin (for detecting keratinocytes), collagen type IV (for detecting the basement membrane) and CD20 (for detecting germinal center/non-plasmacytic B-lymphocytes). The monoclonal antibodies included anti-human CD38 (clone SPC32, diluted 1:300; Novocastra, New Castle, UK), anti-pan-cytokeratin (clone AE1/AE3, diluted 1:8; Nichirei Bioscience), anti-collagen type IV (clone CIV22, diluted 1:600; Dako) and anti-human CD20 (clone L26, diluted 1:400; Dako). After a brief dip in running water and endogenous peroxidase quenching, prefixed frozen sections were incubated with the respective monoclonal antibodies overnight at room temperature. Proteinase K (5 µg/mL) treatment at room temperature for 15 min was added prior to immunostaining for localizing pan-cytokeratin and collagen type IV. The secondary reagent of amino acid polymer type (Simple Stain-Max-PO, Nichirei Bioscience) was incubated for 30 min at room temperature, followed by DAB coloring reaction and brief hematoxylin counterstaining.

By examining consecutive sections, the percentage of plasma cells producing antibodies against Strep A of all CD38-positive plasma cells was evaluated. CD38 is known to be expressed by both plasma cells and immature (activated) B-cells (25). CD38-positive germinal center B-lymphocytes were excluded from counting.

Double immunofluorescence using biotinylated Strep A and fluorescent antibodies to human immunoglobulins

To clarify the immunoglobulin class of specific antibodies to Strep A produced by plasma cells in the tonsils, double immunofluorescence staining was performed using biotinylated Strep A and DyLight 488-labeled goat antibodies against human α -chain, γ -chain or μ -chain (Abcam, Cambridge, MA, USA). After a brief dip in running water, prefixed frozen sections of the tonsils were treated with TBS containing 5 μ g/mL proteinase K at room temperature for 15 min. The sections were incubated with biotinylated Strep A (50 μ g/mL) overnight at room temperature, followed by incubation with a mixture of Alexa Fluor 488 (red)-labeled streptavidin (diluted 1:300; Molecular Probes) and DyLight 488 (green)-labeled goat antibodies against human α -chain (diluted 1:900); γ -chain (diluted 1:300), or μ -chain (diluted 1:300) at room temperature for 1 hr. The nuclei were stained blue with DAPI.

By examining consecutive sections, (i) the ratio of immunoglobulin classes to total plasma cells (the sum of IgA + IgG + IgM plasma cells) in the tonsils and (ii) the percentage of Strep A-reactive plasma cells of the respective immunoglobulin class were evaluated.

Double immunofluorescence for secretory component and IgA

It is known that, after forming a complex structure with a secretory component, IgA is secreted through the mucosal membrane of the tonsil: an immunoreactive secretory component has been observed in some keratinocytes of the reticular mucosa of the tonsil (24). After a brief dip in running water, prefixed frozen sections of the tonsil were treated with TBS containing 5 μ g/mL proteinase K at room temperature for 15 min. The sections were incubated with rabbit antiserum against secretory component (diluted 1:500; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at room temperature, followed by incubation with a mixture of Alexa Fluor 568 (red)-labeled goat anti-rabbit IgG (diluted 1:300; Molecular Probes) and DyLight 488 (green)-labeled goat anti-human α -chain (diluted 1:900; Abcam) at room temperature for 1 hr. The nuclei were stained blue with DAPI.

Statistical analysis

Values in tables are presented as the mean \pm SEM. The number of plasma cells in the lymph nodes before and after *S. pyogenes* immunization was compared with two-tailed Student's *t*-test. Correlations between the detection of Strep A-reactive plasma cells and the frequency of recurrence of tonsillitis were evaluated by Pearson's product-moment correlation coefficient analysis. Values of $P < 0.05$ were considered to indicate statistical significance.

RESULTS

Detection of *S. pyogenes* in recurrent tonsillitis

With the PCR assay, the 121 and 257 bp bands of the *S. pyogenes* genes (panel A: putative V-type Na⁺ ATPase subunit B gene, panel B: streptococcal pyrogenic exotoxin B gene) were identified in the DNA samples from all 12 specimens of recurrent tonsillitis (Fig. 1a,b). The M protein gene (band size around 1300 bp) was also demonstrated in all specimens (Fig. 1c). No positivity was demonstrated in the two control tonsils (Cases 13 and 14). The *emm* genotypes of *S. pyogenes* belonged to *emm12* in nine specimens (75%) and *emm1* in three (25%) (Table 1).

The enzyme-labeled antigen method applied to rat axillary and popliteal lymph nodes and evaluation of the percentage of plasma cells reactive with Strep A among total plasma cells

Plasma cells showing cytoplasmic positivity for immunoglobulins were richly distributed in the axillary and popliteal lymph nodes of both *S. pyogenes*-immunized and control (non-immunized) rats (Fig. 2a,c,e,g). As shown in Table 2, the total number of immunoglobulin-reactive plasma cells increased significantly after immunization ($P < 0.05$). Plasma cells producing antibodies reactive with Strep A were evaluated in prefixed frozen sections of the rat nodes with the enzyme-labeled antigen method employing biotinylated Strep A as a probe (Fig. 2b,d,f,h). Plasma cells with cytoplasmic anti-Strep A reactivity were dispersed in the popliteal and axillary lymph nodes of *S. pyogenes*-immunized rats (Fig. 2b,d), but not of control rats (Fig. 2f,h). No distinct cluster formation by Strep A-reactive plasma cells was noted. The specific plasma cells comprised $3.4 \pm 0.8\%$ of the immunoglobulin-positive total plasma cells in the axillary lymph nodes and $2.9 \pm 0.7\%$ of the total plasma

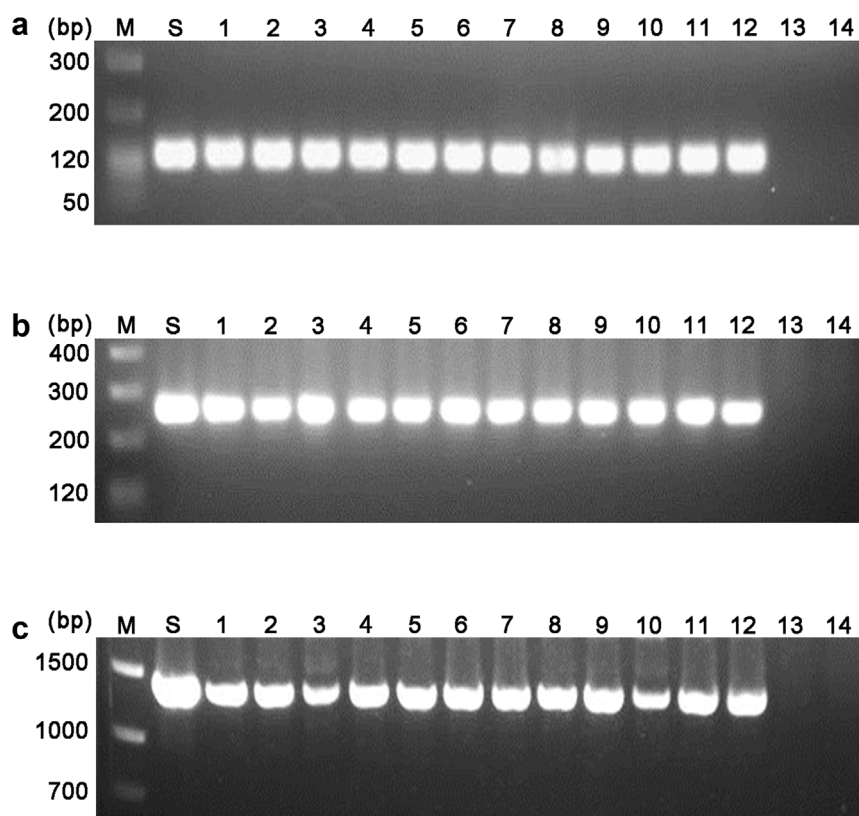


Fig 1. Electrophoretic demonstration of *S. pyogenes*-specific PCR products after ethidium bromide staining. The target genes are (a) putative V-type Na⁺ ATPase subunit B (product size 121 bp), (b) streptococcal pyrogenic exotoxin B gene (product size 257 bp) and (c) M protein (product size around 1,300 bp). Lane M indicates molecular weight markers. Lane S demonstrates the PCR product amplified from a sonicated extract of cultured *S. pyogenes* (positive control). Lanes 1 through 12 show the corresponding bands amplified from tissue extracts from patients with recurrent tonsillitis (Cases 1–12, respectively). Lanes 13 and 14 represent negative control tonsils; lane 13, sleep apnea syndrome (Case 13); lane 14, IgA nephropathy (Case 14).

cells in the axillary lymph nodes (Table 2). The reaction specificity was confirmed by the absorption experiment (data not shown).

The enzyme-labeled antigen method for visualizing Strep A-reactive antibody-producing cells in recurrent tonsillitis and confirmation of the specificity with the absorption experiment

The enzyme-labeled antigen method using biotinylated Strep A was applied to prefixed frozen sections of human tonsils. Representative results for Cases 3 and 6 are shown in Figure 3a,c, respectively. Specific labeling was detected in 8/12 (67%) cases of recurrent tonsillitis (Table 1). The specificity of the enzyme-labeled antigen method was confirmed by an absorption experiment (Fig. 3b,d). An excess amount of unlabeled Strep A was used to abolish specific anti-Strep A signals. In two negative control tonsils (Cases 13 and 14), there were

relatively few CD38-positive plasma cells and no positive anti-Strep A signals were detected (Table 1).

Double immunofluorescence for anti-Strep A reactivity and CD79a

Figure 4 illustrates double immunofluorescence staining for anti-Strep A reactivity and CD79a in tonsils from patients with recurrent tonsillitis (Case 6, top panels: a–c), sleep apnea syndrome (Case 13, middle panels: d–f) and IgA nephropathy (Case 14, bottom panels: g–i). A small percentage of CD79a-positive plasma cells showed anti-Strep A reactivity only in the recurrent tonsillitis specimen.

Distribution of plasma cells producing Strep A-reactive antibodies in recurrent tonsillitis

Plasma cells producing antibodies reactive with Strep A were distributed within the reticular squamous mucosa

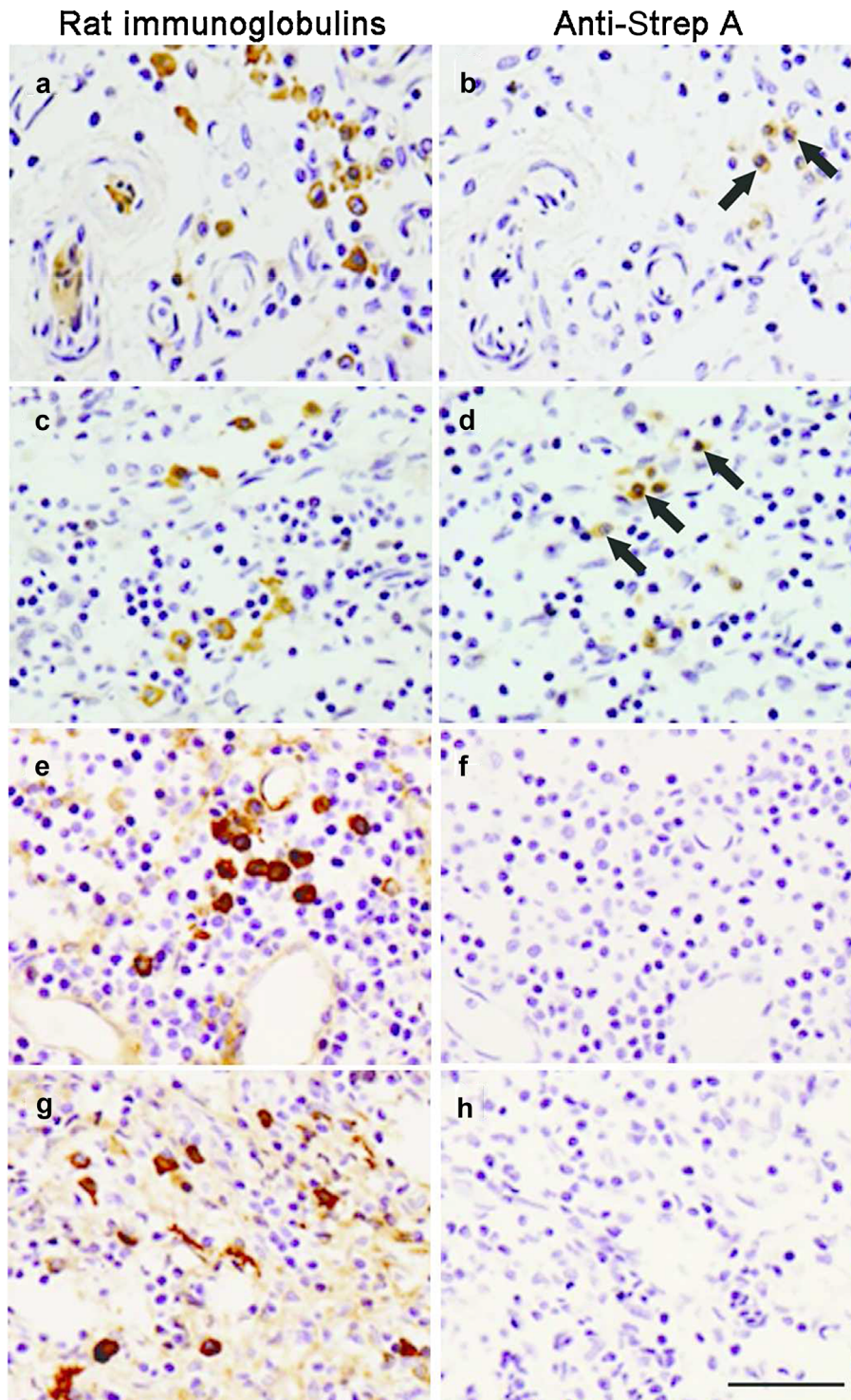


Fig. 2. Visualization of plasma cells producing (a, c, e, g) immunoglobulins and (b, d, f, h) Strep A-reactive antibodies in the lymph nodes of (a–d) *S. pyogenes*-immunized and (e–h) non-immunized rats. Panels a, b, e, and f show axillary node tissue and panels c, d, g, and h popliteal node tissue. Plasma cells producing immunoglobulins are shown by the direct immunoperoxidase method in which a cocktail of HRP-conjugated goat polyvalent antibodies against rat IgG, IgA and IgM is used. With an enzyme-labeled antigen method using biotinylated Strep A, plasma cells producing antibodies reactive with Strep A are observed in the nodes from immunized rats (arrows), but not in those from non-immunized ones. Scale bar represents 50 μ m. Stains: (a,c,e,g) immunoperoxidase; (b,d,f,h) enzyme-labeled antigen method.

Table 2. Number and percentage of plasma cells with anti-Strep A reactivity in the axillary and popliteal lymph nodes of *S. pyogenes*-immunized and non-immunized rats

Rats	Lymph nodes	Number of positive cells (mm ²)		Percentage of positive cells	
		Immunoglobulins-reactive	Strep A-reactive	Strep A/immunoglobulins	
<i>S. pyogenes</i> -immunized	(n = 3) Axillary	(n = 13)	36.0 ± 4.8*	1.2 ± 0.3**	3.4 ± 0.8**
Non-immunized	(n = 3) Axillary	(n = 8)	23.4 ± 4.3	0.0	0.0
<i>S. pyogenes</i> -immunized	(n = 3) Popliteal	(n = 8)	43.1 ± 15.7*	1.3 ± 0.6**	2.9 ± 0.7**
Non-immunized	(n = 3) Popliteal	(n = 6)	29.8 ± 0.9	0.0	0.0

* $P < 0.05$; ** $P < 0.01$. Values indicate the mean ± SEM. *S. pyogenes*, *Streptococcus pyogenes*.

(in Case 3) and just below the mucosa (in Case 4) of tonsils from subjects with recurrent tonsillitis (Fig. 5). The plasma cells were immunoreactive for CD38. The reticular keratinocytes were labeled for pan-cytokeratin and the basement membrane for collagen type IV. Germinal center/non-plasmacytic B-lymphocytes were demonstrated to be CD20-positive cells. In the eight lesions with anti-Strep A reactivity, the percentage of the plasma cells reactive with Strep A ranged from 0.4 to 2.0%

(mean 1.2%) of CD38-positive total plasma cells (Table 1).

Immunoglobulin class of Strep A-reactive antibodies produced by plasma cells in recurrent tonsillitis

Representative findings of double immunofluorescence using biotinylated Strep A and fluorescent-labeled antibodies to IgA, IgG or IgM in the tonsil of Case 3

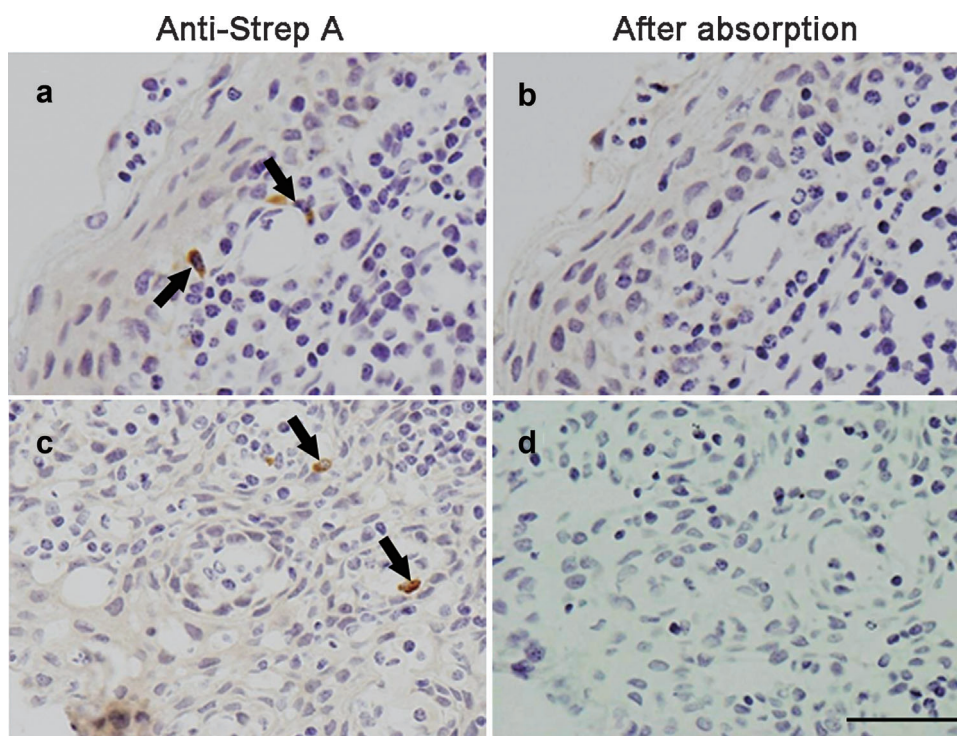


Fig. 3. Confirmation of the specificity of the enzyme-labeled antigen method by absorption experiment. Panels a and b, Case 3; panels c and d, Case 6 (consecutive sections). Arrows indicate plasma cells positively labeled with biotinylated Strep A (panels a and c). The absorption experiment (panels b and d) confirms the specificity of the technique: the anti-Strep A signals have been abolished with an excess amount of unlabeled Strep A. Scale bar represents 50 μ m. Stain, enzyme-labeled antigen method.

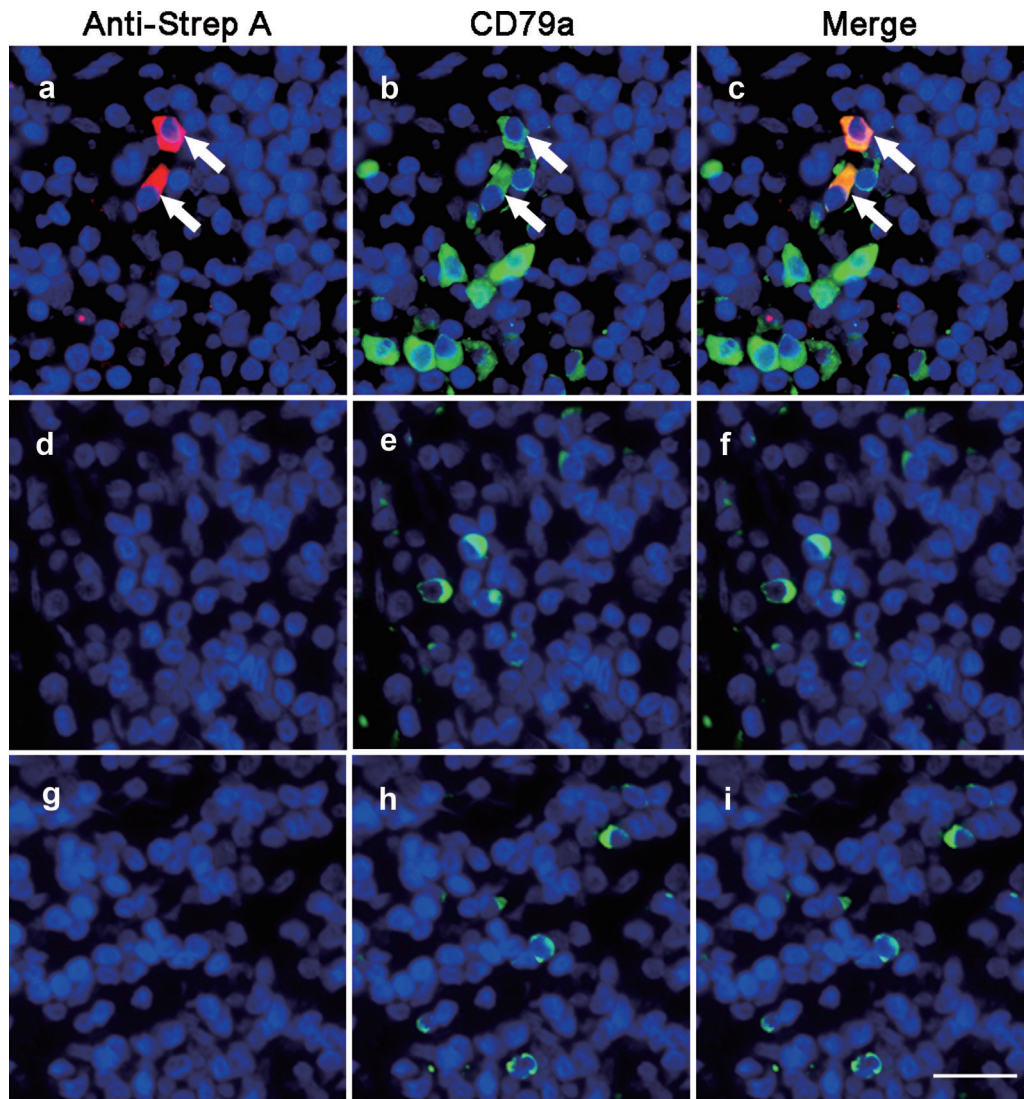


Fig. 4. Double immunofluorescence analysis using biotinylated Strep A and anti-CD79a monoclonal antibody in specimens from patients with recurrent tonsillitis (Case 6, a–c), sleep apnea syndrome (Case 13, d–f) and IgA nephropathy (Case 14, g–i). (a, d, and g) Anti-Strep A reactivity is stained red with Alexa Fluor 568 and (b, e, and h) CD79a is immunolocalized in green with Alexa Fluor 488. The nuclei are stained blue with DAPI. Panels c, f, and i are merged images. Plasma cells producing Strep A-reactive antibodies (arrows) are a minor component. Scale bar represents 50 μm .

are shown in Figure 6. Strep A-reactive antibodies produced by plasma cells in recurrent tonsillitis belonged to the IgA (top panels: a–c) and IgG classes (middle panels: d–f). No IgM class plasma cells were reactive with Strep A (bottom panels: g–i). Eight tonsils with anti-Strep A reactivity were evaluated for the immunoglobulin class of the plasma cells. IgA-type plasma cells accounted for 31% of total plasma cells, IgG-type 58% and IgM-type 11%. Among IgA or IgG-positive plasma cells, Strep A-reactive plasma cells shared 1.2% in

the IgA class and 0.6% in the IgG class on average (Table 3).

Dual immunofluorescent localization of secretory component and IgA in the reticular epithelial cells in recurrent tonsillitis

Double immunofluorescence for secretory component and IgA was analyzed in Case 1. The secretory

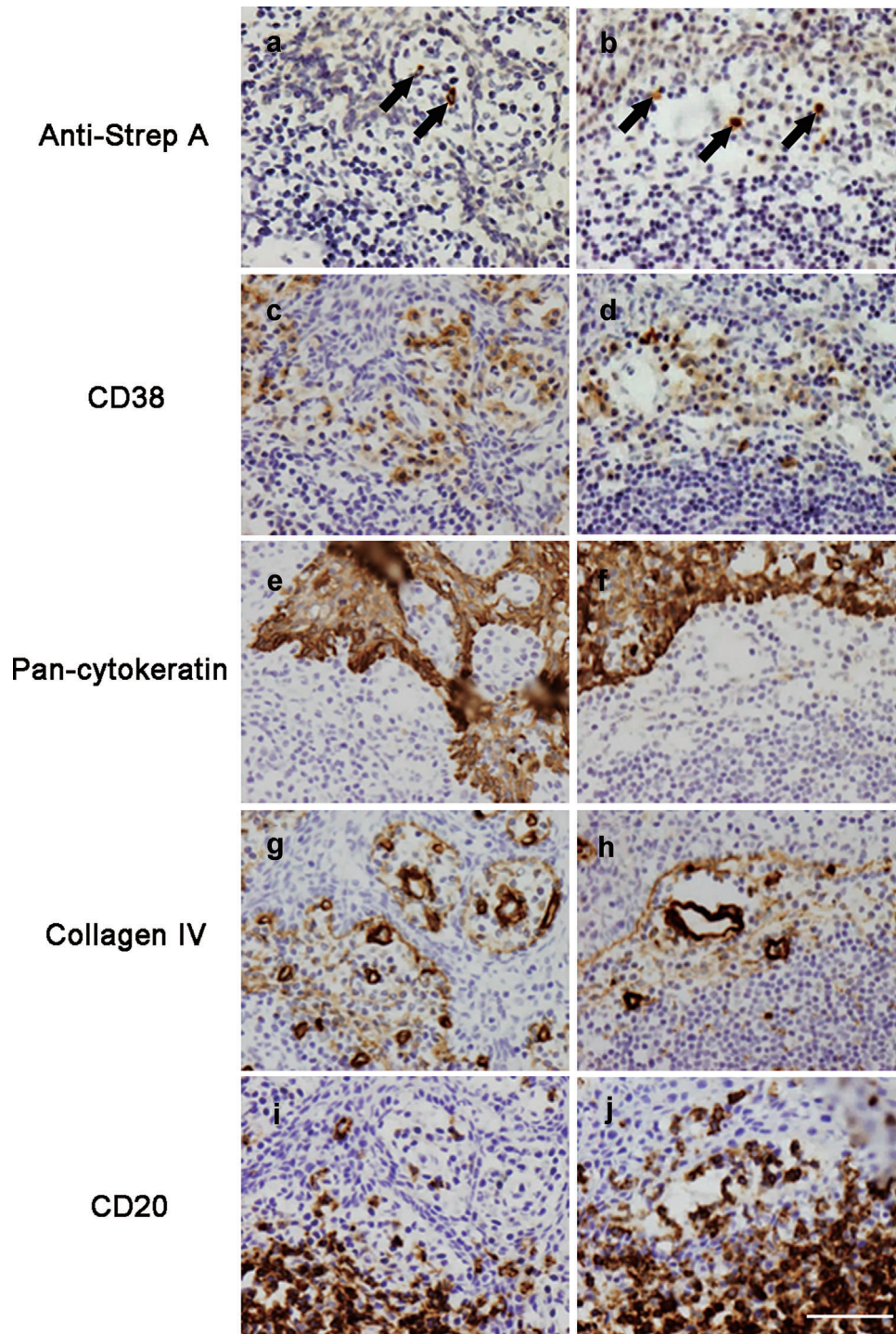


Fig. 5. Distribution of plasma cells reactive with Strep A in recurrent tonsillitis (Case 3 left-sided panels and Case 4 right-sided panels). Consecutive sections demonstrate (a and b) anti-Strep A reactivity, (c and d, showing plasma cells) CD38, (e and f, showing reticular keratinocytes) pan-cytokeratin, (g and h, showing the basement membrane) collagen type IV, and (i and j, showing germinal center/non-plasmacytic B-lymphocytes) CD20. Arrows indicate plasma cells positively labeled with biotinylated Strep A, located (a) in the reticular squamous mucosa and (b) beneath the mucosa. Scale bar represents 50 μ m. Stains: (a, b) enzyme-labeled antigen method; (c, j) immunoperoxidase.

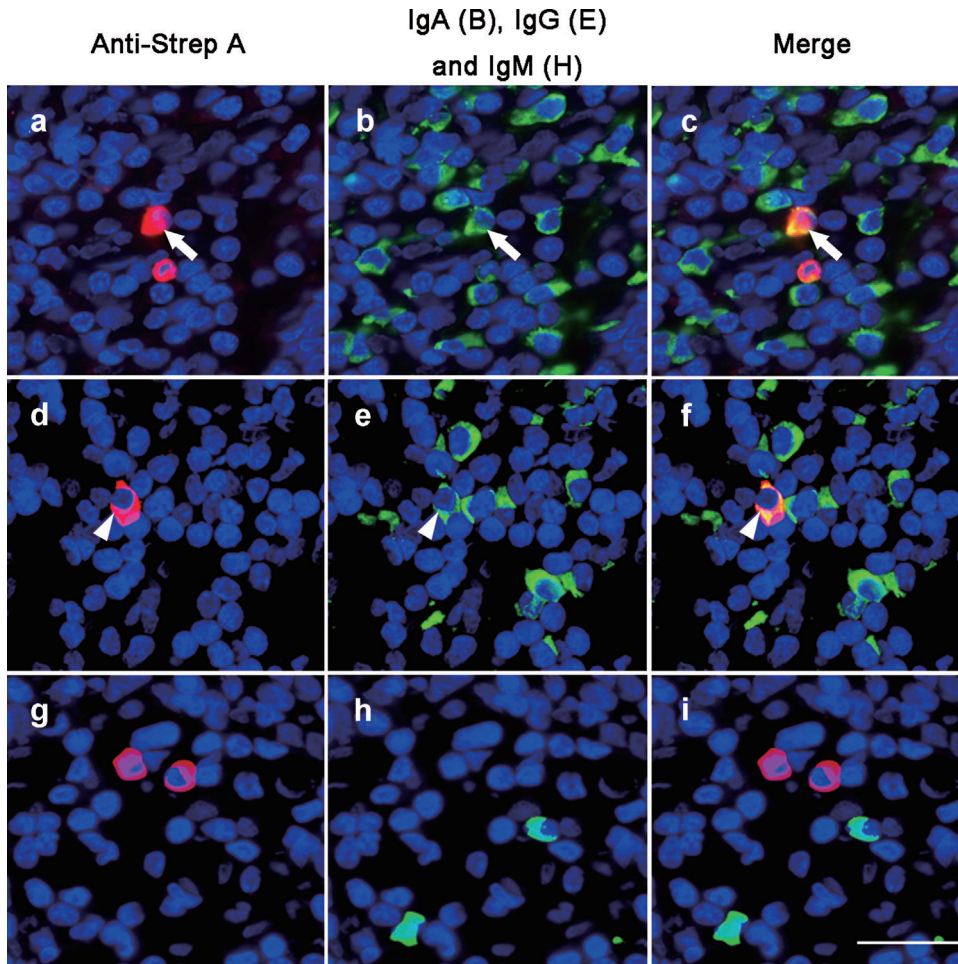


Fig. 6. Demonstration of the immunoglobulin class of Strep A-reactive antibodies produced by plasma cells in recurrent tonsillitis (Case 3). (a, d, and g) Anti-Strep A reactivity is stained red with Alexa Fluor 568, whereas (b, e and h) immunoglobulins are stained green with DyLight 488. The nuclei are stained blue with DAPI. Arrows indicate plasma cells producing IgA-type Strep A-reactive antibodies and arrowheads indicate the IgG-type. No IgM-type plasma cells produce Strep A-reactive antibodies. Scale bar represents 50 μm .

component was immunoreactive in some of the superficially located reticular epithelial cells of the palatine tonsil in this subject with recurrent tonsillitis. IgA (α -chain) immunoreactivity was co-localized in the secretory component-positive epithelial cells, representing transepithelial transportation of secretory IgA (Fig. 7).

Clinicopathological correlations

Identification of anti-Strep A reactivity was correlated with high frequency of recurrence: All six lesions with high frequency of recurrence were positive with the enzyme-labeled antigen method; two of the four cases with moderate frequency of recurrence were also positive. All five tonsils with relatively high density of

anti-Strep A reactivity ($>1.0\%$) belonged to the high frequency group (Table 1). There was a statistically significant correlation between the percentage of plasma cells reactive with Strep A and the frequency of recurrence of tonsillitis. Pearson's product-moment correlation coefficient was calculated as 0.80 ($P < 0.01$).

DISCUSSION

S. pyogenes causes 20–40% of acute tonsillitis (5). In the present series, microbial culture examination was performed only in three cases, all three of which were positive for *S. pyogenes*. Using the *S. pyogenes*-specific PCR assay for the genes of V-type Na^+ ATPase subunit B (19), streptococcal pyrogenic exotoxin B (20), and M protein (21), the bacterial genomes were proven in all 12

Table 3. Strep A-reactive antibody-producing plasma cells and immunoglobulin classes in eight cases of recurrent tonsillitis

Case	Area of section (mm ²)	Number of Strep A-reactive antibody-producing cells/section			Number of immunoglobulin-producing cells/section			Percentage of Strep A-reactive antibody-producing cells	
		IgA	IgG	IgM	IgA	IgG	IgM	Anti-Strep A (IgA)/IgA (%)	Anti-Strep A (IgG)/IgG (%)
1	30	3 (43)	4 (57)	0 (0)	314 (32)	503 (51)	175 (18)	1.0	0.8
2	50	7 (70)	3 (30)	0 (0)	331 (28)	702 (60)	129 (11)	2.1	0.4
3	51	12 (57)	9 (43)	0 (0)	424 (29)	839 (58)	183 (13)	2.8	1.1
4	27	3 (43)	4 (57)	0 (0)	396 (34)	665 (57)	104 (9)	0.8	0.6
5	44	3 (38)	5 (63)	0 (0)	367 (31)	694 (59)	110 (9)	0.8	0.7
6	35	1 (50)	1 (50)	0 (0)	349 (32)	618 (57)	122 (11)	0.3	0.2
7	19	3 (60)	2 (40)	0 (0)	287 (28)	623 (62)	98 (10)	1.0	0.3
8	31	3 (43)	4 (57)	0 (0)	339 (30)	675 (60)	104 (9)	0.9	0.6
Total number		35	32	0	2807	5319	1025		
Mean percentage		52%	48%	0%	31%	58%	11%	1.2	0.6

Parentheses indicate the percentage.

cases of recurrent tonsillitis. The samples used in the present study were from subjects with serious illness who had requested tonsillectomy. Multiple recurrences are observed in 20–30% of subjects with tonsillitis caused by *S. pyogenes* (27, 28). Normal flora inhibits replication of *S. pyogenes* by competing for nutrients, whereas excessive prescribing of antibacterial drugs leads to killing of the normal flora on the throat, which increases susceptibility to *S. pyogenes* infection (8, 29). Reportedly, the normal flora is significantly suppressed in *S. pyogenes*-infected tonsils (30).

Sequence analysis of the 5' end of the amplified *emm* gene encoding M protein of *S. pyogenes* showed that nine lesions (75%) were caused by type *emm12* and three (25%) by type *emm1*. Reportedly, *emm12* is the most common *emm* genotype of *S. pyogenes* provoking tonsillitis (31), whereas *emm1* is intimately associated

with invasive/systemic infections (32). *S. pyogenes* of types *emm12* and *emm1* causes tonsillitis in 3–6 year old infants, while the bacteria of other *emm* genotypes, such as *emm2*, 3, 4, 6 and 28, are often seen in pharyngeal isolates from older subjects (33).

S. pyogenes expresses streptococcal group A (Strep A) carbohydrate antigen on the cell wall (11, 12). An immunochromatography kit that uses Strep A-reactive antibodies for rapid detection of *S. pyogenes* in throat swabs is now commercially available (10). “Dipstick Eiken Strep A” can detect *S. pyogenes* in 1.5×10^5 CFU/swab. No cross reactions were seen among 27 strains from 25 species of microorganisms: The sensitivity of “Dipstick Eiken Strep A” is as high as 92.9% (11).

Strep A is a known potent immunogen of *S. pyogenes* that induces antibodies in humans (34–38). *S. pyogenes*-

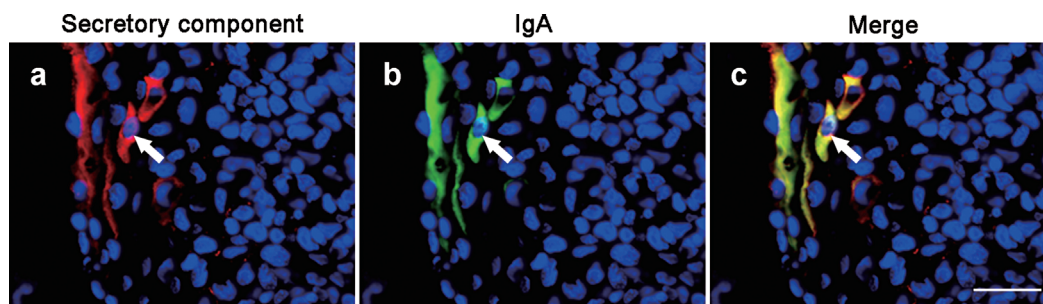


Fig. 7. Double immunofluorescence analysis for secretory component and IgA in recurrent tonsillitis (Case 1). (a) The secretory component is stained red with Alexa Fluor 568 and (b) IgA is stained green with DyLight 488. The nuclei are stained blue with DAPI. Panel C is a merged image. Arrows indicate the superficially located epithelial cells (reticular keratinocytes) co-expressing secretory component and IgA. This illustrates transepithelial transport of secretory IgA. Scale bar represents 50 μ m.

induced acute tonsillitis is common among children (39), and high serum titers of Strep A-reactive antibodies are negatively correlated with *S. pyogenes* colonization of the throat (36). These findings strongly suggest that Strep A-reactive antibodies suppress the growth of *S. pyogenes*.

By employing an enzyme-labeled antigen method (15–18), we histochemically visualized the sites of immune reaction against Strep A in a rat experimental model and also in tonsils from humans with recurrent tonsillitis. Specific antibody responses to Strep A were visualized in eight of the 12 surgically removed tonsils (mainly from children). The specificity of these reactions was confirmed by an absorption experiment and the negativity in both control non-immune rat lymph nodes and in two human control tonsils. The cells with positively signals were CD79a-immunoreactive, confirming antibody production in plasma cells.

The following findings are particularly noteworthy.

1. This is the first report describing histochemical identification of antibody responses to the carbohydrate antigen. Our previous studies of the enzyme-labeled antigen method employed biotinylated proteins as probes (15–18).
2. Frequent recurrences of tonsillitis are considered an important stimulus for induction of Strep A-reactive antibody production locally within the infected tonsil. Reportedly, Strep A-reactive antibodies in the serum accompany neutralizing/killing activity against *S. pyogenes* (36). However, we detected *S. pyogenes* genomes in all 12 lesions evaluated. Further studies are needed to determine whether Strep A-reactive antibodies produced locally within the tonsil can inhibit the colonization and growth of *S. pyogenes*.
3. Plasma cells producing antibodies reactive with Strep A account for 2.2–4.2% of all plasma cells in lymph nodes of rats immunized with boiled *S. pyogenes* and 0.4–2.0% of all plasma cells in *S. pyogenes*-infected human tonsils. In our previous experimental model, locally challenged protein antigens (horseradish peroxidase, ovalbumin and keyhole limpet hemocyanin) actively provoked specific antibody responses in the regional lymph nodes, in which 20–40% of plasma cells produced antibodies against the single protein (15). In the present study, the immunogen (*S. pyogenes*) possesses multiple antigens and antigenic epitopes, probably explaining the smaller percentage of specific antibody response to Strep A. Further histochemical analysis using biotinylated protein antigens on *S. pyogenes* is needed.
4. Strep A-reactive antibodies produced by plasma cells in the human tonsil belonged to both the IgA and IgG classes in an approximately 1:1 ratio. The lack of IgM

antibodies is probably a result of repeated antigenic challenge in the infected tonsils. The co-localization of secretory component and IgA in the superficially located reticular epithelial cells strongly suggests active surface transportation of secretory IgA (26). IgG secretion from the tonsil occurs by passive leakage (40). It has been documented that *S. pyogenes* colonizing the tonsillar surface is coated by both secretory IgA and IgG (41). Tang *et al.* reported that plasma cells are mainly distributed within the vascularized reticular mucosa of the tonsil (14). In our analysis, CD38-positive plasma cells were distributed both in and just beneath the reticular mucosa. Turesson reported that 31% of plasma cells in recurrent tonsillitis are IgA, 61% IgG and 9% IgM (42); these proportions were reproduced in the present study (31% IgA, 58% IgG, 11% IgM). It has therefore been postulated that specific plasma cells located just below the mucosa move into the reticular squamous mucosa to release Strep A-reactive antibodies into this loosely reticulated mucosal structure. The released antibodies of both IgA and IgG types may thus combine to combat *S. pyogenes* and influence microbial growth in the tonsil. In general, IgG class antibodies often accompany neutralizing activity but are not associated with active transportation onto the mucosal surface, whereas secretory IgA promotes clearance of pathogens by blocking their access to epithelial receptors, entrapping them in mucus and facilitating their removal by mucociliary activities (43). Secretory IgA is also able to quench bacterial virulence factors directly (43). The biological significance of secretory IgA and IgG against Strep A locally produced in infected tonsils should be further studied.

In summary, our results indicate that the enzyme-labeled antigen method is a valuable tool for visualizing plasma cells producing specific antibodies against carbohydrate antigen. Our novel technique, which allows microscopic observation of previously unknown disease processes, can be extended to analyzing a variety of lesions with dense infiltration of plasma cells, such as in persistent infections, allergic and autoimmune disorders and certain neoplastic lesions.

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DISCLOSURE

The authors have no conflicts of interest associated with the present study to declare. Eiken Chemical provided the bacteria and biotinylated or unlabeled Strep A purely based on scientific cooperation and without any financial support.

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