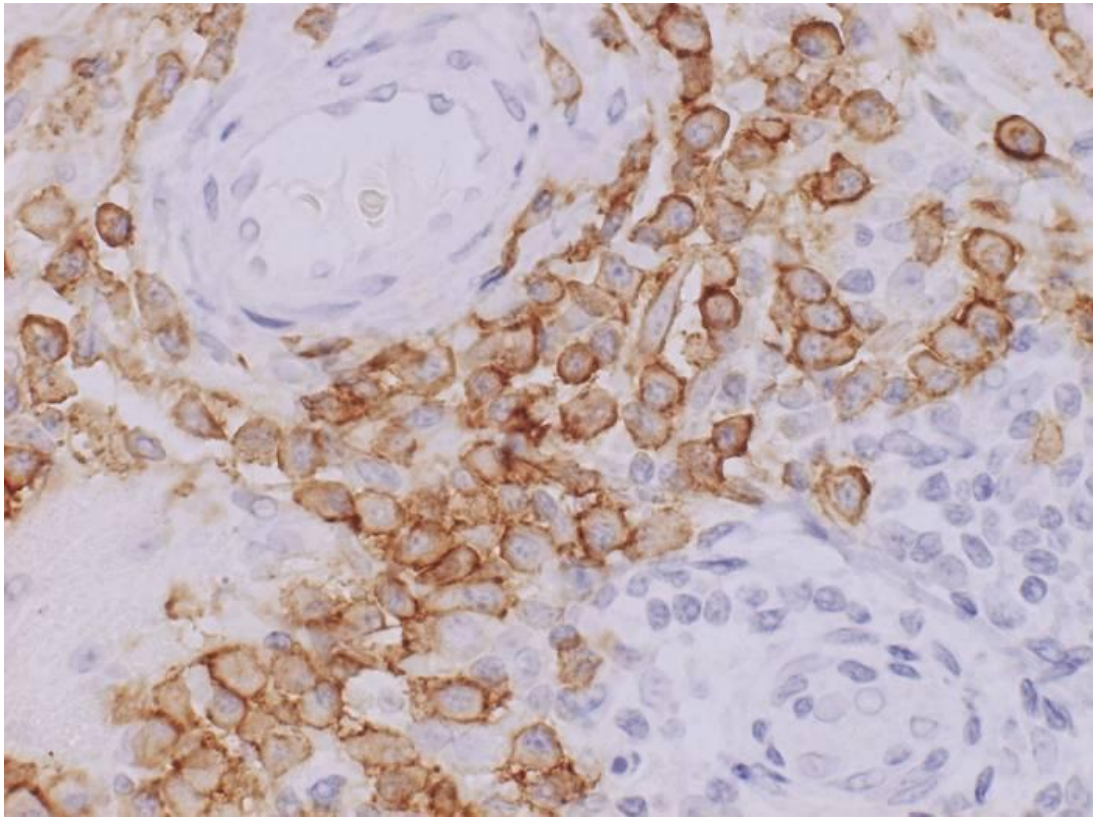
Enzyme-labeled antigen method for detecting specific antibody-producing plasma cells

In chronic inflammatory lesions of autoimmune and infectious diseases, plasma cells are frequently observed. Antigens recognized by antibodies produced by the plasma cells mostly remain unclear. We have developed the enzyme-labeled antigen method. Target biotinylated antigens were reacted to detect plasma cells producing specific antibodies in paraformaldehyde-fixed frozen sections of inflammatory lesions. Our novel approach visualized tissue plasma cells that produced 1) autoantibodies in rheumatoid arthritis, 2) antibodies against major antigens of *Porphyromonas gingivalis* in periodontitis or radicular cyst, and 3) antibodies against a carbohydrate antigen, Strep A, of *Streptococcus pyogenes* in recurrent tonsillitis. This new histochemical technique may give us a breakthrough for understanding the disease from a pathophysiological viewpoint, simply because the immunocytes are seen within the lesion. The technique requires frozen sections, and it is difficult to apply it to formalin-fixed, paraffin-embedded sections.

Ref.-1: Mizutani Y, et al. Enzyme-labeled antigen method: development and application of the novel approach for identifying plasma cells locally producing disease-specific antibodies in inflammatory lesion. Acta Histochem Cytochem 2016; 49(1): 9-19. doi: 10.1267/ahc.15030

Ref.-2: Mizutani Y, et al. Enzyme-labeled antigen method: factors influencing the deterioration of antigen-binding activity of specific antibodies during formalin fixation and paraffin embedding. Acta Histochem Cytochem 2022; 55(5): 129-148. doi: 10.1267/ahc.22-00023

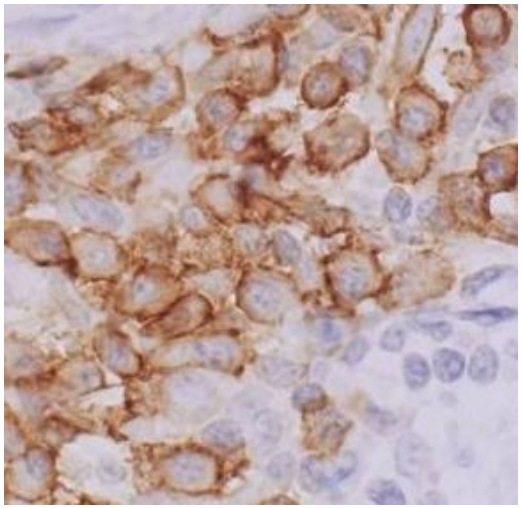
Plasma cells densely infiltrate in the inflammatory lesions, such as autoimmune diseases, infections and malignant tumors



CD138-positive plasma cells in synovial tissue of rheumatoid arthritis

Target antigens of the antibodies produced by plasma cells in the lesion are unknown.

CD138-positive plasma cells



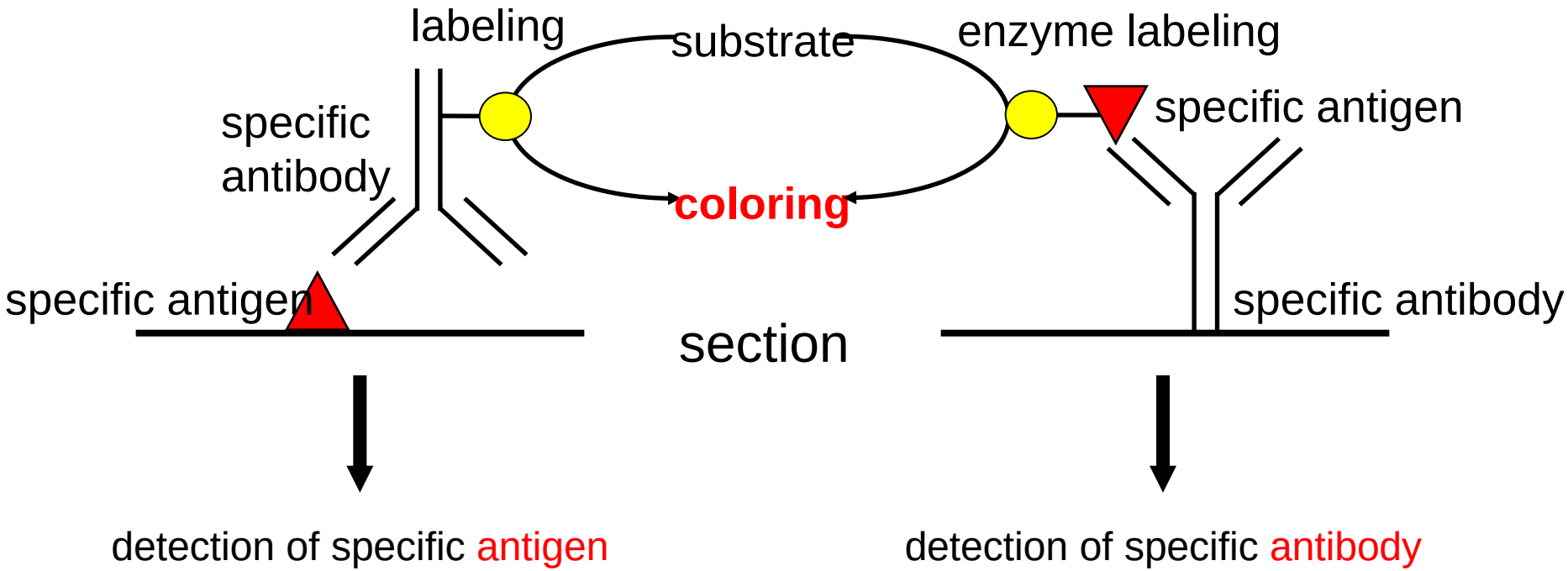
The antibodies produced within the lesion should be related to the pathogensis.

Histochemical detection of the specific antibodies should be useful for the diagnosis and treatment of diseases.

Enzyme-labeled antigen method

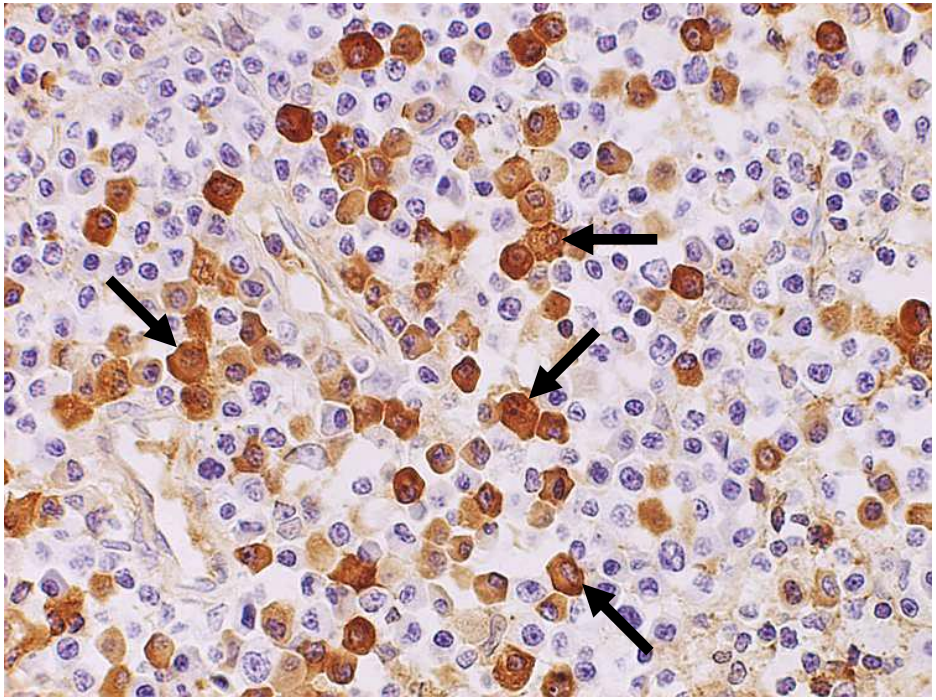
Enzyme-labeled antibody method（immunostaining）

Enzyme-labeled antigen method

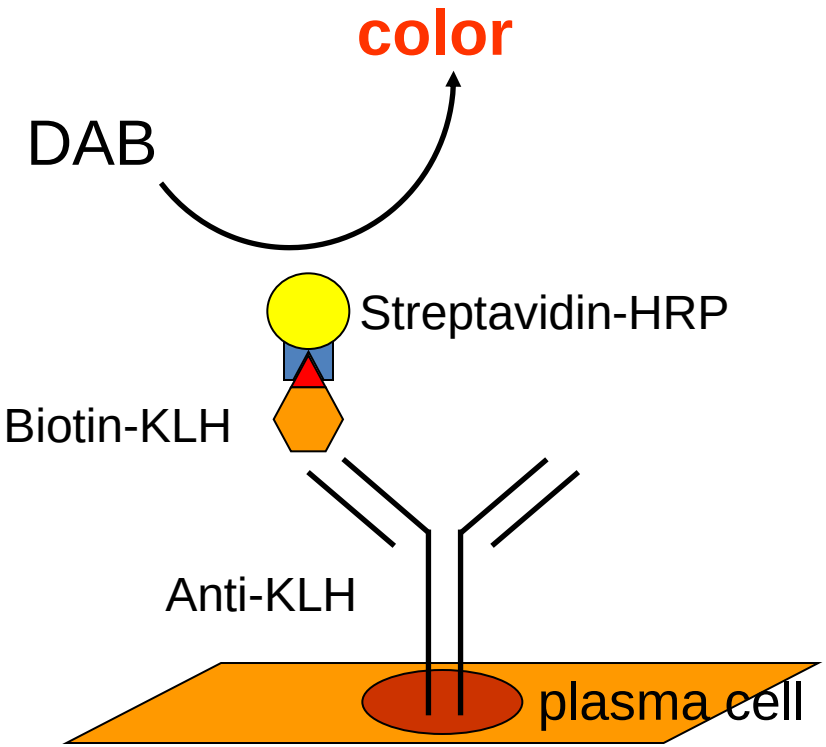


The enzyme-labeled antigen method is a reversed form technique of the enzyme-labeled antibody method.

The enzyme labeled antigen method visualizes the site of antibody production.

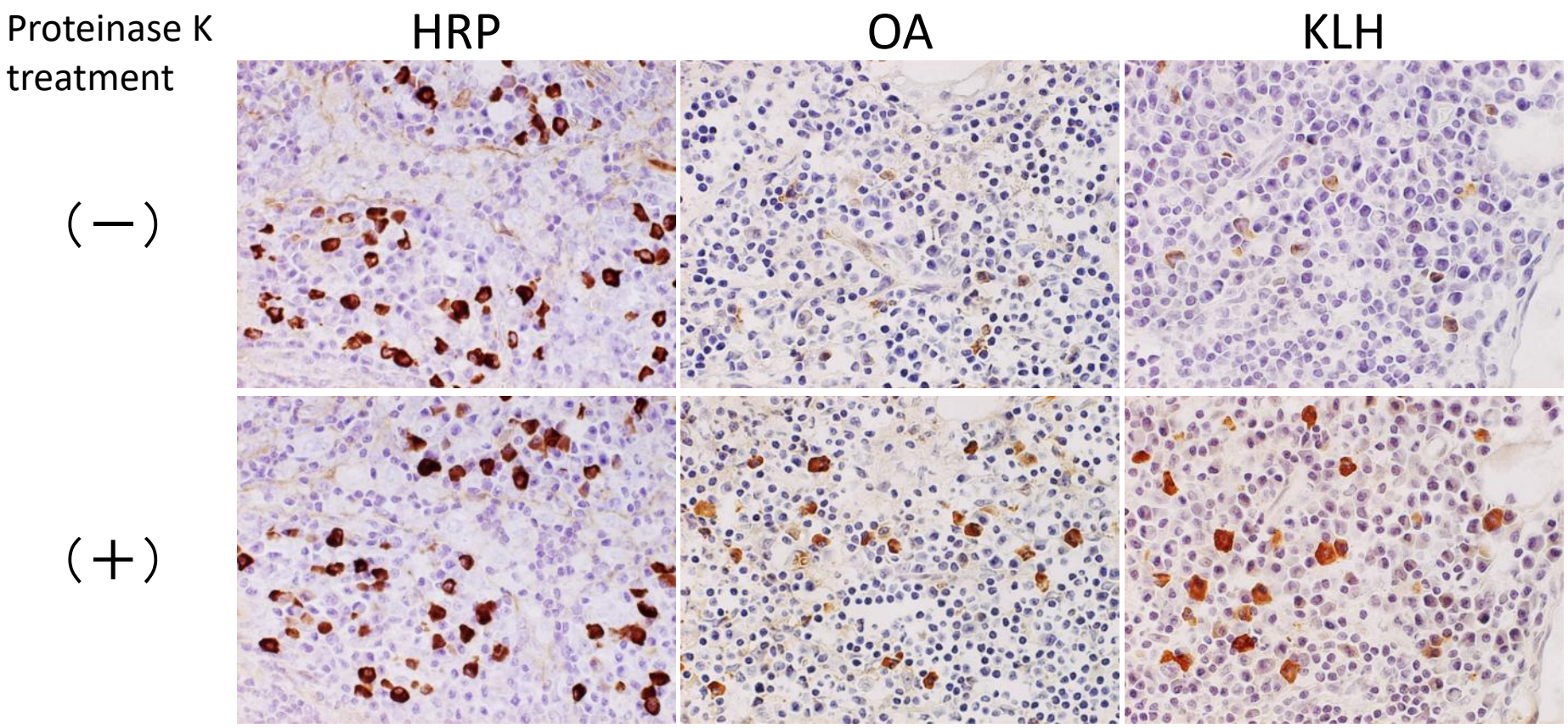


Plasma cells producing antibodies against keyhole limpet hemocyanin（KLH）in the rat lymph node



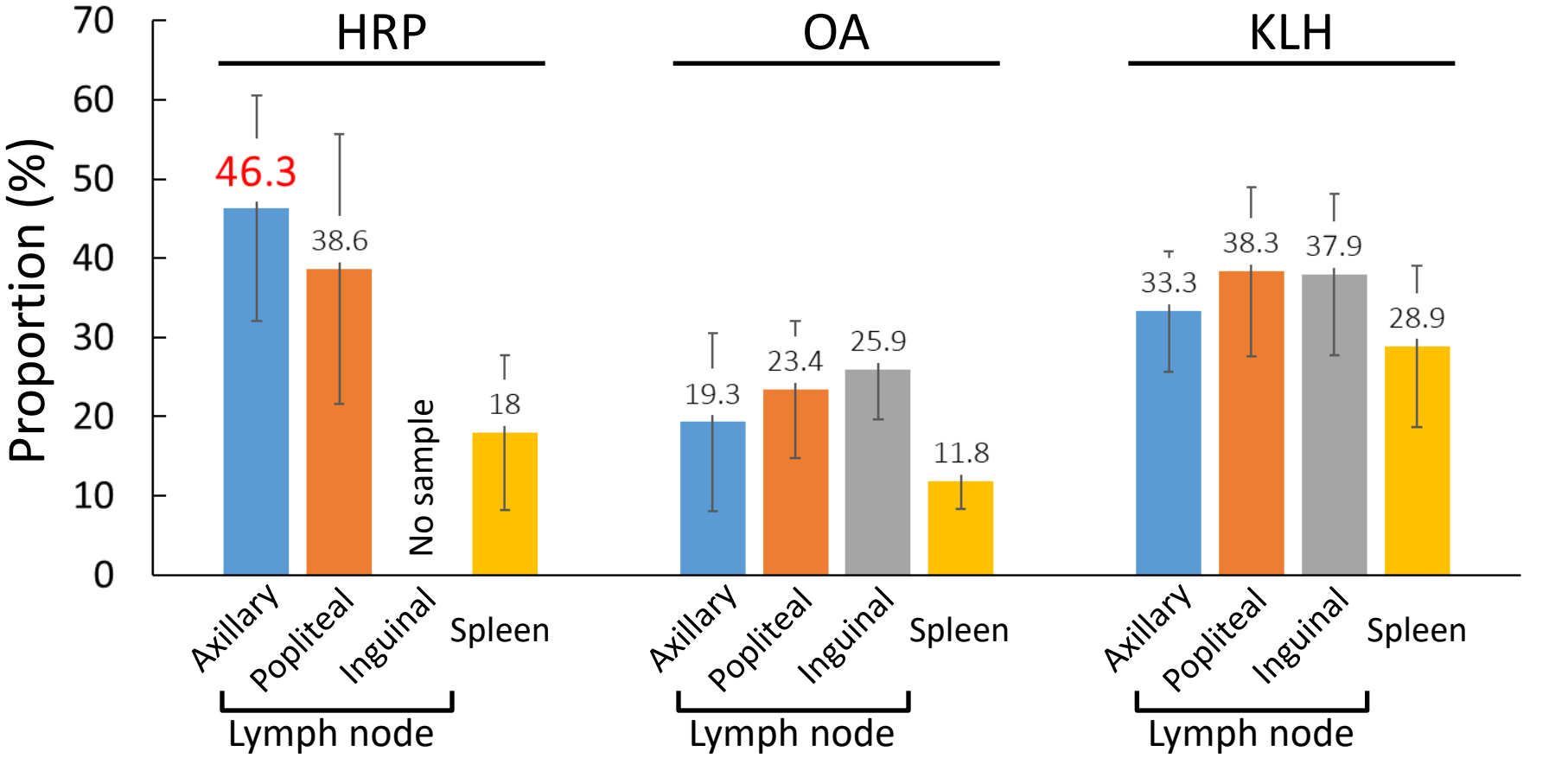
Frozen section of lymph node

The enzyme-labeled antigen method to detect specific antibodies in immunized rat lymph nodes using horseradish peroxidase (HRP), biotinylated ovalbumin (OA) and biotinylated keyhole limpet hemocyanin (KLH)



Except for HRP, pretreatment with proteinase K (5 μg/mL at room temperature for 15 min) is needed to detect specific antibodies in paraformaldehyde-fixed frozen sections.

Percentage of plasma cells producing antigen-specific antibodies among total plasma cells in the lymph node and spleen



（Mean±SD, n=3 or 4）

Under a hyperimmune state, a high percentage of plasma cells produce antibodies against the immunized antigens.

Summary of the enzyme-labeled antigen method in the rat experiment under the hyperimmune state

- The conditions for the enzyme-labeled antigen method using biotinylated antigen as a probe

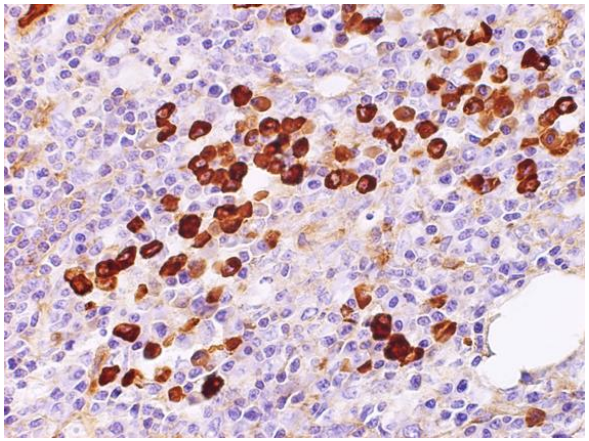
①The use of 4% paraformaldehyde-fixed frozen sections needed

②Retrieval of antibody reactivity by the pretreatment with proteinase K needed

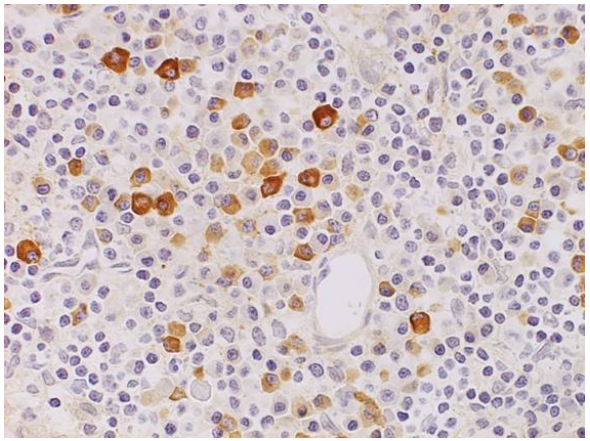
- Numerous plasma cells producing specific antibodies are observed in the regional lymph nodes and spleen.

Mizutani Y et al. J Histochem Cytochem 2009; 57: 101-111.

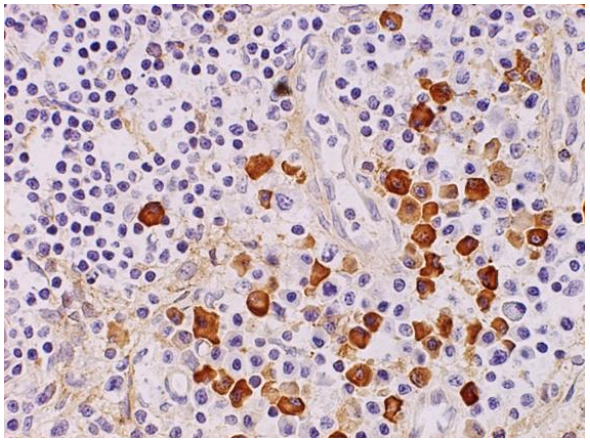
A variety of specific antibodies can be detected



Horseradish peroxidase



Ovalbumin



Keyhole limpet hemocyanin

Lymph nodes of the rat experimentally immunized with these antigens

We need the target antigen!

↓

We should detect and purify the target antigen.

How should we do it?

The method of identification of specific antigens

- In case of autoimmune disease, we should have a library of autoantigens.
- In case of infectious disease, we should have a protein library of the pathogen.

Then, patient's serum or antibodies extracted from the lesion will react with the target antigen.

Techniques of Sawasaki's lab, Ehime University, Matsuyama, Japan

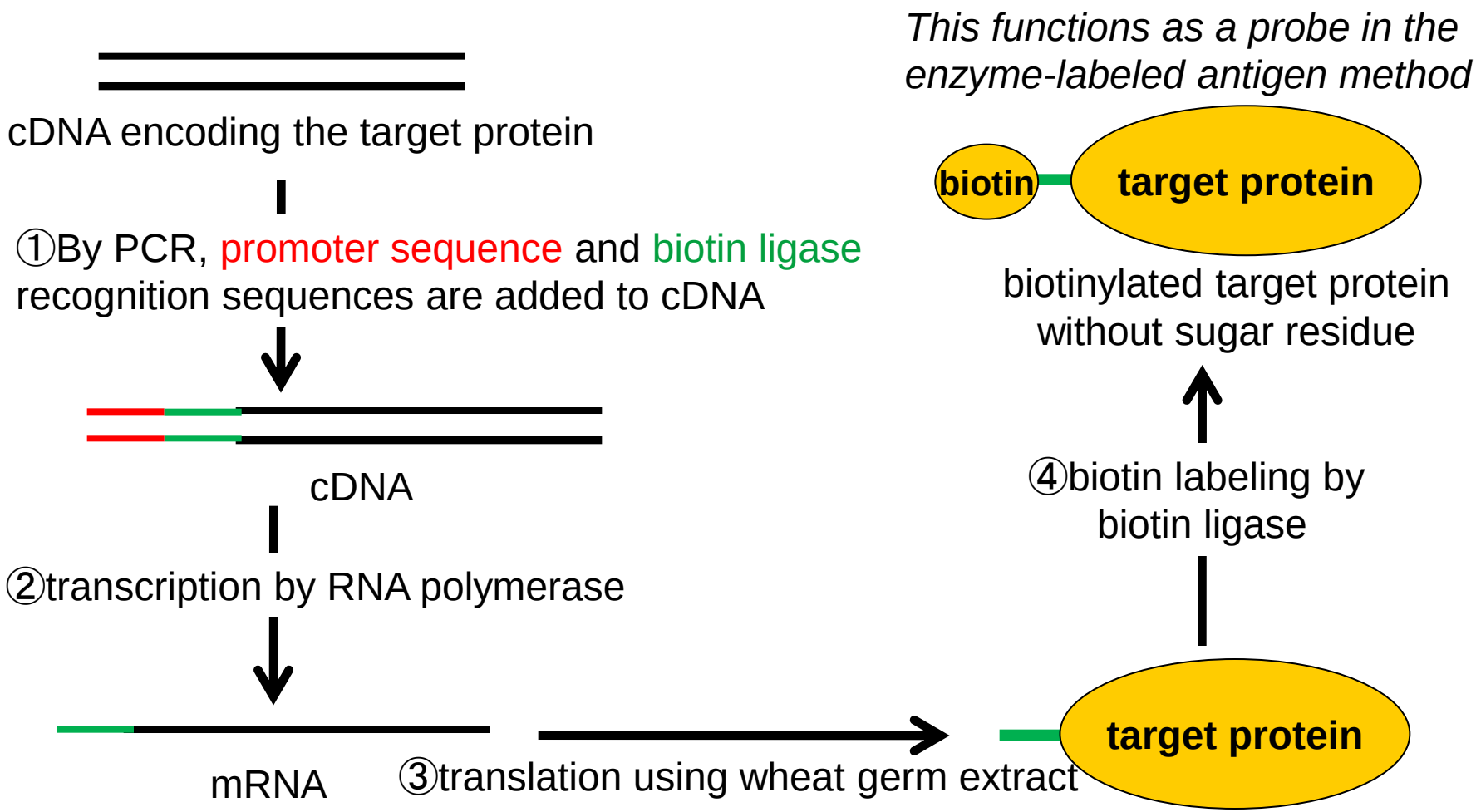
By using the acellular protein synthesis system, Sawasaki's group has established a human/mouse libraries containing more than 2,000 biotinylated proteins.

The target antigens can be screened from the library by using the patient's serum: This is called as the AlphaScreen method.

Acellular protein synthesis system:
Wheat germ extract effectively synthesizes proteins encoded by cDNA amplified by RT-PCR. *E. coli* or cultured cells are NOT used in this system.

We can detect the target antigens of the antibodies produced within the lesion.

The acellular protein synthesis system

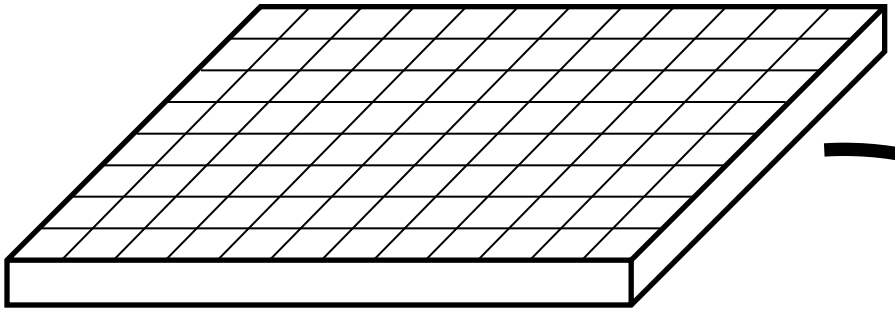


The protein synthesis can be performed automatically

target protein (antigen) detection

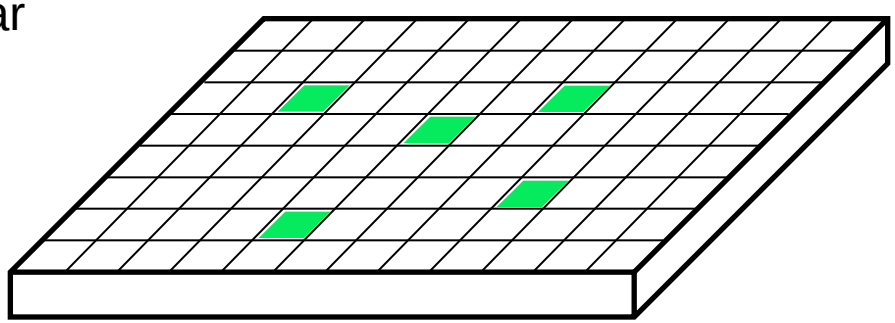
serum

Patient's serum is added to each well.



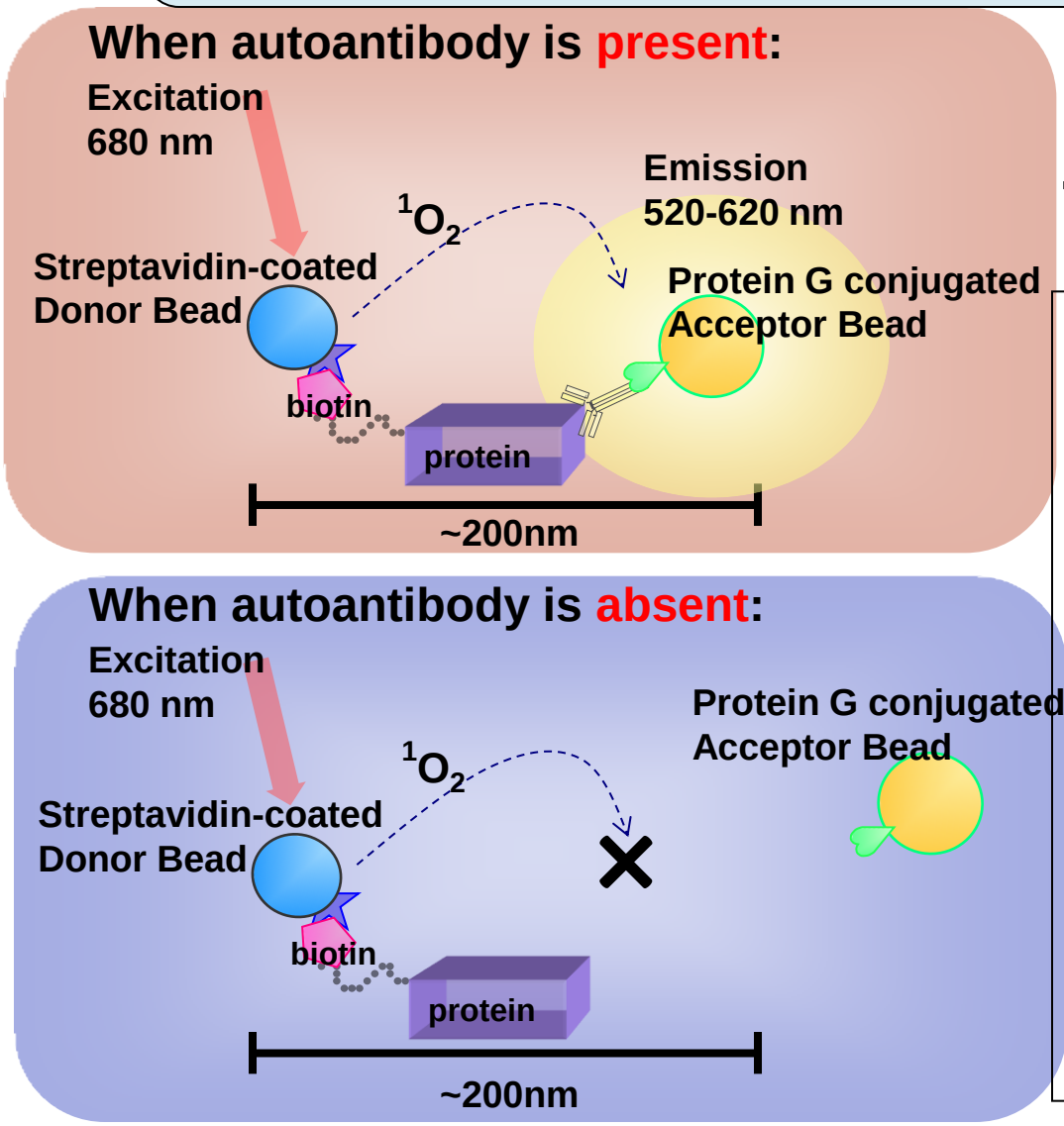
Each well contains different biotinylated protein synthesized by the acellular protein synthesis system.

AlphaScreen method



When the antibody reacts with the antigen, the well shows fluorescent signals.

Detection of the antigen antibody reaction with AlphaScreen method



→ Fluorescent signal Is detectable.

Merit

- Reaction in solution
→The antigens remain intact.

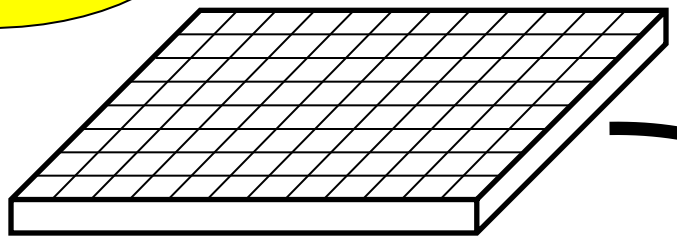
- Small volume of sample needed (serum: 0.025 μL/protein)
- No need for rinsing
- 384-well plate used
→High efficiency detection

- High sensitivity and low background

How can we detect the antigen ?

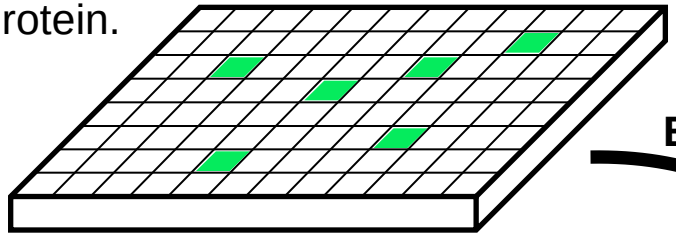
serum
or
tissue extract

Serum/extract added to the well



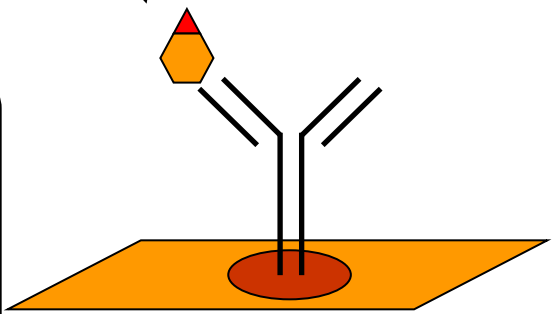
Each well contains different protein.

AlphaScreen method



Antigens reacted with serum/extract detected

Enzyme-labeled antigen method



tissue section

The detected antigens can directly be used as probes for the enzyme-labeled antigen method.

Significance of our idea

1. The target antigens of the antibodies produced within the lesion are detected: →analysis of pathogenesis
2. The site of specific antibody production is demonstrated. →preparation of probes for the enzyme-labeled antigen method
3. This method is applicable to a variety of diseases showing plasma cell infiltration.

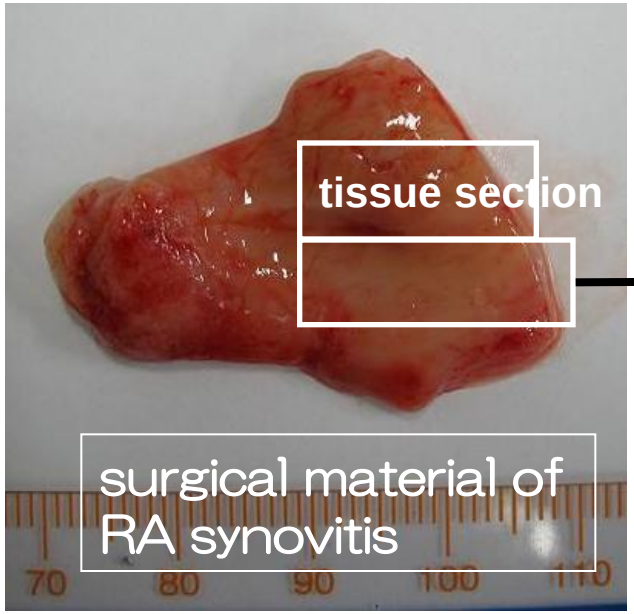
→A novel histochemical technique for analyzing the disease process

Applicability of the enzyme-labeled antigen method

1. Autoimmune diseases, such as rheumatoid arthritis, Hashimoto's thyroiditis, Sjoegren's syndrome and autoimmune gastritis (type A gastritis)
2. Infectious diseases, such as *Helicobacter pylori*-induced gastritis and syphylis
3. Malignant tumors showing heavy infiltration of plasma cells in the stroma, including EBV-related tumors (nasopharyngeal carcinoma, gastric carcinoma with lymphoid stroma, Hodgkin's lymphoma and nasal NK/T lymphoma), HPV-related uterine cervical carcinoma, MALT lymphoma, and even multiple myeloma (plasmacytoma)

Application of the enzyme-labeled antigen method to rheumatoid arthritis

Rheumatoid arthritis (RA) as a model of the enzyme-labeled antigen method



Autoantigen library prepared in the Endo's lab is used.

Homogenization in PBS

↓

Supernatant

↓

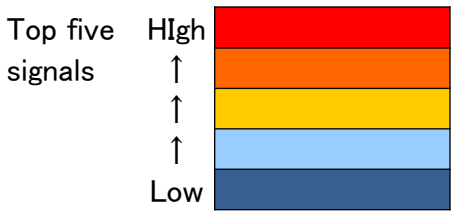
Screening of the antigens reactive to the antibodies in the supernatant by using the AlphaScreen method

↔

Comparison with the result using patient's serum

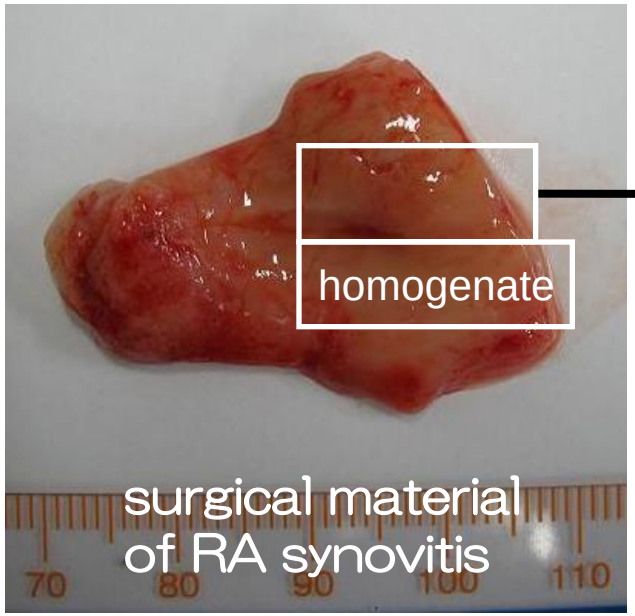
Results using the AlphaScreen method

Ag	Patient 1		Patient 2		Patient 3		Patient 4		Patient 5	
	extract	serum	extract	serum	extract	serum	extract	serum	extract	serum
1	4.3	8.7	4.8	32.8	10.6	6.3	10.1	18.7	5.7	3.8
2	77.6	86.8	1.3	2.0	1.4	1.3	1.4	1.6	1.2	1.3
3	2.9	5.1	2.4	12.7	5.2	4.0	4.9	13.6	6.1	2.0
4	0.9	3.7	13.2	13.3	2.3	6.9	1.8	12.8	1.3	5.8
5	1.9	3.4	6.8	22.5	4.1	4.6	2.1	7.4	1.7	1.8
6	7.2	1.1	1.1	0.9	1.1	1.1	1.1	1.0	1.1	1.2
7	1.1	1.1	7.3	5.3	3.6	2.0	1.4	2.6	1.6	1.4
8	2.7	3.5	6.2	12.0	3.2	2.7	1.8	5.9	1.3	2.1
9	2.7	5.4	5.9	10.4	10.3	3.4	3.4	9.2	6.2	2.5
10	3.4	2.9	5.9	2.7	96.4	1.4	6.3	1.8	1.6	1.2
11	7.9	13.1	6.0	19.1	11.9	5.4	9.4	15.0	6.1	2.3
12	3.3	5.9	4.0	12.8	11.2	2.2	2.8	11.5	3.3	1.5
13	0.9	14.6	6.7	40.1	1.8	10.4	2.5	41.2	0.8	4.7
14	9.1	11.1	3.1	30.4	5.1	8.7	7.7	21.1	5.3	3.8
15	4.4	7.5	3.0	20.1	6.5	4.8	8.1	13.0	5.1	2.1
16	9.0	14.8	1.6	13.9	3.0	5.4	5.1	15.6	4.7	2.8
17	4.8	8.0	5.3	35.6	15.7	11.1	11.2	30.0	5.7	4.4
18	6.9	9.6	5.9	30.9	17.0	11.9	14.9	30.4	9.3	3.7



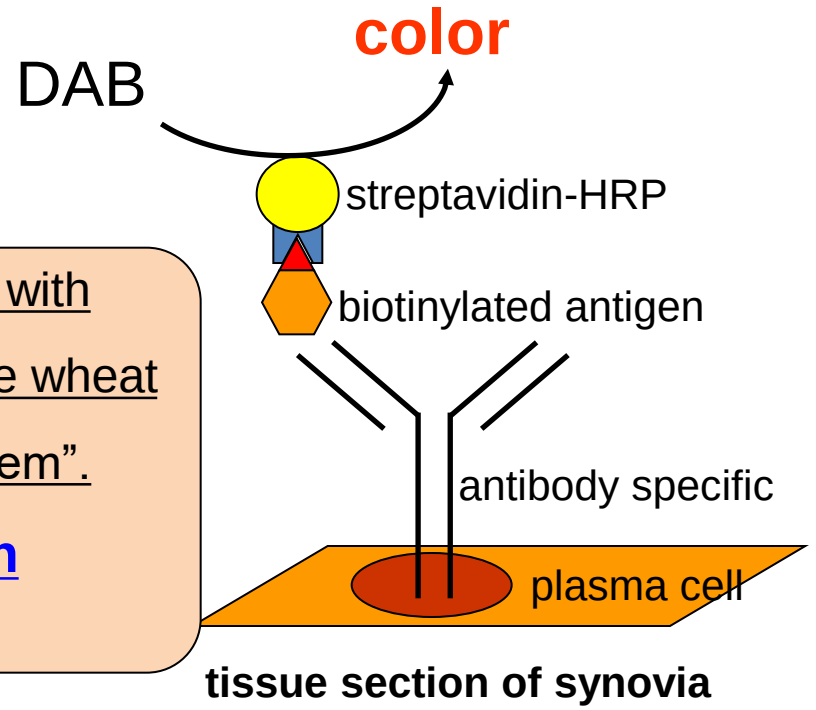
Reaction patterns are similar between the serum and extract, but some antigens are tissue extract-specific.

Use of the screened antigens for the enzyme-labeled antigen method



4% paraformaldehyde-fixed frozen sections

The screened antigens were used as probes for the enzyme-labeled antigen method

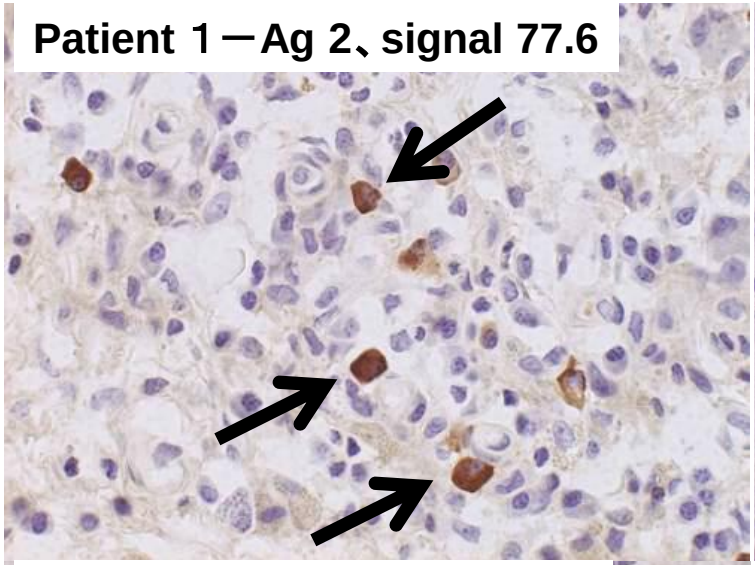


Five biotinylated antigens showing high signals with the AlphaScreen method were expressed by the wheat germ mediated “acellular protein synthesis system”.

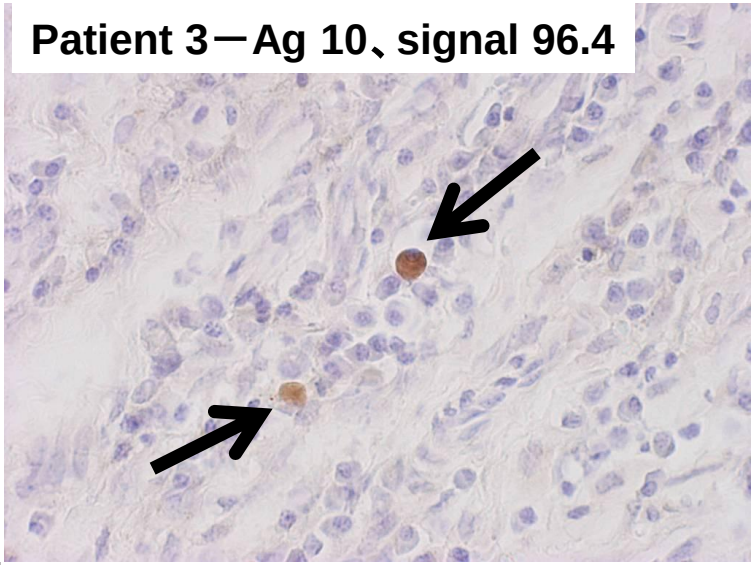
→**probes for the enzyme-labeled antigen method used without purification**

Samples of the enzyme-labeled antigen method in RA synovitis

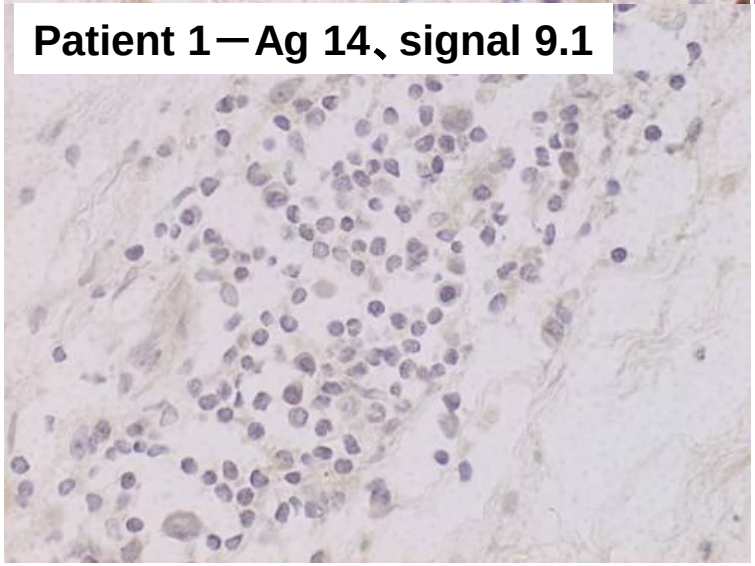
Patient 1－Ag 2、signal 77.6



Patient 3－Ag 10、signal 96.4



Patient 1－Ag 14、signal 9.1



・Plasma cells infiltrating in RA synovitis showed positivity against antigens with the highest signal intensity in patients 1 and 3.

・All the other combinations gave negative results. Enhancement of the detectability of the technique is needed.

Results of our approach to RA lesions

1. Antibodies of tissue origin reacted to multiple antigens in the autoantigen library.
2. Two of them worked as probes for the enzyme-labeled antigen method.
3. For improving the sensitivity of the method, increase of the antigen concentration and the number of biotin molecules labeled to the antigen may be needed.

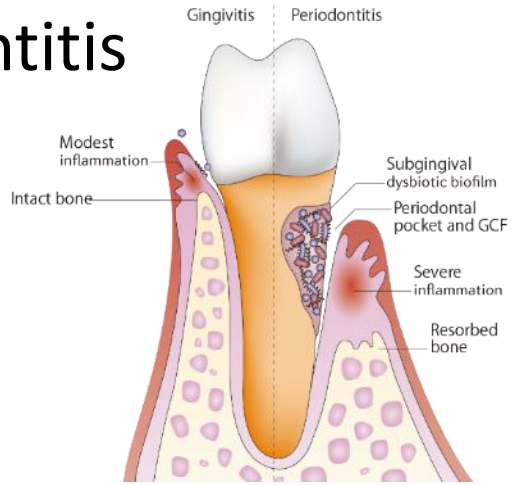
We proved that the target antigens could be detected from the protein library by using antibodies in the serm or those extracted from the lesion.

→This approach functions as a novel technique to analyze the disease mechanism.

Application of
the enzyme-labeled antigen method
to detect *Porphyromonas gingivalis*
antigens in radicular cyst and periodontitis

Studies of the enzyme-labeled antigen method for radicular cyst and periodontitis

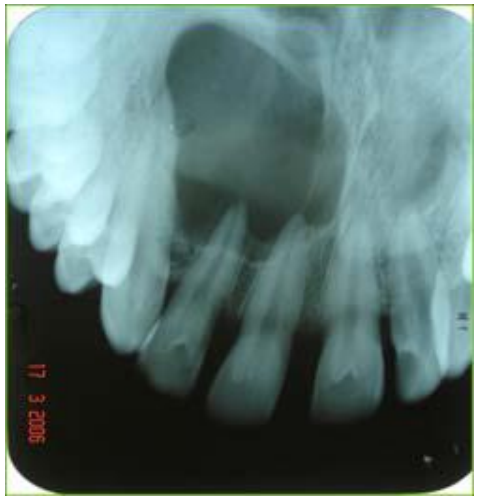
Periodontitis



Hajishengallis G, Nat Rev Immunol, 2015; 15(1):30-

- Periodontitis is a chronic inflammatory dental disorder with gingivitis and destruction of the alveolar bones of the jaw, caused by infection in the perdental pocket

Radicular cyst



Latoo S et al, J Med Edu Res 2009; 11(4): 187-189.

- Radicular cyst is caused by chronic apical periodontitis secondary to dental caries.
- Infection of anaerobic bacteria in the root canal is observed.

***Porphyromonas gingivalis*（Pg）is a pathogenic anaerobic bacterium in periodontitis**

Both lesions show marked infiltration of plasma cells, and the probability of the production of antibodies to Pg is high.

Analysis of antibody production against *Porphyromonas gingivalis* (Pg) antigens

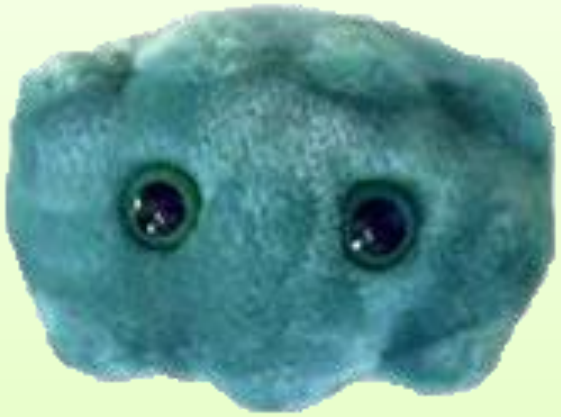
Target antigens of Pg

- Envelope protein "**Ag53**"

  (humoral immune responses confirmed)

- Pathogenic protease: gingipains

  Arg-gingipain and Lys-gingipain



Proteolytic enzyme (pro) and hemagglutiinin (hgp) domains of the gingipain

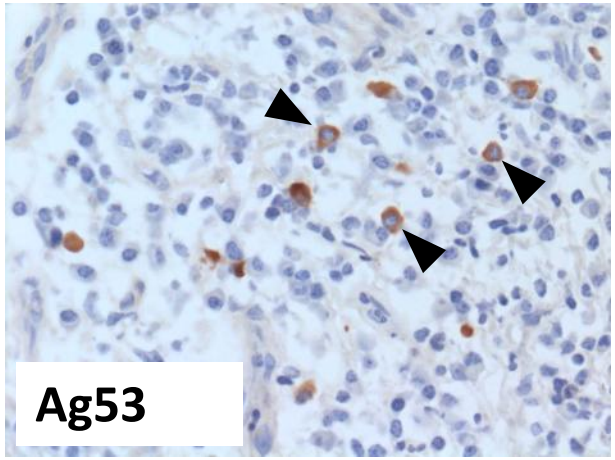
Arg-gingipain	Proteolytic enzyme 「**Arg-pro**」	hemagglutniin 「**Arg-hgp**」
	Homology: 76%	
Lys-gingipain	Proteolytic enzyme 「**Lys-pro**」	hemagglutnin 「**Lys-hgp**」

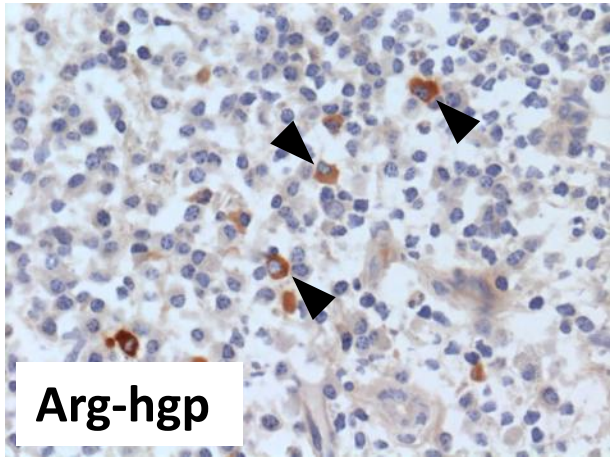
Antibody responses to the five kinds of Pg antigens were analyzed.

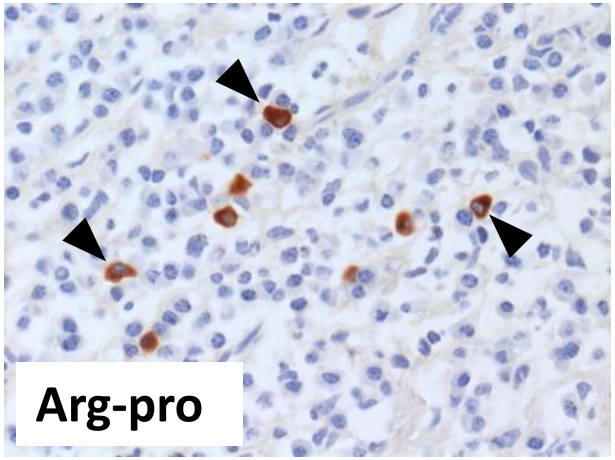
Anti-Pg antibody positive rates in the AlphaScreen method and enzyme-labeled antigen method (ELAM)

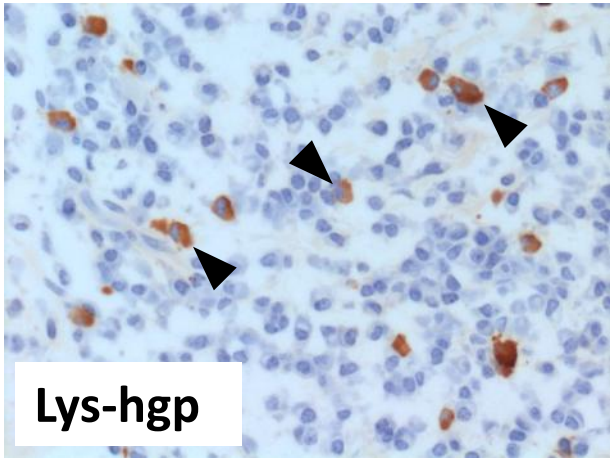
Antigen	AlphaScreen method				ELAM	
	Periodontitis (n=18)		Radicular cyst		Peri-odontitis (n=18)	Radicular cyst (n=8)
	Serum	Gum lesion	Serum (n=10)	Gum lesion (n=6)		
Ag53	0%	33%	10%	0%	28%	0%
Arg-hgp	22%	66%	20%	33%	83%	25%
Lys-hgp	11%	78%	20%	33%	89%	13%
Arg-pro	33%	89%	0%	0%	89%	0%
Lys-pro	28%	17%	10%	0%	22%	0%

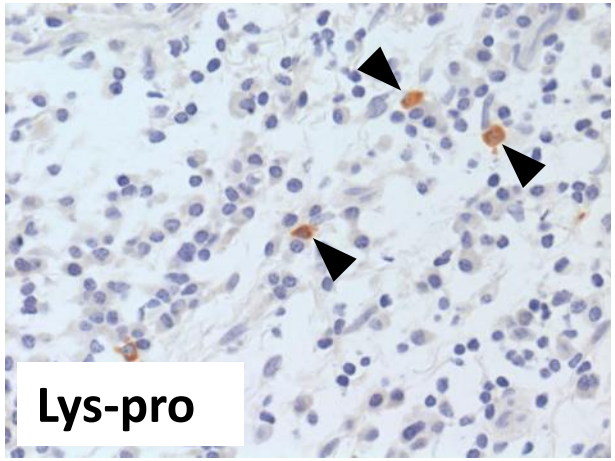
Enzyme-labeled antigen method in periodontitis





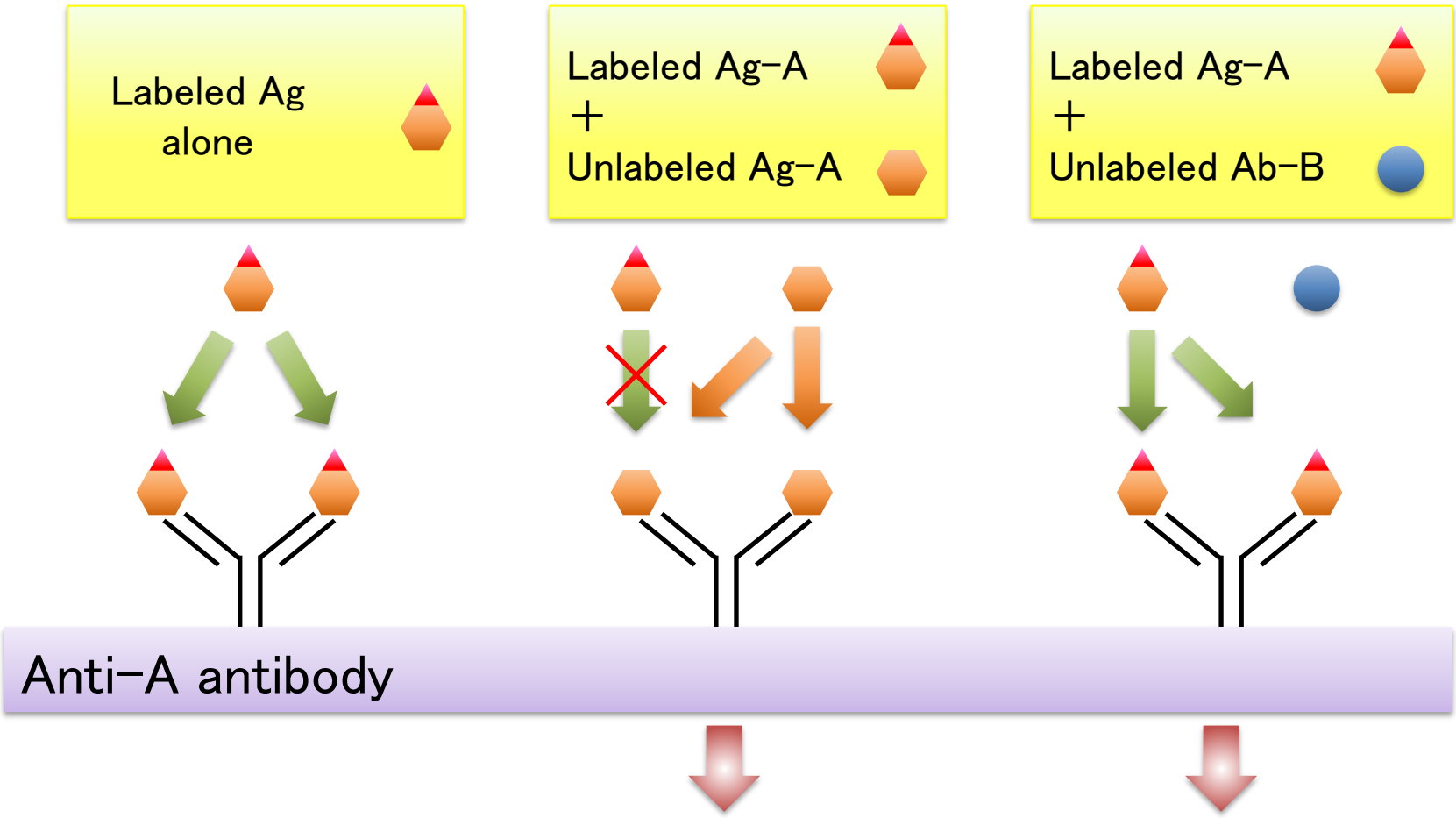




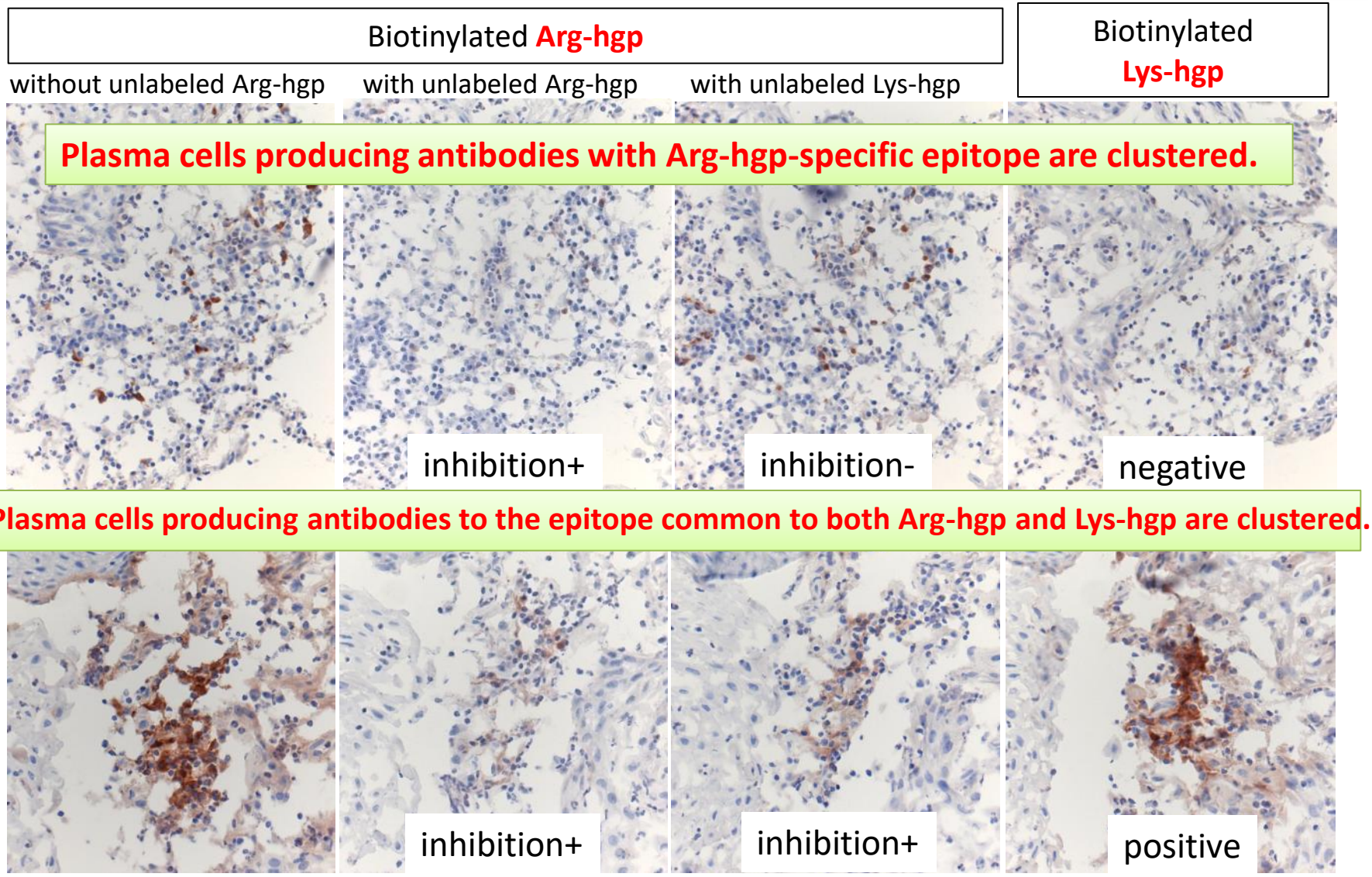


Plasma cells containing antibodies against all 5 kinds of Pg antigens can be demonstrated in the biopsied gum.

Detection of the specificity of antibodies on PFA-fixed frozen sections: *Competitive absorption test using biotin-labeled and unlabeled antigens*



The region specificity of the antibody produced in radicular cyst (Homology between Arg-hgp and Lys-hgp is 76%)

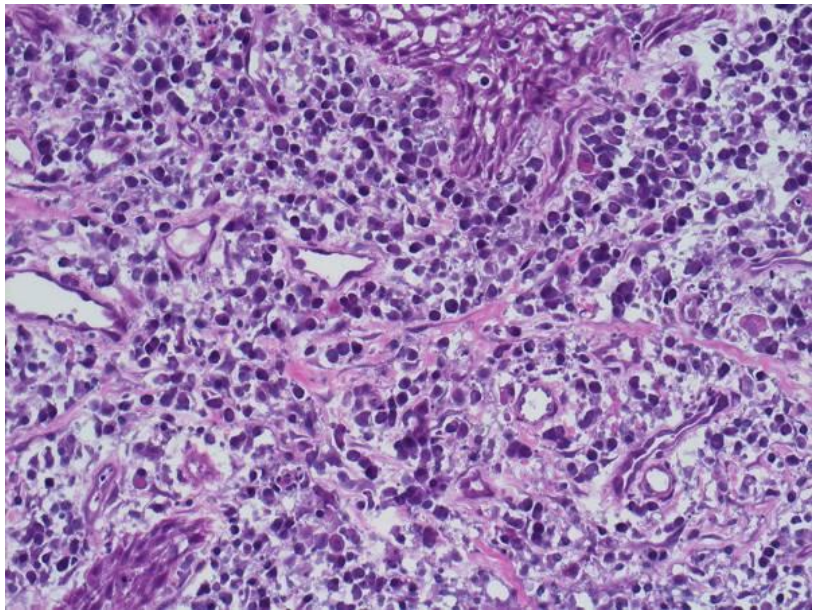


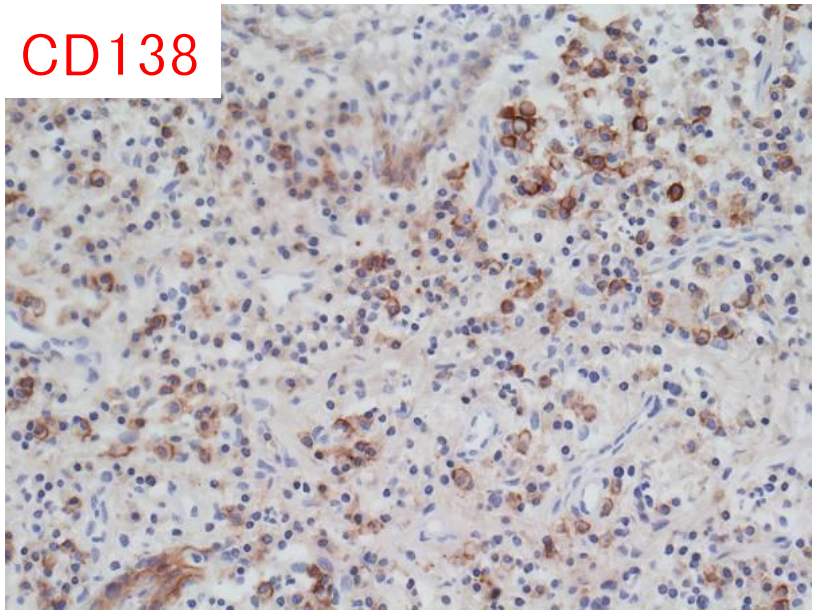
Micromonoclonality (region specificity) in an inflammatory lesion is shown.

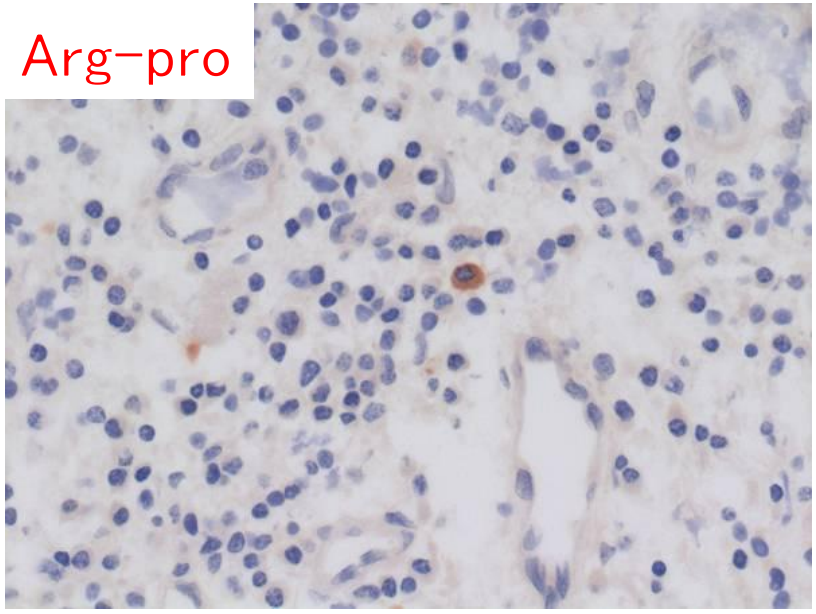
Summary of the results in periodontitis and radicular cyst

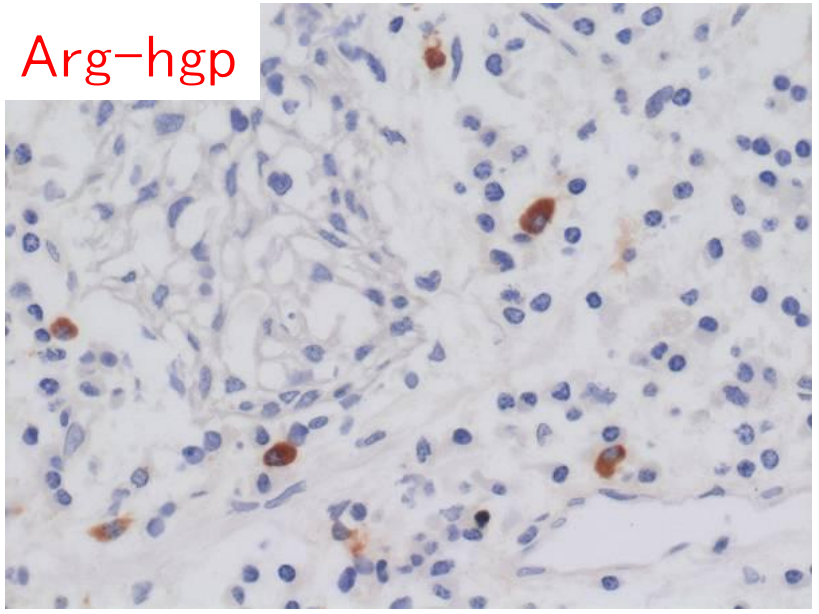
1) Antibodies detectable in the lesions are often negative in the serum.
2) The AlphaScreen signals are more frequently detected in the gum lesions than in the serum.
3) The AlphaScreen signals in the gum lesion roughly corresponds to the positivity of the ELAM.
4) Anti-Pg antibodies are more frequently detected in periodontitis than in radicular cyst.
5) Anti-Arg-pro antibodies are specifically detected in periodontitis, while undetectable in radicular cyst.
6) Pg infection is closely related to periodontitis rather than radicular cyst.

Application of the enzyme-labeled antigen method to periodontitis of the author



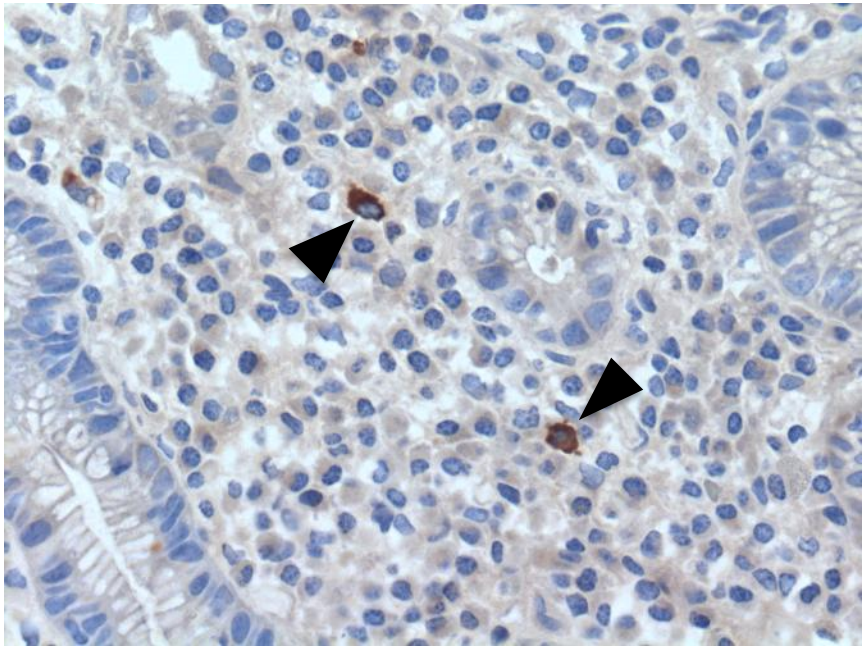






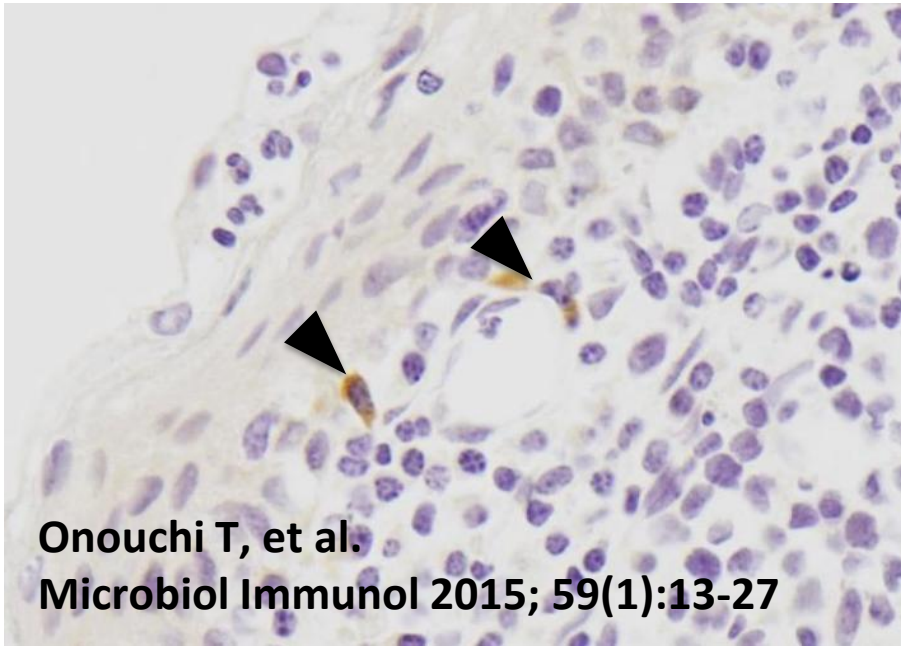
Other application of the enzyme-labeled antigen method

Helicobacter pylori-infected gastric mucosa



H. pylori-derived HSP60

Recurrent tonsillitis



Strep A（*S. pyogenes*-derived carbohydrate antigen）

The enzyme-labeled antigen method is applicable to a variety of inflammatory lesions with plasma cell infiltration.

“Enzyme-labeled antigen method”

- The enzyme-labeled antigen method is applicable to the pathogenic analysis of a variety of inflammatory lesions.
- Antibodies against specific antigens may be localized in the inflammatory lesion but without secretion into the serum. Such antibodies can be visualized with the enzyme-labeled antigen method.
- “Micromonoclonality” is visualizable in the inflammatory lesion such as radicular cyst.
- Problem-1: Paraform-aldehyde-fixed frozen sections are needed for the enzyme-labeled antigen method. Application to formalin-fixed, paraffin-embedded sections is requested.
- Problem-2: The effect of the quality of antigens (pI or molecular weight) on the staining should be evaluated.

Application of the enzyme-labeled antigen method to clinical specimens (1)

Mizutani Y, et al. Enzyme-labeled antigen method: histochemical detection of antigen-specific antibody-producing cells in tissue sections of rats immunized with horseradish peroxidase, ovalbumin, or keyhole limpet hemocyanin. J Histochem Cytochem 2009; 57(2): 101-111. doi: 10.1369/jhc.2008.952259

Tsuge S, et al. Specific in situ visualization of plasma cells producing antibodies against *Porphyromonas gingivalis* in gingival radicular cyst: application of the enzyme-labeled antigen method. J Histochem Cytochem 2011; 59(7): 673-689. doi: 10.1369/0022155411408906

Mizutani Y, et al. Novel approach to identifying autoantibodies in rheumatoid synovitis with a biotinylated human autoantigen library and the enzyme-labeled antigen method. J Immunol Method 2013; 387:57-70. doi: 10.1016/j.jim.2012.09.011

Application of the enzyme-labeled antigen method to clinical specimens (2)

Mizutani Y, et al. *In situ* visualization of plasma cells producing antibodies reactive to *Porphyromonas gingivalis* in periodontitis: the application of the enzyme-labeled antigen method. Mol Oral Microbiol 2014; 29(4): 156-173. doi:10.1111/omi.12052

Onouchi T, et al. 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. Microbiol Immunol 2015; 59(1): 13-27. doi: 10.1111/1348-0421.12213

Mizutani Y, et al. Enzyme-labeled antigen method: development and application of the novel approach for identifying plasma cells locally producing disease-specific antibodies in inflammatory lesion. Acta Histochem Cytochem 2016; 49(1): 9-19. doi: 10.1267/ahc.15030

Mizutani Y, et al. Enzyme-labeled antigen method: factors influencing the deterioration of antigen-binding activity of specific antibodies during formalin fixation and paraffin embedding. Acta Histochem Cytochem 2022; 55(5): 129-148. doi: 10.1267/ahc.22-00023