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*Review*

# Enzyme-labeled Antigen Method: Development and Application of the Novel Approach for Identifying Plasma Cells Locally Producing Disease-specific Antibodies in Inflammatory Lesions

**Yasuyoshi Mizutani[1], Kazuya Shiogama[1], Takanori Onouchi[1], Kouhei Sakurai[2], Ken-ichi Inada[2] and Yutaka Tsutsumi[1]**

*[1]Department of Pathology, Fujita Health University School of Medicine, Toyoake, Aichi 470–1192, Japan and [2]Department of Pathology, Banbuntane Houtokukai Hospital, Fujita Health University School of Medicine, Nakagawa-ku, Nagoya, Aichi 454–8509, Japan*

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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. A new technique identifying these corresponding antigens may give us a breakthrough for understanding the disease from a pathophysiological viewpoint, simply because the immunocytes are seen within the lesion. We have developed an enzyme-labeled antigen method for microscopic identification of the antigen recognized by specific antibodies locally produced in plasma cells in inflammatory lesions. Firstly, target biotinylated antigens were constructed by the wheat germ cell-free protein synthesis system or through chemical biotinylation. Next, proteins reactive to antibodies in tissue extracts were screened and antibody titers were evaluated by the AlphaScreen method. Finally, with the enzyme-labeled antigen method using the biotinylated antigens as probes, plasma cells producing specific antibodies were microscopically localized in fixed frozen sections. 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. Evaluation of local specific antibody responses expectedly contributes to clarifying previously unknown processes in inflammatory disorders.

**Key words:** enzyme-labeled antigen method, plasma cell, wheat germ cell-free protein synthesis system, AlphaScreen method, biotinylated antigen

## I. Introduction

Dense plasma cell infiltration is commonly seen in chronic inflammatory lesions in autoimmune diseases such as rheumatoid arthritis and inflammatory bowel disease and those in infectious diseases such as *Porphyromonas gingivalis* (*P. gingivalis*)-induced periodontitis and *Helicobacter pylori* (*H. pylori*)-associated chronic gastritis. However, antigens recognized by antibodies produced by locally infiltrating plasma cells remain unclear in most cases. If one can histochemically identify the target antigens in tissue sections, a novel breakthrough would be in our own hands for understanding the pathophysiology of inflammatory processes. The reason is quite simple: these plasma cells are observed within the lesion.

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Correspondence to: Yutaka Tsutsumi, M.D., Department of Pathology, Fujita Health University School of Medicine, Toyoake, Aichi 470–1192, Japan. E-mail: tsutsumi@fujita-hu.ac.jp

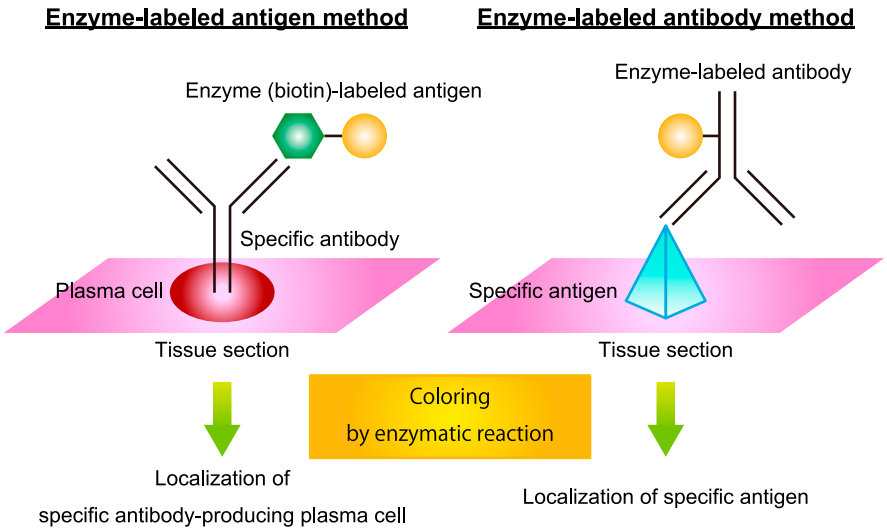


**Fig. 1.** Comparative schematic illustration of the enzyme-labeled antigen method and the enzyme-labeled antibody method. The enzyme-labeled antigen method (left) is a reversed style methodology of the enzyme-labeled antibody method (right). With an enzyme (biotin)-labeled antigen probe, the site of antibody production can be visualized on a tissue section.

We retrieved and developed the enzyme-labeled antigen method, a unique histochemical technique for visualizing antigen-specific antibody-producing plasma cells in fixed frozen tissue sections with biotinylated antigens as probes. As illustrated in Figure 1, the method is a just reverse style of the enzyme-labeled antibody method or immunohistochemical staining [33, 37, 38]. An original form of this technique was documented by two groups in 1968, for visualizing plasma cells producing antibodies against horseradish peroxidase (HRP) in lymph nodes of HRP-immunized rabbits [31, 62]. HRP functioned here as both an antigen and an enzyme. Thereafter, the idea has been little focused, and only a few applications using fluorescence have been described [10, 36, 61].

For establishing and applying the enzyme-labeled antigen method to biopsy or surgical human samples, the following subjects must be overcome: a) identification of target antigens recognized by antibodies produced in the inflammatory lesion, and b) preparation of biotinylated antigen probes, and c) technical development of the histochemical methodology.

We firstly describe an animal model for histochemically demonstrating specific antibody-producing plasma cells in the lymphoid tissue of rats repeatedly immunized with protein antigens. In the next step, the enzyme-labeled antigen method was applied to fixed frozen sections of human inflammatory lesions. Target biotinylated proteins were prepared by the wheat germ cell-free protein synthesis system or through chemical biotinylation. The AlphaScreen (Amplified Luminescent Proximity Homogeneous Assay Screen) method for detecting target antigens in tissue extracts was combined when necessary.

## II. Technical Development of the Enzyme-labeled Antigen Method Using an Animal Model Hyperimmunized by Protein Antigens

For evaluating the eligibility of the enzyme-labeled antigen method, we used an animal model. Rats were hyperimmunized with horseradish peroxidase (HRP), ovalbumin (OA) or keyhole limplet hemocyanin (KLH) [33]. Regional (axillary, inguinal and popliteal) lymph nodes and spleen were sampled after repeated immunization with the emulsified antigens, and 4% paraformaldehyde (PFA)-fixed frozen sections and 10% buffered formalin-fixed paraffin-embedded (FFPE) sections were prepared. The fixation conditions included 4 hr at 4°C for frozen sections and overnight at room temperature for paraffin sections. The enzyme-labeled antigen method was performed using HRP, biotinylated OA or biotinylated KLH as probes. Proteinase K (5 µg/ml) pretreatment for 15 min at room temperature was usually needed for retrieving an antigen-binding activity of the antibody in fixed tissue sections. HRP-labeled streptavidin consisted of the secondary reagent for detecting biotinylated OA or KLH, and localization of HRP was visualized with diaminobenzidine (DAB) reaction. Our standard protocol of the enzyme-labeled antigen method using biotinylated antigens is summarized in Table 1.

Production of antibodies specific to HRP was clearly demonstrated in the cytoplasm of plasma cells in 4% PFA-fixed frozen sections of lymph node and spleen with or without proteinase K pretreatment (upper panel of Fig. 2). Signals against OA and KLH were weak without proteinase K pretreatment, which significantly amplified the signals (middle and lower panels of Fig. 2).

Proportions of the antigen-specific antibody-producing plasma cells among total plasma cells in the regional lymph

**Table 1.** *The standard protocol for the enzyme-labeled antigen method employing biotinylated antigen probes in 4% PFA-fixed frozen sections*

| Step | Procedures |
|---|---|
| 1 | Preparation of 4% PFA-fixed frozen section on aminomethoxysilane-coated glass slide |
| 2 | Rinse in running water for 5 min |
| 3 | Endogenous peroxidase blockage in methanol containing 0.3% hydrogen peroxide for 30 min |
| 4 | Rinse in running water for 5 min |
| 5 | Immersion in 0.05 M Tris-buffered saline (TBS), pH 7.6 |
| 6 | Pretreatment with 5 μg/ml proteinase K in TBS for 15 min at room temperature |
| 7 | Rinse in 0.01 M phosphate buffered saline (PBS), pH 7.2 |
| 8 | Incubation with a biotinylated antigen probe for 1 hr at room temperature |
| 9 | Rinse in PBS three times |
| 10 | Incubation with HRP-labeled streptavidin for 1 hr at room temperature |
| 11 | Rinse in PBS three times |
| 12 | Coloring reaction in 0.05 M Tris-HCl buffer, pH 7.6 containing 0.02% diaminobenzidine and 0.006% hydrogen peroxide |
| 13 | Rinse in running water |
| 14 | Nuclear counterstaining with hematoxylin |
| 15 | Dehydration through graded ethanol, penetration with xylene, and mounting in hydrophobic mounting medium |

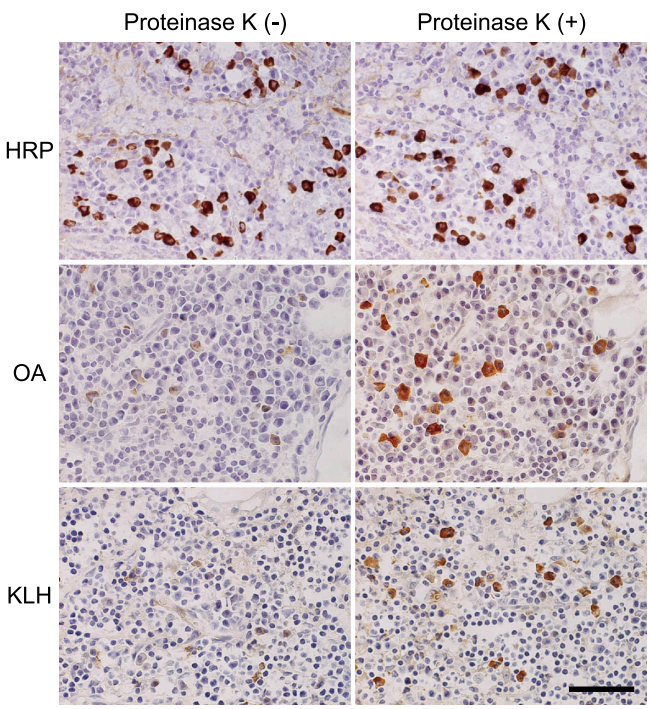


**Fig. 2.** Visualization of plasma cells producing antibodies specific to horseradish peroxidase (HRP), ovalbumin (OA), or keyhole limpet hemocyanin (KLH) in paraformaldehyde-fixed frozen sections of popliteal lymph nodes of hyperimmunized rats using biotinylated protein probes. A considerable number of plasma cells show cytoplasmic positivity in the medulla of the node. Proteinase K pretreatment effectively retrieves positive signals for OA and KLH in plasma cells. Reactivity against HRP remains comparable with or without proteinase K pretreatment. Bar=50 μm.

nodes were surprisingly high, reaching 50% [33]. The spleen showed a lower percentage of specific plasma cells around 15%. The findings suggest that in the hyperimmune state, immunocytes producing antibodies against a single protein can extensively expand within the lymphoid tissue. The specific signals were significantly weakened by the corresponding unlabeled proteins but not by indifferent

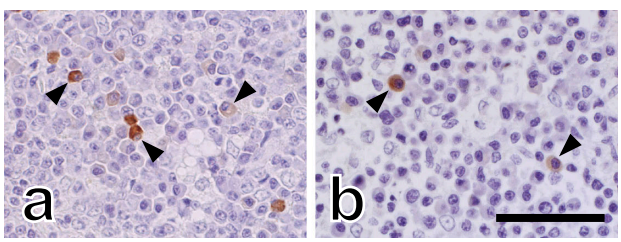


**Fig. 3.** Detection of plasma cells producing antibodies against HPR and KLH in formalin-fixed, paraffin-embedded sections of the hyperimmunized rat lymph node. A small number of positive signals reactive to HRP (**a**) and biotinylated KLH (**b**) are observed even after paraffin embedding. Digestion by a high concentration (80 μg/ml) of proteinase K is requested for the enzyme-labeled antigen method. Arrowheads indicate the positive plasma cells. Bar=50 μm.

unlabeled proteins. We thus confirmed the effectiveness of this histochemical technique for identifying specific plasma cells in microscopic sections.

In FFPE sections, positive cells were not detected without proteinase K pretreatment. After digestion by a high concentration (80 μg/ml) of proteinase K, a small number of positive signals against HRP and KLH were observed (Fig. 3). In case of OA, a high background hampered the detection of the positive cells. The results indicated that antibody activities were not totally abolished in FFPE sections. Because of unstable stainability, however, the use of FFPE sections was not suitable for analyzing the results. Signal amplification technology aiming at retrieving antigen-binding activity of antibodies in FFPE sections should be an important homework to be solved.

## III. Screening of Antigens Binding to Antibodies Locally Produced by Plasma Cells in the Lesion

For screening antigens binding to antibodies locally produced by plasma cells in inflammatory lesions, we developed an approach as described below. Firstly, a bio-

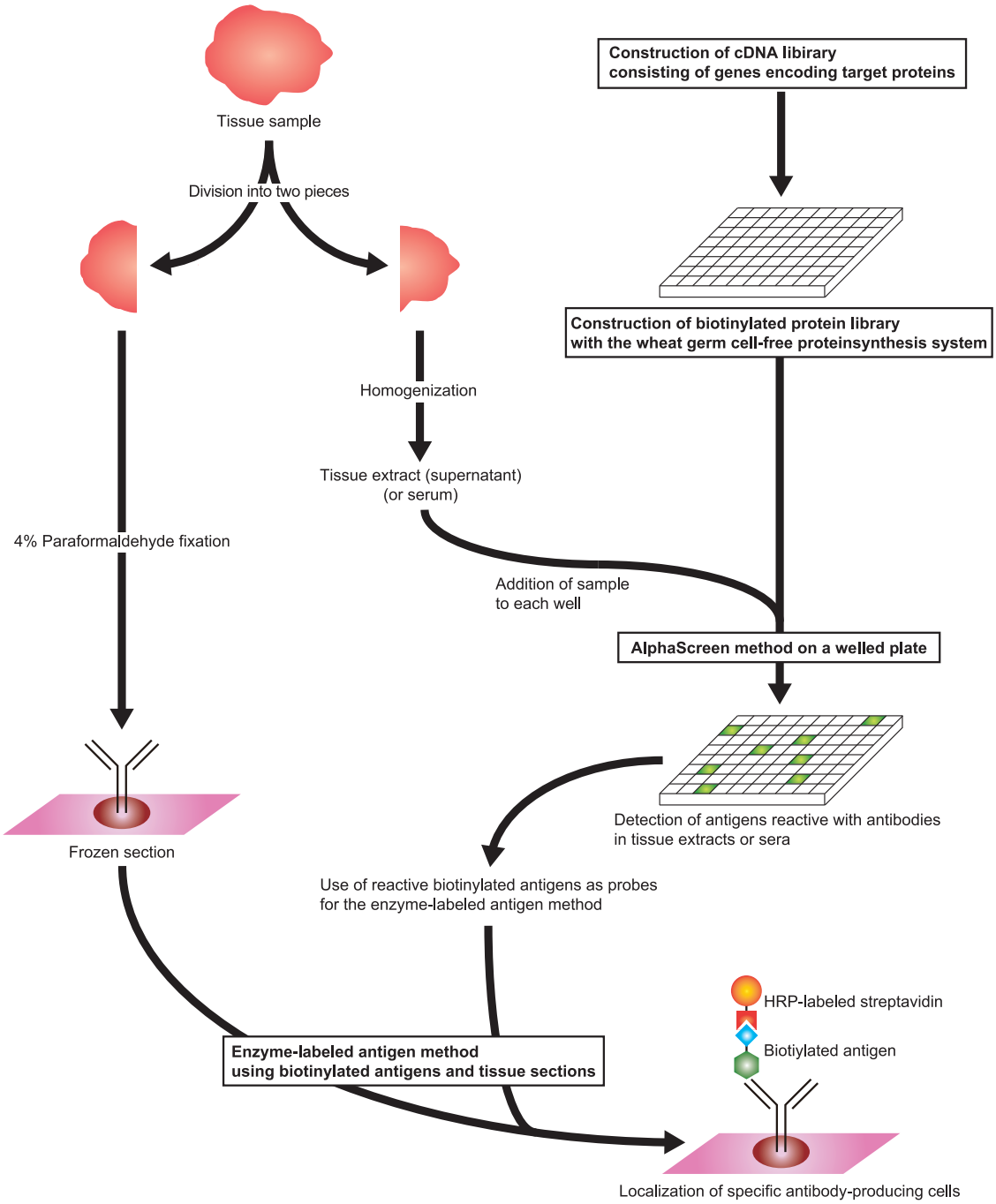


**Fig. 4.** Sequential approach for evaluating antigens recognized by antibodies produced in plasma cells in the inflammatory lesion. A tissue sample is divided into two pieces. One of them is processed for 4% paraformaldehyde-fixed frozen sections for the enzyme-labeled antigen method. The other part is homogenized in PBS and the supernatant is collected as tissue extract for the AlphaScreen assay. The biotinylated protein library is prepared as follows. Firstly, a complementary DNA library consisting of genes encoding target proteins is constructed. Secondary, a biotinylated protein library is constructed with the wheat germ cell-free protein synthesis system using a cDNA library as transcription templates. Protein antigens reactive with antibodies in the serum or tissue extract are then screened with the AlphaScreen method. Finally, the enzyme-labeled antigen method, employing the screened biotinylated protein antigen as a probe and HRP-labeled streptavidin, is applied to localizing specific antibody-producing plasma cells on a fixed frozen section of the inflammatory lesion. This sequential approach enables us to identify antigens recognized by antibodies locally produced within the lesion, and to visualize plasma cells producing specific antibodies in tissue sections.

tinylated protein library of target proteins was constructed or the target proteins were biotinylated by the wheat germ cell-free protein synthesis system [11, 32]. Next, biotinylated antigens reacting with antibodies in tissue extracts were screened or assayed by the AlphaScreen method [32]. Finally, antigen-specific antibody-producing plasma cells were demonstrated in 4% PFA-fixed frozen sections by the enzyme-labeled antigen method employing the biotinylated

antigen as a probe. The sequence of our approach is schematically illustrated in Figure 4.

***1) Synthesis of biotinylated proteins by the wheat germ cell-free protein synthesis system***

*N*-terminally biotinylated proteins were prepared by the wheat germ cell-free protein synthesis system established by Sawasaki *et al.*, Proteo-research Center, Ehime University, Matsuyama [52, 53]. Using plasmid-inserted complementary DNA encoding a target protein, transcription templates were synthesized with the split-primer polymerase chain reaction [53, 54]. The constructs contained a promoter sequence and a transcription enhancer sequence for SP6 RNA polymerase, and pEUE01 plasmid vector, incorporated with the biotin ligase recognition sequence and the open reading frame sequence of the target protein, was used as a transcription template [34, 67]. After transcription by SP6 RNA polymerase to yield messenger RNA, translation to protein products was performed using the wheat germ extract under the bilayer diffusion system [32, 52, 55]. Synthesized proteins were *N*-terminally biotinylated automatically. Conveniently, the translation mixture was able to be used as a biotinylated target protein in the AlphaScreen method and a probe in the enzyme-labeled antigen method without further purification or concentration. Upon request, the biotinylated or unlabeled proteins were synthesized in a large scale, and purified for detailed analysis.

***2) Screening by the AlphaScreen method***

The AlphaScreen method is a simple, high-sensitivity and high-throughput technique for detecting an intermolecular interaction such as an antigen-antibody reaction [32, 72]. Biotinylated antigens were admixed with the serum or tissue extract to incubate on a 384-well plate, and both streptavidin coated donor beads and protein G coated accepter beads were then added to the well. Excitation light provoked chemiluminescence signals, sensitively representing antigen-antibody interaction signals. The principal of the AlphaScreen method is illustrated in Figure 5. The advantages of the AlphaScreen method are as follows. 1) A three-dimensional conformation of the antigen is kept throughout the assay, since the reaction is performed in a liquid phase. 2) The assay can be done with a small amount of sample. In the assay with a serum, the sample volume needed is just 0.025 µl per well, and the assay can be performed on a 384 well-plate without washing steps.

## IV. Application of Our Approach to Biopsy and Surgical Human Samples

Our approach has been applied to localizing disease-specific antibodies in fixed frozen sections of biopsy/surgical human specimens of rheumatoid arthritis, periodontitis and radicular cyst [34, 35, 67]. Antibodies against a carbohydrate antigen, Strep A, on group A *Streptococcus* were

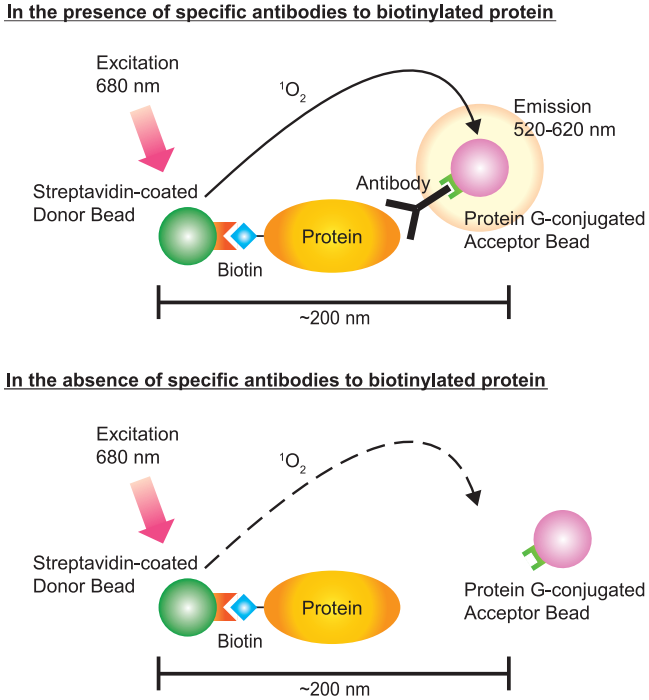


**Fig. 5.** Schematic illustration of the AlphaScreen assay for detecting a biotinylated protein-antibody interaction. The streptavidin-coated donor beads interact with the biotinylated target protein. The protein G-coated acceptor beads interact with the target protein via the specific antibodies. When the 680 nm excitation light is given, the donor beads generate singlet oxygens. The singlet oxygens promote the acceptor beads to emit fluorescent light at 520–620 nm. This reaction occurs only in the presence of the specific antibodies in the solution, since the antigen-antibody reaction shortens the distance between both beads within 200 nm (top panel). No fluorescence is obtained without specific antibodies (bottom panel).

also specifically visualized in fixed frozen sections of recurrent tonsillitis using a biotinylated sugar probe [43]. Regrettably enough, positive signals were scarcely obtained in FFPE sections of these human samples.

***1) Application of the enzyme-labeled antigen method to rheumatoid arthritis***

Rheumatoid arthritis (RA) is a systemic autoimmune disorder featured by multifocal and symmetrical arthritis with progressive bone destruction [64]. Histologically, the pannus in RA synovitis shows dense infiltration of plasma cells [73]. Although multiple autoantibodies have been identified in the serum of RA patients [39, 57], the site of autoantibody production has not been clarified. We tried to identify autoantibody-producing plasma cells in RA synovitis [34].

The autoantigen protein library containing 2,183 biotinylated nuclear, cytoplasmic or membrane proteins was established in Proteo-research Center, Ehime University, Matsuyama. The AlphaScreen method was applied to evaluating reactivity of antibodies in the serum and tissue extract of the synovium surgically removed from five RA patients. Multiple proteins showed positive AlphaScreen

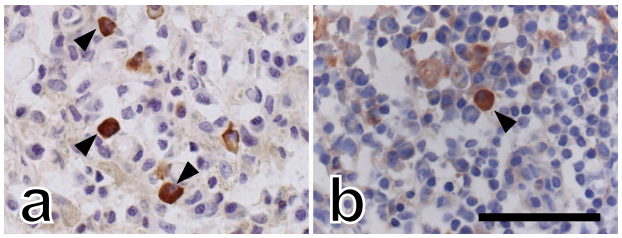


**Fig. 6.** Visualization of autoantibody-producing plasma cells in fixed frozen sections of RA synovitis with the enzyme-labeled antigen method. Arrowheads indicate plasms cells labeled with biotinylated TRIM21 (**a**) and biotinylated FBXO2 (**b**). The site of autoantibody production can be visualized histochemically. Bar=50 μm.

signals reactive with the serum, tissue extract or both of them. Five proteins showing the highest AlphaScreen reactivity to the tissue extract in each case were chosen, and a total of 18 biotinylated autoantigens were obtained. Fixed frozen sections of RA synovitis were then evaluated with the enzyme-labeled antigen method.

A small number of plasma cells producing autoantibodies were demonstrated in two of five RA synovitis lesions, as illustrated in Figure 6. The target autoantigens included tripartite motif-containing 21 (TRIM21) and F-box only protein 2 (FBXO2). Molecular weights are 54.2 kDa for TRIM21 and 33.3 kDa for FBXO2. The positive signals were abolished with unlabeled antigens. No specific plasma cells reactive to the other 16 proteins were seen, partly because of high background staining. Some of the proteins possessed acidic or basic charges. The stainability of the enzyme-labeled antigen method seemed to depend on the property of probe antigens.

The AlphaScreen signals of the tissue extract were especially high for the above two antigens: 77.6 for TRIM21 and 96.4 for FBXO2, while those of the other 16 proteins were less than 17. The comparative findings of the AlphaScreen signals and the enzyme-labeled antigen method are summarized in Figure 7. TRIM 21, also called SSA or Ro52, is known to be a major autoantigen in Sjögren's syndrome [2, 25]. In fact, the RA case with autoantibodies against TRIM21 in the diseased synovium was clinically complicated with Sjögren's syndrome accompanying positive SSA antibodies in the serum.

The association between FBXO2, a member of F-box protein family mediating ubiquitination of proteins targeted for degradation by the proteasome [6], and RA has not been documented. Of note is that the AlphaScreen signal for FBXO2 was detected only in the tissue extract, but not in the serum. This suggests that certain autoantibodies locally produced within the lesion are not secreted to the serum, hence implying the importance of the detailed analysis of the tissue extract for detecting such non-secretory autoantibodies of unclarified clinical significance.

We are confident that our approach, employing a) the wheat germ cell-free protein synthesis system, b) the AlphaScreen method and c) the enzyme-labeled method, is useful for identifying autoantigens recognized by antibodies locally produced in the inflammatory lesion of autoimmune diseases.

### *2) Application of the enzyme-labeled antigen method to periodontitis and radicular cyst*

*P. gingivalis* is a black-pigmented, anaerobic Gram-negative bacillus normally residing in the human oral cavity and colonizing the lesion of periodontitis or pyorrheal gingivitis [7, 21, 29, 59]. It is well known that *P. gingivalis* is a major agent causing chronic periodontitis [30]. Periodontitis results in erosions of the alveolar bone around the teeth, and is a major cause of tooth loss in adults

| Gene Symbol | Case 1 | | | Case 2 | | | Case 3 | | | Case 4 | | | Case 5 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AS signal | | Enzyme-labeled antigen method | AS signal | | Enzyme-labeled antigen method | AS signal | | Enzyme-labeled antigen method | AS signal | | Enzyme-labeled antigen method | AS signal | | Enzyme-labeled antigen method |
| | Tissue | Serum | | Tissue | Serum | | Tissue | Serum | | Tissue | Serum | | Tissue | Serum | |
| ABCF1 | 4.3 | 8.7 | - | 4.8 | 32.8 | BG | 10.6 | 6.3 | - | 10.1 | 18.7 | BG | 5.7 | 3.8 | - |
| ZNF207 | 2.9 | 5.1 | - | 2.4 | 12.7 | - | 5.2 | 4.0 | - | 4.9 | 13.6 | - | 6.1 | 2.0 | - |
| S100A13 | 0.9 | 3.7 | - | 13.2 | 13.3 | - | 2.3 | 6.9 | - | 1.8 | 12.8 | - | 1.3 | 5.8 | - |
| NOL1 | 1.9 | 3.4 | - | 6.8 | 22.5 | - | 4.1 | 4.6 | - | 2.1 | 7.4 | - | 1.7 | 1.8 | - |
| PNRC2 | 7.2 | 1.1 | - | 1.1 | 0.9 | - | 1.1 | 1.1 | - | 1.1 | 1.0 | - | 1.1 | 1.2 | - |
| PMVK | 1.1 | 1.1 | - | 7.3 | 5.3 | - | 3.6 | 2.0 | - | 1.4 | 2.6 | BG | 1.6 | 1.4 | - |
| ING4 | 2.7 | 3.5 | BG | 6.2 | 12.0 | - | 3.2 | 2.7 | BG | 1.8 | 5.9 | BG | 1.3 | 2.1 | BG |
| FKBP4 | 2.7 | 5.4 | - | 5.9 | 10.4 | BG | 10.3 | 3.4 | BG | 3.4 | 9.2 | BG | 6.2 | 2.5 | BG |
| FBXO2 | 3.4 | 2.9 | - | 5.9 | 2.7 | - | 96.4 | 1.4 | + | 6.3 | 1.8 | - | 1.6 | 1.2 | - |
| PRM2 | 7.9 | 13.1 | - | 6.0 | 19.1 | - | 11.9 | 5.4 | BG | 9.4 | 15.0 | - | 6.1 | 2.3 | - |
| IMPACT | 3.3 | 5.9 | BG | 4.0 | 12.8 | - | 11.2 | 2.2 | BG | 2.8 | 11.5 | BG | 3.3 | 1.5 | BG |
| MAP1LC3B | 0.9 | 14.6 | - | 6.7 | 40.1 | - | 1.8 | 10.4 | - | 2.5 | 41.2 | - | 0.8 | 4.7 | - |
| SRP19 | 9.1 | 11.1 | BG | 3.1 | 30.4 | BG | 5.1 | 8.7 | BG | 7.7 | 21.1 | BG | 5.3 | 3.8 | BG |
| KCTD18 | 4.4 | 7.5 | BG | 3.0 | 20.1 | BG | 6.5 | 4.8 | BG | 8.1 | 13.0 | BG | 5.1 | 2.1 | BG |
| GABARAPL2 | 9.0 | 14.8 | - | 1.6 | 13.9 | - | 3.0 | 5.4 | - | 5.1 | 15.6 | - | 4.7 | 2.8 | - |
| TRIM21 | 77.6 | 86.8 | + | 1.3 | 2.0 | - | 1.4 | 1.3 | - | 1.4 | 1.6 | - | 1.2 | 1.3 | - |
| KIAA0409 | 4.8 | 8.0 | - | 5.3 | 35.6 | - | 15.7 | 11.1 | BG | 11.2 | 30.0 | BG | 5.7 | 4.4 | - |
| HIST1H1C | 6.9 | 9.6 | BG | 5.9 | 30.9 | BG | 17.0 | 11.9 | BG | 14.9 | 30.4 | BG | 9.3 | 3.7 | BG |

**Fig. 7.** Heat map of the AlphaScreen (AS) signals against 18 autoantigens in the serum and tissue extract of five RA patients. Signal/noise ratios are written on the map, and the results of the enzyme-labeled antigen method are given (BG indicates high background staining). As boxed in yellow in each case, top five proteins showing high signals in the tissue extract were chosen for analysis, resulting in a total of 18 proteins listed here (gene symbols shown on the leftmost column) for evaluating with the enzyme-labeled antigen method. In case 1, high-titer signals for TRIM21 are seen in both the serum and tissue extract. In case 3, high-titer FBXO2 signals are observed in the tissue extract, but little signals seen in the serum. (borrowed from Mizutani *et al.*, 2013; http://dx.doi.org/10.1016/j.jim.2012.09.011 on ELSEVIER Journal)

**Table 2.** *Comparative percentage presentation of AlphaScreen-reactive antibodies against five antigens of P. gingivalis in the serum and tissue extract, and results of the enzyme-labeled antigen method in gingival tissue sections of periodontitis (PO) and radicular cyst (RC)*

| Antigen | Percentage of positive cases (%) | | | | | |
|---|---|---|---|---|---|---|
| | AlphaScreen method | | | | Enzyme-labeled antigen method | |
| | Serum | | Tissue extract | | | |
| | PO (n=20) | RC (n=10) | PO (n=20) | RC (n=6) | PO (n=18) | RC (n=8) |
| Any of the five antigens | 55 | 20 | 85 | 33 | 94 | 25 |
| Ag53 | 0 | 10 | 30 | 0 | 28 | 0 |
| Arg-hgp | 20 | 20 | 65 | 33 | 83 | 25 |
| Arg-pro | 30 | 0 | 85 | 0 | 89 | 0 |
| Lys-hgp | 10 | 20 | 75 | 33 | 89 | 13 |
| Lys-pro | 25 | 10 | 15 | 0 | 22 | 0 |

PO: Periodontitis, RC: Radicular cyst

[47]. Histologically, the gingival tissue of periodontitis is characterized by dense infiltration of plasma cells [26]. Antibodies to *P. gingivalis* have been detected in the serum, gingival crevicular fluid and the saliva of patients with periodontitis [28, 58]. Reportedly, the immunocytes isolated from the inflamed gingiva of periodontitis secrete antibodies against *P. gingivalis* [42], but these cells have not been visualized microscopically.

Radicular cyst is seen as a sequela of severe dental caries, and frequently shows marked plasma cell infiltration [9]. Microbiological analysis of radicular cyst indicated bacterial colonization of some 50 species, mainly anaerobes such as *Porphyromonas*, *Fusobacterium*, *Tannerella*, *Prevotella*, *Peptostreptococcus*, *Actinomyces*, etc. [9, 23, 40, 63, 70]. Local production of *P. gingivalis*-specific antibodies is expected also in radicular cyst. The local immune reaction against *P. gingivalis* should be consistent in periodontitis but inconsistent in radicular cyst.

By using the wheat germ cell-free protein synthesis system, five biotinylated proteins were prepared as representative pathogenic antigens on *P. gingivalis*. These included Ag53, a 53 kDa membrane-associated protein [28, 44], and four gingipain components, such as the proteinase domain of Arg-gingipain (Arg-pro), the hemagglutinin/adhesin domain of Arg-gingipain (Arg-hgp), the proteinase domain of Lys-gingipain (Lys-pro), and the hemagglutinin/adhesin domain of Lys-gingipain (Lys-hgp) [5, 19]. The molecular weights of these gingipain components are 44 kDa, 103 kDa, 51 kDa and 103 kDa, respectively.

We analyzed 20 lesions of biopsied periodontitis and 10 lesions of surgically removed radicular cyst [35, 67]. The AlphaScreen signals in the serum and tissue extract of periodontitis and radicular cyst and positivity rates of the enzyme-labeled antigen method are comparatively summarized in Table 2. In periodontitis, the AlphaScreen analysis demonstrated antibodies reactive to *P. gingivalis* antigens in 11 (55%) cases in the serum and 17 (85%) cases in the gingival tissue extract [35]. In radicular cyst, the detectability was 2 of 10 (20%) cases in the serum and 2 of 6 cases (33%) in the tissue extract [67]. With the enzyme-labeled antigen method, plasma cells producing antibodies to any of the five *P. gingivalis* antigens were detected in 17 of 18 (94%) fixed frozen sections of periodontitis and 2 of 8 (25%) tissue sections of the radicular cyst [35, 67].

Among five *P. gingivalis* antigens, Arg-hgp, Arg-pro and Lys-hgp consisted of major antigenic components. Positivity rates for these three were high (65–85%) in periodontitis lesions, and antibodies to Arg-hgp and Lys-hgp were occasionally (33%) detected in radicular cyst lesions. Of note is that antibodies to Arg-pro were frequently detected in periodontitis (17/20: 85%), but not in radicular cyst [35]. Lys-pro and Ag53 were infrequently antigenic even in periodontitis. Importantly, the results of the AlphaScreen analysis on the tissue extract and the enzyme-labeled antigen method were comparable in both types of lesions.

The AlphaScreen analysis in periodontitis revealed that antibodies to Ag53, Arg-hgp, Arg-pro and Lys-hgp were more frequently detected in the tissue extract than in the serum. The findings strongly suggest that the locally produced antibodies are not necessarily secreted into the serum, and thus the importance of analyzing antibodies locally produced by plasma cells in the inflammatory lesion should be emphasized.

We further evaluated the proportion of plasma cells reactive with *P. gingivalis* antigens in total plasma cells in the biopsied gingival tissue of periodontitis. The proportion reached 22.3% in gingival tissue. The proportions for the respective antigens were as follows: 0.2–2.3% for Ag53, 0.3–10.2% for Arg-hgp, 0.2–12.9% for Lys-hgp, 0.1–7.0% for Arg-pro and 0.1–0.7% for Lys-pro. The proportion of plasma cells producing antibodies reactive to Ag53, Arg-hgp and Lys-hgp was positively correlated with the AlphaScreen signals in the tissue extract, and the AlphaScreen signals of the serum and tissue extract were significantly correlated [35].

The specificity of this histochemical technique was confirmed by immunoabsorption experiments: unlabeled

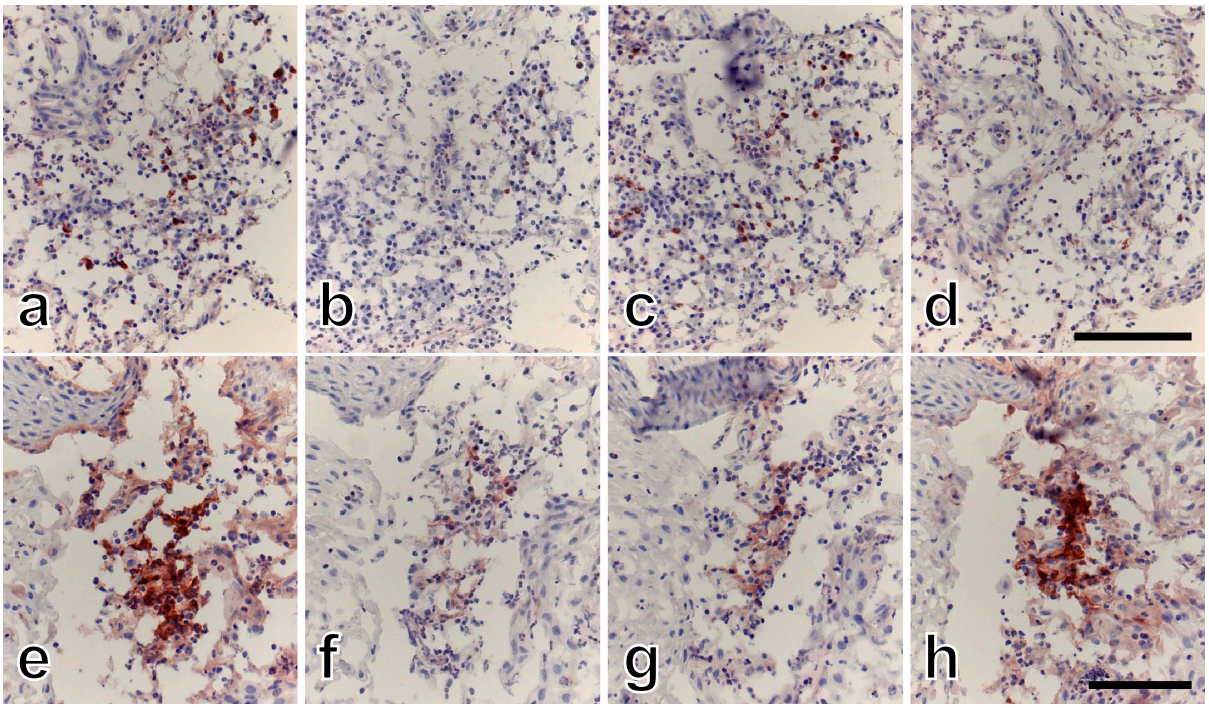


**Fig. 8.** Absorption experiment in radicular cyst, showing region specificity of the antibodies (**a–d**: area 1, **e–h**: area 2). Plasma cells with anti-Arg-hgp reactivity are scattered in area 1 (**a**) and densely clustered in area 2 (**e**). Addition of unlabeled Arg-hgp significantly abolishes the reactivity (**b, f**). Unlabeled Lys-hgp scarcely affects the reactivity in area 1 (**c**) but significantly weakens the reactivity in area 2 (**g**). Anti-Lys-hgp reactivity is not demonstrated in area 1 (**d**), whereas the same clusters are labeled with biotinylated Lys-hgp in area 2 (**h**). It is concluded that the plasma cells in area 1 produce antibodies against an epitope specific to Arg-hgp, whereas another epitope common to both Arg-hgp and Lys-hgp is recognized by the clonally clustered plasma cells in area 2. Bar=100 μm.

proteins abolished the reactivity to the corresponding proteins. In the immunoabsorption experiment for radicular cyst, we identified the "region-specificity" of the antibodies in the plasma cells. The homology of the amino acid sequence between Arg-hgp and Lys-hgp is 76% [41]. When biotinylated Arg-hgp or Lys-hgp was admixed with unlabeled Arg-hgp or Lys-hgp, the positive signals in plasma cells in tissue sections disappeared or become significantly weakened, indicating the production of antibodies to an epitope common between Arg-hgp and Lys-hgp. In some areas, clustered plasma cells were uniformly reactive with the common epitope. However, in other areas, plasma cell clusters showed positivity for Arg-hgp-specific epitope: namely the plasma cells were reactive with biotinylated Arg-hgp but not with biotinylated Lys-hgp, and the reactivity was weakened with unlabeled Arg-hgp but not with unlabeled Lys-hgp (Fig. 8). The region specificity of the antibodies was thus clearly demonstrated with the enzyme-labeled antigen method [67]. In other words, we happened to demonstrate the "micromonoclonality" (local clustering of plasma cells showing the same region-specificity) in the inflammatory lesion. Our experience of the inflammation-related micromonoclonality includes ulcerative colitis (Mizutani, Y., *et al*. unpublished observation) and Russell body gastritis [1]. Clonal expansion of B-lymphocytes has been reported in a variety of chronic inflammatory disorders, including chronic hepatitis C, *H. pylori*-induced chronic gastritis, Sjögren's syndrome and Hashimoto thyroiditis [12, 18, 20, 56]. In our histochemical analysis of periodontitis lesions, however, such clonal clustering was scarcely observed.

In summary, our approach for identifying specific antibodies locally produced in the inflammatory lesion is applicable to infectious diseases. The importance of analyzing the inflamed tissue itself should again be emphasized, since the local immune reaction to *P. gingivalis* is often undetectable by examining the serum.

### *3) Application of the enzyme-labeled antigen method to recurrent tonsillitis*

In recurrent tonsillitis of the palatine tonsil, the major causative agent is *Streptococcus pyogenes* (group A *Streptococcus*), and the histology consistently shows lymphoid follicle formation and dense infiltration of plasma cells [48]. Strep A, a 20 kDa carbohydrate molecule of the cell wall of *S. pyogenes*, is the potent immunogen provoking an antibody response in the human [27, 50]. Biotinylated Strep A, provided by Biochemical Research Laboratory, Eiken Chemical, Nogi, Tochigi, was used as a probe for visualizing plasma cells producing antibodies against Strep A. Fixed frozen sections of 12 surgically removed recurrent tonsillitis lesions were stained with the enzyme-labeled antigen method.

Plasma cells producing antibodies against Strep A

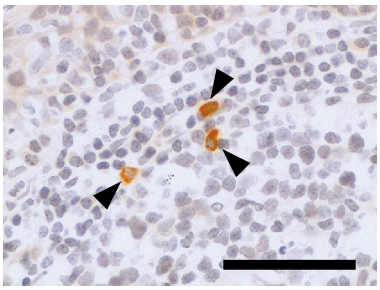

**Fig. 9.** Visualization of Strep A antibody-producing plasma cells in fixed frozen sections of streptococcal tonsillitis with the enzyme-labeled antigen method. Strep A is a highly antigenic carbohydrate substance expressed on type A *Streptococcus*. Arrowheads indicate plasms cells labeled with biotinylated Strep A antigen. Bar=50 μm.

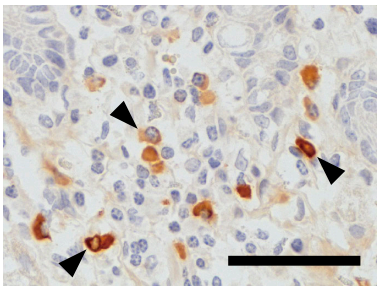

**Fig. 10.** Plasma cells producing antibodies against *H. pylori* components in fixed frozen sections of the human gastric mucosa (surgical specimen). Biotinylated crude extract of cultured *H. pylori* was employed as a probe for the enzyme-labeled antigen method. The cytoplasm of a good number of plasma cells reveals distinct positive signals (arrowheads). Bar=50 μm.

were detected in 8 of 12 recurrent tonsillitis lesions accompanying *S. pyogenes* infection, as shown in Figure 9 [43]. The specificity of staining was confirmed by an immunoabsorption experiment using unlabeled Strep A. The higher the frequency of recurrence of tonsillitis, the higher number of Strep A-reactive plasma cells. Immunoglobulin classes of the plasma cells were either IgG or IgA, and the positively stained plasma cells were distributed within the reticular squamous mucosa or just below the mucosa. It was shown here that the enzyme-labeled antigen method is applicable to detecting antibodies against the carbohydrate antigen.

## V. Scope and Prospect in Analyzing Antibodies Locally Produced in a Variety of Lesions

Antigens recognized by antibodies locally produced from plasma cells in inflammatory tissues have rarely been studied, and the pathophysiological significance of plasma cell infiltration in the diseased site remains unclear. By applying the enzyme-labeled antigen method, the following themes and questions related to the plasma cell response are expected to be clarified, we hope.

1) In *H. pylori*-induced chronic active gastritis, the coiled bacteria colonize the surface of the foveolar epithelium of the gastric mucosa. Interestingly, the bacteria are absent from the focus of intestinal metaplasia [3]. IgA and secretory component are closely associated with intestinal metaplasia, where IgG-producing plasma cells richly distributed in the non-metaplastic gastritis mucosa disappear [68]. It seems that the intestinal metaplastic mucosa contributes to suppress *H. pylori* infection by active secretion of secretory IgA specific to *H. pylori*. The specific IgA antibodies should be secreted from plasma cells onto the intestinal metaplastic foci. With the enzyme-labeled antigen method, we can identify certain target antigens of *H. pylori* specifically produced in the metaplastic lesion. Expectedly, different *H. pylori*-related antigens may be the target of immunoglobulins of IgG and IgA classes. Figure 10 illustrates plasma cells in *H. pylori*-infected gastric mucosa producing antibodies against biotinylated crude extract of the bacteria (unpublished observation).

The protective reactions against *H. pylori* infection in the diseased gastric mucosa may thus be elucidated.

2) Infection of *H. pylori* causes a wide variety of gastric disorders, including chronic active gastritis, peptic ulcer, gastric polyp, gastric lymphoma and gastric carcinoma [46]. Clarification of the difference in the antibody response against various *H. pylori*-related antigens can be a clue for understanding previously unknown mechanisms of inflammatory reactions in the respective gastric disorders. The enzyme-labeled antigen method may contribute to this approach.

3) Antibody responses to *H. pylori* occasionally cause systemic autoimmune disorders such as idiopathic thrombocytopenic purpura and Guillain-Barre syndrome [14]. Induction of causative autoantibodies in the infected gastric mucosa can be visualized with our technique. In type A autoimmune gastritis [45], we can see the site of local production of proton pump antibodies in the actively inflamed and atrophic oxyntic gastric mucosa, when biotinylated $H^+$-$K^+$ ATPase is available as a probe.

4) In the gastric mucosa-involved by mucosa-associated lymphoid tissue (MALT) lymphoma, numbers of plasma cells are commonly observed, and monoclonality (light chain restriction) may be detected. *H. pylori* is a known causative pathogen of gastric MALT lymphoma, since antibiotic eradication of *H. pylori* is often effective in inducing regression of this lymphoid neoplasm [71]. It is highly likely that chronic antigenic stimuli by *H. pylori* infection induce the neoplastic transformation of gastric mucosal B-cells, but the tumorigenic antigens have not been evaluated. The analysis of target *H. pylori*-related antigens recognized by the monoclonal or polyclonal plasma cells in the lymphomatous lesion may contribute to analyzing causative mechanisms of gastric MALT lymphoma.

5) In the lamina propria of normal intestinal mucosa, plasma cells are commonly observed [13]. It is assumed that these immunocytes produce IgA or IgM-type anti-

bodies against intestinal bacterial flora such as *Escherichia coli*, *Enterococci* and anaerobic *Clostridia*, in order to protect the intestinal mucosa from their abnormal growth or to keep the homeostasis between the host and flora. The target bacterial antigens of secretory IgA/IgM secreted from the physiologically observed mucosal plasma cells remain unknown. By preparing biotinylated antigen library for the respective intestinal flora, we can utilize the enzyme-labeled antigen method. The histochemical analysis of the antibody-producing immunocytes may help us understand the homeostatic mechanisms functioning in the normal gut tissue.

6) Detection of pathogen-related antibody production within the biopsied or autopsied inflammatory lesion may lead pathologists to appropriate histopathological diagnosis of the infectious disorders. In biopsy specimens, dense infiltration of plasma cells is a diagnostic hallmark of syphilis [69], but little is known whether or not plasma cells produce antibodies to *Treponema pallidum*. The enzyme-labeled antigen method can be a potential diagnostic tool, particularly when pathogens are scarcely demonstrated by conventional or immunohistochemical techniques in the infectious lesions.

7) In the lesion of allergic rhinitis, numbers of eosinophils and plasma cells are distributed. Pollen and mites comprise representative allergens, and allergenic protein antigens have been clarified [4, 74]. Theoretically, in the nasal lesion of type I allergy, IgE-type antibodies are locally pathogenic, while IgG-type antibodies may protect the disease process. The enzyme-labeled antigen method using fixed frozen sections of surgically removed nasal polyps may contribute to elucidating the role of plasma cells in this common allergic disorder.

8) In the serum of normal human individuals of blood group A type, blood group B antibodies of IgM category are consistently observed. IgM-type antibodies against blood group A are physiologically seen in the individuals of blood group B [65]. It is also well known that IgG- and IgM-type antibodies to galactose-α-1,3-galactose-β1,4-*N*-acetylglucosamine (α-gal), a carbohydrate molecule related to the blood group B antigen, are normally seen in the serum of all the human individuals, and they provoke hyperacute rejection in xenotransplantation [17]. No one knows the site of production of such natural antibodies. Although antibody titers are low, serum autoantibodies such as anti-nuclear or anti-smooth muscle antibodies are detected in healthy individuals [66]. The site and cause of the production of these antibodies is also unclear. Once biotinylated sugar or protein antigens are in hand, the enzyme-labeled antigen method may be useful for clarifying the distribution of specific antibody-producing plasma cells within the human body.

9) The site of autoantibody production can be elucidated when the tissue specimen of autoimmune diseases and labeled known autoantigens are available. Examples include antibodies to SSA (TRIM21) or related antigens in the salivary gland of Sjögren's syndrome [2, 25], anti-DNA antibodies in the lymph node of systemic lupus erythematosus [49], anti-myeloperoxidase antibody in necrotizing vasculitis of microscopic polyarteritis nodosa [51], and antibodies to desmogleins-1 and 3 in the skin of pemphigus vulgaris [8]. The enzyme-labeled antigen method may further contribute to specifying the corresponding antigens in IgG4-related diseases [24].

10) In certain kinds of malignant tumors such as medullary carcinoma of the breast and Epstein-Barr virus-related carcinomas (i.e.: nasopharyngeal lymphoepithelioma and gastric carcinoma with lymphoid stroma) [16, 22], dense plasma cell infiltration within the tumor tissue is quite characteristic. The pathophysiological significance of the antibodies produced by these tumor-related plasma cells remains unclear. It is highly expected that the plasma cells distributed within the Epstein-Barr virus-related gastric lymphoepithelioma-like carcinoma secrete antibodies against the viral antigens. Plasma cell reaction is also often seen in the stroma of human papillomavirus-induced uterine cervical cancer tissue [15]. If such plasma cell reaction is a histological hallmark of virally provoked neoplasms, the antibodies produced in medullary mammary carcinoma may hopefully have activities against an unknown human mammary tumor virus. Our technique may have a role in analyzing the mechanism of tumor immunity.

11) It is known that serum antibodies to tumor suppressor p53 protein are detected in various malignant tumors, and are thus useful as a diagnostic marker for esophageal, colorectal and breast cancers [60]. The production site of the p53 antibodies is unknown. Accumulation and overexpression of p53 protein in tumor cells are intimately related to the p53 gene mutation and may also lead to antibody production. With the biotinylated p53 protein as a probe, the enzyme-labeled antigen method can detect the distribution of p53 antibody-producing plasma cells within the tumor tissue or in regional lymph nodes. In some lymphoproliferative lesions, production of autoantibodies is accelerated, and the surgical removal of the tumor results in the disappearance of tumor-related paraneoplastic signs and symptoms. A representative example included paraneoplastic pemphigus, provoking skin lesion and serious airway symptoms [75]. By applying our technique, the site of production of autoantibodies to desmogleins 1 and 3 and desmoplakin may be specified in or around the thymoma or non-Hodgkin's lymphoma.

## VI. Conclusions

The enzyme-labeled antigen method is a useful histochemical tool for identifying plasma cells producing specific antibodies to the target antigen and for evaluating local antibody reactions against causative agents. The wheat germ cell-free protein synthesis system was effective in preparing biotinylated antigen probes. The AlphaScreen method was convenient for screening target antigens and

detecting the antibody activity. The present approach is applicable to a variety of inflammatory lesions histologically accompanying significant plasma cell infiltration. We sincerely hope and expect that our novel approach contributes to evaluating local and specific antibody responses within the lesion and to clarifying pathophysiological processes of the inflammatory disorders.

## VII. Conflict of Interest

There is no conflict of interest in the present review.

## VIII. Acknowledgments

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