Simultaneous Demonstration of Two Antigens with Immunogold-Silver Staining and Immunoenzymatic Labeling

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Abstract

A novel technique for independent and simultaneous labeling of two antigens expressed on individual cells (referred to as mixed labeling) is presented. The staining procedure combined three-step (streptavidin-biotin) immunogold-silver staining with three-step immunoenzymatic labeling. To ensure both high specificity and high sensitivity, particular emphasis was placed on designing a protocol that avoids immunological crossreactivity between the antibody reagents and overlapping of the final color products. Two examples for usage of this mixed labeling technique are described: lymphocyte subpopulations were identified in inflammatory lesions of human skin and infected host cells were characterized in the skin of mice infected with the obligatory intracellular parasite *Leishmania major*, a cause of human cutaneous leishmaniasis. (*The J Histotechnol* 16:223, 1993)

Key words: alkaline phosphatase, FITC-anti-FITC system, immunogold-silver staining, immunohistochemistry, labeling, leishmaniasis, mixed labeling, two color staining

Introduction

Various approaches have been successfully applied for studying the distribution of multiple antigens within one histological section. They are based on the successive performance of two independent single labeling procedures with contrasting signals. Double immunoenzymatic labeling (IEL) or two-color immunofluorescence labeling have gained wide acceptance (1–3). However, both possibilities present immanent, although different problems.

Double IEL allows satisfactory antigen detection only as long as the molecules are expressed by different cells in the section. If the antigens are colocalized on the very same cell, mixing of the dyes finally obscures the individual single color signals. This mostly results in poor visibility of the less abundant antigen and impedes appropriate evaluation. Thus, mixed labeling with double IEL has not gained much application.

On the other hand, double immunofluorescence is a well-suited and widely used method for mixed labeling. Major disadvantages of immunofluorescence procedures are the fading of signals, different illumination and filter systems for viewing, invisibility of the general pathology during fluorescence microscopy, autofluorescence of tissue components, and a lower sensitivity than IEL.

For these reasons, an alternative approach has been envisaged that avoids these disadvantages by combining immunogold-silver staining (IGSS) with IEL (4–7). This article describes examples of mixed labeling procedure. For analysis of lymphocyte subpopulations in human tissues, primary antibodies of the same species (mouse) that were either unconjugated or linked to fluorescein isothiocyanate (FITC) were used for staining the respective antigens. For analysis of immunocompetent cells in mouse tissues, primary antibodies from different species were used for double staining.

Materials and Methods

Preparation of Tissue Sections

For the mixed labeling technique, biopsy material from inflammatory lesions of human skin (eg, atopic dermatitis, chronic eczema) or skin lesions from mice infected with *Leishmania major* (a cause of human cutaneous leishmaniasis) were used. Immediately after collection, the tissue samples were snap-frozen in cryomolds (Miles Scientific, Naperville, IL) containing OCT compound (Miles Scientific) with isopentane precooled in liquid nitrogen or dry ice. Cryostat sections (4–8 μm) were cut at a temperature of −12°C to −20°C and thawed onto adhesive slides. The adhesive was selected according to its lack of metal ions, which would interfere with the silver enhancement procedure of the IGSS. Histostik (Accurate Chemical and Scientific, Westbury, NY) or 0.5% gelatin solution without further additives were both suitable for coating the slides. For some tissues, it was sufficient to thaw the sections onto slides that had been rinsed thoroughly with 25% hydrochloric acid (HCl). Subsequently, the sections were airdried and fixed in acetone (−20°C, 10 min).

Before labeling, the sections were rinsed with phosphate buffered saline (PBS) to wash off OCT compound. Immediately thereafter, they were incubated in a skim milk solution (Blotto) with 20% heat-inactivated sheep serum to avoid both nonspecific and specific background labeling. Blotto was pre-

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pared by vigorously stirring 5% skim mild powder and 0.1% Tween 20 into PBS and adjusting the pH to 7.4–7.6. Alternatively, PBS containing 0.1% bovine serum albumin (BSA), 0.1% gelatin and 0.1% Tween 20 can be used for washing. To block nonspecific binding, this buffer was supplemented with 20% heat-inactivated fetal calf serum (FCS).

Antibodies, Reagents, and Labeling Procedure for Human Tissues

For the tissues examined in this paper, unconjugated mouse monoclonal antibodies (MAbs) directed against CD45 (Becton-Dickinson, Sunnyvale, CA) were used for detection of the first antigen, and FITC-conjugated mouse MAbs recognizing CD3 (Becton-Dickinson) served for demonstration of the second antigen (8). However, a variety of other antibodies may be combined. As second- and third-step reagents (see also Figure 1 and staining procedure given below), the following reagents are recommended: biotinylated anti-mouse immunoglobulin (Ig) at 1:200 (Amersham, Amersham, UK), streptavidin-conjugated gold at 1:30–1:50 (Amersham), silver enhancement mixture (Amersham), rabbit anti-FITC Ig (East Acres Chemicals, Southbridge, MA), alkaline phosphatase (AP)-conjugated goat anti-rabbit Ig at 1:200 (Accurate). Alternative products from other manufacturers were equally suitable in our hands. The appropriate concentrations had to be adjusted carefully by checkerboard titrations.

As suitable color substrates for the AP staining of human and mouse tissues, fast blue or new fuchsin (Sigma, St Louis, MO or Merck, Darmstadt, Germany) is recommended.

Fast blue solution. Fast blue solution contained 0.2 mg/ml naphtol AS-MX phosphate (Sigma) dissolved in N,N-dimethylformamide (final concentration 2%), 1 mM levamisole and 1 mg/ml fast blue BB salt in 0.1 M Tris-buffered saline buffer at pH 8.2.

New fuchsin solution. The new fuchsin substrate solution consisted of 0.5 mg/ml naphtol AS-BI phosphate (Sigma) dissolved in N,N-dimethylformamide (final concentration 0.5%), 6 mM sodium nitrite, 1 mM levamisole, and 4% (w/v) new fuchsin (stock solution diluted in 2 M HCl) in Tris-buffered saline (200 mM, pH 9.0). The substrate solutions were filtered onto the slides and color development was stopped after 5–20 min.

Staining Procedure (Figure 1)

1. Preincubate slides with Blotto containing 20% sheep serum (blocking solution) at room temperature (RT) for 20 min.
2. After removal of excess blocking solution (no washing!), incubate slides with first-step MAb at RT for 2 hr or, alternatively, at 4°C overnight.
3. Rinse thoroughly with Blotto (× 3).
4. Incubate slides with biotinylated sheep anti-mouse Ig at RT for 1 hr.
5. Rinse thoroughly with Blotto (× 2) and then with PBS containing 5% BSA and 0.1% Tween 20.
6. Incubate with streptavidin-conjugated gold at RT for 1 hr.
7. Rinse thoroughly with PBS/BSA/Tween 20 (× 2), and then with Blotto (× 2).
8. Block with Blotto containing 10% normal mouse serum at RT for 30 min to avoid binding of the FITC-conjugated mouse MAb to be applied in Step 9 to the biotinylated anti-mouse Ig (see step 4).
9. Incubate slides with FITC-conjugated mouse MAb directed against the second antigen to be demonstrated at RT for 2 hr or at 4°C overnight.
10. Rinse thoroughly with Blotto (× 3).
11. Incubate with rabbit anti-FITC Ig at RT for 1 hr.
12. Rinse thoroughly with Blotto (× 3).
13. Incubate with AP-conjugated goat (or sheep) anti-rabbit Ig at RT for 1 hr.
14. Rinse thoroughly with Blotto (× 3).
15. Incubate with AP-specific substrates (fast blue or new fuchsin solutions). Monitor the AP-substrate reaction under a microscope for optimal signal to noise ratio. Stop the enzyme reaction by rinsing with Blotto.
16. Rinse with distilled water (D/W).
17. Visualize immunogold labeling by incubation of slides with silver-enhancement mixture until the desired signal intensity has developed (2–5 incubation periods of 3–10 min each). Rinse thoroughly with D/W between the incubation periods.
18. Rinse thoroughly with D/W.
19. Counterstain with non-interfering dyes (eg, nuclear fast red) and mount.
20. Evaluate sections with brightfield and epipolarization microscopy (the latter results in clear and sensitive demonstration of IGSS).

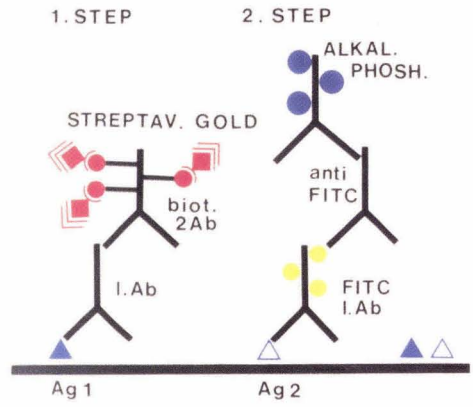

Figure 1. Schematic presentation of the mixed labeling procedure with primary antibodies from the same species. The first antigen (Ag1), to be visualized by IGSS, is detected by an unconjugated mouse antibody. The corresponding biotinylated secondary antibody has multiple spacer arms to avoid steric hindrance of the reaction with the tertiary reagent (gold-conjugated streptavidin). Size and signal of the gold particles (red squares) are enlarged by silver enhancement (red angles). The second antigen (Ag2), to be labeled by IEL, is detected by a FITC-conjugated antibody (FITC, yellow circles), followed by rabbit anti-FITC Ig and AP-conjugated anti-rabbit Ig (AP, blue circles).

Antibodies, Reagents, and Labeling Procedure for Mouse Tissues

For the experiments depicted in this study, a rabbit serum raised against *L. major* was used for demonstration of the first antigen. Rat MAbs to non-lymphoid dendritic cells (NLDC-145) served for identification of the second antigen on this particular type of host cell (9). As secondary and tertiary re-

agents, the following material was used: biotin-conjugated mouse anti-rat Ig (Dianova, Hamburg, Germany) at 1:500; gold-conjugated goat anti-rabbit antibodies (Dako, Hamburg, Germany) at 1:100; streptavidin-biotin AP complex (Dako); fast blue solution as substrate for AP; and immunogold-silver enhancement mixture for visualization of IGSS.

Staining Procedure

1. Rinse slides with Blotto. Follow by preincubation with Blotto containing 20% sheep serum or FCS at RT for 20 min.
2. After washing in Blotto, incubate sections with a mixture of both primary antibodies (eg, rabbit anti-*L. major* antiserum and monoclonal rat anti-NLDC antibodies) at RT for 2 hr or, alternatively, at 4°C overnight.
3. Rinse thoroughly with Blotto (× 3).
4. Incubate sections with gold-conjugated anti-rabbit antibodies at RT for 1 hr.
5. Rinse thoroughly with Blotto (× 3).
6. Block with Blotto containing 20% normal rabbit serum at RT for 30 min.
7. Rinse thoroughly with Blotto (× 3).
8. Incubate sections with biotinylated anti-rat Ig antibodies at RT for 1 hr.
9. Rinse thoroughly with Blotto (× 3).
10. Incubate sections with streptavidin-biotin AP complex at RT for 1 hr.
11. Rinse thoroughly with Blotto (× 3).
12. Apply fast blue solution and incubate until the desired color intensity has developed. Rinse with Blotto to stop the reaction.
13. Rinse briefly with D/W.
14. Incubate slides with PBS containing 2% glutaraldehyde at 4°C for 10 min (postfixation).
15. Rinse briefly with D/W.
16. Incubate slides with PBS containing 50 mM glycine at 4°C for 10 min for quenching endogenous AP activity. *Note*: Steps 14–16 may also be included in the protocol for labeling human tissue.
17. Rinse briefly with D/W.
18. Apply immunogold-silver enhancement mixture and incubate until the desired signal intensity has developed (2–5 incubation periods of 3–10 min each). Rinse thoroughly with D/W between the incubation periods.
19. Rinse thoroughly with D/W.
20. Counterstain with nuclear fast red and mount.

Optimal Concentration Determination

Prior to combining IGSS with IEL, the optimal concentration of the relevant antibodies and reagents were worked out by checkerboard titration with the respective single color stains. Subsequently, the mixed labeling procedure was performed for simultaneous detection of two antigens with a partly overlapping expression pattern.

Results

Figure 2A shows an example of a human skin section that was stained for the T cell-associated antigen CD3 (IEL, blue) and for the leukocyte common antigen CD45 (IGSS, brown). Under epipolarization illumination (Figure 2B), IGSS single-labeled cells displayed yellow bright grains, whereas cells additionally stained by IEL showed a shift towards a bright bluish color. In Figure 3A, an example is depicted of a mouse skin section stained for non-lymphoid dendritic cells (IEL, blue) and for the obligatory intracellular protozoan parasite *L. major* (IGSS, brown). The skin is heavily infiltrated by parasites (Figure 3B), some of which are localized within dendritic cells (10).

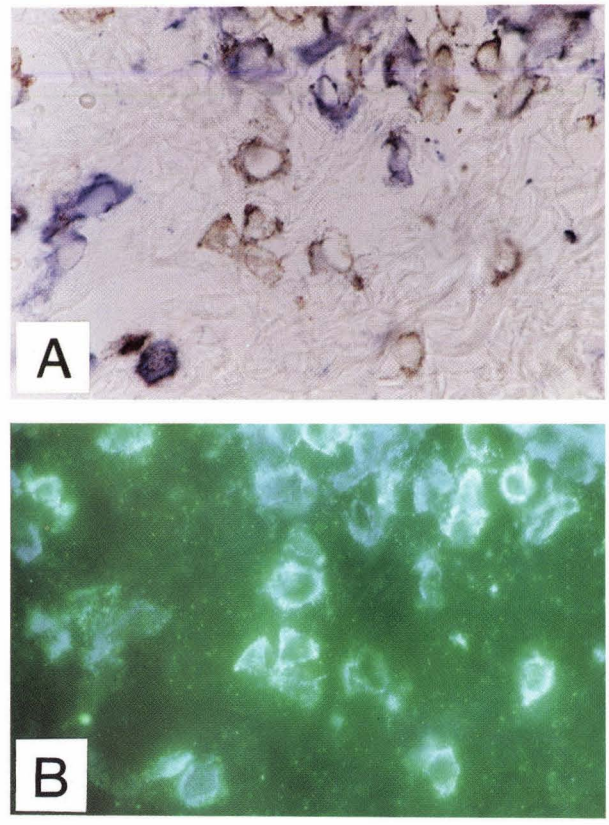

Figure 2. Double staining for CD45 RA (IGSS) and CD3 (AP labeling with naphthol AS-MX/fast blue) in a section of human skin with chronic inflammation. (A) Under brightfield illumination, the brown cells were $CD45RA^+$ $CD3^-$, whereas the dark blue cells were double positive ($CD45RA^+$ $CD3^+$). (B) This is verified under epipolarization illumination, which exclusively showed the immunogold silver-labeled $CD45RA^+$ cells. Original magnification × 1,000.

Discussion

The presented protocols for simultaneous demonstration of two tissue antigens are based on comprehensive studies with a large series of combinations of IGSS and IEL (7). The sensitivity of a three-step IGSS is three- to five-fold lower than that of a three-step IEL. Consequently, mixed labeling was started with IGSS and followed by IEL because the detection of the second antigen in double labeling procedures is generally associated with a reduced sensitivity. This sequence of double labeling posed some restrictions on the choice of reagents to be utilized for the IEL procedure. In general, peroxidase labeling with the substrates 3-amino-9-ethylcarbazole (AEC) or diaminobenzidine (DAB) displays a higher sensitivity than AP labeling and is, therefore, used more frequently. The red end product of the substrate AEC, in contrast to the brown reaction

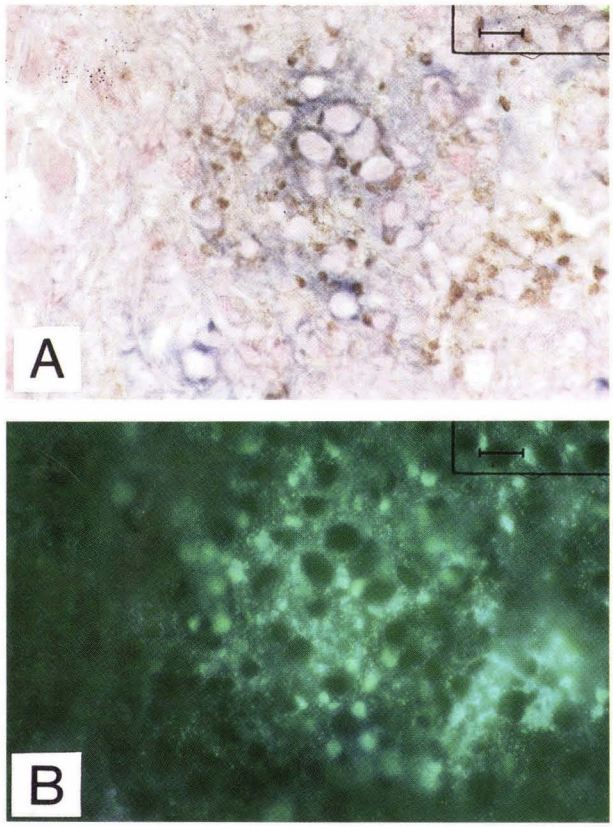

Figure 3. Double labeling for identification of parasitized cells in the skin of mice infected with *L. major*. (A) The obligatory intracellular parasites were labeled with IGSS (brown) and the host cells (non-lymphoid dendritic cells detected by rat MAbs NLDC-145) were identified by AP labeling with naphthol AS-MX phosphate/fast blue as substrate; brightfield illumination. (B) The distribution of parasite antigen was visible under epipolarization illumination. Original magnification × 1,000.

product of DAB, would contrast well with IGSS under brightfield illumination. However, our earlier studies showed that peroxidase labeling is incompatible with IGSS (7). The silver enhancement procedure resulted in nonspecific silver grain precipitation on the AEC end product. On the other hand, performance of the substrate reaction after silver enhancement prevented the reflection of silver-enhanced gold particles under epipolarization illumination.

For these reasons, AP labeling appears to be better suited for mixed labeling. Nevertheless, the substrate-chromogen reagents have to be selected carefully. We have previously shown that the reaction product of naphthol AS-MX phosphate/fast red reflects polarized incident light in a way similar to IGSS and that the substrate bromochloro-indolyl-phosphate/nitro blue tetrazolium is poorly discernible from IGSS under brightfield illumination (7). Therefore, only the substrates naphthol AS-MX phosphate/fast blue (Figure 2) and naphthol AS-BI phosphate/new fuchsin seem to be appropriate for mixed labeling combining IEL and IGSS. They do not interfere with and can be easily discriminated from IGSS.

Multistep procedures strongly amplify the reactions of the primary antibodies with the respective tissue antigens and result in a high sensitivity. However, if it is not possible to use primary antibodies from different species, undesired reactions may occur between the various Igs. This cross-reactivity can be drastically reduced by using hapten-coupled antibodies (such as FITC-conjugated MAbs for detection of the second antigen) and the corresponding hapten-specific Ig (8). Such a technique allows the independent detection of two tissue antigens with MAbs derived from the same species (eg, combining unconjugated and hapten-conjugated mouse MAbs). In addition to antibody conjugation with FITC, sulfonation or coupling with digoxigenin may be utilized. FITC offers major advantages because a large variety of FITC-linked antibodies are commercially available, and FITC conjugation can be achieved with standard protocols. We have shown an example of mixed labeling via the FITC/anti-FITC-system (Figure 2). In the other example, crossreactivity was prevented by using primary antibodies from different species (Figure 3). An advantage of the latter technique is the time-saving possibility of simultaneous application of both primary antibodies.

When FITC-conjugated antibodies and anti-FITC Ig are used for detection of the second antigen, there is only one possibility for crossreactivity that should be kept in mind: after completion of the first-step staining (Figure 1), the biotinylated sheep anti-mouse Ig used as secondary reagent may still have free binding sites. Thus, the FITC-linked antibody for detection of the second antigen may be nonspecifically trapped. This can be minimized by optimal dilution of the biotin-conjugated anti-mouse Ig and, of even greater importance, by blocking with normal mouse serum before starting with the second-step staining.

The described protocols for mixed labeling with a combination of IEL and IGSS offer a method which allows the highly specific and sensitive labeling of shared epitopes. In addition to the immunological characterization of cells, the method is well suited for such other fields of research as neuroscience, cell biology, and tumor biology.

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References

1. Larsson LI: *Immunocytochemistry: Theory and Practice*. CRC Press, Boca Raton, FL, 1988
2. Pryzwansky KB: Applications of double-label immunofluorescence. In *Techniques in Immunocytochemistry*, Bullock GR, Petrusz P (eds). Academic Press, London, 1982, pp 77–89
3. Tidman N, Janossy G, Bodger M et al: Delineation of human thymocyte differentiation pathways utilizing double-staining techniques with monoclonal antibodies. *Clin Exp Immunol* 45:457–465, 1981
4. De Waele M, De Mey J, Renmans W et al: Potential of immunogold-silver staining for the study of leucocyte subpopulations as defined by monoclonal antibodies. *J. Histochem Cytochem* 34:1257–1263, 1986
5. De Mey J, Hacker GW, De Waele M, Springale DR: Gold probes in light microscopy. In *Immunocytochemistry: Practical Applications in Pathology and Biology*, Pollak JM, Van Noorden S (eds). Wright PSG, Bristol, 1986, pp 71–88
6. Holgate CS, Jackson P, Cowen PN, Bird CC: Immunogold-silver staining: new method of immunostaining with enhanced sensitivity. *J Histochem Cytochem* 31:938–944, 1983
7. Gillitzer R, Berger R, Moll H: A reliable method for simultaneous demonstration of two antigens using a novel combina-

tion of immunogold-silver staining and immunoenzymatic labeling. *J Histochem Cytochem* 38:307–313, 1990
8. Van der Loos CM, Das PK, Van den Oor JJ, Houthoff HJ: Multiple immunoenzyme staining techniques: use of fluoresceinated, biotinylated and unlabeled monoclonal antibodies. *J Immunol Methods* 117:45–52, 1989
9. Kraal G, Breel M, Janse M, Bruin G: Langerhans cells, veiled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody. *J Exp Med* 163:981–997, 1986
10. Blank C, Fuchs H, Rappersberger K, Röllinghoff M, Moll H: Parasitism of epidermal Langerhans cells in experimental cutaneous leishmaniasis. *J Infect Dis* 167:418–425, 1993