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Detection of Intracellular Immunoglobulin in Formalin-Paraffin Sections of the Chicken Spleen Using the Immunoperoxidase Technique*

Andrew Chang-Young Fei[1] Pen-Heng Chang[2] And King-KiangYoung[3]

A direct immunoperoxidase technique for the identification of immunoglobulin-containing cells in the section of chicken spleen is described. This is applicable to formalin fixed, paraffin-embedded material. It is based on an immunohistochemical method using peroxidase-labelled antibodies.

Rabbits were immunized by purified chicken IgG. The IgG fraction of rabbit anti-chicken IgG antiserum was conjugated with horseradish peroxidase. The presence of immunoglobulin-containing cells in chicken spleen sections was revealed by staining the tissue-bound peroxidase-labelled antibody with diaminobenzidine, than briefly counterstained with hematoxylin.

The endogeneous peroxidase was satisfactorily inhibited by treating the dewaxed section with a fresh 0.5% solution of hydrogen peroxide in absolute methanol for 20 minutes.

The structure of immunoglobulin-containing cells in chicken spleen sections is clear, preparations are permanent, and retrospective studies of stored paraffin-embedded tissue are possible.

The diagnostic pathologist is usually in receipt of tissues that have been fixed in formalin and embedded in paraffin. However, when the need for immunologic studies becomes aparent, the requirement for fresh frozen unfixed tissue or cold alcohol-fixed, paraffin-embedded material limits the application of immunofluorescence methods (Sainte-Marie, 1962; Heron, 1970). The use of antibody labelled with horseradish peroxidase has resolved some of these difficulties. An immunoperoxidase method for demonstrating a variety of antigens in formalin-fixed paraffin-embedded tissues has been described (Taylor, 1978). This technique has the advantage that the materials stored for several years after processing can be retrospectively analyzed.

This technique has been successfully performed on mammalian tissue sections (

1. Taiwan Provincial Research Institnte for Animal Health, Tansui, Taiwan, R. O. C.

2. Department of Veterinary Medicine, National Taiwan University, Taipei , Taiwan, R. O. C.

3. Department of Medical Research, Veterans General Hospital, Taipei, Taiwan, R. O. C.

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Taylor, 1978). However, no investigation dealt with this technique in chicken tissue sections was mentioned.

This report presents the direct immunoperoxidase method for demonstrating intracellular immunoglobulins in formalin-fixed paraffin-embedded chicken spleen sections and the related techniques.

MATERALS AND METHODS

Antibodies to chicken IgG

Briefly, whole chicken serum was firstly salted out in ammonium sulfate solution. Then the IgG or IgM were purified with DEAE cellulose and gel filtration separately (Lebacq, 1979).

Rabbits were immunized with purified chicken IgG. The antisera was absorbed with purified IgM in Sepharose CL-4B column to get specific rabbit anti-chicken IgG antisera. The IgG fraction of specific rabbit anti-chicken IgG antisera was prepared with DEAE cellulose, based on the "batch technique" of Reif (1969). The rabbit IgG was used for conjugation with horseradish peroxidase (HRP)*.

HRP Conjugation

Conjugation was carried out by the method of Wilson and Nakane (1978). Briefly, 8mg IgG was mixed with 4mg HRP. After reaction, the mixture was applied on top of a 1.0×115 cm column of Sephacryl S-200. The elutes corresponding to the first two thirds (tube 12—17) of the first peak, with high absorbance at 280nm and 403nm (Fig. 1), were collected and used for the detection of intracellular immunoglobulin.

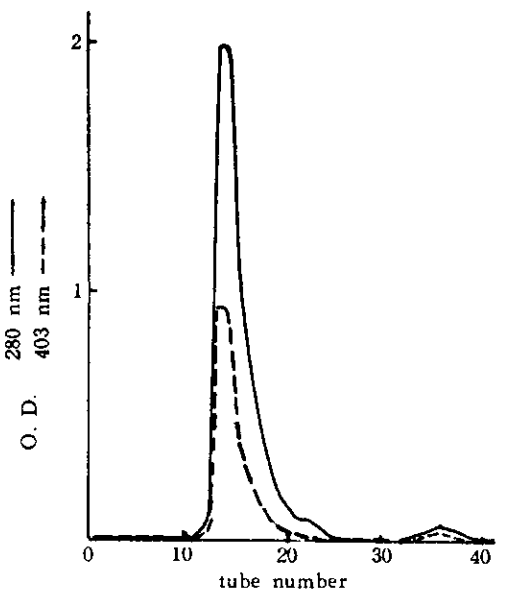

Fig. 1. Separation of HRP-IgG conjugation mixture on a Sephacryl S-200 column (1×115cm, in PBS).

*Sigma Chemical Company, Type IV. P. O. Box 14508, ST. Louis, MO., 63178, U. S. A.

**Sigma Chemica Company.

Stainin technique

Five μm thick setions were obtained from neutral formalin-fixed, paraffin-embedded blocks of chicken spleen tissue. Three groups of sections were dewaxed with xylol and the endogeneous peroxidase activity was blocked by one of the following 3 different treatments: (1) fresh 0·5% solution of hydrogen peroxide in methanol for 20 min. (Burns, 1975); (2) 0.074% hydrochloric acid in methanol for 15 min. (Weir *et al.*, 1974); (3) methanol for 20 min., followed by 0.0125% hydrogen peroxide in Phosphate Buffered Saline, PBS (Taylor and Mason, 1974).

The sections were then taken through the alcohols to the water. The extracellular proteins were digested with 0.1 % trypsin solution in PBS at pH 7.2 and 37°C for 30min.. They were then washed in cold PBS with agitation for 3 times and exposed to a range of HRP-conjugated rabbit anti-chicken IgG antibody. A dilution of 1/10 of the antibody with an exposure of 1 hour in moist chamber at 37°C was satisfactory. Sections were then thoroughly washed in 3 changes of PBS.

The tissue-bound HRP-conjugated antibody was then washed with 0.05M Tris-HCl buffer at pH 6.0, then stained with 0.05% 3, 3′-diaminobenzidine tetrahyrochoride** and 0.01% hydrogen peroxide in 0.05M Tris-HCl buffer at pH 6.0 for 10min. Finally the sections were counterstained with a weak hematoxylin. dehydrated, cleared in xylol and mounted in Canada balsam.

RESULTS

HRP Conjugation

The rabbit IgG was coupled to HRP by the sodium periodate oxidation. After reaction, the mixture was passed through a Sephacryl S-200 column. The first peak with high absorbance at 280nm and 403nm (Fig. 1) was collected and used in this study. The Reinheit-Zahl value, abbreviated as RZ value, of HRP-conjugated IgG collection was 0.4.

The inhibition of endogeneous peroxidase activity

Only the treatment of fresh 0.5% solution of hydrogen peroxide in methanol-H_2O_2 can inhibit the endogeneous peroxidase activity. The endogeneous peroxidase activity under that treatment was completely blocked, allowing the most precise identification of immunoglobulin-containing cells (Fig. 2). The other two treatments of inhibiting the endogeneous peroxidase activity exert little effect on the reduction of nonspecific (pseudopositive) staining (data not shown). In addition to the endogeneous peroxidase, the serum residue in sections was also a factor to produce pseudopositive background staining; the intensity of nonrelevant positive brown color in background increased if diminishing the digestion time of trypsin.

The immunoglobulin-containing cells

The immunoglobulin-containing cells were clearly identified by the strongly positive reaction of brown color in the cytoplasma (Fig. 2). The brown color provided a sharp contrast with surrounding negative cells. The morphology of the positive cells corresponded to the typical plasma cell (Fig. 2). The intensity of the brown color of

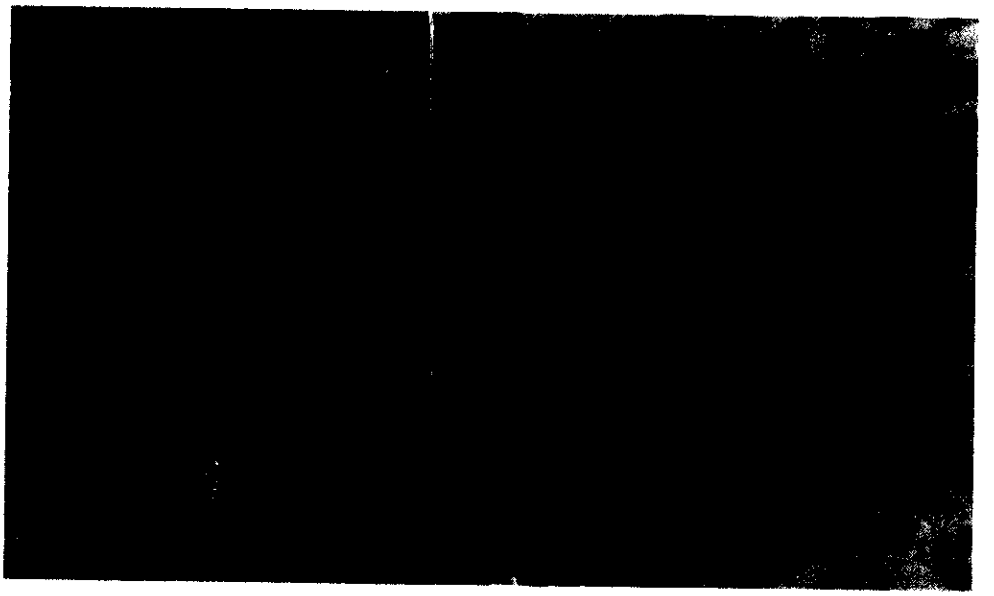

Fig. 2. Photomicrographs of positive cells which in the final preparation was shown in the cytoplasma. left×165, right×1,000.

positive cells diminished if diluting HRP-conjugated antibody.

DISSCUSSION

In this study the RZ value of HRP-conjugated IgG collection was 0.4. The best HRPIgG conjugation should have an RZ value of approximately 0.3 to 0.6. The values correspond to conjugate having an average of 1 to 2 HRP molecules bound to each IgG molecule (Wilson and Nakane, 1978).

The immunoperoxidase technique usually meets the difficulty that the pseudopositive background staining of endogeneous peroxidase confuses the result. This study compared three commonly used methods of inhibiting the endogeneous peroxidase. It shows that only fresh 0.5% solution of hydrogen peroxide in methanol for 20min, exerts satisfactory result for chicken tissue. Streefkerk (1972) reported that methanol-H_2O_2 strongly inhibits the endogeneous peroxidase activity without inhibiting the antibody reaction; and this is most advantageous when using diaminobenzidine as the indicator. The inhibition method may be used not only in the direct immunoperoxidase techniques, as reported here but also for the indirect method, the peroxidase-antiperoxidase PAP method and in study in which HRP is used as a protein marker.

The following facts indicats that the positive cells of brown color was not resulted from the extracellular serum IgG: (1) The morphology of the positive cells corresponded to the typical plasma cells. (2) The contrast of brown positive cells and the surrounding negative cells was very sharp. Additionally. the fact that the intensity of the brown color of positive cells could be diminished by diluting HRP-conjugated antibody indicates that the positive cells indeed contain intracellular chicken IgG. Thus this method could detect the intracellular immunoglobulins.

The method described here is believed to be a general procedure for the detection of most protein antigen in chicken tissue. This method has advantages over the more commonly used immunofluorescent one. The degree of specificity is simillar (Burns *et al.*, 1974), but difficulty arises in differentiating specifically fluoresent cells from autofluorescent cells. Moreover, this peroxidase-labelled method gives a permanent preparation. The result dose not fade with time and thus it is easier to make comparisons between different batches of slides tested. Cell structure is clear and it is accurate for quantitative studies of the easily classified positive cells.

The sensitivity, specificity and convenience of the immunoperoxidase technique have made it a useful tool for routine application and retrospective study in pathological laboratory.

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經福馬林固定之雞脾臟細胞內免疫球蛋白之免疫酶染鑑定法*

費昌勇[1]　張本恆[2]　楊錦江[3]

本文旨在介紹以直接免疫酶染法染鷄脾臟組織內含免疫球蛋白之細胞。其技術乃利用經藁草根過氧化酶（Horseradish peroxidase）標幟之抗體與組織內之特定抗原進行結合，並藉酶染法標出位置。本法之特點爲可適用於經福馬林固定之臘切片組織。

數隻白兎以純化之鷄 IgG免疫。將兎抗鷄 IgG血清中之兎IgG純化出，與藁草根過氧化酶結合。含IgG之鷄脾細胞與經藁草根過氧化酶標幟之兎抗鷄IgG之抗體結合，便可用diaminobenzidine染出含鷄IgG之細胞；背景染色爲蘇木紫。

組織內之藁草根過氧化酶可用含有 0.5%過氧化氫之甲醇處理切片20分鐘，以抑制假陽性反應。含有鷄IgG之陽性細胞形態十分清晰，切片可久存。浸臘之組織塊（block）亦可供日後研究之用。

1.臺灣省家畜衛生試驗所

2.臺北市家畜衛生檢驗所

3.臺北市榮民總醫院醫學部

*中央研究院動物研究所集刊23卷2號，199—203頁。