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Research Article

CHARACTERISTICS OF ENZYME-ANTIBODY CONJUGATE PRODUCED BY DIFFERENT METHODS IN IMMUNOHISTOCHEMISTRY

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ABSTRACT

The principal development that has taken place in the past years is embodied in the title immunohistochemistry. The change from fluorescent-antibody methods to enzyme-antibody conjugate is necessitated by changes brought about by a single advance which has produced a minor technological revolution.

In immunohistochemistry demonstrating of enzyme-antibody conjugate plays an important role. We have compared two conjugation procedures, the one-step method and two-step method with glutaraldehyde as a cross-linking agent as these novel methods are simple and fast which does not suffer from any cross reactions.. The enzymatic and staining characteristics of both the conjugates were evaluated. HRP was use as an enzyme with AntiHela as a monoclonal antibody.

All the conjugates showed positive reaction with Hela cells stained as brown granular deposits but did not show any reactivity with others. The panel of malignant tissues against which the conjugates were tested revealed a strong reactivity with yellow-brown color in the nuclear region of the malignant cells. However the contrast of the staining was more marked when two step conjugate was used and the molecular weight of it was lower than that of the one-step conjugate. One- step glutaraldehyde method was cheapest as compared to other and time of preparation is also less.

The results have led us that conjugate was the backbone and preparation must be of great attention.

Keywords: Enzyme –Antibody; Glutaraldehyde Conjugates; Immunohistochemistry, Staining.

INTRODUCTION

There has been a spate in the use of immunohistochemistry methods for quantitative and qualitative localization and estimation of antigenic substances with the availability of pure monoclonal antibodies against an ever-increasing number of antigenic substances like hormones, tumor proteins, normal body components, etc. For these, a marker has been attached to the antibody which may include: fluorescent dye, radioactive molecules and recently enzymes. The fluorescent dyes and radioactive substances pose the problem of use of special equipment, carcinogenicity and radiation exposure as well as a short shelf life.

The fluorescence method introduced long back has obtained an important place in the detection of antigens and antibodies in tissues and blood and developed a new technique immunohistoenzyme which offers several advantages over the other methods[1,2]. Several investigators have used the direct and indirect technique like immunoperoxidase for the demonstration of tissue antigens and tissue-bound antibodies[3,5]. The results of some studies suggest that the immunoperoxidase method is very specific and sensitive as the compared to other methods. The sensitivity, the specificity and the reliability of the method depends to a great extent on the quality of the conjugate and its method of preparations. The enzymes most popularly used are Horseradish- peroxidase (HRP) and Alkaline Phosphatase (ALP) with a variety of substrates Diamine benzidine (DAB) and p-nitrophylphosphate (PNP) repetitively, these being used both for enzyme immunoassay as well as immunohistochemistry[6-8].

In the present study HRP was employed because the enzyme was commercially available in relatively pure form, the cytochemical and histochemical methods have been well established and successfully adapted to microscopy. We have compared two conjugation procedures, the one-step method and two-step method with glutaraldehyde as a cross-linking agent as these novel methods are simple and fast which does

not suffer from any cross reactions. The enzymatic and staining characteristics of both the conjugates were evaluated.

MATERIALS AND METHODS

Preparation of conjugation: Labeling of monoclonal antibody Anti-Hela against Hela cells, human cancer cervix that was originated in Balb/c mice produced in Department of Pathology, SN Medical College, Agra by hybridoma technique. HRP was commercially purchased by sigma chemicals, USA and conjugation was done by two different techniques (a) one step glutaraldehyde and (b) two-step glutaraldehyde. The coupling of antibody to enzyme was as:

a) The one-step glutaraldehyde method:

1) 3 mg of antibody Antihela was mixed with 10 mg of Enzyme (HRP) in 1 ml of 0.1 M sodium phosphate buffer, (pH 6.8).
2) 0.1 ml of 1% glutaraldehyde was slowly added while the mixture was stirring. Stir for an additional 5 minutes was done and left at room temp (22° C.) for 3 h.
3) 0.1 ml of 1 M lysine at pH 7 to the solution was added and left at room temp for 2 h.
4) The mixture was dialyzed against 0.01 M phosphate, 0.85% NaC1, pH 7 phosphate buffer saline (PBS).
5) Centrifuge at 40,000×g, 4° C. for 20 min to remove debris.
6) Filtered through a 0.22 um membrane and store.

b) The two-step glutaraldehyde method:

1) 10 mg of HRP was dissolved in 0.2 ml of 1.25% glutaraldehyde in PB. The solution was incubated for 18 h at 22° C. Excess glutaraldehyde was removed by dialysis or gel filtration. 1 ml final volume was obtained.
2) Dialyzed against 0.1 M sodium carbonate buffer (pH 9.5).
3) 0.5 mg of AntiHela /0.1 ml saline was added to the above solution (pH was above 9). Incubated 24 h at 4° C.
4) 0.1 ml of 0.2 M ethanolamine was added and incubated for 2 h at 4° C.

Purification and identification of conjugates: The conjugates prepared according was purified by column chromatography and dialyzed against Phosphate buffer saline (PBS). The reactivity of the antibody-conjugate was assessed by testing with Hela cells and also tested against the paraffin embedded tissue sections obtained from routine histological reported. (a) Carcinoma cervix sections grade I to III (b) Non-malignant cervix used as controls.

The conjugates were assessed against all the above by direct Immunoperoxidase technique[9-12].

The positive staining reaction was observed as brown color of substrate (DAB) taken by the cells. The intensity of the staining reaction was graded as +++ strong positive reaction, in more than 50% cells, ++ Moderately positive staining reaction in 25-50% cells, + weakly positive staining reaction in less than 25% cells _ Negative staining reaction, where none of the cells show any type of staining.

RESULTS AND DISCUSSION

The enzymatic and physical properties of the conjugates are shown in Table 1.

All the conjugates showed positive reaction with Hela cells stained as brown granular deposits but did not show any reactivity with others. The panel of malignant tissues against which the conjugates were tested revealed a strong reactivity with yellow-brown color in the nuclear region of the malignant cells. The results are shown in Table 2.

Immunohistochemical methods acts as valuable tools for both routine biochemical and research permanence of the reaction product and usefulness in fixed tissue sections together with the facility for simultaneous pathological diagnosis make the immunoperoxidase method the technique of choice in laboratories at the present time.

In our study the enzymatic and staining characteristics of HRP-antibody conjugates prepared with two methods were compared specifically the staining characteristics of the conjugates. Both the conjugates gave specific staining of the immune complexes. However the contrast of the staining was more marked when two step conjugate was used.

These disappointing results obtained in study involving a comparison of one and two techniques might be attributed to the use of preparation and other reagents. In the one step method the conjugate prepared were composed of a highly heterogeous population of complexes but that free antibody was absent in the reaction mixture. As reported by other the average ratio of antibody to the enzyme in the active complexes varied between 1:3 in this method[13, 14]. While in the two step method HRP alone cannot be insolubilized even with excess of glutaraldehyde which posses few lysine residues and helps to block amino group. As reported by others the average ratio of antibody to the enzyme in the active complexes varied between 1:1 in this method[15].

Chromatography of the two step conjugate does not show proteins eluting with the void volume, and thus the polymerization observed in the one-step conjugate did not occur. So the molecular weight of the two-step conjugate is lower than that of the one-step conjugate. In immunohistochemistry low molecular weight conjugate are the best choices[16].

CONCLUSION

The conjugate prepared by one step glutaraldehyde method are cheapest as compared to other and time of preparation is also less. Since the cross-linking reagents in two step conjugate are more this affects the cost and time.

Table1: Enzymatic properties of conjugates

	One-step glutaldehyde	Two-step glutaldehyde
Working dilution (Chess-board Titration)	1:100	1:800
Protein content (By Colorimeter)	1.2 mg/dl	6.9mg/ml
Molecular weight (Polyacrylamide Gel electrophoresis)	$4x10^5$	$2x10^5$
Cost to prepare conjugate (Indian Rupees)	400	450

Table 2: Reactivity of conjugates

	One-step glutaldehyde	Two-step glutaldehyde
Cancer cervix grade I	+	++
Cancer cervix grade I	++	++
Cancer cervix grade I	+++	+++
Hela-cells	+++	+++
Non-malignant Tissues	--	--

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