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Utility of gold enhancement of the diaminibenzidine reaction product to characterize activated cells in rejecting kidneys

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SUMMARY

The study of lymphocyte activation markers in kidney biopsies and other tissues has a great theoretical interest. Until now, it has been difficult to stain these antigens in tissue, partly because their expression is transient and probably because their density on the cell surface is low.

Using an indirect peroxidase method enhanced with gold salts ($NaCl_4Au$ added at a concentration of 0.25 % after the diaminobenzidine (DAB) for 30 seconds), we have been able to stain the activation and other lymphocyte markers even in some cases in which there was no detectable staining using the common indirect method.

We conclude that this is a useful and simple method to improve both the efficiency and the sensitivity of immunoperoxidase when DAB is used, especially to stain receptors which are not consistently expressed in tissue such as interleukin-2 or transferrin receptors.

Key words: ***Immunoperoxidase. Diaminobenzidine. Lymphoxytes. Interleukin 2 receptor. Gold.***

TINCION DE LAS CELULAS ACTIVADAS DEL ALOINJERTO RENAL MEDIANTE LA INTENSIFICACION DEL PRODUCTO DE REACCION DE LA DIAMINOBENCIDINA CON SALES DE ORO

RESUMEN

El estudio de los marcadores de activación en las biopsias renales y en otros tejidos tiene un gran interés teórico. Hasta ahora ha sido difícil la tinción de estos antígenos debido a la expresión transitoria de los mismos, así como a la baja densidad de los mismos en la superficie celular.

Mediante la utilización de una técnica de inmunoperoxidasa indirecta modificada mediante la adición de sales de oro ($NaCl_4Au$ a una concentración del 0,25 % durante 30 segundos después de revelar la diaminobencidina) hemos podido demostrar en injertos renales con rechazo la presencia de células activadas incluso en los casos en que estas células no eran visibles con la técnica de inmunoperoxidasa normal.

Concluimos que este método es útil para incrementar la sensibilidad de las técnicas de inmunoperoxidasa en el estudio de receptores que se expresan en una baja densidad, como los receptores de interleukina 2 y transferrina.

Palabras clave: ***Inmunoperoxidasa. Diaminobenzidina. Linfocitos. Receptor de interleukina 2. Oro.***

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Introduction

The oxidative polymerization of 3,3-diaminobenzidine (DAB) is a realiable marker for the detection of labelled antibodies in tissue. DAB has a known affinity for the salts of some metals and this property has been used to modify the color of the DAB precipitation product [1] and to enhance the end product of the DAB reaction both in optical and electron microscopy [2, 3].

The study of lymphocyte activation markers using monoclonal antibodies (MoAb) against the interleukin 2 and the transferrin receptors in kidney biopsies and other tissues has a great theoretical interest. Until now, it has been difficult to stain these antigens in kidney biopsies, partly because their expression is transient and probably because their density on the cell surface is low.

Using an indirect peroxidase method enhanced with gold salts we have been able to intensify the staining of the activation and other lymphocyte markers in tonsil and kidney biopsies. By means of progressive dilutions of the primary antibody we have studied the degree of enhancement obtained with gold, and we have found that at the dilution of the primary antibody at which there was no detectable staining using the normal indirect method, there were clearly positively stained cells when gold was added.

Material and methods

After completing the indirect immunoperoxidase method as described elsewhere [4], the staining procedure to enhance the DAB product with gold was as follows:

1. After completing the DAB reaction, wash the tissue in running tap water for 5 minutes.
2. Add a freshly prepared solution of $NaAuCl_4$ at a concentration of 0.25 % in PBS for 30 seconds.
3. Wash again in tap running water for 1 min.
4. Counterstain with Meyer's hemalum.
5. Wash in running tap water for 5 minutes.
6. Dehydrate in graded alcohols and mount in DPX (Raymond Lamb, London).

Serial sections of tonsil from the same block of tissue have been incubated with three different antibodies. The monoclonal antibodies (MoAb) used were: a) Leu3a [5]: an IgG1 mouse MoAb against CD4 antigen present on helper-induced lymphocytes (Becton and Dickinson); b) UCHT4 [6] an IgG1 mouse MoAb against CD8 that identifies cytotoxic-supressor lymphocytes (gift of Dr. Beverly); and Ki-67 [7]: an IgG1 mouse MoAb that stains the nuclei of all proliferating cells (Dako). Serial sections of the same biopsy from a rejecting allografted kidney have been incubated with to different antibodies: anti-Tac [8]: an IgG2 mouse MoAb against CD25 that identifies IL-2 receptor bearing cells (gift of Dr. Waldmann) and anti-Transferrin receptor antibody [9]: an IgG2 MoAb that stains transferrin receptor (Becton and Dickinson).

The localization of the antibodies was revealed using DAB with and without gold salts, using progressive dilutions of the primary antibody. For all the antibodies the first section was stained with the primary antibody at a concentration of 1/10. Te kidney used for the titrations was choosen out of 15 rejecting kidneys because it yielded the highest number of anti-Tac and anti Transferrin receptor positive cells. As negative controls we ommitted the primary antibody.

We counted the cells at a magnification of 400× using a graticule in the eyepiece that measured a surface of 0.058 mm^2 in a Leitz Orthoplan microscope. In every biopsy we have counted 10 fields corresponding to a surface of 0.58 mm^2. The number of cells counted is expressed per mm^2. In the case of tonsil, UCHT4 positive cells have been counted between follicles. Ki-67 and Leu3a positive cells have been counted inside the follicle. We have decided to count the cells inside the follicle when using Leu3a, because the density of positive cells was too high in the interfollicular space. For anti-Tac and transferrin we have counted the cells in the renal interstitium. Only cells that showed at least half of their membrane stained were recorded as positive cells.

To compare the means of the counts obtained at different dilutions with and without gold the Student T test has been used.

Results

The effect of intensification of the reaction product with gold salts was to change the brown deposits of DAB to red-brown or dark-grey. In order to avoid background staining after intensification, sections should be incubated with DAB-peroxidase for as short a time as possible. In our case the optimal time for incubation has varied between 45 and 60 seconds.

We have observed a significant increase in the number of positive cells counted for a given dilution in the section stained with gold compared to the section stained without gold for all antibodies except UCHT4. In the case of UCHT-4 (fig. 1) when using the concentrations of 1/10, 1/20, 1/40 and 1/80 there was no difference in the number of positive cells in the section stained with gold compared to section stained without gold. At the concentration of 1/160 the difference reached statistical significance ($p \leq 0.005$). The effect of the enhancement was specially evident with Ki-67, and also for anti-Tac (fig. 2).

In the rejecting kidney stained with anti-Tac we observed a surface membrane distribution of the DAB reaction product in some cells but in many of them only

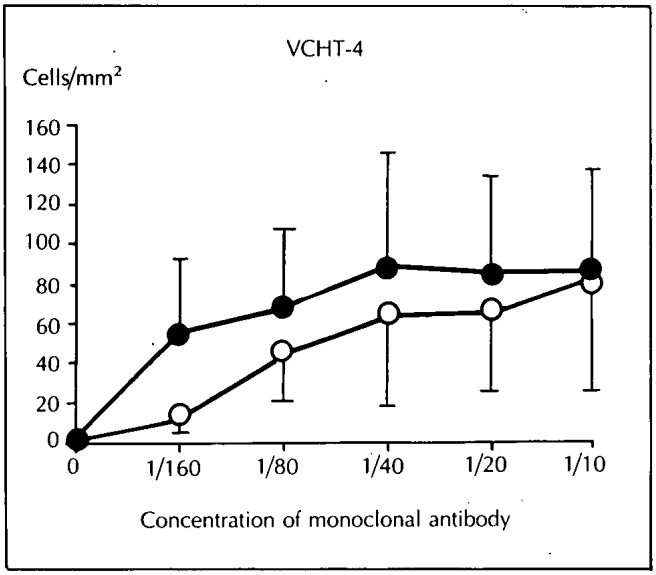


Fig. 1.—Number of UCHT4 positive cells/mm² in the interfollicular area of a normal tonsil when progressive dilutions of the primary monoclonal antibody are employed using the immunoperoxidase indirect method (white circles) and the enhanced method with gold salts (black circles). There is no statistical difference in the number of positive cells except when the primary antibody is employed at a concentration of 1/160 ($p \leq 0.005$).

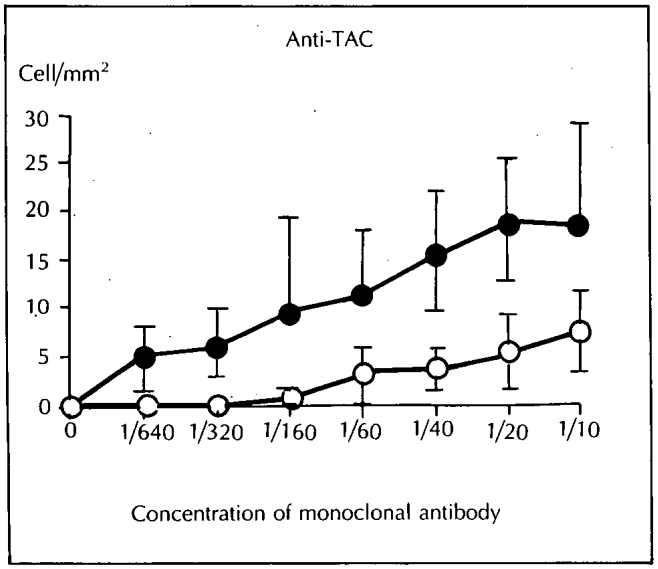


Fig. 2.—Number of anti-Tac positive cells infiltrating the interstitial space of a rejecting kidney. There is a significant increase in the number of positively stained cells for any dilution of the primary antibody when gold is added (black circles), compared to the standard immunoperoxidase method (white circles) ($p \leq 0.005$).

a section or a pole of the membrane was stained (fig. 3). In the most strongly stained cells there was not only staining in the membrane but also in the interstitial space surrounding the cell. It was not usual to see a cluster of three or four positively stained cells surrounded by a heavily stained interstitium. A similar pattern of staining was observed when transferrin receptor was used (fig. 4). In both cases there was some staining of tubular cells.

In every case the use of gold revealed positive cells at dilutions that showed no detectable cells in sections stained using conventional methods (fig. 5).

Discussion

The study of the activation markers of lymphocytes in tissue has been used in different autoimmune diseases [10-13]. Hancock et al. [14] have described interleukin-II receptor bearing cells in kidney biopsies taken during rejection using anti-Tac antibody. In this study the number of anti-Tac positive cells present in the biopsies has been low (between 0 and 18.6 % of all the cells). Despite their theoretical interest, following the publication of this report, the use of monoclonals against activation markers in kidney biopsies has not been frequent [15]. The rarity of reports describing the activation markers in kidney tissue may be due to technical difficulties related to the special properties of these receptors.

The expression of interleukin-2 receptor on lymphocytes reaches the peak level two days after adding Concavalin A to the culture media, and then the number of receptors begins to decrease slowly in number [16]. Due to the transient expression of these receptors, the time at which the biopsy is taken is crucial in the detection of positive cells [14].

The number of stained cells in tissue depends on the technique employed. It is known that the number of positive cells is lower when using an immunofluorescence method than when using an immunoperoxidase technique in the same piece of tissue [17]. It is sensible to assume that the number of receptors present on the cell membrane, in the cytoplasm or in the nuclei of a cell population follows a normal distribution. If the technique employed is sensitive enough, not only the cells that express the receptor at the highest concentration will be visible, but also those at which the receptor is expressed at lower concentrations. In the case of the titrations done with UCHT4 the number of positive cells remains the same when the primary antibody enhanced with gold is used at 1/10, 1/20 and 1/40 as well as when the primary antibody is used at a concentration of 1/10 without gold, suggesting that in this case saturation of the receptor has been achieved.

With all the other antibodies, despite the high concentration at which the primary antibody was employed (1/10), the number of positive cells increased when using the enhancement method. This finding suggests that there was a high proportion of cells that bore too low a density of receptors to

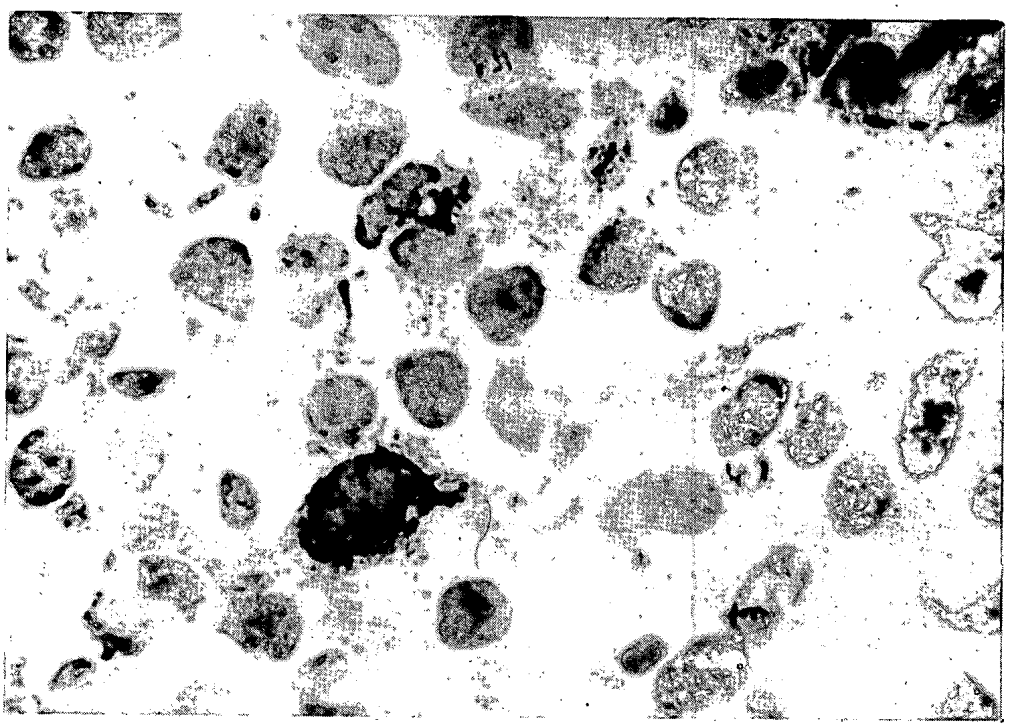


Fig. 3.—Interleukin 2 positive cells in the interstitium of a rejecting kidney stained with anti-Tac at a concentration of 1/10 using gold. There is one cell that shows strong circumferential staining and three cells that show a partial staining of their membrane. (1000× magnification.)

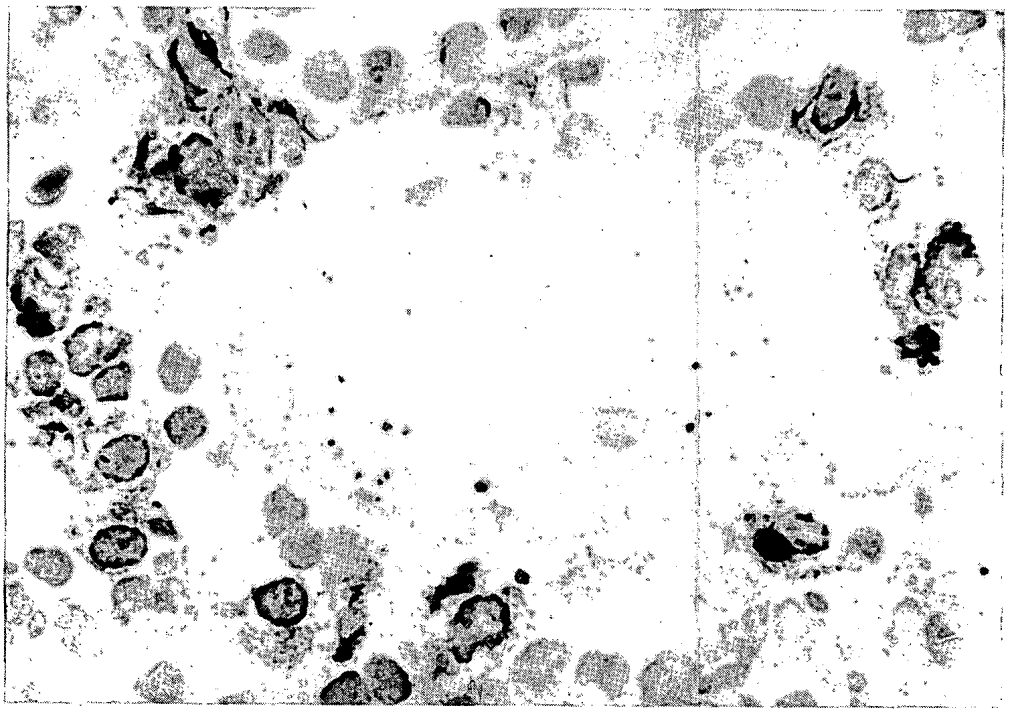
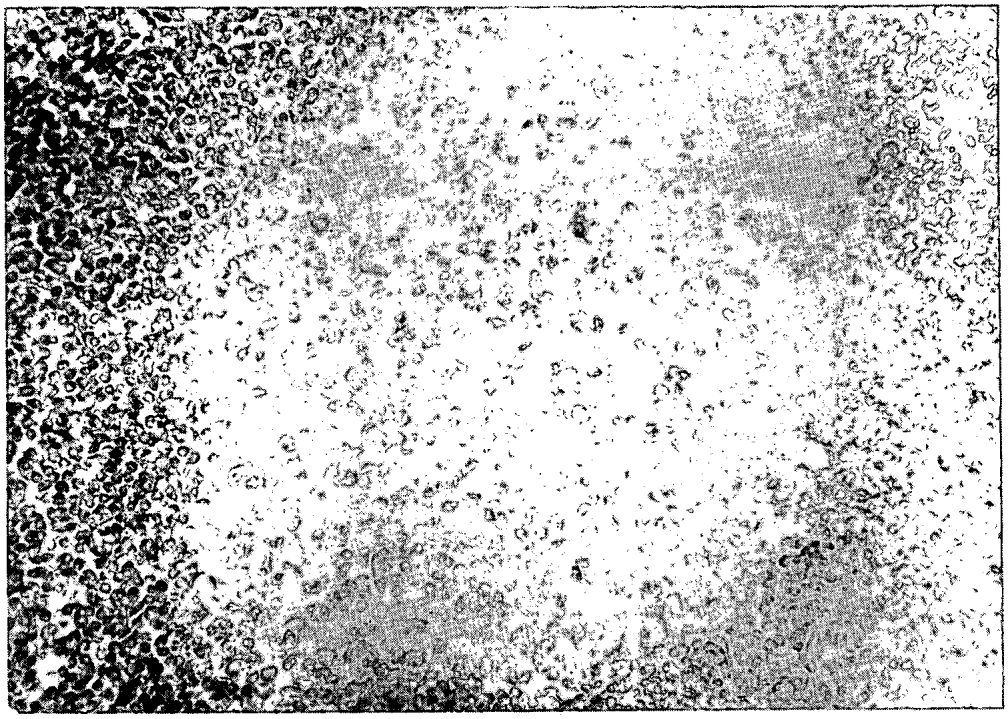


Fig. 4.—Positively stained cells surrounding a tubule from a rejecting kidney stained with human anti-transferrin antibody at a concentration of 1/20. (1000× magnification.)

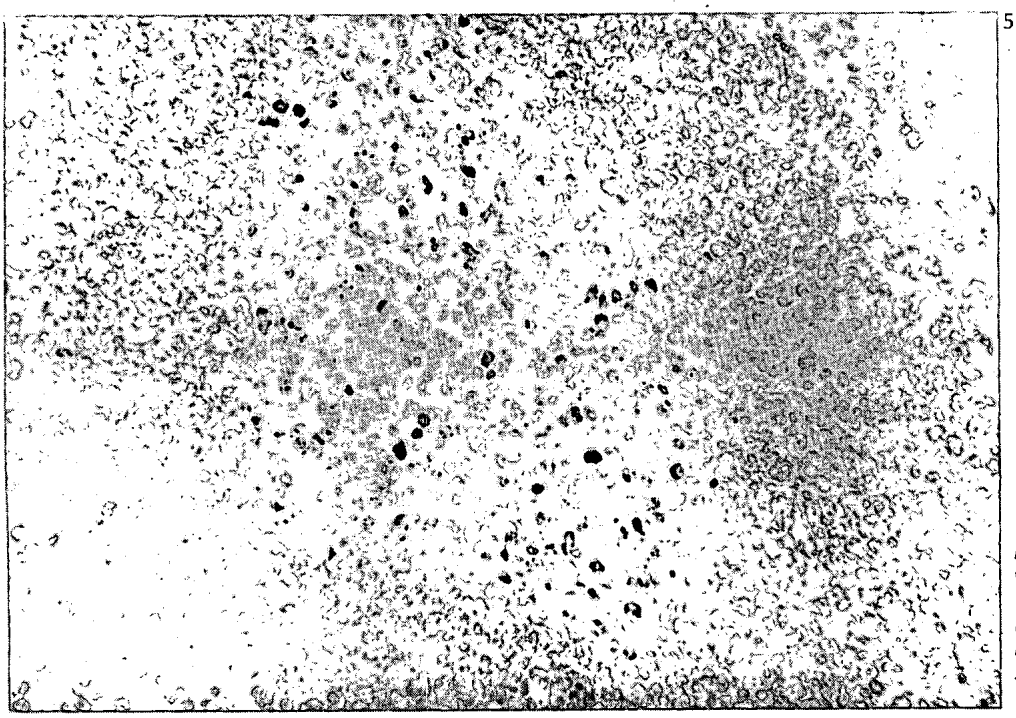
precipitate the minimum amount of DAB needed to visualize the cell.

For this reason, the absence of a positive staining using a specific technique does not necessarilly mean that there are no cells bearing the studied antigen, but may imply that the method is not sensitive enough to make the cells visible. This phenomenon could explain the difficulties encountered when staining the lymphocyte activation markers in kidney biopsies. We have seen (data not shown) that in those kidneys that show very low counts of anti-Tac and anti-Transferrin receptor using the enhanced method there was no staining at all with the standard method.

Interleukin-2 receptor is shed from the cell surface in vitro [18] and has been detected in the blood of transplanted patients during rejection and viral



5A



5B

Fig. 5.—Normal tonsil stained with Ki-67 at a concentration of 1/160. Notice that when the indirect immunoperoxidase method is employed there is no staining at all (5a), but when gold is added there are clearly positive.

infection [19]. The positive staining of the interstitium surrounding positive cells as well as the existence of some degree of background staining in some areas of the interstitium and tubules could be due to the shedding of the receptor.

We conclude that the use of gold salts to enhance the DAB reaction product when using the indirect method is useful in improving the sensitivity of the method especially for the staining of receptors which are not consistently expressed in tissue such as interleukin-2 or transferrin receptor, and are present in low concentrations on the cell surface.

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