IMMOBILIZATION OF ANTI HB_sAg ANTIBODIES ON ARTIFICIAL PROTEIC MEMBRANES

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1. Introduction

In the present paper, we report a study of the immobilization of anti-hepatitis B surface antigen (HB_sAg) antibodies on proteic membranes. Papers dealing with immunoadsorbents were recently reviewed [1] and it appears that membranes covered with specific antibodies may allow the realization of an immunoenzyme device based on the 'sandwich' procedure used in radioimmunology [2], in order to estimate HB_sAg concentration in biological fluids.

Pure immunoglobulins (IgG) were immobilized on collagen membranes using glutaraldehyde. Optimal conditions for antibody immobilization were established using IgG labelled with horse radish peroxidase (HRP). Soluble HRP-labelled antibodies were also used in a 'sandwich' procedure, after immersion of the active membrane in a concentrated solution of highly purified antigen, to confirm the biological activity of the fixed antibodies. This estimation was carried out using a direct spectrophotometric method.

2. Materials and methods

2.1. *Materials*

HB_sAg was prepared and purified from a pool of positive human sera as in [3]. Antisera against HB_sAg raised in rabbits, and IgG, were prepared in our laboratory. HRP, grade I, R Z 3,0, was purchased from Boehringer-Mannheim. Glutaraldehyde (50%), 3,3'-dimethoxybenzidine (DMB), 3,3'-diamino benzidine (DAB), and hydrogen peroxide were bought from Eastman Kodak. Collagens from ossein and pig skin were obtained from Rousselot, Ribécourt. All other chemicals used were of analytical grade.

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2.2. *Preparation and purification of antibodies*

Antisera were obtained after 2 cycles of injections of partly purified antigen emulsified in incomplete Freund's adjuvant, as in [4]. During the first cycle, intraperitoneal injections of emulsion containing 5 mg antigen were made at 1 week intervals. At the end of the first series, after a rest of 7 days, the rabbits were submitted to a second cycle of injections. The blood was collected 7 days after the last injection by heart puncture.

Specific antisera were obtained by absorption of non-specific antibodies by proteins prepared from a pool of normal human sera. The antiserum was then submitted to a Sephadex G-200 preparative electrophoresis in a 0.02 M phosphate buffer (pH 8.2). Cathodic fractions were collected and chromatographed on DEAE-cellulose with the same buffer. IgG were quantitatively eluted in the first peak.

2.3. *Preparation of (anti-HB_sAg)–HRP conjugates*

Coupling was effected by the '2-step method' in [5]. The reaction between peroxidase and glutaraldehyde was realized during the first step and IgG was then chemically linked to the activated enzyme. Labelled IgG was purified by gel chromatography on Sephadex G-200. The labelling was checked by spectrophotometric measurement of peroxidase activity at 460 nm in presence of H_2O_2 and DMB. The labelled IgG were dialysed against distilled water and stored at −30°C.

2.4. *Artificial proteic membranes*

The study of the immobilization of a biologically active material has been vastly expanded in the past few years, notably in the field of enzyme research. These studies have resulted in numerous publications particularly concerning immobilized enzymes [6–8]. We considered using the proteic membranes normally used for enzyme immobilization to fix the anti-HB_sAg antibodies and have principally studied 2 types of membrane.

The procedure for making these membranes is the same for each of the 2 types of collagen considered: alkaline-treated ossein collagen, (pH_i from 4.7–5.0) and acid-hydrolysed pig skin collagen, ($pH_i \sim 8.0$). Collagen, 10 g was dissolved in 100 ml distilled water for 24 h at 37°C. This solution, 1 ml, is spread onto a 20 cm^2 surface of polystyrene, a material chosen for its non-adherence. After air drying at room temperature, the membrane was peeled off and immersed in a freshly prepared solution of 5% glutaraldehyde in 0.02 M phosphate buffer (pH 6.8) for 5 min. The membrane was washed several times in distilled water, then saturated with specific antibodies for 30 min at 37°C, and carefully washed again. To estimate the quantity of IgG fixed onto the membranes, we used HRP-labelled antibodies.

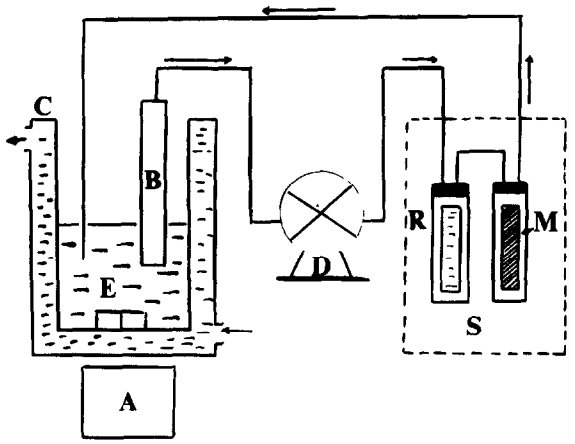

Fig.1. Schematic diagram of the system used for determination of enzymatic activity of membranes: A, stirrer; B, trapping column of silica; C, thermostatted container; D, peristaltic pump; E, reaction medium; M, membrane; R, reference; S, spectrophotometer.

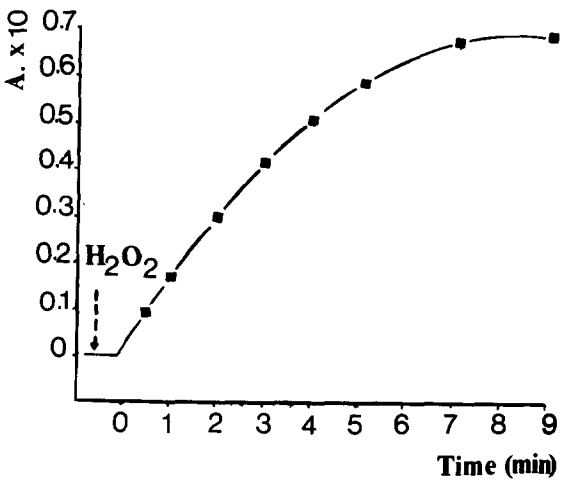

Fig.2. Peroxidase activity of HRP-labelled antibodies in absorbance units as a function of time, in the presence of DAB and H_2O_2 in phosphate buffer, pH 6.8.

2.5. *Determination of peroxidase activity*

The rate of decomposition of hydrogen peroxide by HRP in the presence of DMB was determined by measuring the rate of colour development at 460 nm. A solution of 8.8×10^{-4} M H_2O_2, 2.9 ml containing 25 µl 1% DMB were transferred into a 10 mm wide cuvette. After addition of 0.1 ml peroxidase solution to be determined, the absorption was read every 15 s for 2 min, the peroxidase activity being given by the rate of change of absorption.

When HRP-labelled antibodies were fixed on a membrane, the direct determination of the enzymatic activity was carried out in the presence of DAB (2.5 nM) and H_2O_2. The membrane was placed in the center of the cuvette, perpendicular to the optical beam (fig.1), then immersed into the reaction medium containing DAB in phosphate buffer 0.02 M, pH 6.8. After base line stabilization, reaction was started by addition of 20 µl 30% H_2O_2. The slope of the curve obtained as a function of time is proportional to the quantity of HRP-labelled antibodies fixed onto the membrane (fig.2).

3. Results and discussion

The yield of fixation of the anti-HB_sAg antibodies

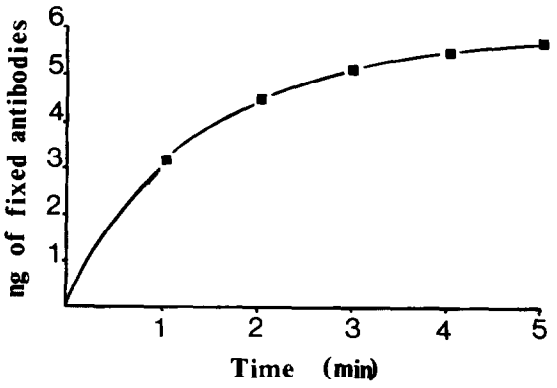

Fig.3. Effects of activation time on binding of labelled antibodies to the artificial membranes.

was studied in relation to different parameters, such as collagen concentration, glutaraldehyde concentration and contact time with the bifunctional agent. It appears that the optimal concentration of collagen is 5%, and that the maximal fixation occurs when the membrane is immersed 5 min in a solution of 5% glutaraldehyde. The amount of immobilized antibodies increased with the time of contact with the solution to 4 min. Then the membrane seems to be saturated with aldehyde functions (fig.3). The quantity of anti-HB_sAg antibodies fixed onto ossein and pig skin membranes was estimated by immobilization of HRP-labelled antibodies.

3.1. *Estimation of immobilized antibodies on ossein membranes*

Activated ossein membranes were immersed into a diluted HRP-labelled antibody solution. The dilution of this solution was such that absorption due to the coloured complex of the enzymatic reaction was between 0.1 and 0.5 absorbance units, at 2 min, under the described conditions. After 30 min immersion, the enzymatic activity of the supernatant was determined. After immersion of 5 membranes in the solution of labelled antibodies, the absorbance difference in optical density was 0.11, corresponding to the loss of 7.0 ng HRP linked to IgG (fig.4A). Since the HRP/IgG molecular ratio was 1–4, we were able to estimate the amount of immobilized antibodies corresponding approximately to 6.0 ng for each membrane. Nevertheless, this was only an estimate because the amounts of labelled and free IgG which might have been immobilized on the membrane were not necessarily identical.

3.2. *Estimation of immobilized antibodies on pig skin collagen membranes*

The quantity of labelled antibodies fixed onto these collagen membranes in similar conditions was ~26.5 ng/membrane (fig.4B). The variation of this amount was ± 0.4 ng after 15 assays. The spectrophotometric study of the enzymatic kinetics showed that HRP fixation to IgG and, indirectly, to membranes, did not significantly modify the K_m of the enzyme (fig.5), which allowed a correct evaluation of the quantity of immobilized IgG.

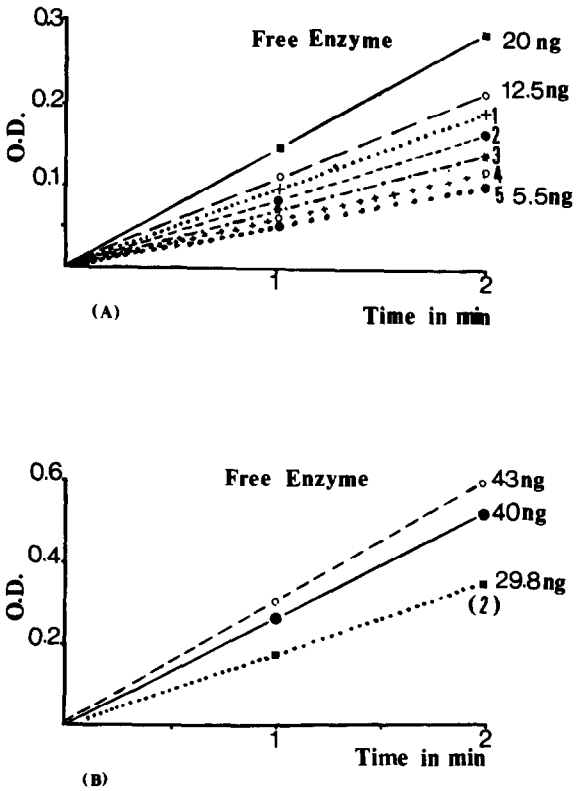

Fig.4. Determination of the amounts of HRP-labelled IgG immobilized on: (A) ossein membranes; (B) pig skin membranes.

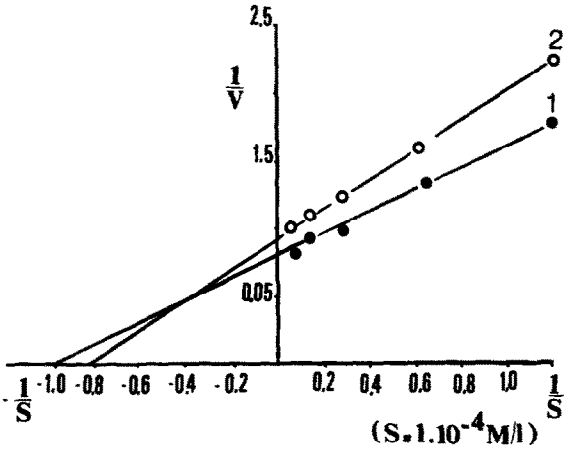

Fig.5. Determination of Michaelis constants (K_m and K_a) of peroxidase free in solution (1) and bound to IgG (2), according to the Lineweaver Burk representation.

3.3. *Approach of a new immunoenzymologic procedure*

After immobilization of anti-HB_sAg antibodies, pig skin collagen membranes were immersed in concentrated solution of purified antigen for 30 min, then washed several times and allowed to react for 30 min with labelled antibodies, according the 'sandwich' procedure. After several experiments, it appears that the immunological properties of the immobilized antibodies were not strongly altered. Nevertheless, the lack of sensitivity of the classical method used for the estimation of HRP activity emphasised the need for another technique for accurate determination of the enzymatic activity of membranes.

Recently we elaborated a more sensitive device, using for determination of the HRP activity, an iodide-selective electrode, modified by fixation of the antibody membrane on the crystal sensor [9,10]. This new tool should easily allow the determination of substances present in trace amounts in biological fluids.

References

[1] Masseyeff, A. and Maoilini, R. (1975) J. Immunol. Meth. 8, 223–234.
[2] Wide, L. and Porath, J. (1966) Biochim. Biophys. Acta 130, 257–265.
[3] Desmet, G. and Boitieux, J. L. (1977) Clin. Chim. Acta 74, 59–69.
[4] Boitieux, J. L. and Desmet, G. (1977) personal communication, IV Forum des Biochimistes, Lyon.
[5] Avrameas, S. and Ternynck, T. (1971) Immunochemistry 8, 1175–1179.
[6] Thomas, D., Bourdillon, C. and Broun, G. (1974) Biochemistry 13, 2995–2999.
[7] Thomas, D. and Caplan, S. R. (1976) in: Membrane separation processes (P. Meares, ed) p. 231, Elsevier, Amsterdam, New York.
[8] Barbotin, J. N. and Thomas, D. (1974) J. Histochem. Cytochem. 22, 1048–1054.
[9] Nagy, G., Von Storp, L. H. and Guilbaut, G. G. (1973) Analyt. Chim. Acta 66, 443–455.
[10] Guilbaut, G. G. and Nagy, G. (1973) Analyt. Lett. 6, 301–312.