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Enzyme-Linked Immunosorbent Assay for A and B Water Soluble Blood Group Substances

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ABSTRACT: The conditions affecting an enzyme-linked immunosorbent assay for salivary blood group substances were investigated. It was found that A, B, and O secretor saliva samples would each bind both anti-A and anti-B typing reagents. The conditions that affected the assay response were optimized for maximum sensitivity and to give the highest resolution possible between the result for an antiserum binding to homologous antigen and the response for heterologous antigen-antibody combinations. Monoclonal antibodies eliminated the heterologous binding indicating that this binding was due to a lack of specificity of the routine typing reagents. A sensitive assay using the monoclonal antibodies to distinguish between samples of A and B secretor saliva is described.

KEYWORDS: pathology and biology, immunoassay, antigen systems, genetic typing

Since their introduction in 1971, enzyme immunoassays [*1,2*] have been used to quantitate a wide variety of antigens and antibodies [*3,4*].

Enzyme immunoassays are based on the same principles as the established techniques of radioimmunoassay [*5*]. The two assays show comparable sensitivities down to about 1 ng/mL [*6-8*]. However, using enzymes as labels, rather than radioisotopes, avoids the hazards and reagent instability of the latter. In addition to high sensitivity, enzyme immunoassays have the advantages of ease of operation and the potential for automation [*9*]. Enzyme immunoassays could therefore be useful in forensic science and have been developed for the species identification of blood and saliva stains [*10*].

Currently, ABO grouping of nonblood body fluids is performed by inhibition of hemagglutination [*11*] and absorption-elution [*12*], but enzyme immunoassays would provide greater sensitivity and objectivity. This paper explores the parameters affecting an enzyme-linked immunosorbent assay (ELISA) for salivary blood group substances.

The ready availability of enzyme-antibody conjugates specific for many antibody classes, both human and animal, led to the selection of a noncompetitive or indirect assay. Homogeneous enzyme immunoassays involve the purification and labelling of the antigen [*6*] and show limited feasibility for the assay of macromolecules [*13*]. The alternative heterogeneous protocol involves the separation of bound and free label using a solid phase, in this case microtitre plates, to which the samples are adsorbed. This also means that the label is separated from any endogeneous enzyme activity or inhibitors present in the sample [*6*].

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Materials

Titertek flat-bottomed sterile polystyrene and flexible activated polyvinylchloride plates are used (Flow Laboratories, Irvine, Scotland).

Buffers

All solutions were prepared from "Analar" grade reagents.

Coating Buffers

The coating buffers were:

(1) pH 6 to 8, potassium dihydrogen phosphate/sodium hydroxide;
(2) pH 8.5 to 9, sodium carbonate/bicarbonate;
(3) pH 10 to 11, sodium bicarbonate/sodium hydroxide; and
(4) pH 11 to 12, disodium hydrogen phosphate/sodium hydroxide.

All were prepared at ionic strengths of 0.03, 0.3, and 3*M* [*14*].

Washing Buffer

The washing buffer was: 0.15*M* phosphate buffered saline (PBS) pH 7.3 + 0.05% v/v Tween 20 (polyoxyethylene sorbitan monolaurate, Sigma Chemical Co.).

Antibody Diluent

The antibody diluents were:

pH 6.5 to 8.5, PBS/sodium hydroxide or
pH 8.5 to 9.6 sodium carbonate/bicarbonate.

Each buffer was made at ionic strengths of 0.04, 0.4, and 2*M* and with 0.5% v/v bovine serum albumin (BSA, Sigma). Detergent was added at 0.05%: v/v Tween 20, w/v Nonidet P40 (BDH Chemicals, Poole, England), or w/v CHAPSO (3-[(3-chlolamidopropyl) dimethyl ammonio] 2-hydroxy-1-propane sulphonate, Calbiochem, San Diego, CA).

Enzyme Substrate

The enzyme substrate was 0.1*M* phosphate citrate buffer pH 4.5 + 0.04% *o*-phenylene-diamine (Sigma) + 0.012% hydrogen peroxide.

Saliva Samples

Saliva samples were collected from A, O, and B secretor individuals, whose red blood cells were typed as Le (a− b+). The samples were boiled for 10 min and centrifuged to remove debris. Each person's saliva was separately pooled and stored at −20°C.

Antisera

The anti-A and anti-B human antisera were routine typing reagents from Immuno AG, Vienna, and Ortho Diagnostic Systems, New Jersey.

Monoclonal anti-A (LM 34/43) and anti-B (LM 30/31.5) were mouse hybridoma culture

supernatants kindly donated by the Glasgow and West of Scotland Blood Transfusion Service, Law Hospital, Lanark.

The antibody-enzyme conjugates used were anti-human IgG-horseradish peroxidase and anti-mouse IgM-horseradish peroxidase (Sigma).

Methods

Published procedures [*8*] were used to set the starting conditions. Each condition, in turn, could then be varied and the response would be governed only by the parameter under investigation.

Tenfold serial dilutions of each saliva from 1 in 10 to 1 in 10^6 were used to determine a working dilution. Each sample of saliva (300 μL) in pH 9.6 carbonate/bicarbonate coating buffer, ionic strength 0.3*M*, was pipetted into the wells of polystyrene micro-ELISA plates. The plates were covered [*15*] and incubated on damp towels [*16*] in plastic boxes for 4 h at 4°C.

The plates were inverted and shaken dry. The wells were filled with PBS + Tween 20 and left for 5 min. This procedure was repeated twice and the plates were shaken dry before adding 250 μL of anti-A or anti-B reagent to each well. The polyclonal antisera were used diluted 1 in 10 in PBS (pH 7.3 and ionic strength 0.4*M*) containing Tween 20 and BSA. When monoclonal antibodies were used they were diluted in an equal volume of the same buffer omitting the BSA. The plates were incubated overnight at 4°C and then washed as before.

Antibody-enzyme conjugate was diluted 1 in 500 with PBS (pH 7.3, ionic strength (I) = 0.4*M*) containing Tween 20 and BSA, then 200 μL was pipetted into each of the required wells. Anti-human IgG-horseradish peroxidase was used to detect the polyclonal antisera and anti-mouse IgM-horseradish peroxidase for the monoclonal antibodies. The plates were incubated for 2 h at 4°C and then the wash procedure was repeated.

Freshly prepared substrate (150 μL) was added to each well, then the plates were incubated in the dark for 30 min at room temperature. The reaction was stopped by adding 50 μL of 2*N* sulfuric acid to each well and the optical density at 490 nm was recorded. The optical densities were measured using a Dynatech MicroELISA Minireader MR590. The zero level was set on substrate solution which had been incubated and stopped in an otherwise empty well.

All possible antigen-antibody combinations were used in the ELISA, such that anti-A, for example, was reacted with B and O samples, giving "heterologous" assays, as well as with A saliva, giving "homologous" assays.

The validity of the assays was ensured by incorporating control tests in all experiments. These controls were to assay with all constituents except sample and also to include assays which had all constituents except the blood group specific antibodies.

All assays were replicated at least three times.

Optimization of ELISA

The conditions affecting sample coating on to micro-ELISA plates, the subsequent reaction with blood group specific antibodies, and the binding of the detecting antibody-enzyme conjugate were investigated. Assays were performed with various incubation times up to 24 h at each stage and temperatures varying from 4 to 38°C for the two antibody binding steps and from 4 to 56°C for sample coating.

The blood group substances were partially purified by ethanol precipitation [*17*] and redissolved in coating buffer for comparison with untreated saliva. Coating buffers of various pH and ionic strengths were used with all the samples.

Manufactured ELISA plates are usually made of polystyrene or activated polyvinylchloride. Plates of both types were compared in this assay.

Some assays included an extra blocking step after the samples had been coated. For this step 1% v/v BSA in either PBS (pH 7.3 and I = 0.4*M*) or pH 9.6 coating buffer (I = 0.3*M*) was incubated for either half an hour or overnight before washing and adding the anti-A and anti-B. The antisera diluents were also varied in pH and ionic strength and three different detergents were assessed. As well as two different sources of typing antisera monoclonal antibodies were used in some assays. These were used at dilutions from 1 to 1 down to 1 in 10 in pH 7.3 PBS (I = 0.4*M*), containing Tween 20, whereas the polyclonal antisera were compared at dilutions from 1 in 10 to 1 in 200.

Antibody purification methods were applied to the polyclonal antisera to judge the effect upon the ELISA responses. This involved ammonium sulphate precipitation [*18*] and ion-exchange chromatography on DEAE-cellulose [*19*]. An alternative was to absorb the blood group specific antibodies with red blood cells of the heterologous specificity and then use the remaining supernatant fraction in ELISA.

The antibody-enzyme conjugates were compared at dilutions from 1 in 500 to 1 in 2000 in PBS (pH 7.3 and ionic strength of 0.4*M*) containing Tween 20 and BSA.

Results and Discussion

Saliva Dilutions

Figure 1 illustrates the optical densities produced in ELISA using polyclonal antisera and a tenfold dilution series of saliva. Clearly, the response increases with concentration up to a maximum at a dilution of 1 in 100. It may be that at higher saliva concentrations so much antigen coats to the plate that it subsequently "leaks off" [*8,20-22*], or the antibody is sterically hindered from binding to an increasing number of antigenic determinants.

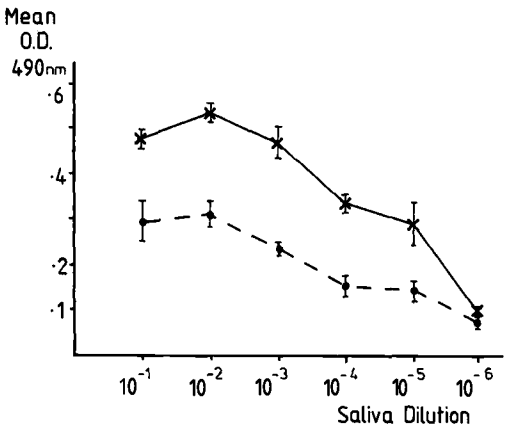

FIG. 1—*Mean of the optical densities at 490 nm produced by four replicates of an assay using polyclonal antisera to detect blood group substances in saliva. A tenfold dilution series of samples of saliva in pH 9.6 buffer were used in the ELISA described in the text. Samples of saliva from A or B secretor individuals gave the same result.*

—×— *Results of ELISA combining samples of saliva with antisera which were specific for the blood group substance which the samples contained.*

- -●- - *Results of assays of salivary blood group substances with heterologous polyclonal antisera.*

I *Indicates one standard deviation either side of the mean optical density.*

In subsequent assays, a dilution of 1 in 1000 was used "so that the range of the assay would not be exceeded by the increase in response resulting from optimization" [*23*].

ELISA Optimization

In the absence of a detergent and 0.5% BSA, the typing antisera and the antibody-enzyme conjugates both adhered to uncoated plates. Using the detergents and BSA in the antibody diluents ensured that the control assays always gave zero optical densities.

The assay was found to be particularly sensitive to the choice of antiserum and to incubation times. The choice of monoclonal antibodies or routine typing reagents had little effect upon the response generated by the homologous reaction if monoclonal antibodies were not diluted beyond 1 in 5. There was a considerable difference in the response generated by the heterologous reactions: with routine typing reagents, the heterologous reaction could generate a response as great as the homologous reaction; with monoclonal antibodies, the response to heterologous reactions was hardly detectable. The effect is shown in Fig. 2 (compare with Fig. 1). The responses were partly dependent upon the dilutions of routine typing reagents used. The homologous reaction was unaffected over the dilution range 1:10 to 1:100 but the heterologous reaction decreased slightly. A dilution of 1:100 was chosen for subsequent tests using these antisera.

The antibody-enzyme conjugates produced a uniform response to a dilution of 1:1000 but it decreased upon further dilution. A working dilution of 1:500 was selected to ensure that an excess was present.

The duration of sample coating, of reaction with type specific antisera, and of binding of antibody-enzyme conjugates had a major influence upon the response. Figure 3 shows the results for various sample coating times. We selected 16 h as the most convenient time, but 8-h coating gave the same results. Using longer times the antigens may have become denatured [*24*] or less able to bind antibody, perhaps because of overcrowding of the antigen molecules.

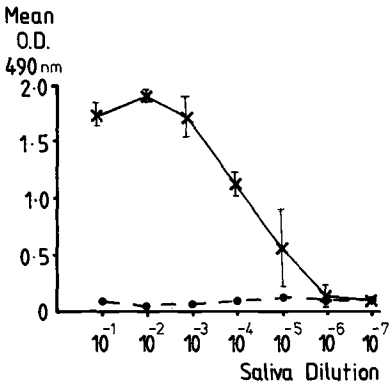

FIG. 2—*Results of ELISA using mouse monoclonal antibodies to detect blood group activity in samples of saliva. A tenfold dilution series of samples in pH 12 buffer were used in the assay under the optimum conditions described in the text. Samples of saliva from A or B secretor individuals gave the same results.*

—✱— *Mean optical densities at 490 nm, and one standard deviation either side of the mean, of four replicate assays with homologous samples of saliva and monoclonal antibody.*

- —•— - *Mean optical densities at 490 nm of four replicates of assays with heterologous saliva and antibody combinations.*

The maximum standard deviation was 0.029.

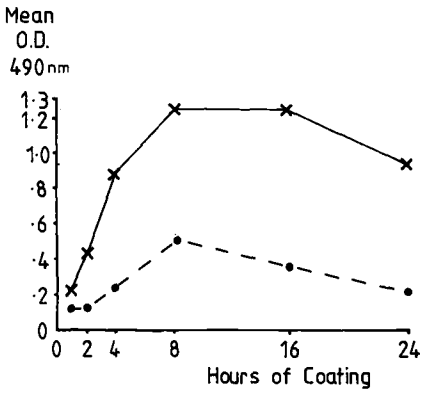

FIG. 3—*Results of ELISA for A and B salivary blood group substances using polyclonal antisera. The method was the same as that used to produce the results shown in Fig. 1 except that all the samples of saliva were diluted 1 in 1000 and different coating times at 4° C were compared. Each point represents the mean optical density at 490 nm of four replicates of each homologous (—×—) or heterologous assay (- —•— -) at each incubation time.*
Maximum standard deviation = 0.028.

The results shown in Fig. 4 indicate that binding of antibody-enzyme conjugate increased with time up to 8 h. At longer times the response to homologous antigen-antibody combinations is reduced perhaps because some antigen "leaks" off the plate carrying bound antibodies with it.

Figure 5 shows the results for different antisera incubation times. The binding of polyclonal antibodies to heterologous samples is high with short incubation times, but the response indicates that it is reduced with time, presumably as the equilibrium between bound and free antibody is established. The binding to homologous samples is favored in incuba-

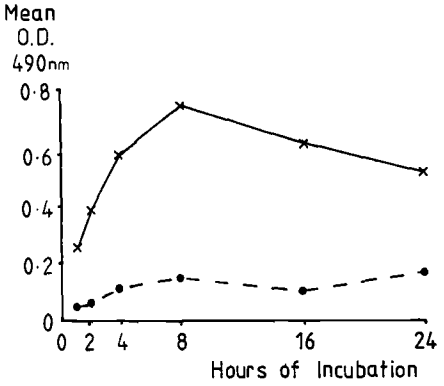

FIG. 4—*Effects on the ELISA response of various incubation times for the anti-human IgG-horseradish peroxidase binding step of an ELISA using polyclonal antisera to detect salivary blood group activity. The samples were diluted 1 in 1000 in pH 12 buffer (I = 0.3M) and coated onto polystyrene micro-ELISA plates overnight at 4° C. The polyclonal antisera were diluted 1 in 100 in PBS (pH 7.3, I = 0.4M), with 0.05% Tween 20 and 0.5% BSA, and incubated in plates at 4° C for 2 h. The antibody-enzyme conjugate was diluted 1 in 500 in the same buffer and incubated at 4° C. Key same as in Fig. 3.*
Maximum standard deviation = 0.073.

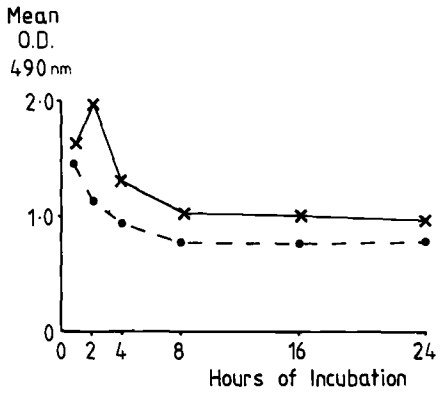

FIG. 5—*Results of ELISA under the optimum conditions described in the text, except using polyclonal antisera, with various incubation times, and anti-human IgG-horseradish peroxidase. Key same as in Fig. 1. Maximum standard deviation = 0.072.*

tions up to 2 h but the response then falls dramatically. Perhaps this is a result of antigen or antigen-antibody complex "leaking" off the plate surface [*20*]. The number of antigenic sites available for binding of antibody would then be increased and lead to new equilibria between free antibody and antibody bound to free antigen and antibody bound to antigen that is adsorbed to the plate surface. Low responses would result if the equilibria favored free antigen-antibody complexes.

For buffers above 0.3*M* ionic strength, the ELISA response to homologous assay combinations was four times higher than the corresponding response with lower ionic strength buffers. The heterologous reaction response rose twofold at 0.3*M* compared to the response with 0.03*M* buffers at any stage. Any further increase in ionic strength had no significant effect on the ELISA results. If the pH of the antisera diluents was outside the range pH 7 to 8, the assay responses were lowered. Inclusion of Tween 20 and BSA in these antibody buffers resulted in lower ELISA responses to heterologous reactions. Nonidet P40 gave lower responses to both reactions but did prevent antibody binding to the plate in the absence of samples. CHAPSO gave assay responses to both reactions which were as high as the homologous assay response in the presence of Tween 20. Antibody diluents were afterwards used at pH 7.3, ionic strength 0.4*M*, and with 0.05% Tween 20 and 0.5% BSA. The extra blocking step using 1% BSA solutions had no significant effect on the ELISA responses compared to those of untreated controls if all the plates were washed the same number of times.

Varying the incubation temperatures gave slightly different ELISA responses to otherwise identical assays. Higher coating temperatures had no significant effect on the homologous assays but increased the response to heterologous antigen-antibody combinations. Higher temperatures for the antisera and antibody-enzyme conjugates' incubations did not affect the heterologous reaction responses but resulted in a slight lowering of the homologous responses. A temperature of 4°C was selected as the optimum temperature for all the incubations in subsequent assays.

The ELISA response in all assays was increased slightly with higher pH of sample coating buffer. Samples were thereafter coated at pH 12. Similarly polystyrene plates gave slightly higher optical densities than when activated polyvinylchloride plates were used to immobilize the antigen.

The purification of blood group substances and of the polyclonal antisera had no significant effect on the assay responses when the purified fractions were compared to unpurified samples and antisera in the assay.

Conclusion

We have described a sensitive assay for salivary blood group substances. The assay gave a significant optical density for samples of saliva which had been diluted up to 10^5 times (see Fig. 1). If the concentration of blood group substance in secretor saliva is taken to be 100 mg/mL [*25,26*], then this ELISA detected these substances at about the nanogram per millilitre level. Maximum sensitivity was achieved by coating the samples at pH 12 ($I = 0.3M$) onto polystyrene plates for 16 h at 4°C. After washing, monoclonal anti-A or anti-B antibodies, which had been diluted in an equal volume of PBS (pH 7.3 and $I = 0.4M$) with Tween 20, were added. The plates were incubated for 2 h at 4°C and then washed again. Anti-mouse IgM-horseradish peroxidase was diluted 1 in 500 in PBS (pH 7.3, $I = 0.4M$) with Tween 20 and BSA, and added to the wells. The plates were incubated at 4°C for 4 h and then washed again. Substrate solution was added to each well and the plates were incubated at room temperature for 30 min before the reaction was stopped and the optical density was recorded.

The sensitivity revealed a lack of specificity in the polyclonal antisera routinely used for ABO typing of saliva samples by absorption-elution and hemagglutination-inhibition. Davies et al [*27*] state that the absorption-elution technique, when used to type seminal stains, is sensitive enough to detect "the fraction of antiserum capable of reacting with "acquired" B." Similarly ELISA detected the fractions of anti-A and anti-B antisera that were capable of reaction with substances in nonhomologous saliva samples, thereby giving results which indicated "aberrant" A and B activity.

The monoclonal antibodies showed complete specificity for the A or B blood group substances. This degree of specificity would be necessary to determine the ABO groups of unknown samples of nonblood body fluids by ELISA. Anti-H monoclonal antibodies are becoming available and are needed in ELISA for testing unknown samples. This paper has demonstrated some of the controlling features of an ELISA assay for A and B blood group substances and that ELISA can provide a very sensitive assay.

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