ENZYME-LINKED IMMUNOSORBENT ASSAY FOR RED CELL ANTIBODY QUANTITATION

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Signed ..... *Director of Studies*

Date 6 October 1999

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ABSTRACT

The aim of the project was to develop a solid phase enzyme-linked immunosorbent assay (ELISA) for the quantitation of red cell antibodies of any specificity. Binding of negatively charged reagent red blood cells (RBC) was achieved in microplate pre-coated with poly-L lysine (PLL). The optimum concentrations of PLL and RBC were between 10 and 20 mg/ml and 0.5% volume for volume, respectively.

Experiments using serial dilutions of monoclonal anti-A, anti-B and anti-D incubated in microplate wells coated with the corresponding antigen positive or negative red cells showed that the RBC remain bound throughout the test and the assay can be shown to be specific. The absorbance values obtained were proportional to the concentration of antibody.

When human polyclonal antibodies (anti-A, anti-B and anti-D) and AB serum (which contained no RBC antibodies detectable by conventional techniques) were used, high background reactions were obtained and the discrimination between positive and negative reactions was very poor. Experiments have shown that a significant proportion of the background reactions in the solid phase tests were due to non-specific binding of antibodies in the test serum to PLL and to the plastic of the microplate wells.

Attempts were made to reduce non-specific binding with varying concentrations of $alpha_1$ acid glycoprotein, bovine serum albumin, bovine and goat casein, foetal calf serum (FCS), rabbit serum and Tween 20. Of these, 20% FCS gave the best reduction in background noise but the assay was still not discriminating enough for routine testing. Neither the replacement of PLL with lectins nor manipulation of the pH for blocking and incubation reactions did not reduce the non-specific reactions significantly.

In conclusion, the principle of solid phase ELISA can be shown to work for monoclonal antibodies to red cell antigens. With polyclonal antibodies there were high non-specific background reactions which could not be reduced enough to allow a useable dose response relationship for antibody quantitation. Hence, the study of the correlation of

the concentration of red cell antibodies by this technique with haemolytic disease of the newborn (HDN) could not be performed.

INTRODUCTION

HISTORICAL BACKGROUND

The ideal characteristics of an immunoassay are that it should be technically robust, reproducible, accurate, sensitive, specific, quantitative and easy to perform. The reagents should be inexpensive, safe and stable, so giving a long shelf life. Also the immunoassay should give objective results that can be determined preferably by the use of simple equipment.

The direct agglutination (saline), enzyme, albumin, polyethylene glycol (PEG), manual polybrene and anti-human globulin techniques used in red cell (RBC) serology rely on haemagglutination as an indication of antigen-antibody reactions. These conventional serological techniques (reviewed by Knight & Poole, 1995), which are usually performed in glass or plastic tubes, provide a convenient and generally reliable means of detecting and identifying RBC antibodies and antigens. However, the subjective readings limit their use in providing accurate quantitative information. Batch testing in tubes can be cumbersome. Also, it was suggested that these techniques may lack the sensitivity to detect small quantities of RBC bound, clinically significant antibodies (Dupuy et al, 1964, Romano et al, 1973, Masouredis et al, 1977). Batch testing was made easier by the use of microplates and automatic samplers although washing of the RBC prior to the addition of anti-human globulin (AHG) for the antiglobulin test, can be tedious. The attachment of RBC to microplate wells has simplified the technique by making the washing between incubations less labour intensive. However, this method still relies on agglutination to provide an indication of reaction. The problems of lack of sensitivity and subjective recording of results were overcome in the liquid phase enzyme-linked antiglobulin tests (ELAT) (Brunner & Kissling, 1979, Leikola & Perkins, 1980a). In these tests, the sensitisation phase was performed in a liquid phase and the results were indicated by the use of enzyme conjugated anti-human globulin. The addition of a suitable substrate produced a coloured end product, the intensity of which was proportional to the number of RBC bound antibody molecules.

The conventional tube techniques are increasingly being replaced in Europe by column technologies. Cards of six microtubes containing a Sephadex gel (Lapierre et al, 1990)

or glass bead microparticles (Reis et al, 1993) suspended in a buffer are being used for blood grouping, phenotyping and RBC antibody screening and identification. The buffer may contain specific antibodies or anti-human globulin (AHG). These test cards are commercially available from Diamed-GB Limited (Dalkeith, Midlothian, Scotland) and Ortho-Clinical Diagnostics (Amersham, Buckinghamshire, England).

An affinity column technology consisting of a strip of 6 or 8 microcolumns containing protein A and G chemically bound to agarose (ReACT Microcolumn test system, developed by Gamma Biologicals, USA), or immobilised protein G, anti-IgM and anti-C3d (Biotest AG, Dreieich, Germany) are currently being evaluated. "Scangel" (Sanofi Diagnostics Pasteur, Marne La Coquette, France under licence from Diamed) which also uses gel filtration technology, is also being evaluated.

Some of these column technologies have found a place in general serological use because they make reading of the test results easier by trapping the reactions within the matrix and removes the need for post incubation washing required for conventional antiglobulin test. However, they are unsuitable for RBC antibody quantitation.

Solid phase techniques for the isolation of antibodies were reported by Campbell et al (1951) who chemically coupled antigens onto diazotised aminocellulose and polyaminostyrene. These immunoadsorbents were used to specifically adsorb antibodies with the subsequent elution of purified antibodies at acid pH. Other workers have since developed assays using cellulose derivatives and synthetic polymers as a supporting matrix of antigens (Avrameus & Ternyck, 1966).

An important part of any solid phase immunoassay is the selection of an indicator system or label. Among the indicator systems available are:

(1) Radioisotopes, can be used as labels in immunoassays for the detection of either antibodies or antigens (Yallow & Berson, 1959; Yallow & Berson, 1960). Radioimmunoassays (RIA) combine sensitivity and specificity and allow the assay of many compounds of biological importance, which often occur in low concentrations in body fluids, e.g. hormones. The use of radiolabels is however becoming less common because of the limited shelf life and the expertise and equipment required for measuring.

(2) Electron dense compounds such as ferritin coupled to antigens or antibodies are used in electron microscopy (Singer & Selick, 1961).

(3) Protein bacteriophage conjugates were used in the detection of antigens or antibodies in solution (Hamovitch & Sela, 1969).

(4) Stable free radicals, which behave differently when coupled to small molecules. This difference in behaviour was measured in spin-immunoassays (Lente et al, 1972).

(5) Fluorescent dyes (Aarlberse, 1973, Cukor et al, 1976) were coupled to antigens or antibodies and used as labels in immunochemical assays.

(6) Enzymes were first used as labels in the enzyme linked antiglobulin conjugates for the localisation of immune reactants in histological preparations (Nakane & Pierce, 1966). Van Weeman & Schuurs (1971) and Engvall & Perlman (1971) pioneered the Enzyme Linked Immunosorbent Assay (ELISA). They combined the use of immobilised antigen or antibody onto solid phase with enzyme labelled antibody or antigen conjugates.

Enzyme immunoassays employ antibodies or antigens conjugated to enzymes in such a way that the enzymatic and immunological activity of each moiety is maintained. Reagents for ELISA are cheaper than those for RIA, have a long shelf life and the technique lends itself to automation.

RIAs are very sensitive and allow precise quantitation. ELISAs are also extremely sensitive and assays of increasing precision have been developed. Radioisotopes, which are used in biological assays, decay rapidly resulting in the reagent having a short shelf life. Because radioactivity constitutes a medical hazard and complex equipment is required for its measurement, highly trained personnel must perform these assays.

In principle, ELISA satisfies most if not all the ideal characteristics of immunoassay. Because of this, the present study aimed to investigate the possibility of employing a solid phase enzyme-linked immunoassay with a view to overcome the deficiencies of conventional serological tests. Conventional techniques do not provide sufficient convenience and precision to quantitate antibodies sufficiently to help with the in vivo prognosis of haemolytic disease of the newborn (HDN). It was intended that the

proposed ELISA would be used to quantitate red cell antibodies and the results would be compared with the disease severity.

PRINCIPLE OF THE ELISA TECHNIQUE

Use of a solid phase permits the separation of immunologically reacted from unreacted material after completion of tests. Cellulose, polyacrylamide, cross-linked dextrans, silicone rubber, microcrystalline glass and plastics have been used as solid phases. These materials can be used in the form of beads, tubes, discs and microplates. The material must be carefully chosen as some tend to have high non-specific binding of the test materials resulting in high background readings (Voller et al, 1979).

Indirect ELISA can be broken down into five steps.

1. Antigens or antibodies are adsorbed onto the solid phase through hydrophobic interactions. Variability of adsorption can be reduced by pre-treatment of the solid support with glutaraldehyde (Hurd, 1984). Glutaraldehyde has also been shown to reduce the amount to desorption from the solid phase. This can be further reduced by treating the antigens or antibodies attached to the solid phase with ethyl chloroformate (Place & Schroeder, 1982).

   Adsorption of proteins onto a solid phase is affected by the initial concentration, pH, time and temperature of incubation and the type of solid support. Polysaccharide antigens adsorb less well to polystyrene plates and pre-treatment with poly-L lysine (Kelsoe & Weller, 1978) is often necessary to achieve binding. Excess antigen or antibody must be washed off before proceeding to the next step.

2. Incubation of the test sample with material bound to the solid phase: the optimum concentration, time and temperature of incubation must be determined. Any unbound test material must be washed away before proceeding to the next step.

3. Incubation with the conjugate: horseradish peroxidase, alkaline phosphatase and glucose oxidase are the enzymes most frequently used in conjugates. The optimum dilution of the conjugate, time and temperature of incubation are important. Excess conjugate must be washed away before proceeding to the next step.

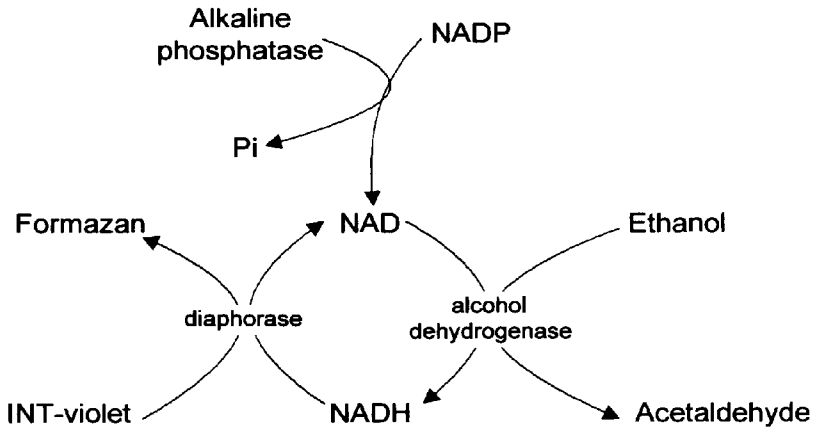
4. Addition and incubation with the substrate: reaction of any bound enzyme conjugate with the appropriate substrate results in a coloured product which can be measured using a spectrophotometer.

5. The endpoint: this is dependent on the solvent for the substrate, concentration, pH, time and temperature of incubation. The reaction can be stopped at a predetermined time or absorbance of a control. Alternatively, kinetic studies can be performed with the optical densities measured at set intervals.

The sensitivity can be improved by causing the conjugated enzyme to produce a catalytic activator for a secondary system (Self, 1985; Gatley, 1986). In this enzyme amplification system (figure 1), alkaline phosphatase dephosphorylates nicotinamide adenine dinucleotide phosphate (NADP) to produce nicotinamide adenine dinucleotide (NAD) in proportion to the amount of bound enzyme. NAD is then reduced to NADH by alcohol dehydrogenase. NADH is reoxidised to NAD by diaphorase with the simultaneous reduction of a tetrazolium salt to a measurable intensely coloured formazan dye with each turn of the cycle.

The sensitivity can also be increased by using the biotin-avidin system (Jackson et al, 1982, Gerrard, 1985). Because of its small size, several molecules of biotin can be conjugated to antihuman globulin or other protein. Avidin, which has four binding sites and a high affinity for biotin, can be conjugated to an enzyme such as alkaline phosphatase. This system offers the advantage of a lower background noise and a high signal amplitude due to amplification.

Figure 1: Enzyme amplification system



APPLICATION OF LIQUID PHASE ELISA TO RED CELL SEROLOGY

In these assays the red cells were sensitised in one test tube (or microplate well), washed and then transferred to another tube (or microplate well) for conjugate and substrate reactions in order to reduce background reactions. Greenwalt et al (1975) developed a liquid phase enzyme-linked antiglobulin assay to quantitate the number of antibody molecules on sensitised red blood cells. Horseradish peroxidase labelled antihuman globulin conjugate and 3,3' diaminobenzene substrate were used to produce a stable precipitate which was dissolved in 1,1,1,3,3,3-hexafluoroisopropanol resulting in a quantitative colorimetric endpoint. Preliminary studies showed that the number of molecules of anti-D bound to Rh D positive red cells were within the range reported using radioiodinated antibodies (Masouredis, 1960, Rochna & Hughes-Jones, 1965). The minimum number of molecules of anti-D detected was 350 per red cell. Greenwalt et al (1975) suggested that the background reaction due to the conversion of the substrate by haemoglobin peroxidase can be reduced by starting the assay with intact red cells then lysing and washing them prior to the addition of the substrate. This would remove the haemoglobin peroxidase and prevent the trapping of the conjugate in the ghost cells, if these were used at the start of the experiment.

The sensitisation of erythrocytes possessing the corresponding antigens by anti-Fy^a, anti-D, anti-K, anti-B and anti-e was visualised using alkaline phosphatase conjugated anti-human globulin (Brunner & Kissling, 1979). This was also a liquid phase assay in which intact red cells were used. Haemolysis, which occurred on addition of p-nitrophenyl phosphate (p-NPP), was reduced when this substrate was prepared in a carbonate-bicarbonate buffer. Also, a second problem of the conjugate binding non-specifically to red cells and the glassware was considerably reduced by addition of 1% bovine serum albumin to the wash solution and by transferring the washed enzyme-coated cells to a separate substrate reaction test tube. The authors concluded that the sensitivity of this test was comparable but did not exceed that of the standard antiglobulin test. It could not detect anti-Fy^a with less than a 2+ reaction.

Leikola & Perkins (1980a) improved the enzyme linked antiglobulin test (ELAT) and showed that the absorbance was linearly proportional to the concentration of the test antibodies incubated with the cells. Their assay was shown to be more sensitive than the manual antiglobulin test. The uptake of anti-D, anti-Fy^a and anti-Jk^a on to red cells with the corresponding antigen was accelerated by the use of low ionic strength solution (LISS), with near maximum reaction occurring within 10 minutes of incubation (Leikola & Perkins, 1980b). However, LISS did not increase the uptake of IgG anti-A or anti-Le^a nor the rate of binding of the conjugated antiglobulin to IgG sensitised cells. These researchers also showed that increased amount of anti-D was bound to papain pre-treated red cells, in a manner independent of the use of LISS.

Osborne & Giblett (1980) used 4 methylumbelliferylphosphate as a substrate for alkaline phosphatase and measured the fluorescence released in the enzyme-linked immunosorbent assay. This liquid phase assay was sensitive, reproducible, quantitative and suitable for automation.

Reppun et al (1983) were the first to perform the ELAT in microtitre plates. The alkaline phosphatase/substrate reaction was stopped with EDTA instead of NaOH. The sensitivity of the enzyme-linked assay was slightly better than conventional antiglobulin tests but occasional antibodies failed to show any specificity by this assay.

Gilman et al (1982), Bessos & Yule (1983) and Lown et al (1984) also confirmed the increased sensitivity of the ELAT over the standard antiglobulin test. Lown et al (1984) also showed that there was a wide variation between alkaline phosphatase conjugated AHG from different manufacturers.

Several workers examined the use of glutaraldehyde as a means of improving the ELAT. Both Gilman et al (1982) and Lown et al (1984) showed that the use of glutaraldehyde fixed red cells significantly reduced the sensitivity of the assay. Greenwalt et al (1992a, b) were able to stabilise the antibody-antigen bond and eliminate the problem of haemolysis without affecting the sensitivity of the test by using 0.05% glutaraldehyde to fix sensitised cells. Makler et al (1981) used glutaraldehyde-fixed red cells in an ELISA assay to quantitate anti-D. By using an insoluble substrate formed by mixing 2-naphthol phosphate and fast blue RR salt, the resulting enzyme-linked antigen distribution assay (ELADA) in combination with electron microscopy was used to study the distribution of antigens on the cell membrane.

The variables affecting the ELAT test were studied by Leikola and Perkins (1980a) and Postaway et al (1985). Postaway et al (1985) found that by incubating the conjugate and substrate at 37°C, the incubation times could be reduced to 60 and 30 minutes, respectively. The assay was reproducible with a coefficient of variation (CV) of approximately 7%. Anti-D could be detected at a level of 4 ng/ml compared with 10 ng/ml needed to give a 1+ reaction for the indirect antiglobulin test.

Kumpel (1990) solubilised sensitised RBC and incubated dilutions of the lysate in anti-IgG and anti-IgM coated microplates in the SOL-ELISA. The results of this quantitative assay correlated well with that of a radiometric assay for IgG1 antibodies but higher sensitisation levels were obtained for IgG3. The assay is reproducible with intra-assay and inter-assay coefficients of variation of 6.6% and 8.1% respectively.

DISADVANTAGES OF ELAT

One of the main disadvantages of the liquid phase ELAT is the number of stages which involve washing using centrifugation. This makes it an extremely time consuming for routine application. Also, for antibody quantitation the amount of sensitised cells used must be kept constant, i.e. there should be no loss during the washing procedure, which could easily occur during the aspiration of the supernatant.

Haemolysis, which often occurs on the addition of the substrate, is undesirable as the release of intracellular enzymes, such as alkaline phosphatase and haemoglobin peroxidase caused substrate conversion. This resulted in false positive reaction and a decrease in the sensitivity of the assay (Brunner & Kissling, 1979). The false positive substrate conversion can be measured by including a haemolysis control in each assay (Postaway et al, 1985) or minimised using carbonate-bicarbonate buffer, pH 10 to dilute the substrate (Brunner & Kissling, 1979). Rigal et al (1984) used glucose oxidase conjugated to anti-IgG in an ELAT which they found to be 10 times more sensitive than the conventional indirect antiglobulin test. The absorbance of the base line using glucose oxidase was lower than alkaline phosphatase and horseradish peroxidase. As glucose oxidase is not an intrinsic red cell enzyme, non-specific reaction with the substrate was avoided. The assay detected anti-D at a level of 1 ng/ml.

Douglas et al (1987) prepared a solid phase in 3.5 ml capped polystyrene containers. The solid phase was prepared by coating the tube with 5 g l^{-1} of fibrinogen then incubating with 100 µl of 290 mg ml^{-1} of immunoglobulin solution and 50 µl of 1% glutaraldehyde. The AHG test was performed in a separate tube and after washing the contents were resuspended and transferred to the washed immunoglobulin coated tube, centrifuged and any unbound cells decanted. Modified Drabkin's solution was added to the tube and the absorbance measured. There was a good correlation between this test and the conventional AHG test.

SOLID PHASE TESTS IN RED CELL SEROLOGY

Blood group substances were successfully used as immunosorbent in ELISA for red cell antibody detection. The use of a purified synthetic red cell antigen Le^a adsorbed

onto microplate wells (Spitalnik et al, 1983) was the basis of a solid phase ELISA for the detection of anti-Le^a. Kinetic rather than end point determination was used to calculate the amount of specific antibody. This assay was shown to be specific, sensitive and could be used to quantitate anti-Le^a. IgG anti-Le^a was demonstrated in cord blood when the maternal samples contained this antibody. Spitalnik et al (1985) identified anti-Le^a in the cord blood which was not detected using routine haemagglutination techniques. Buchs & Nydegger (1989) adsorbed blood group A and B substances onto microplates and used this to quantitate anti-A and anti-B.

Yared et al (1997) extracted soluble antigenically active Rh D from red cells. The soluble Rh D antigens were stable in zwitterionic buffers for extended periods of time with increased affinity for plastics. The Rh D antigens were successfully bound to plastic beads and microplate wells and could be used for quantitative ELISA and for purifying Rh antibodies.

Immobilisation of erythrocytes onto a solid phase was described for the detection of antibodies against cell-surface antigens (Heusser et al, 1981). This was later adapted for antibody screening and compatibility testing (Plapp et al, 1984, Beck et al, 1984). In the screening assay, test serum was incubated with red cells, which were previously adsorbed onto microplate wells, and the reactions were visualised by the addition of AHG and anti-D sensitised RBC. Effacement of the microplate wells with the sensitised cells was indicative of a positive reaction while a negative reaction resulted in a button of red cells in the centre of the wells. These results can be read visually or using a spectrophotometer. This RBC screening assay was reported to be 2 to 4 times more sensitive than the conventional haemagglutination tests but it could not be used for quantitating red cell antibodies.

Gemke et al (1986) developed a microimmunofluorescence technique for ABO and Rh phenotyping of fetal red cells obtained by chorionic villus biopsies in the first trimester of pregnancy. Red cells were attached to wells of Terasaki plates (which are usually used for lymphocytotoxicity test) via positively charged poly-L-lysine (PLL). The blood groups were determined by incubation with specific antisera. This test was used by Brouwers et al (1987) to determine the subclasses of IgG anti-A and anti-B in group O mothers with a group A or B child. Sallander et al (1995) also used PLL to immobilise

RBC onto microplate wells to screen antenatal samples for atypical antibodies using antiglobulin and enzyme enhanced techniques. The reactions were visualised using anti-D sensitised red cells, suspended in anti-human globulin reagent immediately prior to use, resulting in effacement for a positive reaction.

With the solid phase antiglobulin (SPAG) test (Uthemann and Poschmann, 1990), sera and cells were incubated in the wells of a microplate, centrifuged at 600g for 2 minutes then washed using a specially developed plate washer. The cells were resuspended in 100 µl of modified AHG, transferred to the corresponding wells of an activated microplate and allowed to stand at room temperature for 45 minutes then read visually or with a plate reader. Monolayers are formed for positive reactions and discrete button of cells for negative reactions. A solid phase antiglobulin test (SPAT - a modification of the SPAG) uses IgG coated wells for the complete test. After incubation, the RBC were washed by centrifugation and decanting, anti-IgG was added followed by centrifugation to cross-link the antibody on sensitised cells with the IgG coated wells for positive reactions or a button of cells for negative reactions.

Llopis et al (1996) immobilised RBC suspended in a cell fixation buffer onto microplate wells using centrifugation. This formed the basis of the microplate monolayer test (MMT) used for screening and identifying red cell antibodies. Llopis et al, (1997) modified the MMT to form the microplate coagglutination method (MCM) for solid phase ABO and Rh typing. For the MCM, bromelainised A_1B, R_1R_2 RBC were immobilised on the microplate wells.

The MMT was adapted by Boult et al (1997) to quantitate anti-D using a competitive radioimmunoassay.

Nagamine et al (1996) developed a solid-phase direct hemadherence assay (SPDHA) to identify red cell bound antibody without elution. Commercial panel RBC were immobilised onto microplate wells and centrifuged at low speed with 22% polymerised bovine serum albumin and test RBC. After incubating the microplates were centrifuged and the results read macroscopically. The SPDHA detected antibodies to D, C, c, E, e, Fy^a, Fy^b, K, k, A and B antigens but failed to detect antibodies to the Jk^a, Jk^b, s, S and Diego antigens.

A magnetic-mixed passive haemagglutination test was developed by Ohgama et al (1996) to detect irregular RBC antibodies. Test RBC were immobilised onto wheat germ lectin coated microplate wells. The plates were washed after incubation with test sera. Coloured magnetic fish gelatine beads coated with affinity purified antihuman IgG were added and the plates were placed on a magnet. After 3 minutes there was effacement of the microplate wells for positive reactions and buttons of cells for negative reactions. The authors concluded that the sensitivity of the test system was better than conventional spin tube AHG methods.

For the solid phase methods described, three methods of measuring or visualising the reactions were used:

1. Anti-human globulin and anti-D sensitised RBC (or magnetic gelatine beads precoated with antihuman IgG) resulting in effacement for positive reactions and buttons of cells for negative reactions. These rely on haemagglutination and offer no advantage over the continuous flow analyser,
2. Competitive radioimmunoassay - the use of radioisotopes is on the decrease, and
3. Purified RBC antigens were used instead of whole red cells in the ELISA.

Method 3 is preferable but purified red cell antigens other than group A and B are not readily available.

NON-SPECIFIC REACTIONS

Non-specific adsorption of immunoglobulins in test sera onto the solid phase is the major cause of high background reading. This is due to the high concentration of immunoglobulins which is normally found in human serum. In order to detect 10 ng/ml of antigen-specific IgG in human serum containing approximately 10 mg ml^{-1} of IgG, one must select one IgG molecule out of a million, based on a difference of idiotypic binding (Graves, 1988). Graves (1988) suggested that in designing a solid phase assay, the non-specific binding sites must be minimised and any residual non-specific reaction must be measured and subtracted from the total signal to yield a noise free signal.

Voller et al (1976) in a review of the applications of ELISA to endocrinology, immunopathology, haematology, microbiology and parasitology, and Spitalnik et al (1983) reported that the use of Tween 20 in the wash solution eliminated non-specific binding. Some workers (Leikola & Perkins, 1980a, Postaway et al, 1985, Greenwalt et al, 1992a and b) used bovine serum albumin (BSA) in the wash solution in liquid phase ELAT. The initial sensitisation was carried out in one tube and after washing, the contents were transferred to a clean tube for the antiglobulin reaction. Thus, non-specific binding of test antibodies to the reaction tube did not affect the antiglobulin reaction.

Kenna et al (1985) observed significant non-specific reactions with rabbit IgG when PBS-Tween 20 was used as wash and diluent buffers. This background noise was reduced when the serum was diluted in casein. Further reduction was obtained when casein was also used as the wash buffer. Casein was found to be superior to BSA, gelatin and PBS-Tween 20. The authors also found the inclusion of the antimicrobial agent Thiomersal in the casein wash buffer was essential for maximal blocking. Buchs and Nydegger (1989) used a solution containing casein, Tween 20, sodium azide and sodium chloride to block non-specific reactions in a solid phase assay for the quantitation of anti-A and anti-B.

Graves (1988) used alpha$_1$ acid glycoprotein (AGP) and Tween 20 to block non-specific reactions in a solid phase assay for the detection of antibodies to the prostate-specific antigen p30. AGP is an abundant negatively charged serum glycoprotein and was selected because it resembles typical cell surface sialoglycoproteins (including red cells) in its charge and high carbohydrate and sialic acid content. After coating the p30 antigen and AGP, the microplates were washed and blocked with Tween 20 followed by the successful testing for antibodies to the p30 antigen.

The background can also be increased by non-specific binding of antibodies in the test serum onto the test cells (Wilson et al, 1985). Thus cells negative for the antigen being tested must be included in each assay to measure the non-specific uptake of antibodies and this must be deducted from the test results.

In conclusion, there has not been any published reliable method of reducing the non-specific reactions when whole or lysed red cells were used as solid phase in an ELISA.

HAEMOLYTIC DISEASE OF THE NEWBORN

Haemolytic disease of the newborn (HDN) occurs when the life span of the infant's red cells is shortened by antibodies passively acquired from the mother. This is a condition in which there is great need for more convenient, precise, non-invasive methods to quantitate and/or determine the clinical significance of maternal antibodies, to aid the prediction of the clinical outcome of the disease.

The two main routes of antibody stimulation in a mother are blood transfusion and fetal blood transfer. There is evidence that fetal red cells enter the circulation of pregnant women. The trauma of childbirth sometimes results in feto-maternal haemorrhage. However, immunisation and the production of sufficient antibody to cause HDN in the first child is rare in previously untransfused women. This is probably due to the volume of fetal blood in the maternal circulation being too small to cause sensitisation (Issitt, 1985). Also, the concentration of the antibody produced is often too low in the early stages of such pregnancies (Mollison et al, 1987).

Blood transfusion may result in the production of antibodies or sensitisation if the recipient lacks antigens which are present on the donors red cells. In the first pregnancy, if these mothers are exposed to fetal red cells with antigens to which they are sensitised, then detectable antibodies may be produced as a result of the secondary immune response and the baby may be affected. Antibodies to the Kell, Duffy, Kidd and Rh blood group antigens other than anti-D are more often stimulated by blood transfusion.

Anti-D was by far and still is the major red cell antibody implicated in HDN. In order to reduce the number of Rh D negative mothers becoming sensitised by fetal Rh D positive red cells, anti-D prophylaxis was introduced as a result of the work of Clarke et al (1963) and Freda et al (1964). The principle of the treatment is that Rh D negative mothers are given 500 IU/ml of anti-D gammaglobulin within 72 hours of delivery of Rh D positive babies. 250 IU/ml of anti-D gammaglobulin is given to Rh negative women

when any invasive procedure, such as amniocentesis, fetal blood sampling or intravascular transfusion is carried out. The passively acquired anti-D binds to Rh D positive fetal red cells leaked into the maternal circulation and are removed by the reticuloendothelial system before there is an immune response. This treatment has resulted in the number of perinatal deaths due to anti-D declining by 96% by 1983 (Tovey, 1986). The failures are due to immunisation occurring during the pregnancy or previously due to abortion or miscarriage, massive transplacental haemorrhage, anti-D immunoglobulin not given or given too late after delivery or insufficient anti-D given. In order to reduce or even eliminate sensitisation during pregnancy, the panel at a Consensus Conference on anti-D prophylaxis (Urbaniak et al, 1997) concluded that because all Rh D negative are at risk from hidden bleeds, they should be given anti-D IgG prophylactically. A decision of whether to give 500iu at 28 and 34 weeks or a single larger dose early in the third trimester is yet to be made.

The severity of the disease varies from intrauterine death due to hydrops fetalis to infants requiring no therapy. Treatment after birth varies from top-up transfusion to alleviate anaemia to one or more exchange transfusions.

Recent therapeutic advances have enabled clinicians to gain access to the fetal circulation mostly by ultrasound-guided fetal blood sampling from the umbilical vein (Rodeck & Letsky, 1989). This has allowed fetal blood testing and intravascular transfusion or intravascular exchange transfusion. However, this invasive procedure has the risk of feto-maternal haemorrhage and fetal death.

ANTENATAL ASSESSMENT OF THE SEVERITY OF HDN

Hughes-Jones et al (1971) pointed out that the severity of HDN is dependent on:

(i) the amount of antibody on the infant's cells,
(ii) the rate of transfer of antibody across the placenta,
(iii) the nature of the antigen on the fetal tissues,
(iv) the state of development of bilirubin excretion in the infant,
(v) the capacity of the reticuloendothelial system to destroy sensitised cells, and
(vi) the erythropoietic response.

With HDN due to anti-D, infants of the Rh phenotype R_2r (cDE/cde) are more severely affected than R_1r (CDe/cde) (Murray et al, 1965, Morley et al, 1977).

Assays which are available to aid the assessment of the severity of HDN are:

1. The total amount of bile pigment in amniotic fluid when measured spectroscopically was shown to be related to the severity of HDN. However, there are dangers associated with amniocentesis, such as maternal immunisation, miscarriage and damage to blood vessels which can result in the death of the fetus, so it is important that the procedures are not performed unnecessarily.

2. There is some correlation between the severity of the disease and the titre of the maternal antibody (Mollison, 1987) although using conventional manual techniques this correlation is not a good one (Morley et al, 1977). The use of the Autoanalyser for quantitation of anti-D against a recognised standard has improved the predictive outcome of HDN (Sturgeon & Kaye, 1970, Tovey & Haggas, 1971, Fraser et al, 1972, Morley et al, 1977, Bowell et al, 1982). Morley et al (1977) and Bowell et al (1982) suggested that the maternal anti-D level at which amniocentesis should be considered as 4 iu/ml (1.44 ug/ml). The SOL-ELISA was better at distinguishing babies with severe HDN (Hadley et al, 1991).

3. IgG subclass has been shown to influence the outcome of pregnancy. IgG1 crosses the placenta earliest and even exceeds maternal levels whereas levels of IgG3 rose slowly and may never reach adult levels (Schur et al, 1973, Schanfield, 1980). Pollock and Bowman (1990) found that IgG1, IgG3 and IgG1 plus IgG3 were present in 33%, 3% and 64 %, respectively of 98 sera tested. Very severe cases of HDN are associated with the presence of IgG1 plus IgG3, occasionally with IgG1 and rarely with IgG3 alone (Pollock & Bowman, 1990, Zupanska et al, 1989).

4. Cellular based assays such as the antibody dependent cell mediated cytotoxicity (ADCC), monocyte monolayer assay (MMA) or the monocyte phagocytic assay (MPA) and the chemiluminescence test (CLT) have been

used to assess the severity of HDN, with varying degrees of success. In an attempt to determine which bioassay gave a better correlation with the severity of HDN, nine laboratories collaborated in the testing of 14 sera from 11 mothers of infants with Rh HDN, 2 monoclonal anti-D and 3 samples of hyperimmune anti-D (Mollison et al, 1991). The correct results for the assays used were as follows: Monocyte based ADCC - 60%, lymphocyte based ADCC - 57%, CLT - 50%, MMA/MPA - 41% and U937 cells and cultured macrophages - 32%. The study showed no correlation between the IgG subclasses and the severity of HDN. It was difficult to draw any firm conclusion from this study because of the small number of sera tested and the technical variation between the assays used in laboratories.

AIMS OF THIS STUDY

The aim of the project is to improve on current techniques for measuring anti-D concentration. This is usually accomplished by titration using the antiglobulin test or the more reliable autoanalyser. The methods employed for enhancement of agglutination on the autoanalyser are the bromelain methylcellulose used in the United Kingdom or polybrene.

The autoanalyser methods rely on agglutination to measure the concentration of anti-D in test samples against a British Standard and as such results do not accurately reflect the number of anti-D molecules. Hadley et al (1991) showed the SOL-ELISA to be a better predictor of HDN. The method involves sensitisation of RBC, followed by solubilisation of the sensitised cells with Triton and measurement of the released antibody bound to the blood group polypeptide. The amount of released antibody will depend on the solubilisation phase and may not accurately reflect the total bound to the RBC. This method has been used as a research tool and has not found routine application in the United Kingdom.

A solid phase ELISA which is technically simple to perform would perhaps offer precision and convenience and would more accurately measure the concentration of blood group specific antibody.

The aim of this study was to develop a solid phase ELISA to quantitate red cell antibodies. In particular, it was intended to examine the following variables:

1. The use of LISS suspended and enzyme modified cells

2. The effect of varying the various incubation phases

3. The effect of varying the incubation temperatures

4. The effect of using different Rh phenotype or pooled cells on the quantitation results

5. The correlation of the results obtained by ELISA with those obtained using single channel autoanalyser

6. The predictive value of red cell specific IgG levels determined by ELISA technique on the severity of HDN

7. The correlation of the concentration of blood group specific IgG1 and IgG3 with the severity of HDN.

MATERIALS AND METHOD

MATERIALS

Microplates: Titertek activated PVC flat bottom (ICN Flow, High Wycombe, Buckinghampshire)
U well microplates (Alpha Laboratories, Eastleigh, Hampshire)
U well microplates (Bibby Sterilin Ltd, Stone, Staffordshire)
Nunc flat bottom microplates (Life Technologies, Paisley, Scotland)
Immulon flat bottom microplates (Dynatech Laboratories, Billinghurst, West Sussex)

Tween 20 (Bayer Diagnostics, Basingstoke, Hampshire)

Phosphate Buffered Saline and Low Ionic Strength Saline (Inverclyde Biologicals, Bellshill, Scotland)

Bovine serum albumin (BSA) (Organon Teknika, Milton Road, Cambridge)

Foetal calf serum (FCS - Imperial Laboratories)

Rabbit serum (J R Scientific)

Conjugate: Anti-human IgG or IgM linked to horseradish peroxidase - diluted 1 in 500 in PBS for use (Sigma Chemical Company Limited, Poole, Dorset)
Anti-human IgG linked to alkaline phosphatase - diluted 1 in 500 for use (Sigma Chemical Company Limited, Poole, Dorset)

Substrate: For horseradish peroxidase - ortho phenylene diamine (OPD - Sigma Chemical Company Limited, Poole, Dorset) dissolved in citric acid/phosphate buffer containing 0.012% hydrogen peroxide.
For alkaline phosphatase - p-nitrophenyl phosphate (pNPP - Sigma Chemical Company Limited, Poole, Dorset)

Serum: IgG3 human monoclonal anti-D (International Blood Group Reference Laboratory, Bristol)
Human polyclonal anti-A, anti-B, anti-D and AB serum (Welsh Blood Service)
IgM mouse monoclonal anti-A and anti-B (Biotest UK Ltd, Solihull, West Midlands)

All other reagents were purchased from BDH (Poole, Dorset) or Sigma-Aldrich (Poole, Dorset).

Equipment: Heraeus Megafuge 1.0 (Heraeus Equipment Ltd., Brentwood, Essex)
SLT 400ATC plate reader (Tecan UK Limited, Goring-on-Thames, Reading)

METHODS

Preparation of citric acid/phosphate buffer (pH 5.0)

48.5ml of 0.1M citric acid solution was mixed with 51.5ml of 0.2M disodium hydrogen phosphate solution to obtain a buffer of pH 5.0. 40 µl of hydrogen peroxide was added to 100 ml of the buffer immediately before use.

Preparation of pNPP substrate diluent

21.3 ml of 0.2M sodium carbonate solution was mixed with 38 ml of 0.2M sodium bicarbonate solution, 1 ml of 0.1M magnesium chloride solution and made up to 100 ml with distilled water.

Preparation of casein buffer

97 ml of LISS (or PBS) was poured into a beaker containing a magnetic flea. The beaker was placed on magnetic stirrer and 7.5g of Hammarsten casein and 2.5g of Trizma base were gradually added. When they were dissolved 100 µl of 10% thiomersal and 300 µl of 0.1% phenol red were added followed by 5.5g of Tris-HCl. This stock solution was stored at 4°C when dissolved. The stock solution was diluted to the required concentration prior to use.

Preparation of bromelain

A beaker containing 100 ml of PBS was placed on a magnetic stirrer and stirred using a magnetic flea. 20 g of bromelain powder was gradually poured into the beaker and

mixed for a minimum of 15 minutes after which the solution was centrifuged. The supernatant (containing 20% bromelain) was poured into 5 ml tubes, corked, labelled and frozen at -40°C. Prior to use a tube of 20% bromelain was thawed and diluted to 0.1%.

Preparation of 0.5% Tween 20

5 ml of Tween 20 was diluted in a litre of PBS.

Bromelain pre-treatment of red cells

1 ml of thrice washed red cells were mixed with an equal volume of 0.1% bromelain and incubated at 37°C for 15 minutes. The treated cells were washed three times with PBS then suspended to 0.5%.

ELISA PROCEDURE 1

1. 100 µl of poly-L-lysine (3 µg ml^{-1} in PBS) were added to each well of a microplate and incubated at room temperature for 1 hour. The poly-L-lysine was flicked out and the plates were washed 3 times with PBS containing 0.05% v/v Tween 20 (PBS/Tween). The plates were blotted dry.

2. 100 µl of 0.2% red cells were added to each well or left blank, centrifuged at 32g for 2 minutes in a Heraeus Megafuge 1.0 then washed 3 times with PBS/Tween. The plates were blotted dry and observed for effacement of red cells at the bottom of the microplate.

3. The wells were filled with distilled water and left to stand for 1 minute. The supernatants were flicked out and the plates washed a further 2 times with distilled water.

4. 100 µl of 0.25% v/v glutaraldehyde was added to each well and the plates were incubated for 15 minutes. They were washed twice with PBS/Tween then once with the blocking buffer.

5. 250 µl of blocking buffer was added to each well and incubated for 30 minutes at room temperature. After flicking out the blocking buffer the plates were blotted dry.

6. 100 µl/well of test serum (neat or diluted) or other reagent was added then incubated for 60 minutes at room temperature or 37°C. The samples were flicked out and the plates were washed 4 times with PBS/Tween then blotted dry.

7. 100 µl of conjugate (anti-human IgG conjugated to alkaline phosphatase) was added to each well and incubated for 60 minutes at room temperature. The plates were washed 4 times with 0.9% unbuffered saline then blotted dry.

8. 100 µl of substrate (pNPP) was added to each well then incubated for 60 minutes at room temperature. The reaction was stopped with 50 µl per well of 1M NaOH.

9. The absorbances were read at 405 nm.

ELISA PROCEDURE 2

The following changes were made to Procedure 1:

- Step 1 - PBS/Tween contained 0.1% Tween 20.

- Step 6 - The test serum (neat or diluted in LISS) or other reagent was incubated in the microplate well for 30 minutes at room temperature or 37°C.

- Step 8 - 100 µl of substrate (pNPP for alkaline phosphatase or OPD for horseradish peroxidase) was added to each well. The substrate incubation time for alkaline phosphatase conjugate was 60 minutes at 37°C and the reaction was stopped with 50 µl per well of 0.5M NaOH. The substrate incubation time for horseradish

peroxidase was 15 minutes and the reaction was stopped with 100 µl per well of 2M H_2SO_4.

RESULTS

Development of solid phase

The initial experiments were designed to determine the conditions for the binding of the red cells to the microplate wells of flat bottom Titertek microplates. Attempts to bind the RBC to the solid phase by incubation with 0.2% RBC at room temperature and 4°C overnight were unsuccessful, there being no visible evidence of RBC bound to the wells of the microplates after washing. The use of centrifugation for various speeds from 32g to 196g and times from 1 to 5 minutes to assist the binding of RBC to the solid phase was ineffective.

The use of positively charged poly-L-lysine to bind RBC to the solid phase was investigated next. 100 µl/well of 3 µg ml^{-1} of PLL in PBS was incubated for 1 hour at room temperature then washed with 0.05% Tween PBS. After addition of 100 µl of 0.2% RBC and centrifugation at 32g for 2 minutes followed by washing, effacement of the wells with RBC was observed. Overnight and 1 hour incubation of PLL coated plates with 0.2% RBC also resulted in RBC attachment. There was less variability in the tests when centrifugation was used for the attachment of the red cells.

Testing the solid phase with monoclonal antibodies

Microplate wells with and without PLL were incubated with A_1, A_2 and B RBC then washed with distilled water to lyse the RBC and remove haemoglobin and endogenous alkaline phosphatase (or haemoglobin peroxidase). After fixing with glutaraldehyde, the RBC coated wells were incubated with doubling dilutions in 0.5% bovine serum albumin (BSA) of either mouse monoclonal anti-A or anti-B for 1 hour at room temperature and the ELISA performed as in procedure 1. The results of these experiments (figures 2a and 2b) show that:

(a) the RBC remain bound throughout the assay as indicated by the specific reactions obtained.

(b) the principle of ELISA can be shown to work,

(c) the assay can be shown to be specific as judged by the positive reactions for monoclonal anti-A with A_1 and A_2 RBC and negative reactions with B RBC under the conditions chosen.

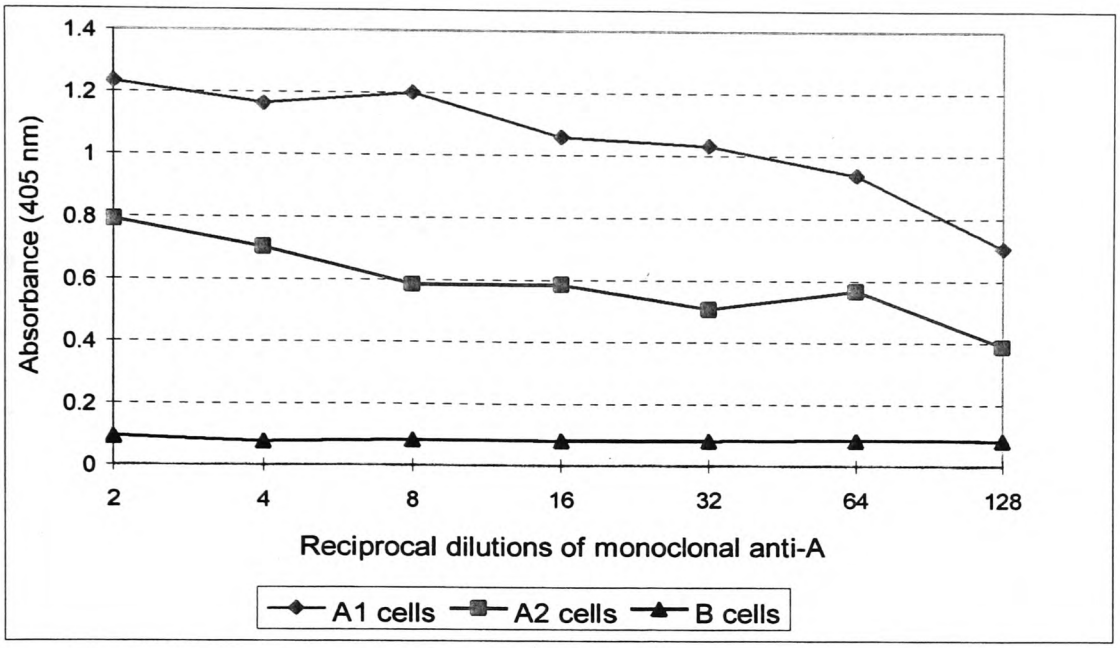
The optical densities obtained in the wells without PLL but with A_1, A_2 and B cells were less than 0.1 for all the dilutions, indicating that the RBC were not or very poorly bound to the wells and that PLL (or some other agent) is required for this.

As the eventual aim of the project was to use the methodology to quantitate anti-D and other red cell antibodies for the study of the HDN, an ELISA for anti-D detection was next evaluated. Rh D positive cells have fewer D sites than groups A_1, A_2 and B RBC and so will present more challenges to the assay. Also the concentration of anti-D by ELISA can be compared to that of the autoanalyser.

Microplate wells coated with 0.2% O R_1R_2 (Rh D positive) or O rr (Rh D negative) RBC via 3 $\mu g\ ml^{-1}$ PLL, were then incubated with serial dilutions of an IgG monoclonal anti-D (30801). The results (see figure 2c) show that the reactions were specific and proportional to the dilution of the antibody. However, the optical densities were lower than those obtained for the monoclonal anti-A and anti-B. This is probably due to there being fewer D sites than A or B sites on the RBC and it is possible that the monoclonal anti-D had a lower concentration of antibody than the monoclonal anti-A and anti-B.

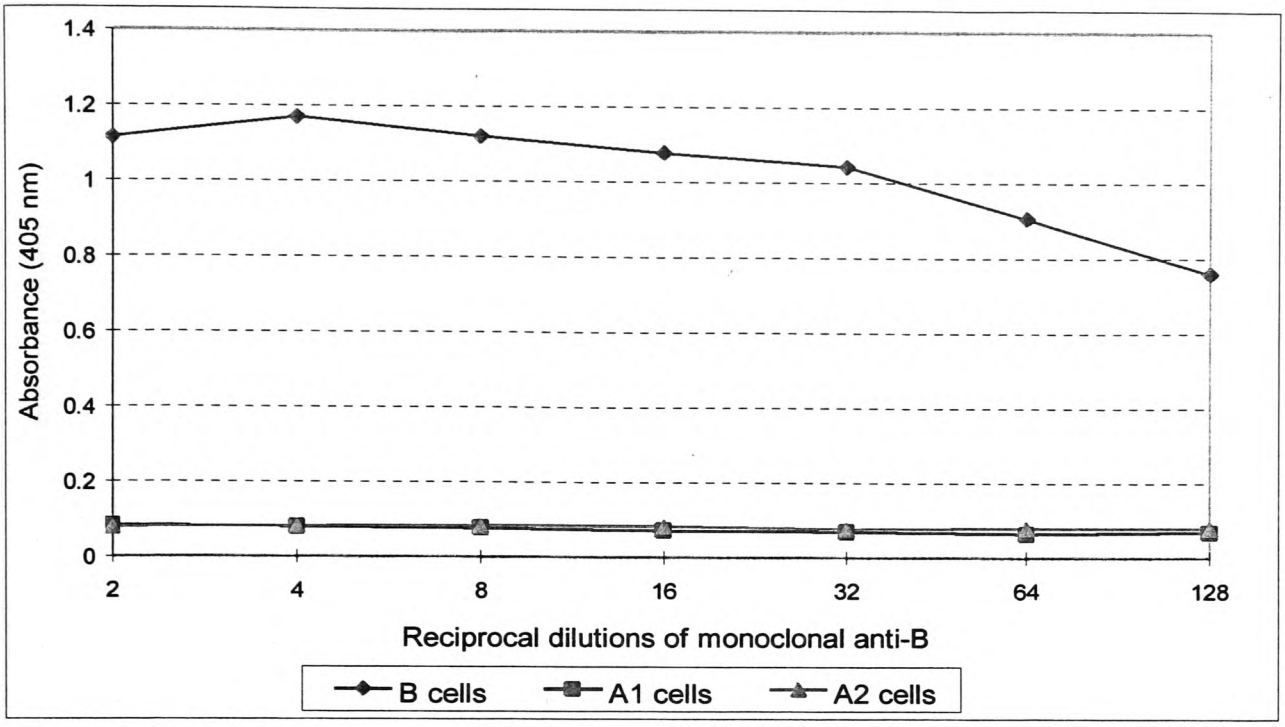
The signal to noise ratios for the monoclonal anti-A, anti-B and anti-D with the appropriate RBC are shown in figure 2d. This ratio (absorbance of test ÷ absorbance of negative control) is a measure of the relative specificity of the assay, i.e., how well the assay distinguishes between specific antigen binding and non-specific binding.

Figure 2a: Effect of testing dilutions of monoclonal anti-A on the specificity of the solid-phase system



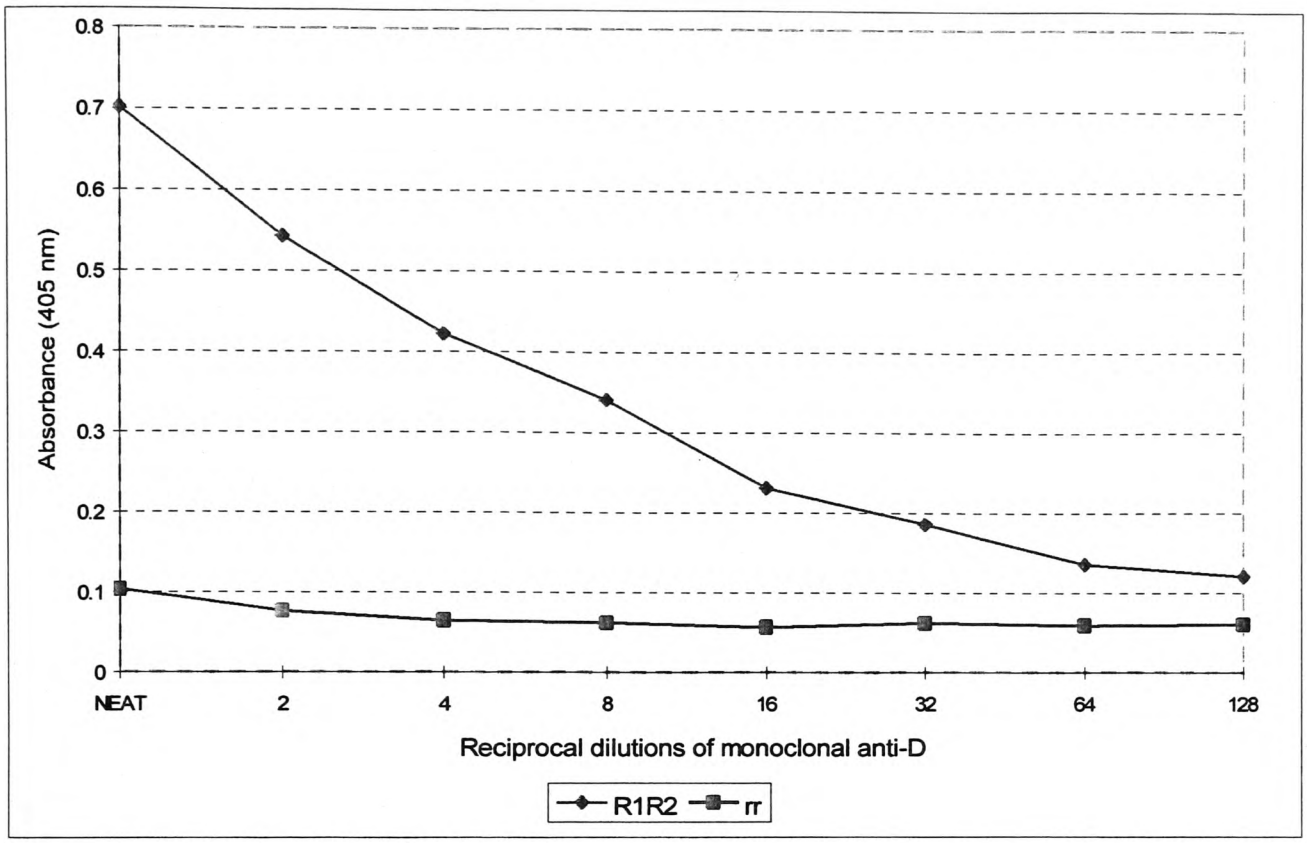
Each microplate well was incubated with 100 ul of 3 ug ml^{-1} of poly-L-lysine (PLL) at room temperature, washed followed by effacement with either A_1, A_2 or B red cells. After lysing and fixing with glutaraldehyde, the microplate wells were incubated with doubling dilutions of mouse monoclonal anti-A. The ELISA test was performed using goat anti-mouse IgM antibody conjugated to alkaline phosphatase with pNPP as substrate. Each dilution was tested in quadruplicate with the mean of the absorbances plotted.

Figure 2b: Effect of testing dilutions of monoclonal anti-B on the specificity of the solid-phase system



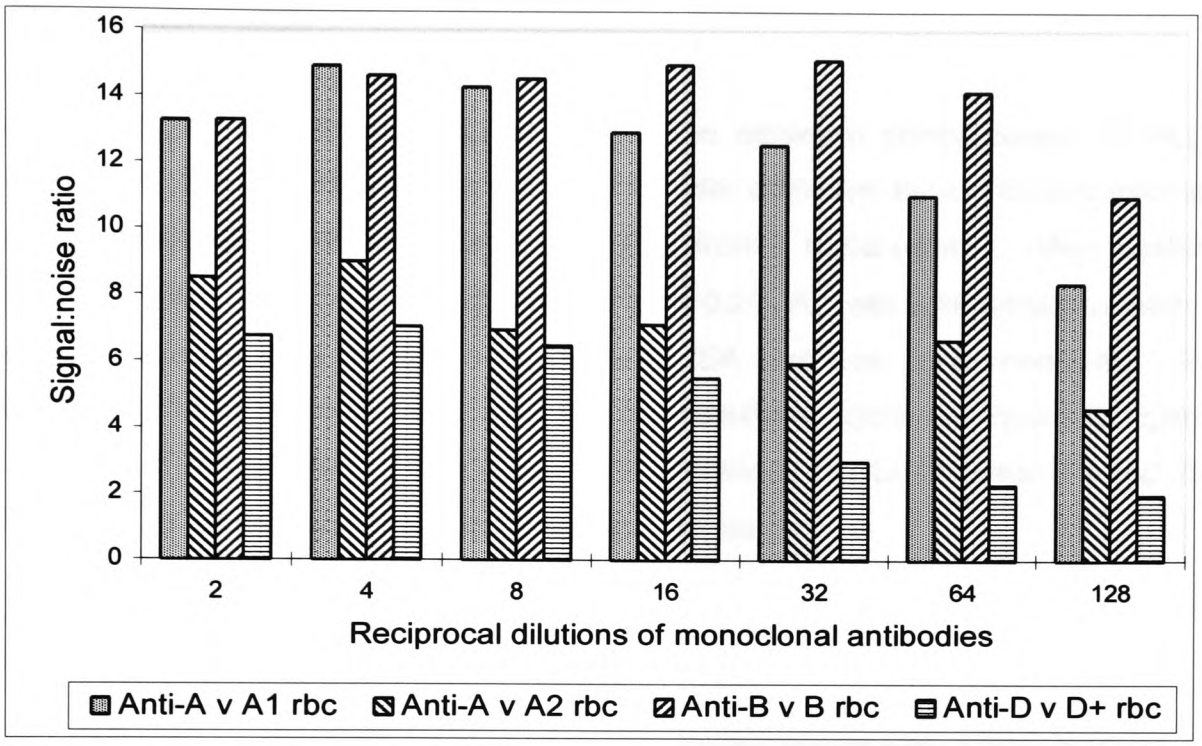
Each microplate well was incubated with 100 ul of 3 ug ml^{-1} of poly-L-lysine (PLL) at room temperature, washed followed by effacement with A_1, A_2 or B red cells. After lysing and fixing with glutaraldehyde, the microplate wells were incubated with doubling dilutions of mouse monoclonal anti-B. The ELISA test was performed using goat anti-mouse IgM antibody conjugated to alkaline phosphatase with pNPP as substrate. Each dilution was tested in quadruplicate with the mean of the absorbances plotted.

Figure 2c: Effect of testing dilutions of monoclonal anti-D on the specificity of the solid-phase system



Microplate wells were coated with 3 ug ml^{-1} of PLL then effaced with 100 µl of either O R_1R_2 (Rh D positive) or O rr (Rh D negative) RBC. These were then tested in quadruplicate with doubling dilutions of human IgG monoclonal anti-D by the ELISA.

Figure 2d: Signal:noise ratio for monoclonal anti-A, anti-B and anti-D



The signal to noise ratio for the monoclonal anti-A, -B and -D was extracted from the data presented in figures 2a to 2c. It is a measure of the relative specificity of the ELISA.

Optimisation of the conditions of the assay

1. PLL concentration

The aim of this experiment is to determine the optimum concentration of PLL for binding of RBC to the microplate wells. This was achieved by incubating microplate well plates with 100 µl of concentrations of PLL from 0 to 50 µg ml^{-1}. After washing 3 times with PBS/Tween and blotted dry, 100 µl of 0.2% A_1 cells was added to each well and centrifuged at 32g for 2 minutes. The ELISA test was performed with 1 in 10 dilution of IgM mouse monoclonal anti-A. The results in figure 3a show that optimum effacement of A_1 cells was obtained with concentrations of PLL between 10 and 20 µg ml^{-1}. 10 µg ml^{-1} of PLL was used for further experiments.

2. Red cell concentration

Microplate wells coated with 10 µg ml^{-1} PLL were incubated with concentrations of A_1 cells from 0.1% to 1.0% and the ELISA performed with 1 in 10 dilution of IgM mouse monoclonal anti-A. The results in figure 3b show that the concentration of red cells that resulted in the highest absorbance in the ELISA test is 0.5%. Chequer board experiments also confirmed 10 µg ml^{-1} PLL and 0.5% RBC as optimum.

3. Effect of time and ionic strength on antibody binding

This experiment was performed to determine the ionic strength (LISS or PBS) and the minimum period of incubation for maximum sensitisation of RBC (coated onto microplate wells) with antibodies in the test serum or dilutions. Human derived polyclonal IgG anti-D was used, as binding was more likely to be affected by ionic strength. 100 µl of 0.5% O R_1R_2 RBC were added to PLL coated wells and centrifuged for 2 minutes at 32g. After washing, 100 µl of 1/2 dilutions of human monoclonal anti-D in either LISS or PBS were added to each well and incubated for 15 to 60 minutes at 37°C. The conjugate and substrate were incubated for 60 minutes each. The same dilution of anti-D was used in each experiment and it showed (figure 3c) that there is

increased binding of anti-D in LISS which has reached maximum binding at 30 minutes. Maximum binding of anti-D in PBS was achieved in 60 minutes.

4. Effect of time and temperature on conjugate binding

The previous experiment was repeated to determine the optimum temperature and the minimum time of incubation for maximum binding of conjugate to the sensitised RBC but with the dilutions of human derived polyclonal anti-D incubated for 30 minutes at 37°C. The conjugate was incubated for times varying from 15 to 60 minutes at temperatures of 18°C, 37°C and 45°C. The substrate was incubated for 60 minutes at 37°C. The maximum absorbance was obtained after 60 minutes incubation at 37°C although it was not significantly greater than that at 45 minutes (figure 3d). A conjugate incubation time of 60 minutes at 37°C was used for further experiments.

5. Effect of time and temperature on substrate conversion

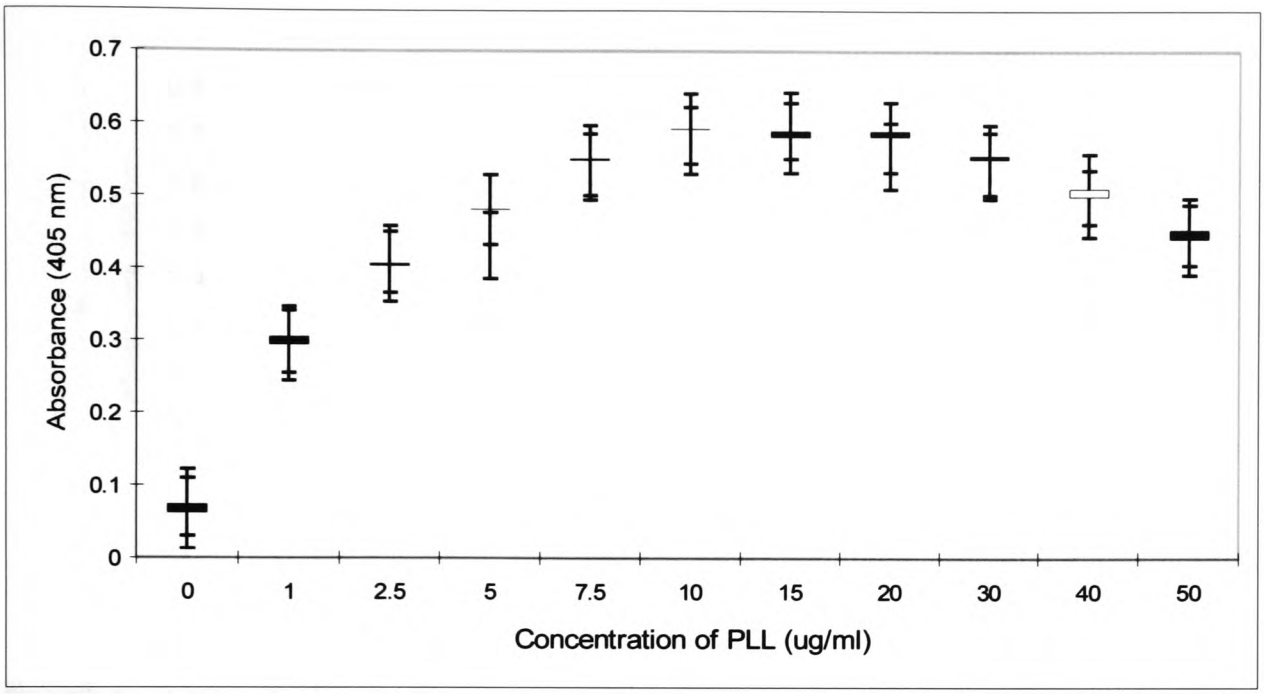
The above experiment was repeated to determine the optimum temperature and time for substrate conversion but with the conjugate incubated for 60 minutes at 37°C. The substrate was incubated for times varying from 15 to 60 minutes at temperatures of 18°C, 37°C and 45°C. Figure 3e shows that maximum absorbance was obtained at 37°C for 60 minutes incubation and these were used in further experiments.

6. Number of washes after serum/cell and conjugate incubations

These experiments were performed to determine the optimum number of washes for removal of unbound immunoglobulin from the test serum to ensure that no neutralisation of the conjugate occurred and hence result in a falsely lower absorbance. The optimum number of washes for the removal of unbound conjugate was determined. Failure to effectively remove the unbound conjugate would result in falsely higher readings. Wells of microplates coated with O R_1R_2 RBC were also used for these experiments. These were tested with human derived polyclonal anti-D because there is a higher concentration of immunoglobulin to test the washing efficiency of the procedure. Any remnant immunoglobulin after optimum washing must either be specifically bound to the D antigen on the RBC (i.e. anti-D) or non-specifically bound to

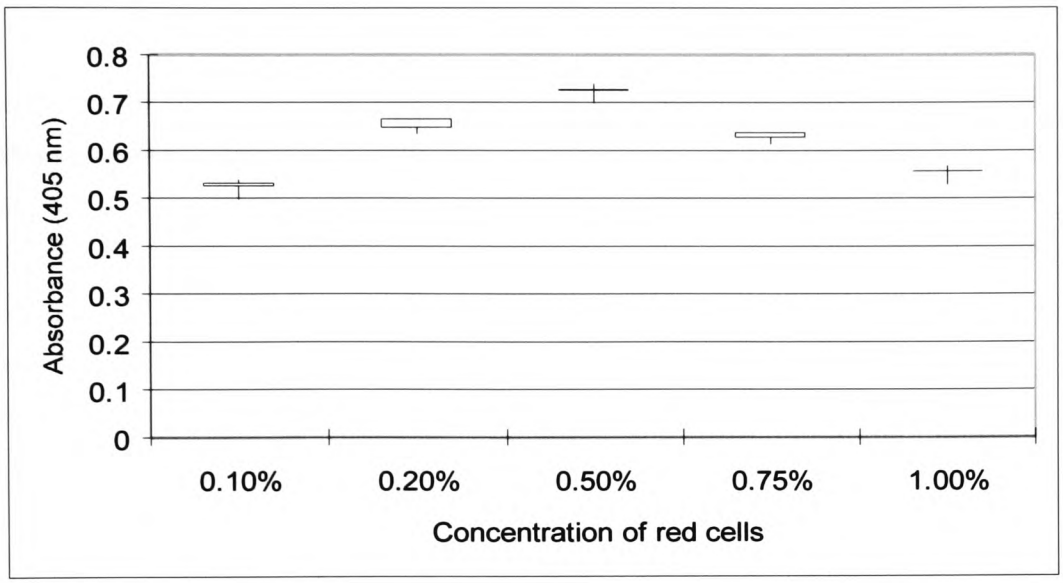
the RBC or solid phase including PLL. The wells were washed between 3 and 6 times with 250 µl each of PBS/Tween. Figure 3f shows that the optimum number of washes was 5. A minimum of three washes with 0.9% saline was required to remove any unbound conjugate before the addition of substrate (figure 3g). With further experiments microplate wells were washed five times with 250 µl of PBS/Tween after serum/cell incubations and 4 times with 0.9% saline after conjugate incubation.

Figure 3a: Effect of PLL concentration on RBC attachment to the solid phase



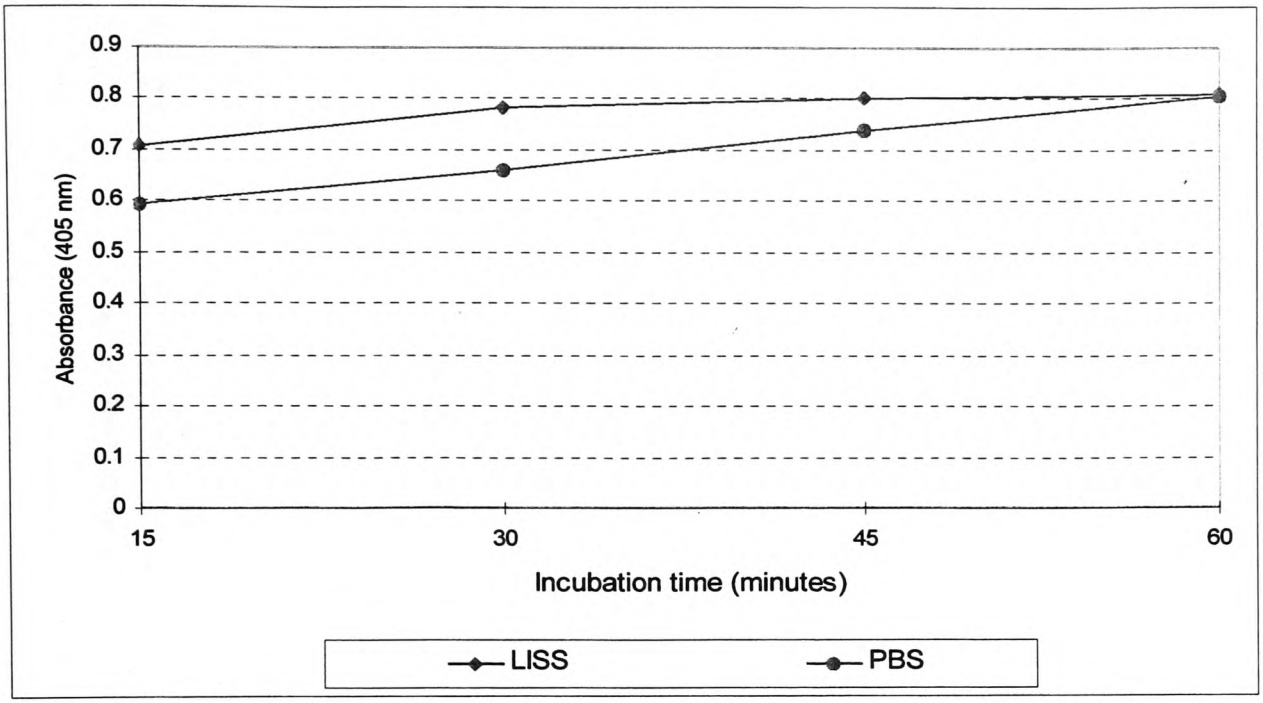
Microplate wells were coated with varying dilutions of PLL followed by effacement with 0.5% A_1 RBC. This was then tested with 1/10 dilution of mouse monoclonal anti-A by the ELISA method. Each dilution of PLL was tested in quadruplicate. Error bars are for 1 standard deviation.

Figure 3b: Effect of the red cell concentration on antibody binding



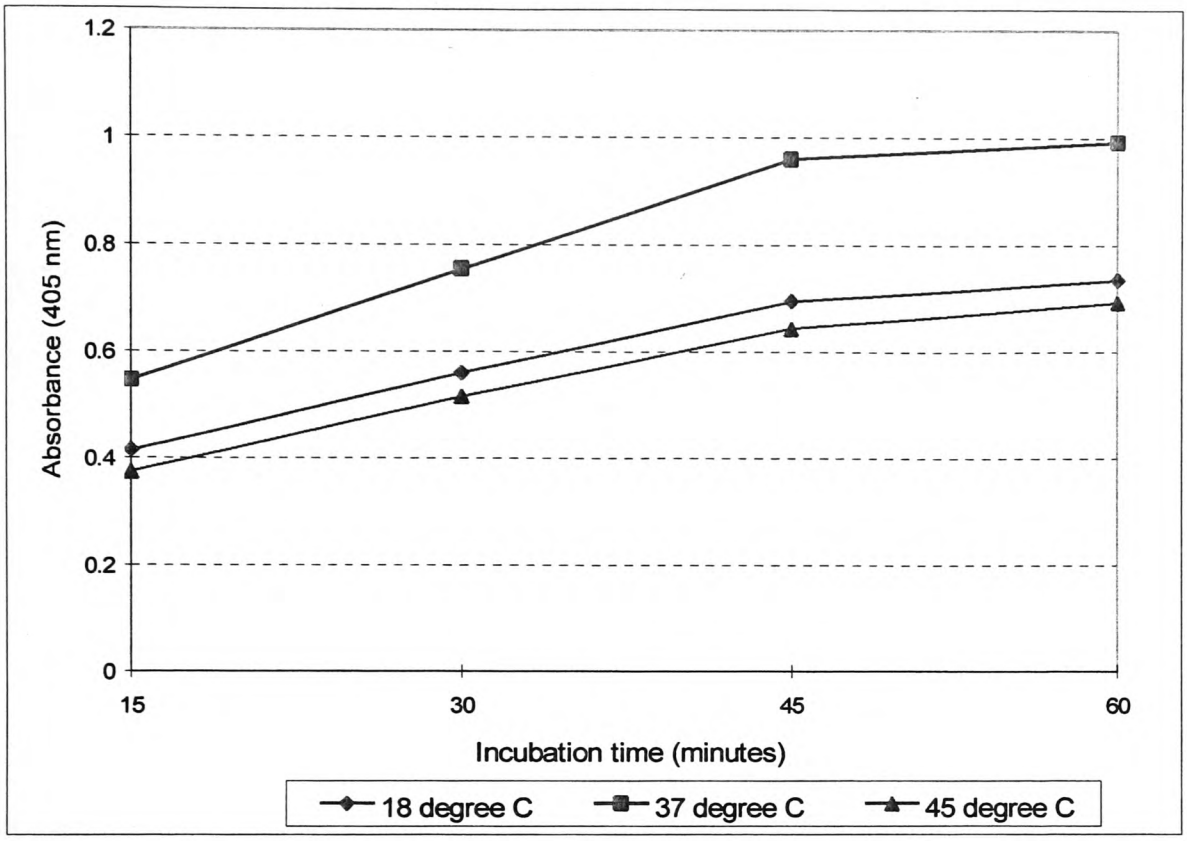
Microplates were coated with 10 mg ml^{-1} of PLL then centrifuged with various concentrations of A_1 RBC. These were then tested with 1/10 dilution of mouse monoclonal anti-A by the ELISA test. Each concentration of A_1 cells was tested in quadruplicate. Error bars are for 1 standard deviation.

Figure 3c: Effect of time and ionic strength on serum/cell incubation



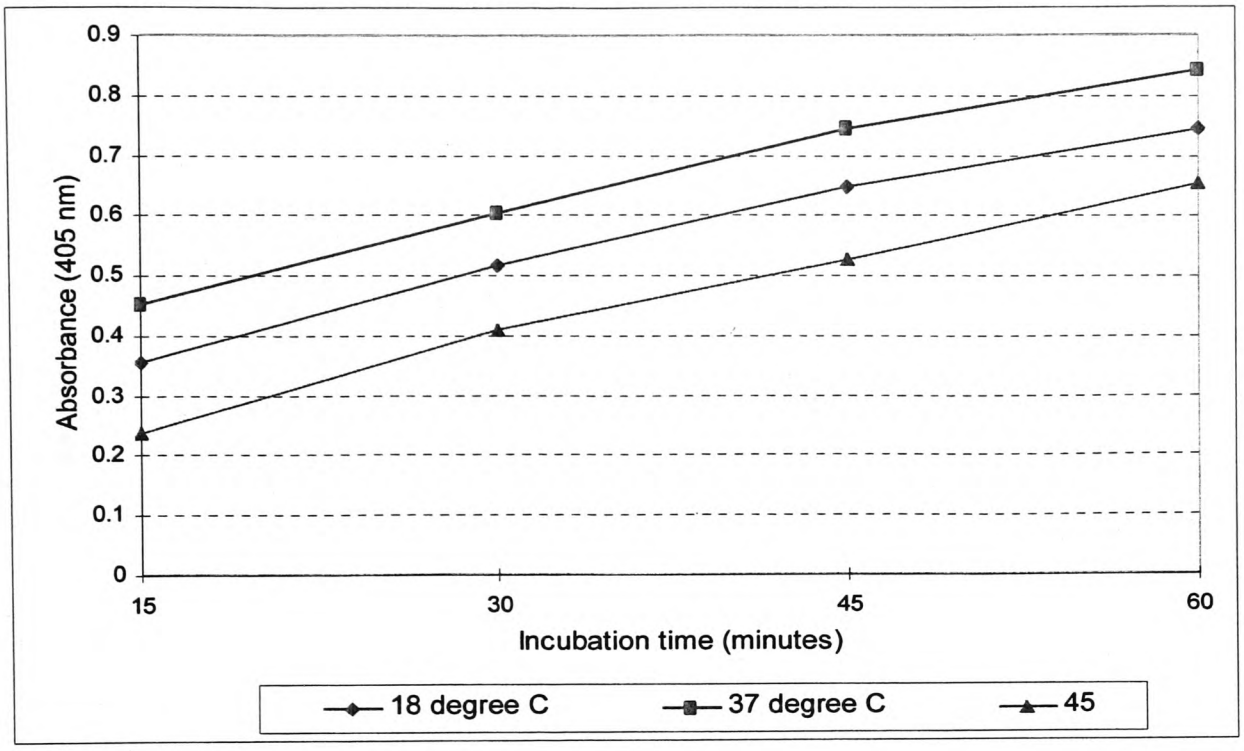
Microplates coated with 10 μg ml^{-1} of PLL were centrifuged with 0.5% O R_1R_2 RBC. After washing, 100 μl of ½ dilutions of monoclonal IgG anti-D in either PBS or LISS were added to each well and incubated for 15 to 60 minutes. The conjugate and substrate incubations were 60 minutes. All tests were performed in quadruplicate.

Figure 3d: Effect of time and temperature on conjugate reaction



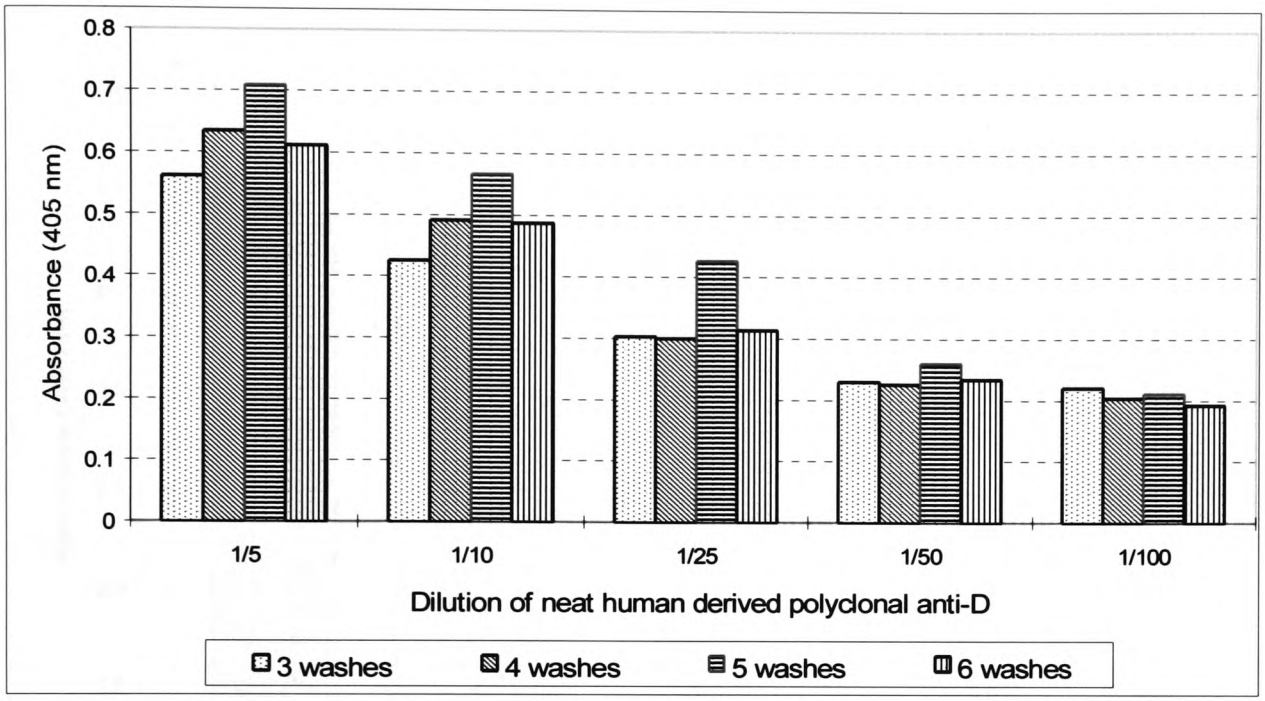
Microplates coated with 10 μg ml^{-1} of PLL were centrifuged with 0.5% O R_1R_2 RBC. After washing, 100 μl of ½ dilution of monoclonal IgG anti-D in LISS were added to each well and incubated for 30 minutes. The conjugate time was varied from 15 to 60 minutes and incubation temperatures of 18°C, 37°C and 45°C were used. The substrate was incubated for 60 minutes. All tests were performed in quadruplicate.

Figure 3e: Effect of time and temperature on substrate incubation



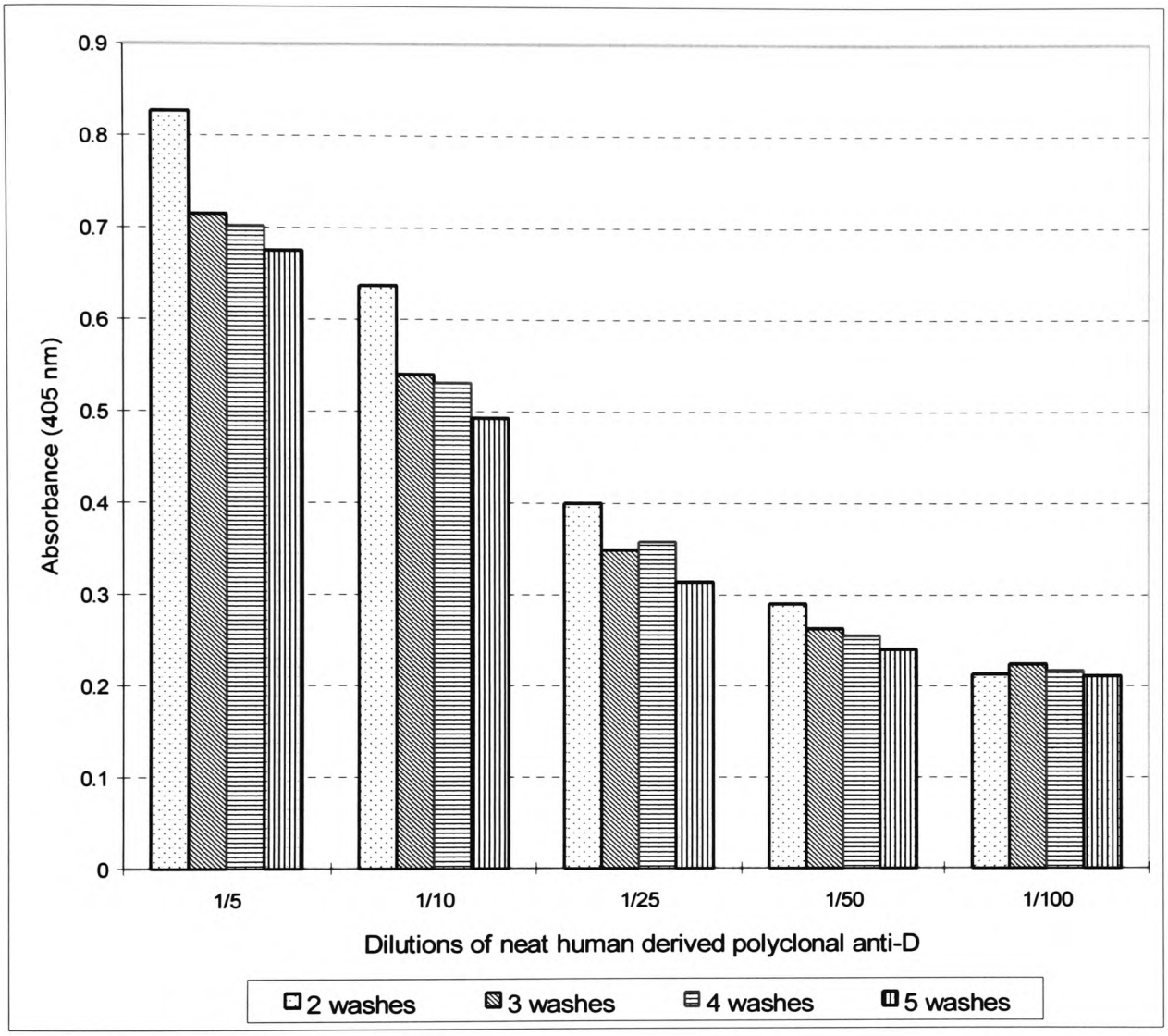
Microplates coated with 10 μg ml^{-1} of PLL were centrifuged with 0.5% O R_1R_2 RBC. After washing, 100 μl of ½ dilutions of monoclonal IgG anti-D in LISS was added to each well and incubated for 30 minutes. The conjugate was incubated for 60 minutes at 37°C. The substrate was incubated for times varying from 15 to 60 minutes and incubation temperatures of 18°C, 37°C and 45°C. All tests were performed in quadruplicate.

Figure 3f: Experiment to determine the optimum number of washes after serum/ cell incubation



Microplates coated with 10 μg ml^{-1} of PLL were centrifuged with 0.5% O R_1R_2 RBC. After washing, 100 μl of dilutions of neat human polyclonal IgG anti-D in LISS were added to each well and incubated for 30 minutes. The wells were then washed between 3and 6 times with PBS/Tween. The conjugate and substrate were incubated for 60 minutes at 37°C. After incubation with the conjugate, the wells were washed 4 times with 0.9% saline. All tests were performed in quadruplicate.

Figure 3g: Experiment to determine the optimum number of washes after conjugate incubation.



Microplates coated with 10 μg ml^{-1} of PLL were centrifuged with 0.5% O R_1R_2 RBC. After washing, 100 μl of dilutions of neat human derived polyclonal IgG anti-D in LISS were added to each well and incubated for 30 minutes. The wells were washed 5 times with PBS/Tween then incubated with conjugate for 60 minutes at 37°C. After incubation with the conjugate, the wells were washed between 2 and 5 times with 0.9% saline. All tests were performed in quadruplicate.

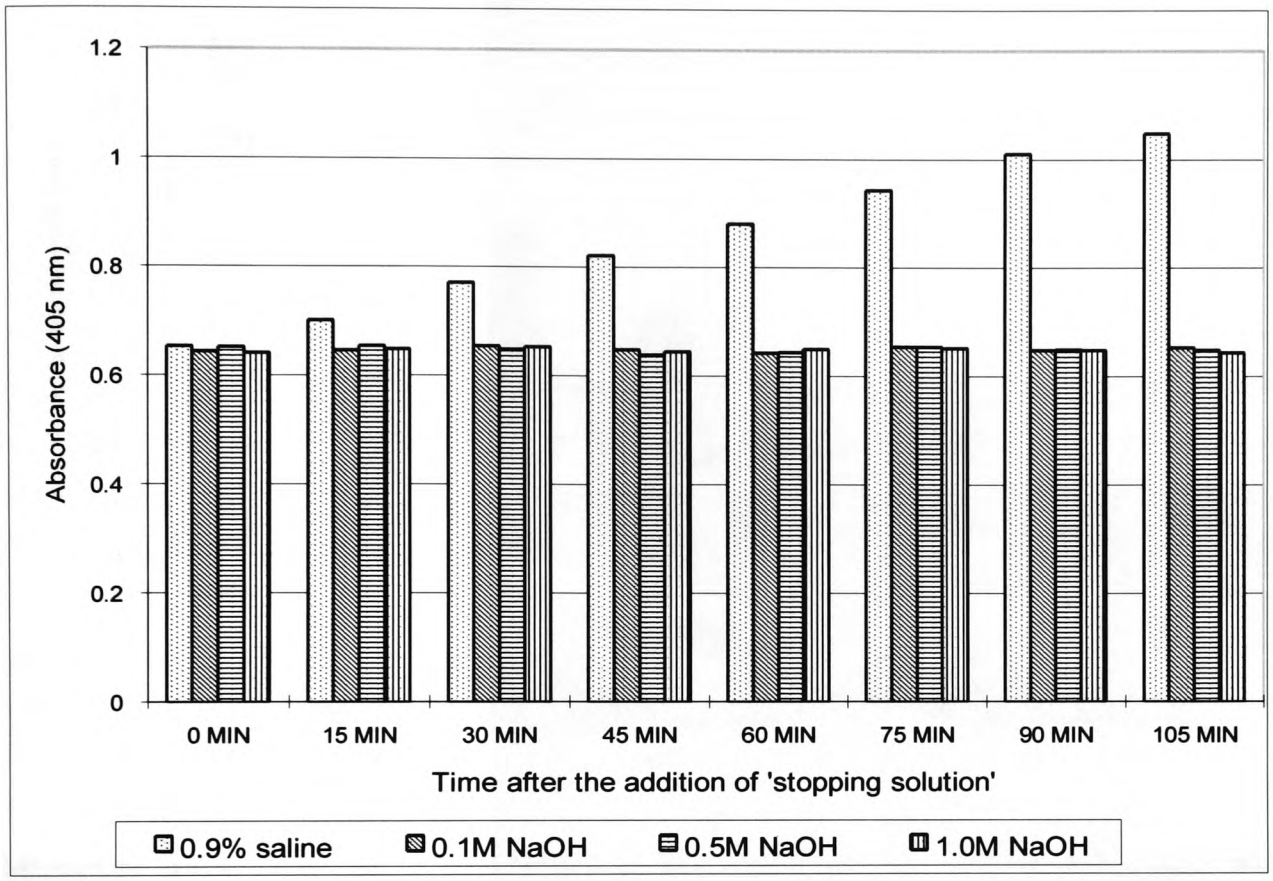
7. Use of EDTA and NaOH as 'stopping solutions' for pNPP

NaOH is the reagent of choice for stopping the enzymatic conversion of pNPP but the successful use of EDTA was reported by Reppun et al (1983). The aim of these experiments was to determine the better stopping solution and the optimum concentration. Wells coated with O R_1R_2 RBC were used in this experiment. 100 µl of 1/5 dilution of IgG monoclonal anti-D in LISS was added to microplate wells and incubated for 30 minutes at 37°C. After incubating with conjugate then substrate, the substrate reaction was stopped with either Na_2EDTA, K_2EDTA or NaOH. Figure 3h shows that both 0.1M Na_2EDTA and K_2EDTA completely reversed the substrate reaction. Similar results were obtained for 0.05M Na_2EDTA and K_2EDTA. 0.01M Na_2EDTA was ineffective at stopping the reaction and 0.01M K_2EDTA partially reversed the reaction. 0.1M, 0.5M and 1.0M NaOH were all effective at stopping the substrate reaction (figure 3i). 0.5M NaOH was used in further experiments.

8. Effect of bromelain treatment of RBC prior to attachment to the microplate wells

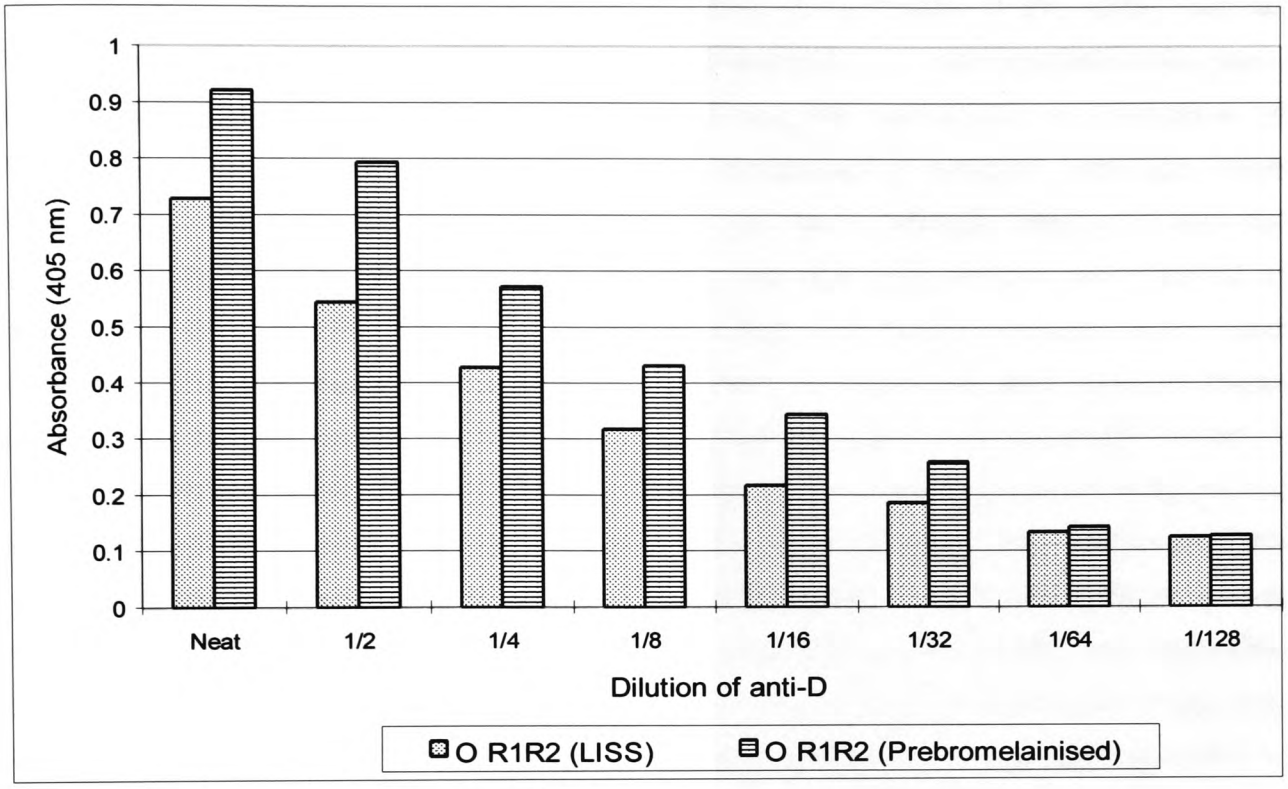
Treatment of red cells with enzymes such as bromelain results in the removal of hydrophilic glycoproteins carrying the negatively charged sialic acid with a reduction in the zeta potential. This leads to an increase in accessibility of some red cell antigens resulting in increased specific antibody binding. The aim of the experiment was to determine the effect of pretreating the RBC with bromelain prior to coating the microplate wells. The reason for choosing bromelain was because the ELISA will be used to quantitate anti-D and the results compared to that of the autoanalyser, which uses bromelain treated cells. Microplate wells were coated with either untreated or bromelain pre-treated RBC. 100 µl of monoclonal anti-D or dilutions (in LISS for the untreated RBC or PBS for bromelain pre-treated RBC) were added to each well and the ELISA test performed. Figure 3j shows that there are higher absorbances with bromelain pre-treatment for most of the concentrations of anti-D but the end point (titre) is the same for both the treated and untreated cells.

Figure 3h: Experiment to determine the concentration of NaOH required to stop pNPP substrate conversion



100 µl of 1/5 dilution of IgG monoclonal anti-D in LISS was added to microplate wells precoated with O R_1R_2 RBC and incubated for 30 minutes at 37°C followed by 5 washes of 250 µl with PBS/Tween. Incubation times of 60 minutes for conjugate reaction and substrate conversion were used. The wells were washed 4 times with 0.9% saline prior to the addition of the substrate. The substrate conversion was 'stopped' with 50 µl of various concentrations of NaOH and with 0.9% saline used as a control.

Figure 3i: Effect of bromelain treatment of the RBC before attachment to the microplate wells



Microplate wells were coated with either O R_1R_2 (untreated) or O R_1R_2 pre-treated with bromelain. These were then incubated with monoclonal IgG anti-D or dilutions (in LISS for the untreated RBC and PBS for the bromelain treated RBC) for 60 minutes. Sixty minutes incubations were used for conjugate reactions and substrate conversion. The substrate reaction was stopped with 50 µl of 0.5M NaOH. All the tests were performed in quadruplicate.

Tests with polyclonal antibodies

The previous experiments have demonstrated that the principle of the assay can be made to work for relatively pure preparations of antibody, i.e. monoclonal antibodies. Human polyclonal antibodies have been used but the specificity, i.e. reactions of polyclonal antibodies with cells lacking the corresponding antigen, has not been determined. As the test is intended to be used as a clinical diagnostic tool for quantitating serum based polyclonal antibodies, the next experiments were aimed at determining how specific the assay is and whether it is robust enough to be used routinely. The optimised ELISA (see procedure 2 in Methods) was used in these experiments but with serial dilutions of a human polyclonal anti-D (DL4542) tested in microplate wells coated with O R_1R_2 and O rr RBC. The results are shown in figure 4ai with the signal (R_1R_2 cells) to noise (rr cells) ratios shown in figure 4aii. Serial dilutions of human polyclonal anti-A were also tested in microplate wells coated with A_1 and B RBC (figures 4bi and 4bii). Both these experiments showed that there was very little discrimination between the reactions of dilutions of the human polyclonal antibodies with the cells carrying or lacking the corresponding antigens. This is quite contrary to expectations as no reaction was expected between anti-D and O rr cells and between anti-A and B cells. This indicates that there is non-specific binding of the test antibody to either the red cells, PLL, microplate or a combination and that this is proportional to the dilution of the antibody. The conjugate may also bind non-specifically to the RBC, PLL and/or microplate and contribute to the background. However, the effect would be the same at all dilutions of the primary antibody. It is also possible that incomplete removal of alkaline phosphatase from the red cells is causing substrate conversion and so contributing to the background reactions.

The above experiment with Rh D positive and Rh D negative cells was repeated but with serial dilutions of AB serum (which contained no detectable anti-D by conventional techniques). Figure 4c confirmed the non-specific reaction found with polyclonal anti-D and anti-A.

Monoclonal antisera suspended in a diluent have specific antibody reactants only and often in considerably lower concentrations than polyclonal sera. There are no antibodies of other specificities as in polyclonal sera which can bind to free sites on the

microplate wells, PLL and/or RBC to cause unwanted reactions. In order to test this hypothesis, monoclonal anti-D was diluted in AB serum and tested using the above protocol. The results in figure 4d show the presence of the non-specific reactions. With neat serum, there is very good discrimination between the reactions of the monoclonal anti-D and the Rh D positive and D negative RBC. However with a decreased concentration of monoclonal anti-D, there is an increased concentration of the diluent contaminating antibodies from the human polyclonal AB serum resulting in the shape of the graph seen in figure 4d.

In order to investigate if the material used in the microplate contributed to the non-specific reactions, Dynatech Immulon II polystyrene microplate was used in the next experiment. The discrimination between the reactions with antigen positive and negative cells did not improve. Also alkaline phosphatase was substituted with horseradish peroxidase (with OPD as substrate) but this did not reduce the unwanted reactions. Dynatech Immulon II microplate and horseradish peroxidase with OPD as substrate were used in further experiments because the plates are more rigid than the Titertek, the conjugate was less expensive and the substrate reaction can be stopped after 15 minutes

The total immunoglobulin content of monoclonal reagent used is very much less than that of human serum. In an attempt to investigate whether the total immunoglobulin content of polyclonal serum has an effect on the non-specific binding, human serum containing anti-D was absorbed with Rh D positive RBC. The sensitised cells were washed to remove any unbound antibody and the bound anti-D was eluted at 56°C. Doubling dilutions of the eluted anti-D was tested by the ELISA method and the results are shown in figure 4e with the signal:noise in figure 4f. The discrimination between RhD positive and RhD negative RBC was good but the OD readings obtained were low.

Figure 4ai: Absorbances of serial dilutions of human polyclonal anti-D with Rh D positive and Rh D negative RBC

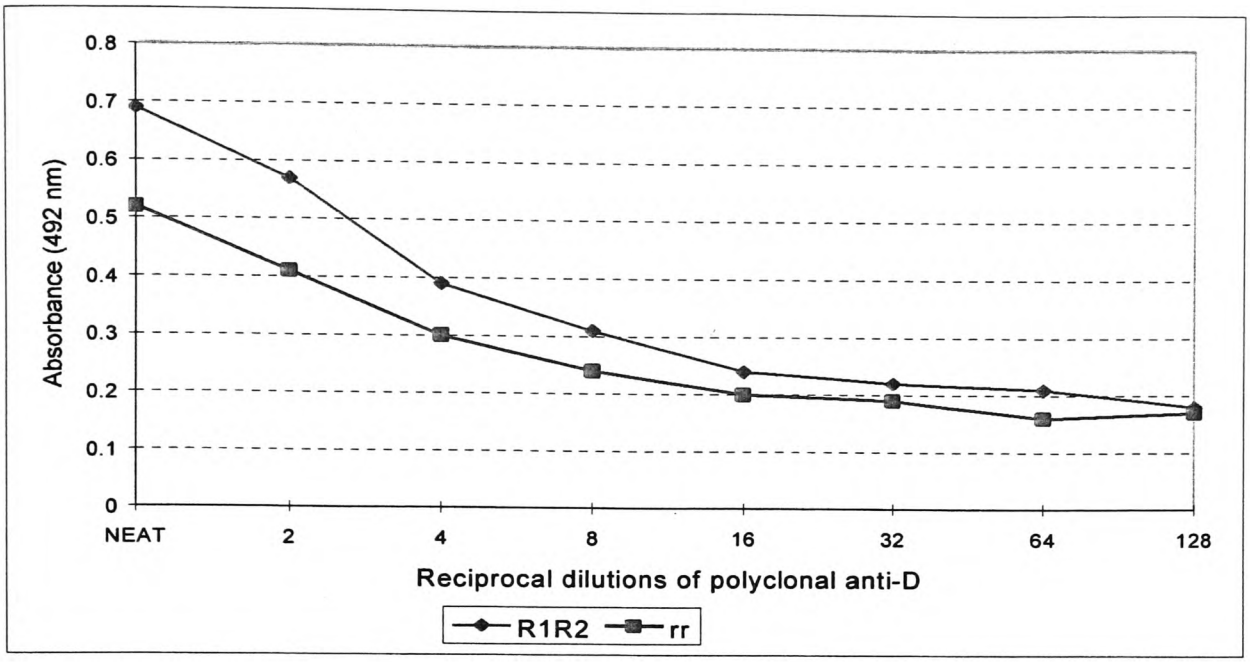
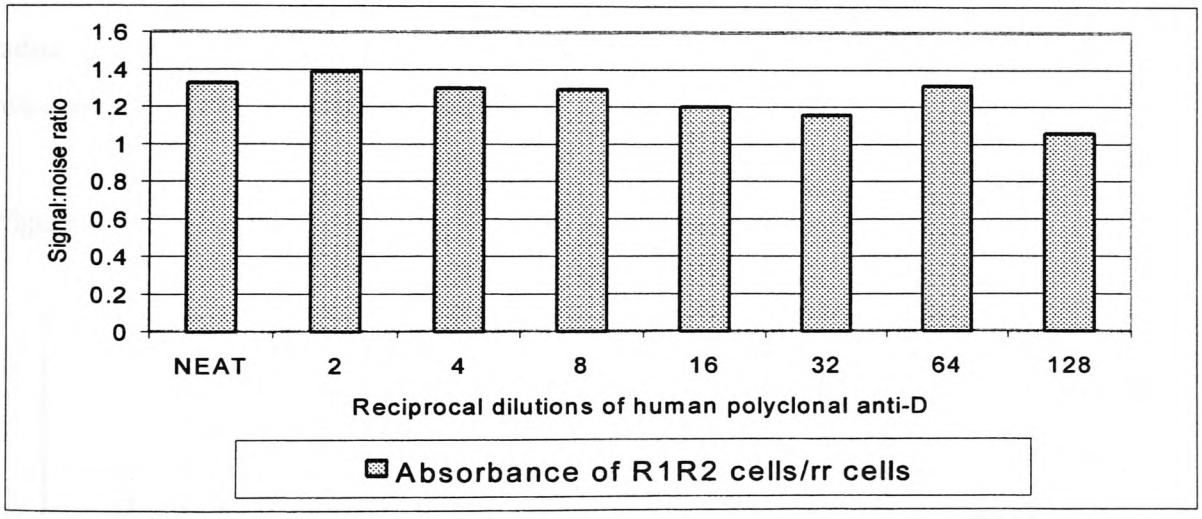
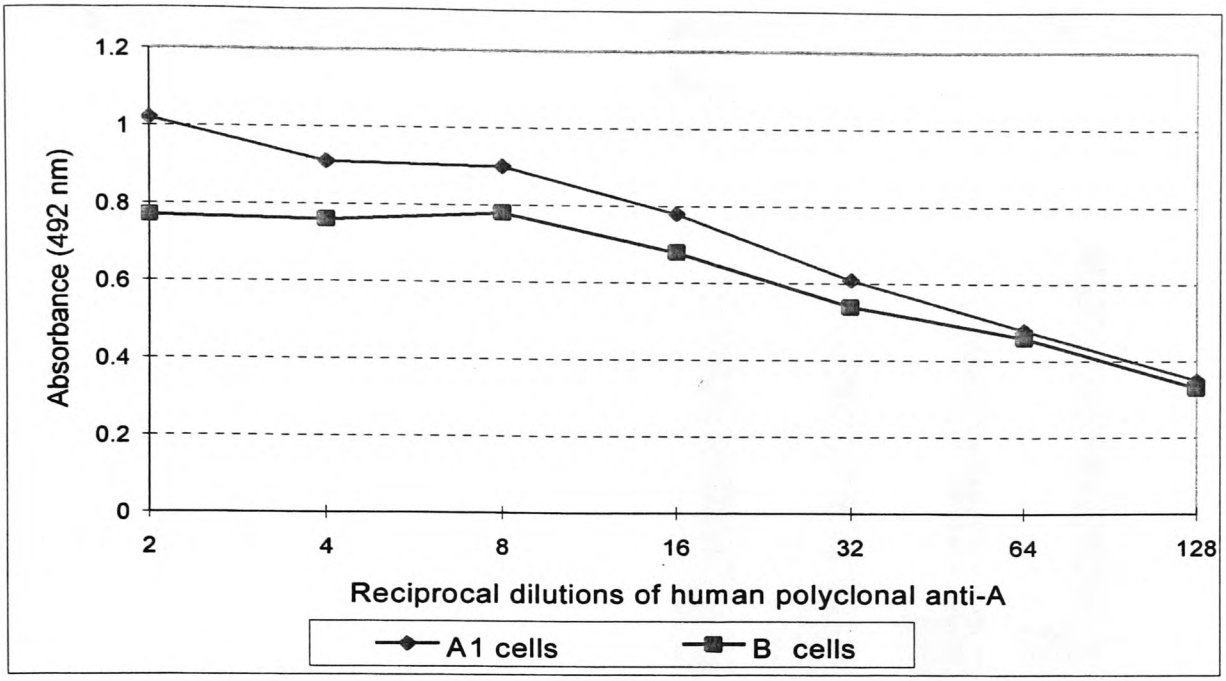


Figure 4aii: Signal:niose ratio of the absorbances of R1R2 and rr cells with polyclonal anti-D



Microplate wells pre-treated with 10 μg ml^{-1} of PLL were effaced with either O R_1R_2 or O rr RBC. These were then incubated with human polyclonal anti-D or dilutions in LISS, for 60 minutes. Sixty minutes incubations were used for conjugate reactions and 15 minutes for substrate conversion. The substrate reaction was stopped with 100 μl of 2M H_2SO_4. All the tests were performed in quadruplicate.

Figure 4bi: Absorbances of serial dilutions of human polyclonal anti-A v A_1 and B cells



Microplate wells pre-treated with 10 μg ml^{-1} of PLL were coated with either A_1 rr or B rr RBC. These were then incubated with human polyclonal anti-A or dilutions in PBS, for 60 minutes. Sixty minutes incubations were used for conjugate reactions and 15 minutes for substrate conversion. The substrate reaction was stopped with 100 μl of 2M H_2SO_4. All the tests were performed in quadruplicate.

Figure 4bii: Signal:noise ratio serial dilutions of human polyclonal anti-A v A_1 and B cells

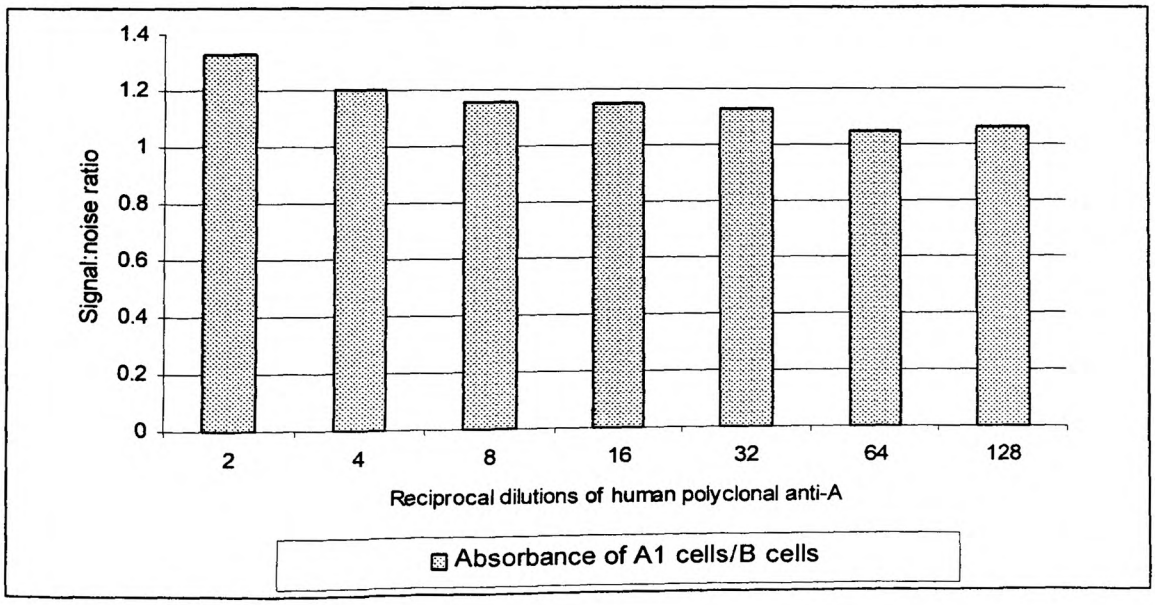
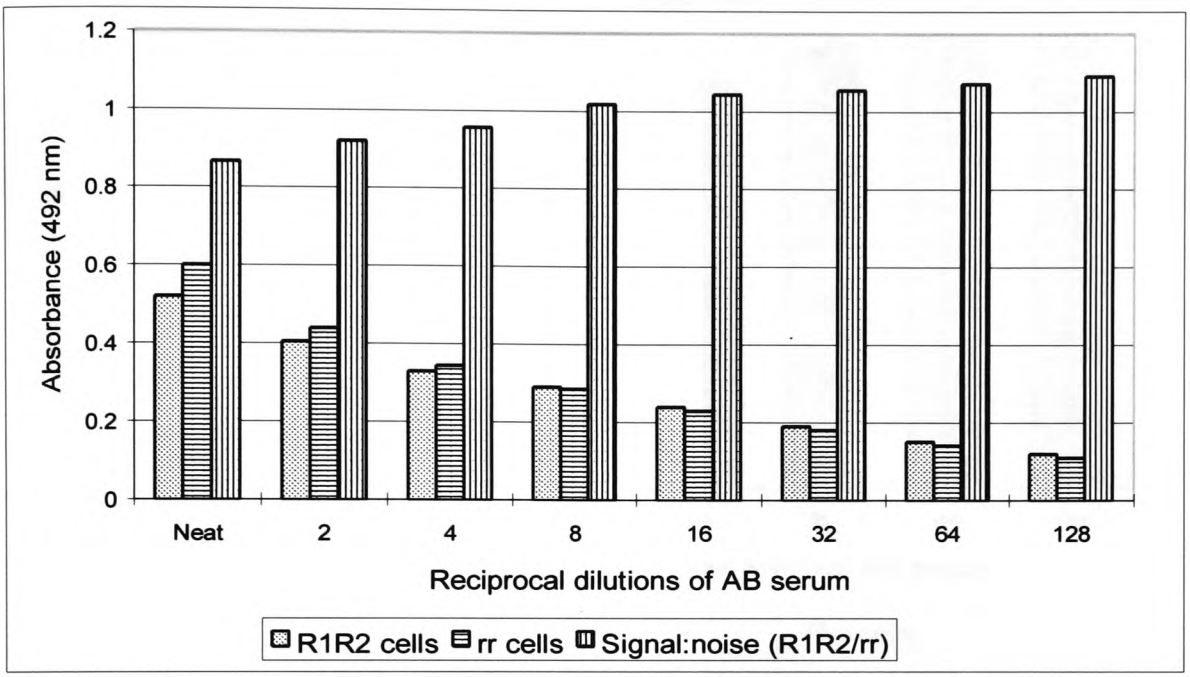
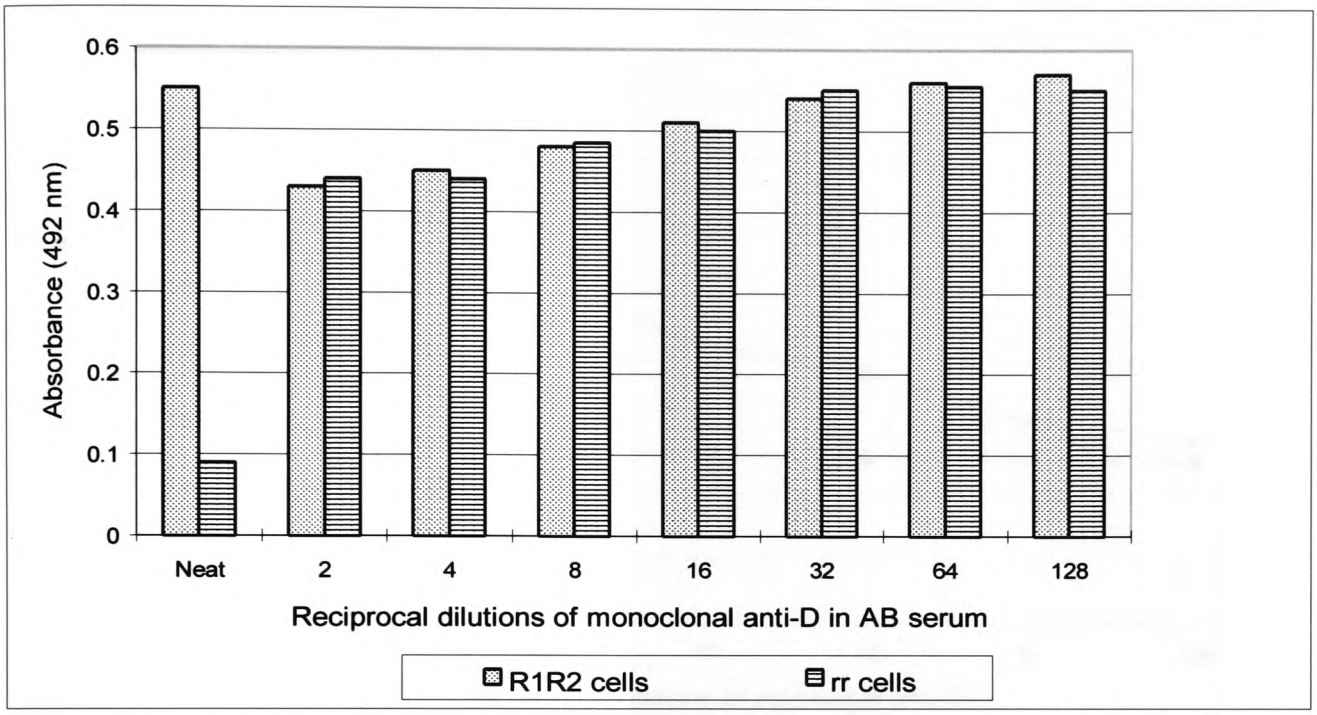


Figure 4c: Reactions of serial dilutions of AB serum on with O R_1R_2 or O rr RBC using a solid phase ELISA



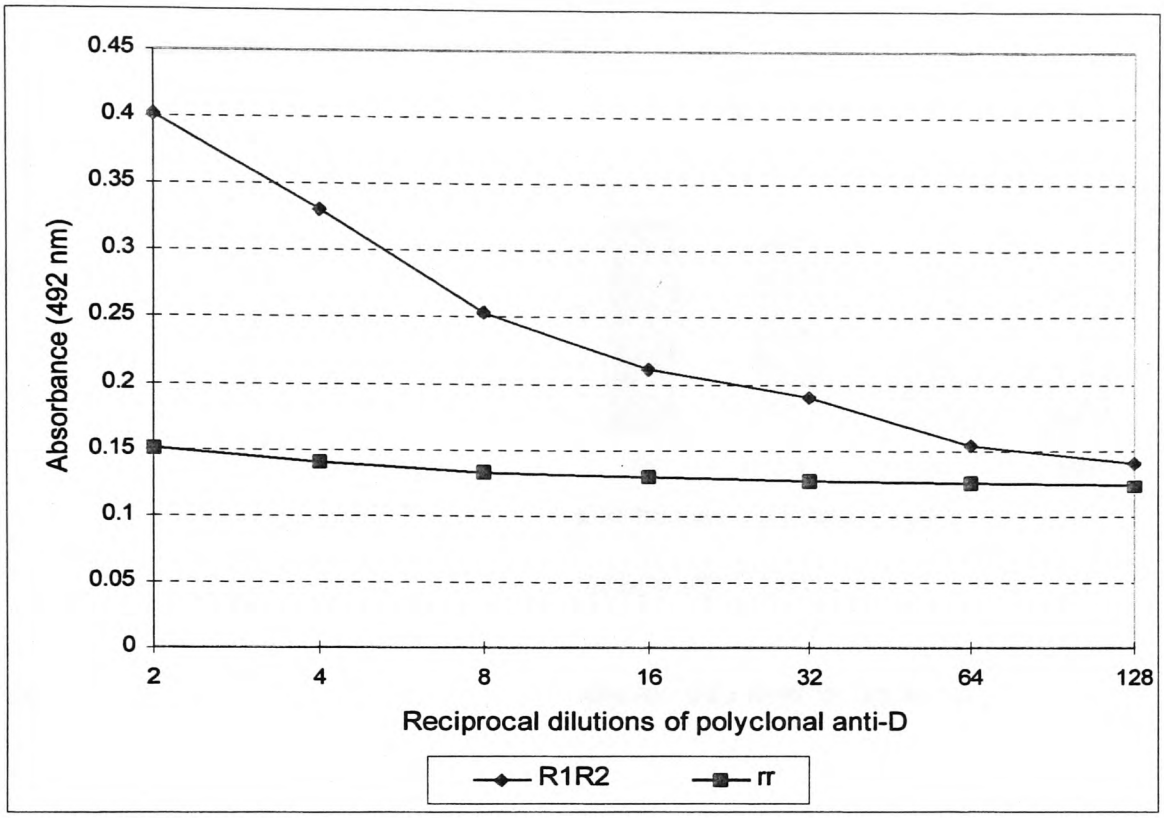
Microplate wells pre-treated with 10 μg ml^{-1} of PLL were coated with either O R_1R_2 or O rr RBC. These were then incubated with AB serum (containing no red cell specific antibody by conventional techniques) or dilutions in PBS, for 30 minutes. Sixty minutes incubations were used for conjugate reactions and 15 minutes for substrate conversion. The substrate reaction was stopped with 100 μl of 2M H_2SO_4. All the tests were performed in quadruplicate.

Figure 4d: Reactions of serial dilutions of monoclonal anti-D diluted in AB serum with O R_1R_2 or O rr RBC



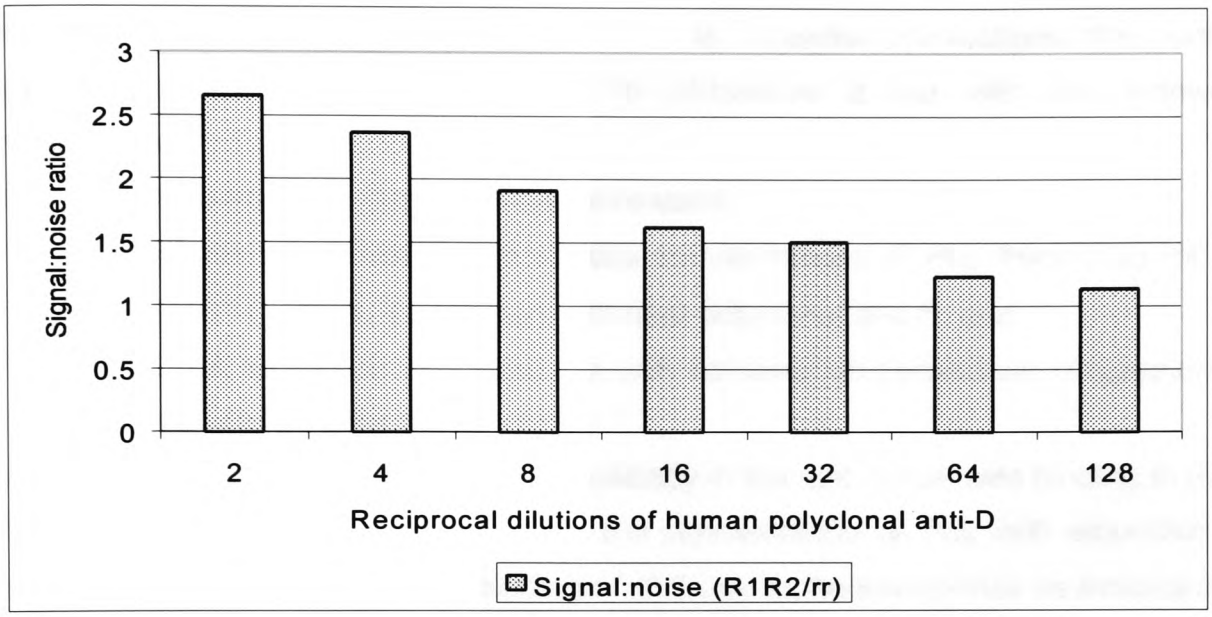
Microplate wells pre-treated with 10 μg ml^{-1} of PLL were coated with either O R_1R_2 or O rr RBC. These were then incubated with monoclonal IgG anti-D or dilutions in AB serum (containing no red cell specific antibody by conventional techniques), for 30 minutes. Sixty minutes incubations were used for conjugate reactions and 15 minutes for substrate conversion. The substrate reaction was stopped with 100 μl of 2M H_2SO_4. All the tests were performed in quadruplicate.

Figure 4e: Reactions of serial dilutions of absorbed/eluted anti-D tested using the solid-phase ELISA



O R_1R_2 RBC was incubated with human polyclonal anti-D in glass test tubes then washed four times with PBS. The bound antibody was eluted at 56°C. Microplate wells were coated with 10 μg ml^{-1} of PLL, effaced with 0.5% O R_1R_2 or O rr RBC then blocked with 20% FCS for 60 minutes at ambient temperature. 100 μl of doubling dilutions of the eluted polyclonal anti-D in 0.1% Tween 20 was added to each well and incubated at 37°C for 30 minutes. Each test was performed in duplicate.

Figure 4f: Signal:noise ratio of R_1R_2 and rr cells tested with serial dilutions of absorbed/eluted anti-D using the solid-phase ELISA



The signal:noise ratio was calculated from absorbance data used in figure 4e.

Investigation of non-specific reactions

Unwanted reactions could be due to antibodies binding non-specifically to the microplate well plastic, PLL and/or to the red cells. In order to investigate this, further experiments were carried as outlined in procedure 2 but with the following modifications:

i) Dynatech Immulon II microplates were used,

ii) microplates were coated with various concentrations of PLL from 0 $\mu g\ ml^{-1}$ to 100 $\mu g\ ml^{-1}$ (in PBS) then tested with neat polyclonal anti-D, and

iii) the ELISA technique was used but with horseradish peroxidase conjugated to anti-IgG with OPD as the substrate.

The results in figure 5a showed that IgG antibody in the test serum was binding to PLL. This binding was initially proportional to the concentration of PLL with saturation at about 75 $\mu g\ ml^{-1}$. There was also binding of antibody to the solid phase as indicated by the significant OD obtained in the absence of PLL.

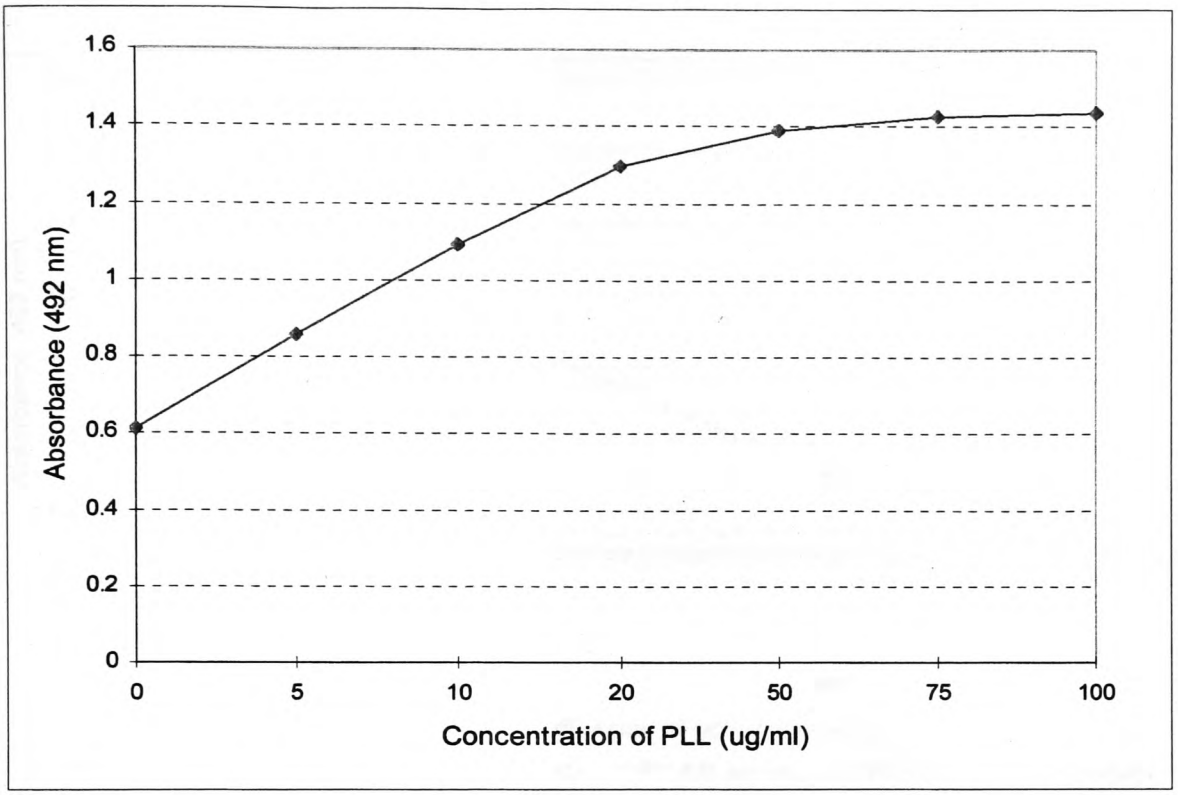
In order to determine if there was non-specific binding onto red cells, equal volumes of 1/10, 1/100, 1/500 and 1/1000 dilutions of polyclonal anti-D and AB serum in PBS were incubated separately with packed O R_1R_2 (Rh D positive) and O rr (Rh D negative) RBC in glass tubes. After washing 4 times in PBS, 0.5% cell suspensions in PBS were prepared. 100 μl of the 0.5% RBC suspensions were added to microplate wells pre-coated with PLL (10 $\mu g\ ml^{-1}$) and centrifuged for 2 minutes at 32g. The microplate wells were then washed, the cells lysed with distilled water then fixed with 0.25% (v/v) glutaraldehyde. 200 μl of 20% foetal calf serum (FCS) in PBS was added to each microplate well and incubated for an hour at ambient temperature in an attempt to reduce non-specific binding of the conjugate. Any test antibody bound to the glass tube would not affect the final result as the sensitised and unreacted RBC was transferred to a microplate for the remainder of the assay. The results, after incubation with conjugate then substrate, are shown in figure 5b. The discrimination between the reaction of anti-D and Rh D positive and Rh D negative cells was very good. There was some non-specific reaction equivalent to absorbance of less than 0.2 units which may be due to non-specific binding of test antibodies to RBC, of conjugate to the solid phase or RBC, of residual haemoglobin peroxidase causing substrate conversion or a

combination of the above. When the blocking phase was omitted there was a slightly higher non-specific reaction, indicating that the conjugate can bind to the solid phase but at a much lower level than human serum.

In another set of experiments, microplate wells were pre-coated with O R_1R_2 red cells which were then lysed and washed. 1/10 dilution of polyclonal anti-D was added to four random wells (as positive controls) and the microplate was incubated for 30 minutes at 37°C then washed. Conjugate (haemoglobin peroxidase and alkaline phosphatase was used in separate experiments) was added to the wells with anti-D and half the wells without antibody. After completion of the tests, the absorbance was measured. There was no significant difference in optical densities in wells without polyclonal antibody but with or without conjugate (data not presented) demonstrating that substrate hydrolysis due to residual haemoglobin peroxidase and alkaline phosphatase when the conjugate was omitted from the test, was not responsible for non-specific reactions. Non-specific reaction due to conjugate binding to the red cells, solid phase or PLL was also negligible.

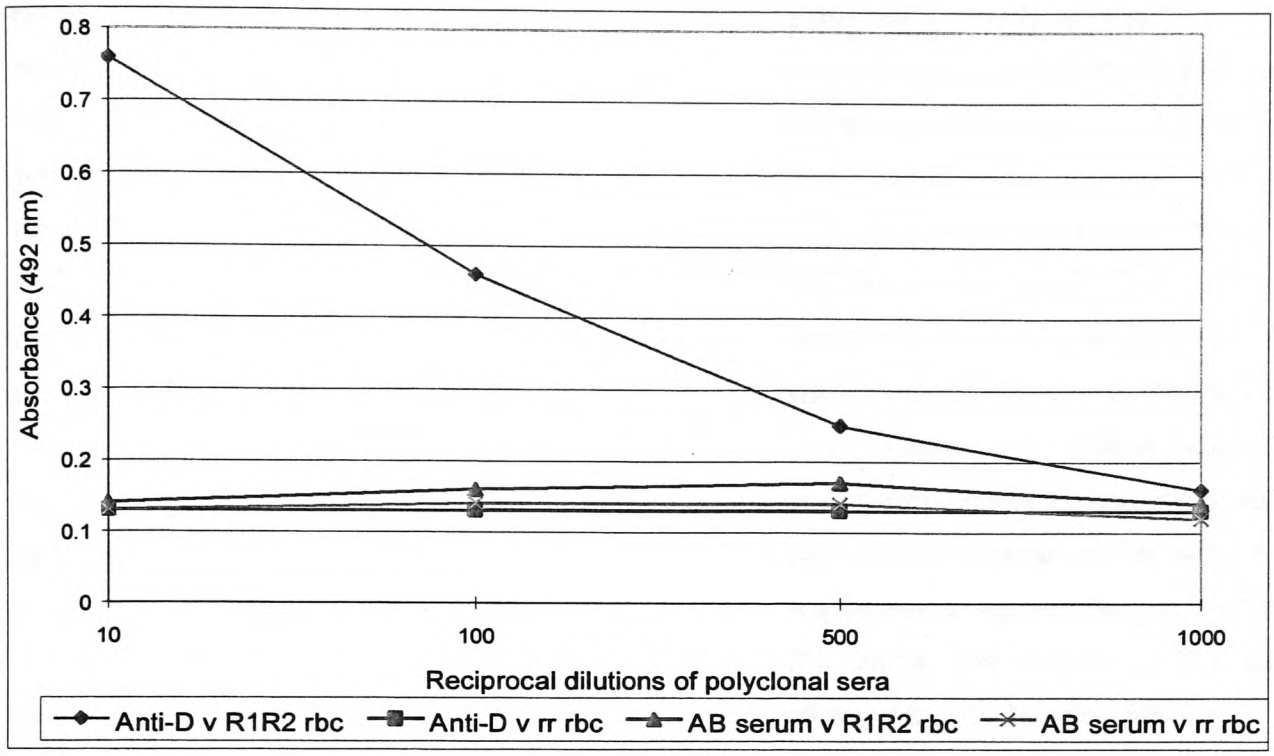
The conclusion that can be drawn from the above experiments is that the non-specific reactivity is mainly due to constituents of the polyclonal serum binding to PLL and/or the microplate.

Figure 5a: Effect of PLL concentration on non-specific binding



Dynatech Immulon II microplate wells were coated with various dilutions of PLL. The effacement with RBC was omitted. This was then tested with neat polyclonal anti-D by the ELISA to examine the amount of non-specific binding of protein occurred according to the varying concentration of PLL. Horseradish peroxidase conjugated to IgG was used with OPD as substrate. The substrate reaction was stopped after 15 minutes with 100 μl of 2M H_2SO_4.

Figure 5b: Effect of pre-sensitising RBC then attaching them onto the microplate wells for the ELISA



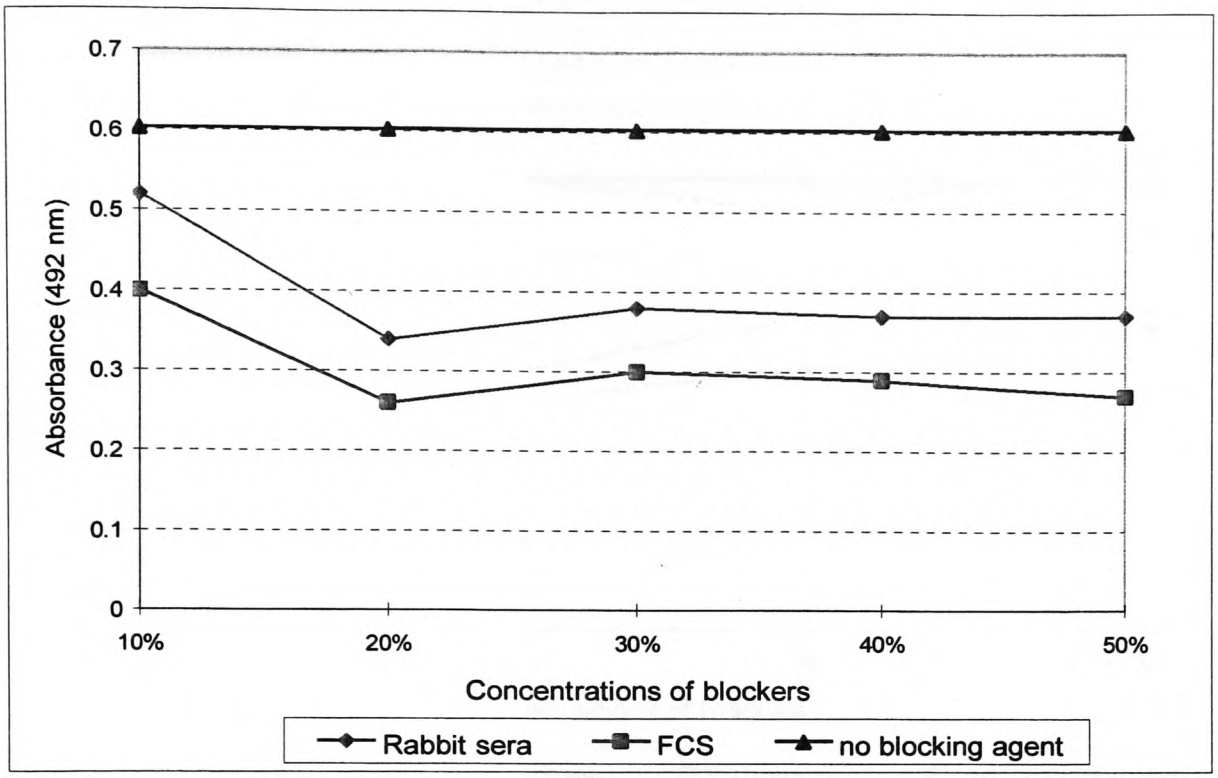
O R_1R_2 and O rr red cells were incubated with one of 4 dilutions of polyclonal anti-D or AB serum in duplicate at 37°C in glass tubes, washed 4 times with PBS then made up to 0.5% in PBS. These were then centrifuged onto microplate wells previously coated 10 μg ml^{-1} of PLL. The microplates were then washed, blocked with 20% FCS and the ELISA performed. Horseradish peroxidase conjugated to IgG was used with OPD as substrate. The substrate reaction was stopped after 15 minutes with 100 μl of 2M H_2SO_4.

Investigations into blocking non-specific binding

The above experiments have shown that a significant proportion of the background noise is due to non-specific binding of test serum to the solid phase and to PLL. The non-specific binding has to be reduced by blocking the potentially reactive sites with materials which will improve the specificity without otherwise influencing the final assay procedure. Experiments to reduce this background noise were carried out as described in procedure 2 but with effacement of RBC omitted from the assay. Prior to the addition of test serum, the microplate wells were incubated with various concentrations of reagents for 60 minutes at room temperature in attempting to reduce non-specific binding. The reagents used were alpha$_1$ glycoprotein (AGP), bovine serum albumin (BSA), bovine and goat casein, FCS and Tween 20. These reagents were selected because other researchers have found them useful in reducing non-specific binding. The microplates were then tested with a 1/10 dilution of AB serum in 0.1% PBS Tween and the results for some of these are shown in figures 6a, 6b and 6c. From these experiments it was deduced that 20% foetal calf serum (FCS) was marginally better at reducing non-specific reactions followed by 1% bovine casein.

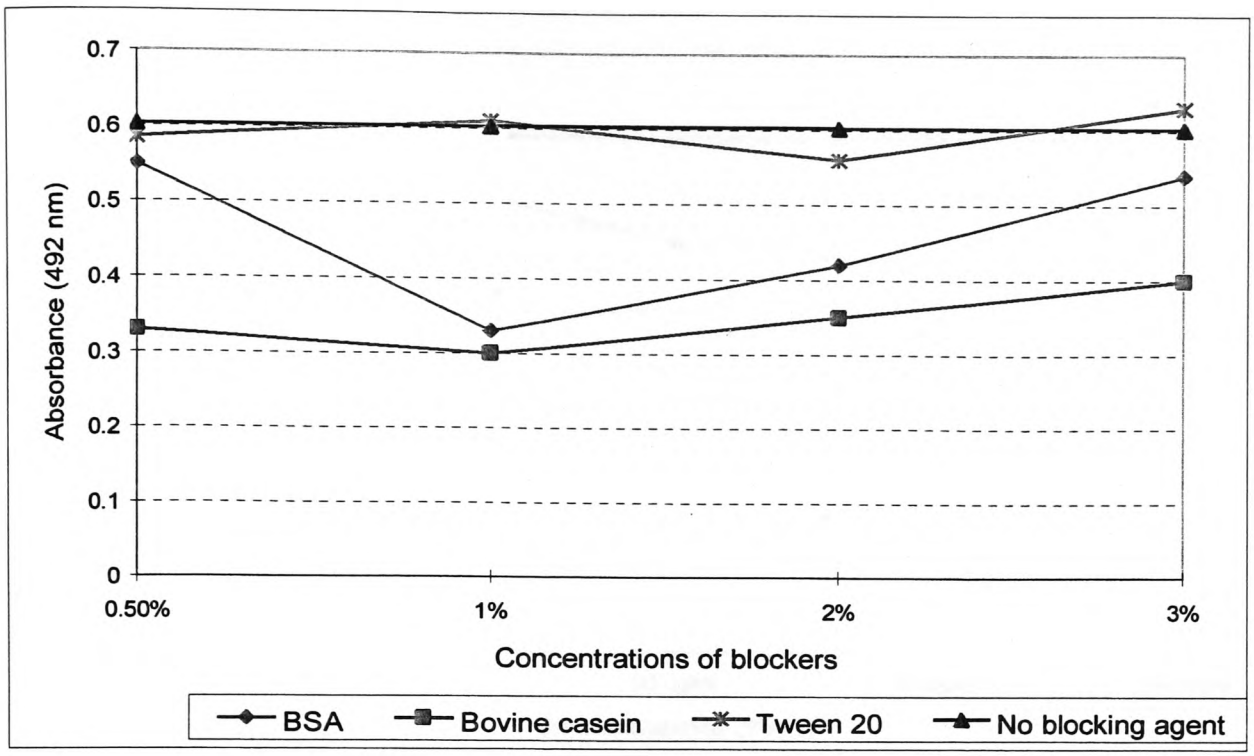
The next set of experiments was carried out as described in procedure 2. After coating with R_1R_2, rr or no RBC, half of the wells were incubated with 20% FCS and no 'blocker' in the others. Dilutions of anti-D in 0.1% PBS Tween were added to the wells and the ELISA performed. This experiment was repeated but with 1% bovine casein used to pre-coat the wells. The results in figure 6d showed that 20% FCS made only a marginal improvement to non-specific reactions. Similar results were obtained when 20% rabbit serum or 1% casein was used to reduce non-specific reactions. However, there was a slight improvement in discrimination between positive and negative reactions when 20% FCS was used to pre-coat the wells and as a diluent (data not shown). The inclusion of Thiomersal in the 'blocking' buffer did not result in any further improvement in the assay (data not shown).

Figure 6a: Effect of FCS and rabbit sera on blocking non-specific reactions



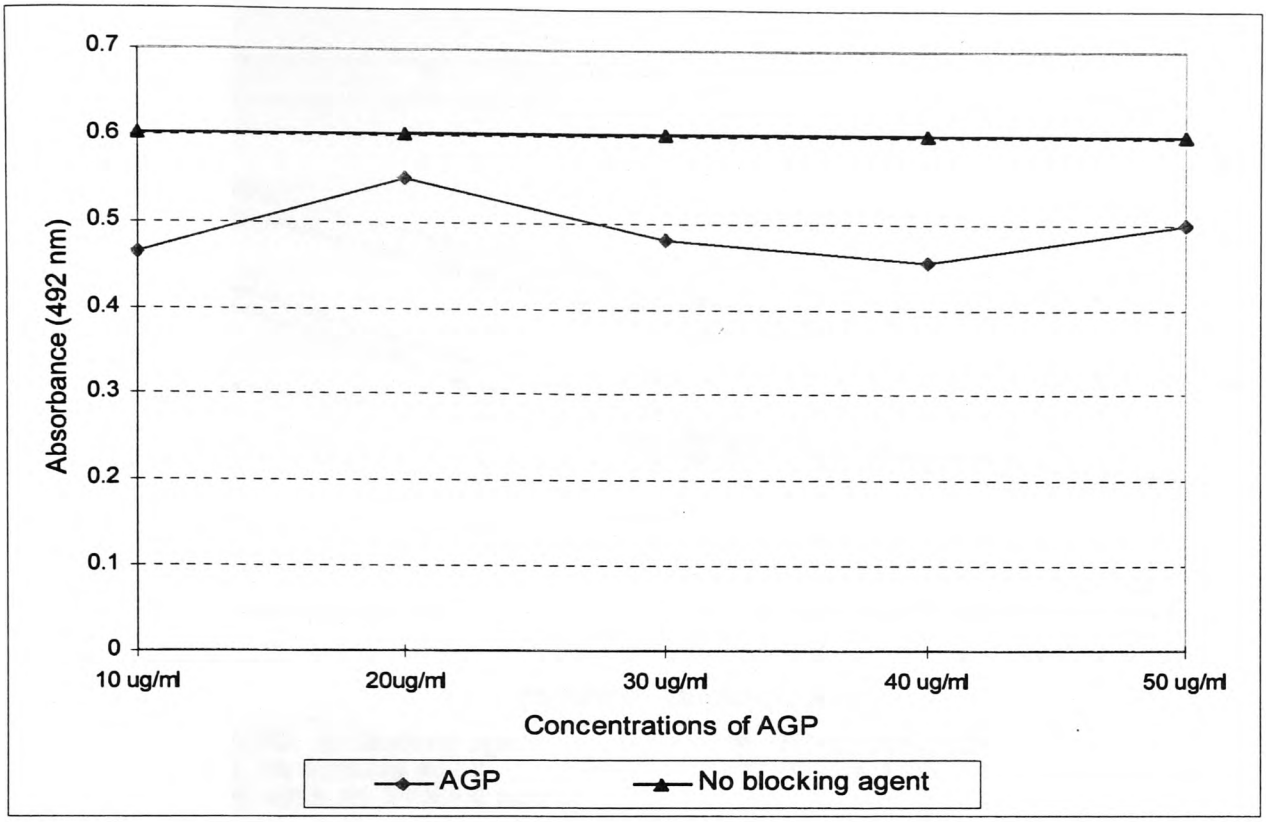
Dynatech Immulon II microplate wells were coated with 10 μg ml^{-1} of PLL. After washing, the wells were incubated with 250 μl of dilutions of fetal calf serum (FCS) or rabbit serum for 60 minutes at ambient temperature then decanted and blotted dry. 100 μl on 1/10 dilution of AB serum in PBS was added to each well and incubated for 30 minutes at 37°C. After washing, horseradish peroxidase conjugated to AHG was used as the conjugate with OPD as the substrate. The substrate reaction was stopped after 15 minutes with 100 μl of 2M H_2SO_4. The tests were performed in quadruplicate.

Figure 6b: Effect of BSA, bovine casein and Tween 20 on blocking non-specific reactions



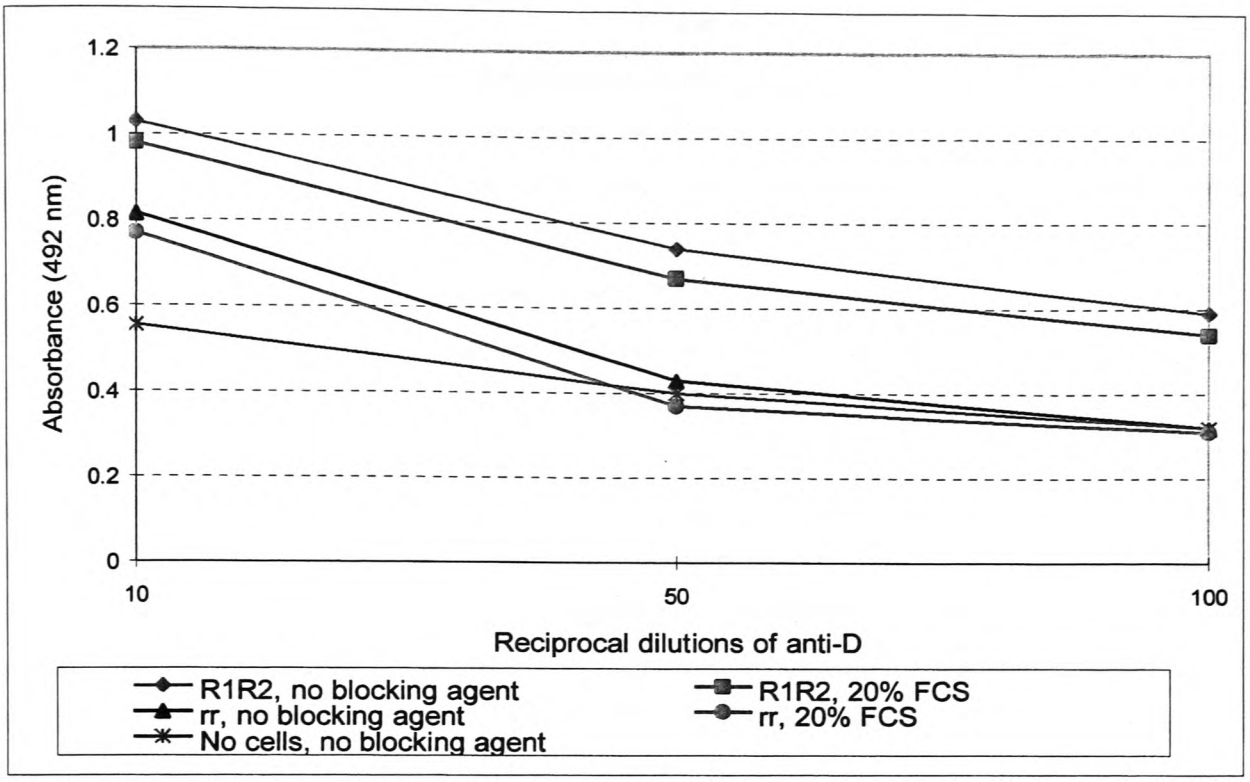
Dynatech Immulon II microplate wells were coated with 10 μg ml^{-1} of PLL. After washing, the wells were incubated with 250 μl of dilutions of bovine serum albumin (BSA), casein and Tween 20 for 60 minutes at ambient temperature then decanted and blotted dry. 100 μl on 1/10 dilution of AB serum in PBS was added to each well and incubated for 30 minutes at 37°C. After washing, horseradish peroxidase conjugated to AHG was used as the conjugate with OPD as the substrate. The substrate reaction was stopped after 15 minutes with 100 μl of 2M H_2SO_4. The tests were performed in quadruplicate.

Figure 6c: Effect of alpha$_1$ acid glycoprotein on blocking non-specific reactions



Dynatech Immulon II microplate wells were coated with 10 μg ml^{-1} of PLL. After washing, the wells were incubated with 250 μl of dilutions of alpha$_1$ acid glycoprotein (AGP) for 60 minutes at ambient temperature then decanted and blotted dry. 100 μl on 1/10 dilution of AB serum in PBS was added to each well and incubated for 30 minutes at 37°C. After washing, horseradish peroxidase conjugated to AHG was used as the conjugate with OPD as the substrate. The substrate reaction was stopped after 15 minutes with 100 μl of 2M H_2SO_4. The tests were performed in quadruplicate.

Figure 6d: Effect of 20% FCS on blocking non-specific reactions of polyclonal anti-D with O R_1R_2, O rr or no RBC



Microplate wells were coated with 10 μg ml^{-1} of PLL then effaced with 0.5% O R_1R_2, O rr or no RBC. After blocking with 20% FCS or PBS for 60 minutes at ambient temperature, the wells were incubated with one of 3 dilutions of polyclonal anti-D in 0.1% Tween 20 and the ELISA performed. Each dilution was tested in quadruplicate

Effect of pH on non-specific reactions

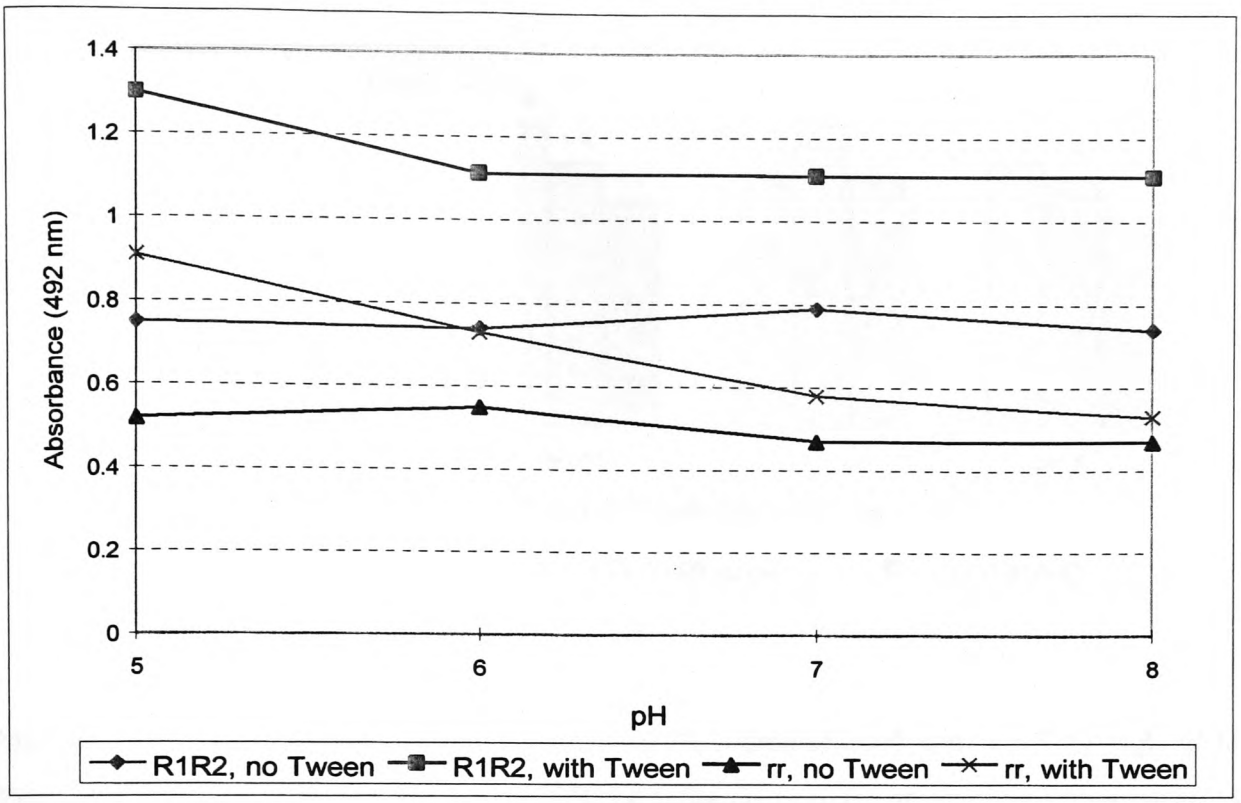
In an attempt to demonstrate the effect of pH on the non-specific reaction, microplate wells were 'blocked' with 20% FCS after RBC were attached. These were then tested with polyclonal anti-D diluted in Sorensons buffer of pH 5.0, 6.0, 7.0 and 8.0. In a separate experiment, the microplate wells were then incubated with 0.5% Tween after 'blocking' with FCS then incubated with 1/10, 1/100 and 1/1000 dilutions of the same polyclonal anti-D. The signal:noise ratio (OD for R_1R_2 cells / OD for rr cells) is shown in figure 2d. The results of the tests with the 1/10 dilution of polyclonal anti-D (figure 7a) show that when the microplate wells were not incubated with 0.5% Tween, variation of pH from 5 to 8 does not significantly affect antibody binding. However, when the microplate wells were incubated with 0.5% Tween, there is increased antibody binding for the three dilutions of anti-D tested. This increased binding is more pronounced at pH 5.0. The signal:noise ratio (figures 7b and 7c) which is an indication of the discrimination between positive and negative reactions shows that the discrimination was improved by pre-treatment with 0.5% Tween. However, the discrimination has not improved enough and the background reaction was still too high for this assay to be used routinely.

Because increased antibody binding was obtained at pH 5.0, RBC were bound to microplate wells which were then blocked with 20% FCS at this pH for maximum blocking. After incubating with 0.5% Tween, dilutions of polyclonal anti-D at pH 7.0 were added to the wells and incubated at 37°C for 30 minutes. This pH was chosen because the experiment above has shown that non-specific reaction was lower and it is the optimum pH for anti-D reaction. There was no improvement in the background reaction nor the discrimination.

Other workers have shown that after coating the microplate wells with antibodies or purified antigens, non-specific reactions in ELISA can be considerably reduced by treatment with the above 'blocking agents'. One of the main differences between this and other successful assays is that whole RBC are being used to coat the microplate wells. It is possible that the red cells in some way prevent the blocking agents from binding to the free sites on the wells or PLL. It was hoped that by centrifuging microplates with wells containing RBC suspended in 1% bovine casein or 20% fetal

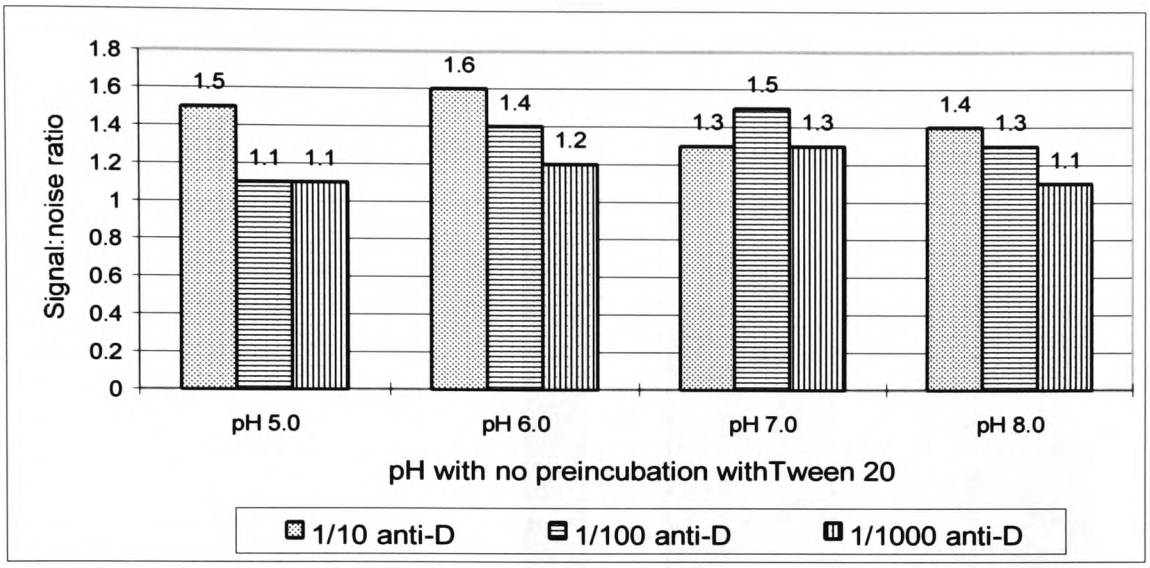
calf serum after PLL incubation, the blocking agents would bind to the free non-specific sites. The RBC did not bind to the PLL and hence the microplate wells. Also, when microplate wells coated with PLL were pre-incubated with 1% bovine casein or 20% FCS red cells did not attach to the wells. This indicates that bovine casein and FCS bind directly to PLL and blocking the attachment of the red cells.

Figure 7a: Effect of pH on non-specific reaction



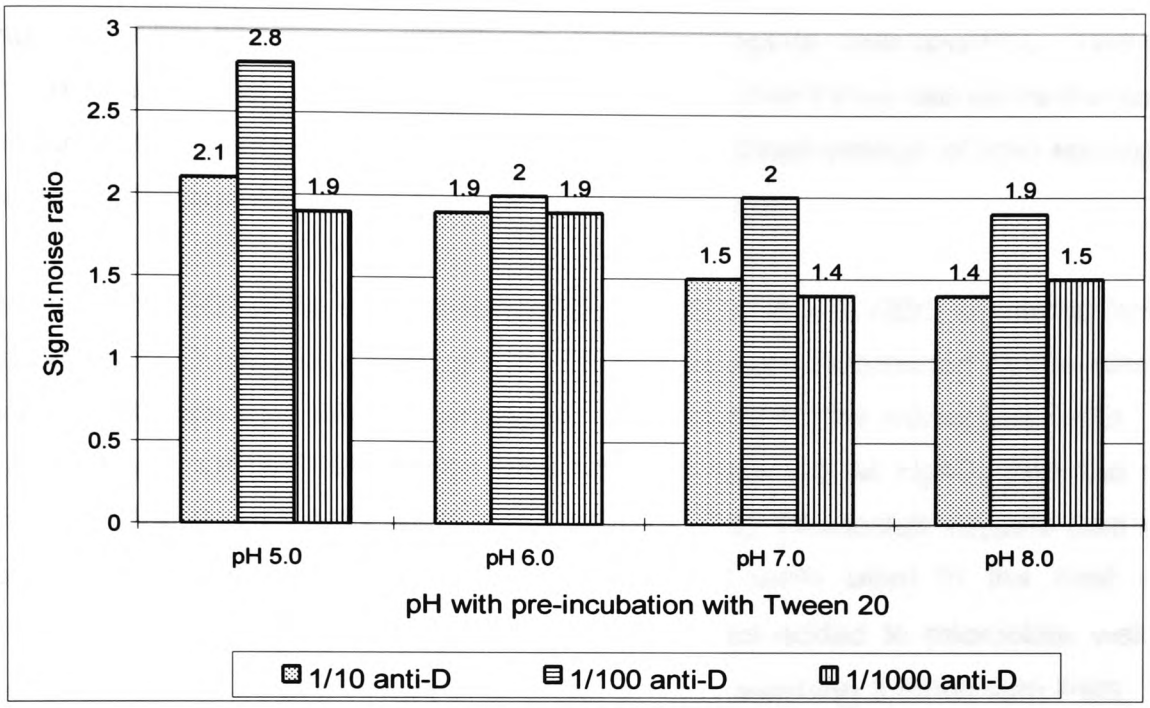
Microplate wells were coated with 10 μg ml^{-1} of PLL then effaced with 0.5% O R_1R_2 or O rr RBC. After blocking with 20% FCS for 60 minutes at ambient temperature, some wells were incubated with 0.5% Tween 20. 100 μl of 1/10 dilution of polyclonal anti-D in isotonic Sorensen's buffer at pH 5, 6, 7 or 8 was added to each well and incubated at 37°C for 30 minutes and the ELISA performed. Each test was performed in quadruplicate.

Figure 7b: Effect of pH on non-specific reactions with dilutions of polyclonal anti-D



Microplate wells were coated with 10 μg ml^{-1} of PLL then effaced with 0.5% O R_1R_2 or O rr RBC. They were then incubated with 20% FCS for 60 minutes at ambient temperature. 100 μl of 1/10, 1/100 or 1/1000 dilution of polyclonal anti-D in isotonic Sorensen's buffer at pH 5, 6, 7 or 8 was added to each well and incubated at 37°C for 30 minutes. Each test was performed in quadruplicate by the ELISA.

Figure 7c: Effect of pH on non-specific reactions with dilutions of polyclonal anti-D - wells blocked for a second time with 0.5% Tween 20



Microplate wells were coated with 10 μg ml^{-1} of PLL then effaced with 0.5% O R_1R_2 or O rr RBC. After blocking with 20% FCS for 60 minutes at ambient temperature, the wells were blocked for a second time with 0.5% Tween 20. 100 μl of 1/10, 1/100 or 1/1000 dilution of polyclonal anti-D in isotonic Sorensen's buffer at pH 5, 6, 7 or 8 was added to each well and incubated at 37°C for 30 minutes. Each test was performed in quadruplicate by the ELISA.

Studies on the use of lectins and antibodies to attach RBC to microplate wells

The experiments so far have shown that most of the background reactions were due to immunoglobulins binding non-specifically to the microplate wells and PLL. This phase of the investigation is to look at alternatives to PLL to see if they can serve the purpose of attachment of red cells, but without suffering the disadvantage of also serving as a suitable site for non-specific immunoglobulin absorption.

Green et al (1993) have successfully used lectins to attach RBC to microplates in a solid phase ABO reverse grouping test. Reactions were determined by the binding of indicator red cells to antibody sensitised RBC bound to the microplate wells. After incubation with OPD, the reaction was stopped with 0.5 M H_2SO_4 and the colour measured at 492 nm. Artocarpus integrifolia (jacalin), Phaseolus vulgaris (red kidney bean), Ulex europaeus (gorse) and lentil lectins were used in the next set of experiments. A 100 µl of dilution of each lectin was added to microplate wells and incubated for 2 hours at ambient temperature. After washing 3 times with PBS, 100 µl of 0.5% RBC was added to each well and the microplates were either centrifuged at 32g for 2 minutes or left overnight at 2-8°C. Red cells attachment was observed with only jacalin and gorse lectins. However, no improvement in the non-specific binding in the ELISA was obtained with this substitution (data not shown).

Microplate wells coated with various concentrations of anti-IgA overnight were incubated with RBC sensitised with affinity purified human IgA protein using the tannic acid method. The RBC were poorly bound and the non-specific reactions were still too high.

One of the constituents of red cell membranes is glycophorin A. The aim of this experiment is to coat the microplate wells with dilutions of anti-glycophorin A and to use this to bind the RBC to the solid-phase. This resulted in poorly bound monolayers and the background readings were still too high.

Red blood cells bind directly to Nunc flat bottom and Nunc and Sterilin U bottom microplate wells through centrifugation. This addressed the issue of non-specific binding to PLL leaving the element of direct interaction between the microplate plastic

and human serum proteins. However, the monolayer formed was dissociated with the use of automated plate washer. Lowering the pressure under which the wash fluid was dispensed and fixing the monolayers to the wells with glutaraldehyde reduced this. There was still variability in the monolayers and the non-specific reactions were still too high with poor discrimination between positive and negative reactors for quantitative work. However, the method is suitable for qualitative work using haemagglutination as an indication of positive reactions.

DISCUSSION

There are situations in medicine where there is a need to quantitate red cell antibodies for the purposes of diagnosis and treatment. This is usually performed by titration or in the case of anti-D and anti-c, quantitation using a continuous flow analyser. The continuous flow analyser relies on haemagglutination as an indication of antigen antibody reaction. Because antibodies in serum are polyclonal and the various species of anti-D do not agglutinate to the same degree, the test does not accurately reflect the concentration of anti-D. Also, because British Standards are only available for anti-D and anti-c, only these specificities can be quantitated using this methodology.

Liquid-phase haemagglutination tests in tubes have been the mainstay of red cell serology work since the discovery of the ABO blood group system by Landsteiner. Although generally reliable, they are cumbersome and their subjective readings limit their use in quantitative assays. The potential advantages of solid-phase techniques offering reliable, objective, quantitative and sensitive alternatives to conventional red cell serology with the ability to automate was discussed by Rosenfield et al (1978).

The use of microplates with automated samplers and readers have improved the reliability of the liquid phase haemagglutination assays. The 1980s saw the development of solid phase tests for routine pre-transfusion testing. These assays were performed in liquid phase in one microplate solid phase antiglobulin test - SPAT (Ross & Gordon, 1986) or in two microplates solid phase agglutination - SPAG (Biotest AG, Frankfurt, Germany). Both rely on the attachment of sensitised red cells onto antibody coated U well microplate to indicate positive reactions. With the true solid-phase test of Beck et al (1984) whole or ghost RBC for antibody screening or identification were coated onto microplate wells and the AHG test performed in them. Both systems rely on haemagglutination to indicate the end result and as such limits their use in providing accurate quantitative information.

The development of an enzyme-linked immunosorbent assay (ELISA) for quantitating rabbit IgG (Engvall and Perlmann, 1971) has led to thousands of publications of its applications in various scientific fields including red cell serology. The majority of the publications for applications in red cell serology were therefore based on the use of the

enzyme conjugated antiglobulin and substrate as an indication of the reactions in liquid phase tests. The published applications of solid-phase ELISA in this field were based on the immobilisation of purified red cell antigens to test for the corresponding antibodies (Spitalnik et al, 1983 & 1985, Buchs & Nydegger, 1988, Yared et al, 1997) or of antiglobulin to test for red cell bound antibodies (Francois-Gerard & Opret-Meunier, 1987).

The aim of this project was to develop a solid-phase ELISA to quantitate for red cell antibodies using RBC immobilised onto microplate wells. Using monoclonal antisera, the initial experiments show (figures 2a to 2c) that:

(i) the principle of the assay does work,

(ii) the assay can be specific, although in these particular tests the specificity may be due more to the nature of the antisera in that they do not contain antibody of other specificities, and

(iii) the red cells remain bound to the solid phase throughout the assay as determined by the specific results.

The reactions of monoclonal anti-A with A_1 and A_2 RBC by the ELISA confirms the observation in conventional tests that stronger reaction (higher optical densities) are obtained with A_1 than A_2 cells because of the larger number of antigen sites per cell of 1000000 and 250000 respectively (Mollison et al, 1987). The maximum absorbance for the monoclonal anti-D was lower than that obtained for monoclonal anti-A and anti-B probably because there are fewer D sites on R_1R_2 cells than A and B sites on red cells of the appropriate blood groups. Also, it is possible that there are fewer anti-D molecules than anti-A and anti-B in the monoclonal sera tested. However, the background noise iridicated by the reactions of the monoclonal antisera with the corresponding cells was 0.1 or less absorbance units.

The signal:noise ratio is a measure of the relative specificity of the assay, i.e., how well the assay distinguishes between specific antigen binding and non-specific binding - the higher the ratio, the better is the discrimination between positive and negative reactions. Figure 2d shows the signal:noise ratios of the three monoclonal antibodies with the corresponding antigens. Higher ratios were obtained for monoclonal anti-A

and anti-B with A_1 and B cells respectively, probably a reflection of the larger number of antigen sites per cell.

The next experiments were performed to determine the optimum conditions for the solid-phase ELISA. The optimum concentrations of PLL and red cells were 10 to 20 μg ml^{-1} (figure 3a) and 0.5% RBC (figure 3b), respectively. Scott (1991) found that the optimum concentrations of PLL for binding red cells were between 1 and 10 μg ml^{-1} and Gemke et al (1986) used 10 μl of 100 μg ml^{-1} in Terasaki plates. Sallander et al 1995) used 0.4% RBC for effacement in a solid-phase test using haemagglutination for visualising the reactions.

Ionic strength has been shown to have a powerful effect on reactions in conventional liquid phase tests. There is an increased rate of antibody/antigen reaction with red cells suspended in LISS, with optimum sensitisation accomplished in between 20 and 30 minutes. Diluting the antisera in LISS or PBS was used to investigate whether the enhanced reactivity with low ionic strengths can be used to advantage in solid phase tests. The experiment showed (figure 3c) that at the dilution tested there was increased binding of anti-D in LISS, which has reached maximum binding at 30 minutes. Maximum binding of anti-D in PBS was achieved in 60 minutes.

The optimum time and temperature for the conjugate incubation (figure 3d) was 60 minutes and 37°C, respectively. The highest absorbance for substrate incubation was obtained at 37°C at 60 minutes (figure 3e). Leikola and Perkins (1980a) did not find a significant difference between conjugate incubation at 21°C and 37°C but there was increased rate of reaction at 45°C. Postaway et al (1985) found that the conjugate and substrate incubation time can be reduced to 60 minutes at 37°C in a liquid-phase assay.

Sodium hydroxide is generally accepted as the reagent of choice to stop the reaction of alkaline phosphatase with pNPP but the successful use of 0.1M EDTA was reported (Reppun et al, 1983). In this study, NaOH at the three concentrations tested was confirmed to be effective at stopping the substrate conversion (figure 3i).

Bromelain is used to enhance antibody binding in conventional liquid phase tests and in anti-D quantitation on single channel analysers. Pre-treatment of the red cells with bromelain before attachment to microplate wells resulted in increased binding of antibody to the red cell (figure 3j) but the end point was the same as with untreated cells. Similar results were obtained by Reppun et al (1983).

When this optimised assay was used for testing polyclonal antibodies, unacceptably high background reactions were obtained (figure 4a and 4b). Serum lacking detectable red cell antibody by conventional techniques also gave comparable background readings (figure 4c). The background reaction was proportional to the concentration of test serum and was independent of the specificity of the antibody. The problem of non-specific reactions was encountered in liquid phase ELISA tests for the detection and quantitation of RBC antibodies (Leikola & Perkins, 1980a, Lown et al, 1984, Douglas et al, 1987, Greenwalt et al, 1992a). This was due mainly to adherence of immunoglobulin to the glass surfaces in which the tests were performed. This was avoided in subsequent tests by transferring the red cells to a different tube after reaction with the enzyme-linked antiglobulin and the use of BSA in the wash solution. Leikola & Perkins (1980a) also found slight adherence of the enzyme conjugated antiglobulin onto unsensitised RBC, which together with intrinsic phosphate membrane enzyme formed the baseline OD values.

Scott (1988) also found high background (non-specific) reactions in solid-phase ELISA. Some of the possible reasons for the high background reactions are the:

a) concentration of the total antibody in the test serum,
b) binding of the test antibody to PLL,
c) binding of the test antibody to the plastic of the wells of the microplate,
d) binding of the test antibody non-specifically to the red cells,
e) binding of the enzyme conjugated antiglobulin to PLL,
f) binding of the enzyme conjugated antiglobulin to the wells of the microplate,
g) binding of the enzyme conjugated antiglobulin to the red cells,
h) conversion of the substrate by remnant red cell haemoglobin peroxidase or alkaline phosphatase.

The concentration of antibody in monoclonal reagent is very low and can be measured in nanograms per millilitre. These antibodies are specific for the antigens being tested. Polyclonal sera have approximately 10 mg ml^{-1} of IgG and in order to test for a specific antibody one must select one out of a possible 1 million IgG molecules based on a difference in idiotypic binding (Graves, 1988). This leaves a considerable excess of antibody molecules, which can bind non-specifically to the red cells or solid phase unless these binding sites are blocked.

Figure 4d shows the results of serial dilutions of monoclonal anti-D in AB serum tested with O R_1R_2 and O rr RBC immobilised on microplate wells. The discrimination between antigen positive and negative cells with undiluted monoclonal anti-D was very good and the non-specific reaction was less the 0.1 OD units. When the monoclonal anti-D was diluted in AB serum, the antibodies in the AB serum seem to be contributing significantly to the non-specific reactions. As the concentration of the monoclonal anti-D becomes lower, the concentration of AB serum increases resulting in the higher non-specific binding seen in figure 4d. This shows that the total concentration of antibody in the test serum does have a significant effect on non-specific binding.

In order to confirm the effect of total immunoglobulin concentration on non-specific binding, O R_1R_2 red cells were used to absorb anti-D from a polyclonal sample. After washing the red cells to remove unbound antibody, the bound antibody was eluted at 56°C and serial dilutions were tested by the solid phase ELISA with both O R_1R_2 and O rr RBC. Figure 4e shows that there was good discrimination and the background noise was low. Scott (1988) has shown that the high background was due to immunoglobulin binding by adding purified polyclonal mouse IgG to monoclonal reagent, which resulted in high non-specific reactions. However, Scott (1988) also found that the background problem persisted when IgG from polyclonal anti-A was purified away from other serum components by ion-exchange chromatography and tested by solid phase ELISA.

In order to determine the cause of the non-specific reactions, experiments were performed without red cell attached to the solid phase. These experiments show (figure 5a) that:

(i) there is substrate conversion in the absence of PLL indicating that test antibody or conjugate has bound to the solid phase,

(ii) in the presence of various concentrations of PLL, the non-specific reaction was initially proportional to the concentration of PLL with saturation around 75 μg ml^{-1}.

In order to determine the contribution of test antibody binding non-specifically to the red cells, the next experiment was carried out in two stages. Firstly, the O Rh D positive and O Rh D negative red cells, were incubated with several dilutions of polyclonal anti-D or AB serum in glass tubes then washed to remove unbound antibodies. Secondly, the cells were resuspended then immobilised onto PLL coated microplate wells, blocked then tested with conjugate and substrate and read on a plate reader. The results in figure 5b shows very good discrimination and that the contribution of test antibody binding non-specifically to the red cells and conjugate binding to the red cells and/or solid phase is less than 0.2 absorbance units.

The above experiments have shown that the high background reaction was due mainly to the test antibody binding non-specifically to the solid phase and PLL. There was also some non-specific binding of test antibody to the test red cells. Additional experiments have shown that non-specific binding of conjugate or substrate conversion in the absence of conjugate (due to residual haemoglobin peroxidase) was negligible. Reppun et al (1983) reported that substrate and/or conjugate without cells contributed little to the background absorbance. Leikola and Perkins (1980) found slight adherence of conjugate onto unsensitised RBC.

The next set of experiments was designed to reduce the non-specific binding of test antibody to the solid phase or PLL. Red cells were omitted and various concentrations of α_1-acid glycoprotein (AGP), bovine serum albumin (BSA), rabbit serum, casein, foetal calf serum (FCS) and Tween 20 were tested to reduce the non-specific binding of test antibodies. Casein is a mixture of milk proteins, of different size, amino acid sequence, degree of glycosylation and sulphation and surface charge. Kenna et al (1985) found casein to be superior to BSA at reducing but not eliminating non-specific binding. The authors found that the addition of a microbial agent, Thiomersal to a casein wash solution was required to achieve maximum blocking. Graves (1988)

showed that rabbit IgG binding was high to positively charged surfaces and low to negative ones. His investigations of the binding of IgG to surface immobilised proteins and synthetic peptides of differing isoelectric points showed that AGP, the most negatively charged substance tested (pI=2.7), was the most effective for improving surface binding specificity. He also studied BSA (pI=4.7), human serum albumin (pI=4.9) and lactoferrin, pI=8.7) which were less effective.

Figures 6a, 6b and 6c show that the best result was obtained with 20% fetal calf serum although it did not completely remove the background reaction nor was there any improvement by including Thiomersal in the wash and 'blocking' solutions. Treatment of the PLL coated wells with casein or fetal calf serum prevented the binding of RBC to the solid phase suggesting that they might be capable of blocking non-specific binding to PLL molecules. PBS containing 0.05% Tween 20 was poor at preventing the attachment of RBC to PLL.

When 20% FCS was used to block non-specific antibody binding in the red cell ELISA assay there was some improvement in the discrimination (figure 6d) but the background reaction was still too high at absorbances greater than 0.4 units for 1/10 dilution of anti-D. Incubating with 0.5% Tween 20 after coating with 20% FCS increased the background noise but improved the discrimination.

There was no significant effect of pH (between 5 and 8) on non-specific antibody binding if the wells of the microplate were blocked with 20% FCS (figure 7a). If the wells of the microplate were blocked first with 20% FCS followed by 0.5% Tween 20, there was increased antibody binding which was more pronounced at pH 5.0 (figure 7b and 7c). Using this knowledge in experiments did not improve the assay.

The use of lectins or antibodies to bind the red cell to the solid phase did not improve the background reactions. Nunc flat bottom ELISA microplates enabled red cells to be bound directly to the solid-phase but this did not result in a reduction in the background reactions. Red cells were also bound directly onto Nunc and Sterilin U well microplates but the monolayer dissociated during washing with a plate washer. The monolayer was stabilised by reducing the force under which the wash fluid was dispensed and by fixing the monolayer to the wells with 0.25% glutaraldehyde. The non-specific reaction was

still too high and the discrimination poor. The use of glutaraldehyde to fix RBC to the solid-phase was shown to reduce the sensitivity of the procedure (Stocker & Heuser, 1979, Lown et al, 1984).

In a true solid phase test all the reactions occur in a single well or tube. The use of BSA, Tween 20 (Voller et al, 1976), casein and AGP (Graves, 1988) have reduced the non-specific binding of test antibodies in other ELISA assays which are sensitive and specific. In the present study these blocking agents have not reduced the non-specific reaction sufficiently in a solid phase RBC ELISA for it to be used routinely. Scott (1988) concluded that the high background reading obtained in a solid phase RBC ELISA is due to non-specific binding of IgG and conjugate to the immobilised red cells. The background reaction was present when the RBC were attached to the solid phase by PLL, anti-red cell antibody or lectin and was not dependent on the type of microplate used. The use of LISS for diluting test antibodies also increased the background binding.

The results of experiments in the present investigation have shown that the amount of non-specific binding due to IgG and conjugate to the red cells is extremely small and does not seem to depend on the concentration of antibody as shown in figure 5b. In this experiment RhD positive and RhD negative RBC were incubated with doubling dilutions of anti-D, washed then adhered to microplate wells via PLL. The background reaction was less than 0.2 absorbance units and the discrimination was very good. This confirmed the findings of Leikola and Perkins (1980a) that antibody proteins are adsorbed onto the solid phase causing high background reactions and this is dependent on the total IgG or IgM concentration.

The results also show that the amount of non-specific binding when red cells were not used was proportional to the concentration of the test serum and PLL. This suggests that unless blocked, test antibody will bind to any free PLL molecules. Replacement of PLL with lectins should reduce this non-specific binding. Experiments have shown that the use of lectins to anchor red cells to the microplate wells has also resulted in a high background reaction.

A successful ELISA is based on antibodies or antigens immobilised onto a solid support. This is also true when purified red cell antigens were used as solid-phases. It is possible that whole or ghost red cells can trap antibody molecules within the red cells or in the intracellular spaces, which are difficult to wash away but accessible to conjugate and substrate, adding to the true background readings.

In conclusion, the high background reaction in the RBC solid phase ELISA is due primarily to test antibody binding to the solid phase and PLL. It is also possible that trapped antibody molecules within ghost red cells and in the intracellular spaces can cause conjugate binding and substrate conversion, adding to the background. There is also some non-specific binding of test antibody to the RBC but that due to the conjugate and substrate conversion by remnant haemoglobin peroxidase and alkaline phosphatase is negligible. The use of casein, Tween 20, FCS, rabbit serum, bovine serum albumin and AGP did not significantly reduce the background noise or the discrimination. Because of the failure to develop a workable solid-phase ELISA for red cell antibody quantitation, the correlation with HDN could not be performed.

FURTHER WORK

As attempts to develop a reliable solid phase ELISA for red cell antibody quantitation were unsuccessful, further work would be based on the use of flow cytometry. Austin et al (1995) and Hilden et al (1997) have shown the use of flow cytometry for anti-D quantitation to be reproducible and reliable. The correlation with the single channel autoanalyser was reported to be good by both authors. This method could be used to quantitate IgG subclass red cell antibody and determine if there is any correlation with the clinical outcome of HDN.

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