Improved antibody detection by the use of range expansion and longer filter wavelength in a low ionic strength-protamine sulphate Auto-Analyzer system

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SYNOPSIS Range expansion, achieved by insertion of a variable resistance between the colorimeter and the recorder together with the use of 550 nm colorimeter filters, has resulted in markedly improved sensitivity for antibody detection, and improved sample identification, in a low ionic strength-protamine sulphate (LISPS) system. Range expansion also permits a lower concentration of red cells to be used, thus economizing on fully typed cells. Glycerol stored frozen cells were found to be only slightly less sensitive than fresh cells in this system.

An automated antibody screening system should detect all antibodies of clinical significance with a sensitivity at least equal to and preferably better than the manual method it replaces. The bromelinmethyl cellulose (BMC) system described by Marsh *et al* (1968) has the disadvantage of not detecting weak antibodies to the enzyme degradable antigens MNS and Fy^a, and in our hands did not detect weak anti-K. Our attention was drawn to the low ionic strength-polybrene/protamine sulphate system described by Lalezari (1967; 1968).

In common with Dawes and Goldsmith (1975), we found that polybrene caused the aggregated cells to adhere to the glass coils, owing to the high degree of surface absorption of polybrene to glass, as noted by Greenwalt and Steane (1973). This resulted in cell baseline fluctuations, excessive 'carry-over', and poor sample identification. Reduction in the polybrene concentration to overcome these problems resulted in a loss of sensitivity. These difficulties were not experienced when protamine sulphate (Lalezari, 1967; Judd, 1971) was used, and this became the reagent of choice. Experiments revealed that the LISPS system enhanced the reaction of MNS and Fy^a antibodies. Anti-K gave reactions equivalent to manual antiglobulin tests, but the system was slightly less sensitive than the BMC system in detecting weak anti-D, thus confirming the findings of Perrault and Högman (1971).

In developing an automated screening system, it is found that working conditions represent a compromise between sensitivity, as shown by changes in optical density between the baseline and top of the peak, and sample identification. Alterations to the manifold or the chemistry did not produce the sought-after improvement in peak height and sample identification. Therefore the suitability of a range expander (Owen, 1970; Annan and Fisher, 1971) for increasing peak height and sample identification in an inverse colorimetric system such as this was investigated. During the course of the experiments it was decided to examine the effect of filter wavelength, in combination with range expansion, on peak height.

Marsh *et al* (1968) have drawn attention to the need for a regular supply of fully typed fresh cells for antibody screening. Habibi *et al* (1973) and Perrault and Högman (1971) overcame this problem by utilizing a selected panel of donors, but the provision of sufficient fully typed fresh cells for antibody screening is often difficult. Marsh *et al* (1968) and Moore (1969) found that glycerol frozen cells failed to form satisfactory rouleaux in the BMC system, although Kolberg and Nordhagen (1975) have reported satisfactory results with frozen cells in this system. Judd (1971) reported that cells frozen in glycerol or liquid nitrogen were satisfactory in the LISPS system. Therefore a study has been carried out comparing fresh cells with cells frozen in glycerol by the method of Mollison (1972).

Material and methods

The LISPS manifold is basically similar to that described by Lalezari (1968). A two-channel machine was assembled around Technicon AA1 modules. Two range expander units were constructed as described by Annan and Fisher (1971).

REAGENTS

Protamine sulphate (Salmine), Fisons Ltd, batch 04 used at a concentration of 0.1% in 0.9% saline. Acid dextrose, pH 3.20, 5.0% dextrose BP in distilled water containing 2.0 ml of 1.0 M citric acid/litre. Resuspending solution 0.2 M trisodium citrate pH 8.20. Wash solution 0.3 M trisodium citrate. Triton X-100 (Koch-Light Ltd) 0.5% in distilled water. Tween 20 (Koch-Light Ltd) 10.0% in distilled water added to resuspending and wash solutions to a final concentration of 0.02%.

TEST CELLS

Packed cells were stored at -20°C in 3.0 M glycerol and recovered for use by overnight dialysis against phosphate buffered saline pH 6.80. They were washed four times and suspended in the same buffer. The strength of the test cells was adjusted according to the colorimeter filter wavelength in use, that is, 14.0% for use with 550 nm filters and 6.0% for 440 nm filters respectively.

Fresh cells were obtained from acid-citrate dextrose blood donations less than 7 days old, washed four times in buffered saline pH 6.80, and suspended in the same buffered solution.

TEST SERA

An anti-D reagent with an albumin titre of 64 (21.9 μg anti-D/ml), a 'papain only' anti-D (approx. 0.01 μg anti-D/ml), and anti-K with an antiglobulin titre of 4, and an anti-Fy^a with an antiglobulin titre of 8 were serially diluted in antibody free serum from a group AB donor.

PROCEDURE

Three sample cups containing the same antibody free group AB serum were placed between each dilution. The anti-D sera were tested $\times$ R_{2r} cells and the anti-K $\times$ Kk cells, and the anti-Fy^a $\times$ Fy (a+b+) cells. The same cell was used in both channels and comparative measurements were made with 440 nm filters in one channel and 550 nm filters in the second channel. The concentration of the test cell suspension was adjusted to keep the cell baseline in the range 0.70-0.95 optical density (OD) units. The dilutions were then resampled with the colorimeter 100% T control set at maximum, and with a No. 1 aperture on the reference photocell to give maximum range

expansion. The cell baseline was adjusted to 1.0-2.0 OD units by means of the range expander.

The cell suspensions were then diluted to half their original concentrations and the dilutions were resampled after the cell baseline was adjusted to 0.70-0.95 OD units using the range expander.

SAMPLING RATE

Sixty samples/hour with a 2:1 sample:wash ratio. The manifold was at ambient temperature, but the resuspending solution was cooled in melting-ice before introduction into the manifold.

Peak height was measured as change in optical density (Δ OD) from the cell baseline.

Results

Figure 1a shows the results obtained with 440 nm

Fig 1 Range expansion and sensitivity of anti-K detection: $\square$ 550 nm filters; $\blacksquare$ 440 nm filters. (a) no range expansion, 550 nm and 440 nm filters, 14.0% and 6.0% cells respectively; (b) maximum range expansion, 550 and 440 nm filters, 14.0% and 6.0% cells respectively; (c) maximum range expansion, 550 nm and 440 nm filters, 7.0% and 3.0% cells respectively.

and 550 nm filters used under their optimum conditions, but without range expansion. In order to maintain the cell baseline within the limits of 0.70-0.95 OD units the cell strengths were adjusted to 14.0% with the 550 nm filters and to 6.0% with the 440 nm filters. The peak heights obtained with the 550 nm filters and 14.0% cells were about one and a half times higher than those obtained with 440 nm filters and 6.0% cells. Figure 1b shows the results

obtained with maximum range expansion using the same cell concentrations as above. Operation of the range expander brought the cell baseline to between 1.0 and 2.0 OD units. The range expander increased peak height by an average of 80% with 550 nm filters and of 60% with 440 nm filters. Figure 1c shows the effect of maximum range expansion when the cell strengths were decreased from 14.0% to 7.0% and from 6.0% to 3.0%.

Reduction in the cell concentration allowed an increase in the amount of range expansion obtained because the variable resistance of the range expander needed to be turned to maximum resistance to bring the cell baseline to 1.0-2.0 OD units. In practice, any further reduction in the cell concentration to increase range expansion does not give any improvement with either set of filters. The use of cell concentrations weaker than 7% with 550 nm filters, or 6% with 440 nm filters, does not reliably improve the sensitivity for antibody detection. Also it has the disadvantage of increasing baseline variations beyond useful limits.

The use of a No. 1 reference photocell aperture and turning the '100% T' colorimeter control to maximum allowed a useful increase in range expansion, giving a 20% increase in antibody sensitivity for the anti-D, anti-Kell, anti-Fy^a sera tested.

With 550 nm filters, 7.0% cells, and maximum range expansion the increase in peak height was on average 170% greater than that obtained with 14.0% cells and no range expansion. With 440 nm filters and 3.0% cells the increase in peak height was on average 100% greater than that obtained with 6.0% cells and no range expansion.

The AB serum negative control in each set of tests indicates the change in optical density between the cell baseline and a serum sample, which provides the sample identification.

Figure 2 shows the antibody profiles obtained with an anti-K with and without maximum range expansion, and using both 550 nm and 440 nm filters at their respective optimum cell concentrations. The best results were obtained by using the 550 nm filters and full range expansion.

Similar results were obtained with the other antibodies tested. The conditions optimum for anti-K were also found to be optimum for anti-D and anti-Fy^a. Range expansion also increased the peak height of the neutral AB results, but this had the advantage of providing better sample identification.

COMPARISON OF FRESH AND GLYCEROL FROZEN CELLS

Experiments with anti-D, anti-K, and anti-Fy^a antibodies under parallel conditions with stored frozen cells and fresh cells of the same genotypes revealed that the sensitivity of antibody detection

Fig 2 Examples of chart profiles obtained with anti-K: (a) maximum range expansion, 550 nm filters, 7.0% cells; (b) no range expansion, 550 nm filters, 14.0% cells; (c) maximum range expansion, 440 nm filters, 6.0% cells; (d) no range expansion, 440 nm filters, 6.0% cells.

with frozen cells was only slightly below that of fresh cells. Peak heights were on average about 10% lower with frozen cells.

Discussion

The three anti-sera used throughout this study were a 'papain only' anti-D, a weak anti-K, and a weak anti-Fy^a. They were selected as the test sera because they are the most difficult type of antibodies to detect. Our experience has shown that a system which will detect these antibodies will give satisfactory results with most other clinically significant antibodies with the possible exception of certain anti-Lewis antibodies. It is important to select 'weak' antibodies, which presumably like 'papain only' anti-D have low association constants (Casey *et al*, 1972) when investigating optimum conditions for antibody detection. The use of dilutions of more avid antibodies is not as satisfactory since they are too easily detected.

The principle of using a resistance in the circuit between the colorimeter and recorder of an Auto-Analyzer to increase peak height has been discussed by Owen (1970). Annan and Fisher (1971) have described a variable range expander unit easily connected to an AutoAnalyzer system.

We have investigated optimum filter wavelength and test cell concentration in combination with maximum range expansion in a LISPS antibody screening system. Initial experiments showed that

without range expansion there was no difference in sensitivity between 440 nm and 550 nm filters, which is in accordance with the findings of Perrault and Högman (1971). However, baseline 'noise' levels were much greater with 550 nm than with 440 nm filters, owing to the smaller change in optical density produced by the lower test cell concentration that must be used with the latter. Range expansion magnified the 'noise' as well as the signal, as noted by Owen (1970). It is important to keep baseline 'noise' levels to the minimum as large fluctuations are manifested as 'false positive' peaks and give poor sample identification. Baseline 'noise' levels are easily brought to an acceptable level by the addition of Tween 20 to the wash and resuspending solutions as recommended by Gunson *et al* (1972). This did not cause any significant reduction in sensitivity for antibody detection, and did not cause the aggregated cells to adhere to the glass coils as Dawes and Goldsmith (1975) observed with polybrene.

Habibi *et al* (1973), Judd (1971), Lalezari (1968), and Perrault and Högman (1971) used 440 nm colorimeter filters with test cell concentrations between 6.5 and 10.0%. Dawes and Goldsmith (1975) described the use of 550 nm filters with a test cell concentration of 15.0%. In this study we have demonstrated that with maximum range expansion the optimum cell concentrations are 7.0% for 550 nm filters and 6.0% for 440 nm filters. Reduction in the test cell concentration from 14.0% to 7.0% allowed a larger degree of range expansion, giving increased antibody sensitivity and improved sample identification while allowing a 50% saving in the quantity of fully typed cells needed to run the machine. Reduction of the test cell concentration below 7.0% resulted in undesirable increased baseline fluctuations. Range expansion also increased peak height of the neutral AB serum results, but this was beneficial, resulting in improved sample identification without the need for antibody containing 'marker' samples.

In common with Marsh *et al* (1968), we found that glycerol frozen cells show greatly reduced sensitivity in the BMC system for anti-K, anti-Fy^a, anti-Jk^a, and 'papain only' anti-D. Our attempts to obtain improved sensitivity in the BMC system by increasing the concentration of methyl cellulose, as suggested by Kolberg and Nordhagen (1975), did not give satisfactory results. Therefore we cannot recommend

the use of frozen cells in a BMC system. However, glycerol frozen cells reacted satisfactorily in the LISPS system, confirming the findings of Judd (1971). This has the advantage that a whole donation may be stored and aliquots recovered for use in the absence of a regular supply of fresh fully typed cells.

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