The effect of ambient temperature on the anti-D assay using the AutoAnalyzer

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SYNOPSIS Using the AutoAnalyzer, the percentage agglutination effected by the anti-D antisera studied showed a varied dependence on the ambient temperature over the manifold subsequent to the incubation period at 37°C. This leads to assays which are a function of the ambient temperature. It is suggested that the entry of a relatively large volume of rouleaux-dispersing agent results in an elution of bound antibody to a new position of equilibrium, the shift being dependent on the particular equilibrium constant of the antibody and the rate of its attainment on the ambient temperature. A constant ambient temperature will lead to greater accuracy of anti-D assay.

Anti-D assay is achieved in the AutoAnalyzer by the decantation of agglutinated cells after the dispersal of rouleaux deliberately induced to potentiate agglutination. With the exception of the incubation coils, the manifold is conventionally at ambient temperature which may vary throughout the working day. Moore and Fernandez (1972) stated that maintenance of a constant ambient temperature of $20 \pm 1^\circ\text{C}$ improved the percentage agglutination obtained with most antibodies, resulted in less drift of the baseline and led to an improvement in the reproducibility of replicate tests, indicating that ambient temperature affected the agglutination reaction in the manifold. For the assay of anti-D to be independent of changes in ambient temperature such changes must have an identical effect on the degree of agglutination produced by the test anti-D antisera and the standard antiserum against which the test sera are assayed.

The purpose of this preliminary communication is to present experimental evidence which reveals that changes in ambient temperature do not affect to the same extent the degree of agglutination produced by three different anti-D antisera.

Materials and Methods

The basic apparatus used in these experiments was the Technicon AutoAnalyzer manifold described

by Marsh, Nichols, and Jenkins (1968) with the modifications previously described (Gunson, Phillips, and Stratton, 1972a). With the exception of the two 28-turn, jacketed incubation coils maintained at 37°C, the manifold was placed in a water bath. Two 14-turn, glass coils were inserted in the rouleaux-dispersal reagent line to enable this reagent to equilibrate, before its entry into the manifold, with the temperature of the water bath which was maintained to within $\pm 0.1^\circ\text{C}$ by a Grant LC 10 circulator (Grant Instruments, Cambridge). The reagents were as described previously (Gunson *et al*, 1972a). The test erythrocytes comprised a pool of three red cell samples, group O R₁R₁ or group O rr, pretreated with bromelain by the method described by Gunson, Phillips, and Stratton (1972b).

Three anti-D antisera were investigated; serum 2 (see figure) is the working standard used in the laboratory for the quantitation of anti-D and comprises a pool of 32 anti-D antisera derived from Rh(D)-negative males deliberately immunized with Rh(D)-positive red cells and from females isoimmunized as a result of Rh(D) heterospecific pregnancy.

Serum 1 was obtained from a Rh(D) immunized male after six spaced injections of Rh(D)-positive red cells, anti-D having been present in the serum of this individual for four years. Serum 3 was obtained from an Rh(D)-negative male early in the immune response to a single injection of Rh(D)-positive red cells.

The presence of anti-D in each serum, together with a weak anti-G component in serum 1, was con-

Fig The percentage agglutination of R_1R_1 test cells obtained with three anti-D antisera in the AutoAnalyzer over the ambient temperature range 5-35°C.

firmed by serological tests using a large panel of test red cells of known phenotype. Antibodies of other specificities were not detected.

The percentage agglutination effected by an antiserum was calculated as the change in haemolysate optical density expressed as a percentage of the optical density of the baseline.

Experimental Results

A single, suitable dilution of each of the three antisera was prepared. Each dilution was aspirated twice over a range of ambient temperatures from 5 to 35°C in steps of five degrees. The average percentage agglutination achieved with R_1R_1 test cells for each serum at each of the ambient temperatures is shown in the figure. From these curves it can be seen that changes in ambient temperature over the range 5 to 15°C produce similar effects on the agglutination achieved by the three antisera, with maximum agglutination at 15°C. Over the range 15 to 25°C, the degree of agglutination obtained with sera 1 and 2 is affected only to a slight degree by the change in ambient temperature. However, with serum 3 there is an obvious decrease in percentage agglutination over this temperature range. Differences in the dependence of agglutination on ambient temperature become marked above 25°C. Investigation of the antisera at dilutions effecting maximum agglutination in the region of 50 and 10% respectively gave similar results.

To exclude a progressive loss in sensitivity of the machine being the cause of the patterns observed in the figure, the experiment was repeated as above but with descending ambient temperatures. Similar results were obtained. Rh(D)-negative test cells failed to elicit detectable agglutination with the

three antisera over the specified range of ambient temperatures. The baseline was unaffected by changes in ambient temperature.

Discussion

The assay of anti-D using the AutoAnalyzer is dependent on the degree of agglutination achieved by a test antiserum relative to that produced by a standard anti-D preparation. It is clear from the experiment described above that serum 3 shows a greater degree of dependence upon ambient temperature than does the standard preparation, serum 2. Thus, the assay of antiserum 3 will be a function of ambient temperature. It is estimated that at 15°C the anti-D assay of this serum will be 16% greater than at 25°C. The dependence of serum 1 upon ambient temperature is less than that of the standard preparation, its assay at 15°C being about 3% lower than at 25°C.

Based on these preliminary studies it appears that anti-D assay should be performed with a constant ambient temperature between 10 and 15°C. The reason for this conclusion is twofold. First, the temperature profile over the range 5 to 15°C for each of the three antisera is similar, so that the behaviour of the test antisera mirrors that of the standard preparation. Secondly, it is in this range of ambient temperature that maximum agglutination is observed with each antiserum.

Moore and Fernandez (1972) noted that the reproducibility of the assay of certain antisera was improved at constant ambient temperature. It is evident that the assay of serum 3 will be affected by small changes in ambient temperature above 15°C, so that its replicate assay, when the manifold is exposed to fluctuations in room temperature, will be greater than that of antiserum 1. Indeed, it has been observed that, with an ambient temperature varying between 22 and 24°C, certain antisera exhibit a variability of assay some fourfold greater than that of the majority (Gunson, Phillips, and Stratton, 1974). This was partly explained by an individual test cell effect, but it is likely that fluctuations in ambient temperature contributed to the increased variability, a premise enhanced by the finding that the assay of serum 3, which shows marked dependence on ambient temperature, was one with an abnormal variability in the previous study.

The large volume of reagent introduced into the manifold subsequent to the incubation coils not only disperses rouleaux but also alters the total concentration of antigen and antibody so that a new position of equilibrium will be defined. The rate of readjustment in the position of equilibrium will be temperature dependent (Arrhenius equation). Calculations (to be

reported in full at a later date) have revealed that the shift in the position of equilibrium is more pronounced with antibodies having low equilibrium constants, in the order of 1×10^7 litre per mole. At higher ambient temperatures antibodies of low equilibrium constant will dissociate rapidly to the new position of equilibrium, whereas the degree of agglutination produced by antibodies of higher equilibrium constant will be affected to a lesser degree. It is known that antibodies developed early in the immune response tend to be of lower equilibrium constant than antibody produced after several antigenic stimuli (Eisen and Siskind, 1964; Holburn, Cleghorn, and Hughes-Jones, 1970). Serum 3 was obtained from a volunteer seven weeks after the first appearance of anti-D following a single antigenic stimulus and thus one would expect this antibody to have a lower equilibrium constant than that in serum 1, obtained some four years after the initial immunization and subsequent administration of spaced antigenic stimuli. Serum 2, comprising a pool of 32 anti-D antisera, would be expected to have an average value of equilibrium constant.

The reduced rate of attainment of the new position of equilibrium with decreasing ambient temperature does not explain the observed reduction in percentage agglutination at temperatures below 15°C. The reason for this is not immediately apparent but the effect does not differentiate between the three antisera studied (see fig).

From the results of the preliminary study, given above, it is evident that ambient temperature must be included with the other important variables which influence anti-D assay in the AutoAnalyzer. It is suggested that using the assay procedures described,

a constant ambient temperature in the range of 10 to 15°C should be maintained. It is realized that at this temperature there is the possibility that contaminating cold agglutinins may contribute to the agglutination and their effect has to be assessed using Rh(D)-negative red cells of the appropriate type. In practice, anti-D assays are carried out, for the most part, at dilutions of antiserum far beyond the endpoint of the agglutinating activity of the cold agglutinins. However, by altering the conditions existing in the manifold it may be possible to quantitate anti-D at an optimum ambient temperature in the region of 20°C. Experiments to this end are in progress and the results will be reported at a later date.

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