Technical method

Bulk preparation of reagents for hepatitis B testing

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Hepatitis B surface antigen (HBsAg) is a marker of one of the causative agents of post-transfusion (serum) hepatitis (Giles et al, 1969) and as such must be identified in donor blood before transfusion. Over the past few years, a number of techniques have been applied to the detection of HBsAg, ranging in sensitivity and sophistication from agar-gel diffusion (AGD, first generation test) to radioimmunoassay (RIA, third generation test). The experience of many laboratories clearly indicates that third generation methodology must be adopted if maximum efficiency of blood donor testing is to be achieved (Ling and Overby, 1972; Cayzer et al, 1974; Hopkins et al, 1975). Unfortunately, third generation reagents are available to the majority of blood transfusion centres only on a commercial basis, the cost ranging from 10 pence per test (Hepatett—Reverse Passive Haemagglutination (RPHA) Wellcome Reagents) to 50 pence per test or more, apparently depending on geographical location (AUSRIA-II, Radioimmunoassay (RIA), Abbott Laboratories).

In 1973 details were published of a tanned-cell haemagglutination inhibition (HAI) technique, utilizing Terasaki tissue culture trays, which achieved a sensitivity similar to RIA but at a fraction of the cost (Hopkins and Das, 1973). This technique has recently been evaluated by three Scottish regional transfusion centres testing over 70000 blood donations together with well documented HBsAg panels (Hopkins et al, 1975). The results indicated that, with appropriate training, the technique could be introduced into most regional transfusion centres, thereby keeping reagent costs to a minimum. A valid comparison between centres was possible only because reagents were prepared in bulk at one centre and were thus completely standardized.

This communication describes the bulk preparation of HBsAg coated glutaraldehyde fixed spherocytes in a quantity sufficient for approximately one million tests.

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Preparation of Fixed Spherocytes

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Reagent standardization should be enhanced if a uniform cell population is used for HBsAg coating. Uniformity is, in this case, achieved by selection of the most robust cells using a controlled hypotonic medium.

One unit (approximately 450 ml) of human O rhesus negative blood collected in anticoagulant (citrate phosphate dextrose), and not more than three weeks old, was obtained from the blood bank. It was first necessary to determine the molarity of phosphate buffer pH 7-2 required for the selection process. Isotonic (0-2 M) phosphate buffer containing 0-1% sodium azide was serially diluted in deionized water, and 0-1 ml of fresh, well mixed blood was added to 2 ml of each dilution of phosphate buffer in a test tube, mixed thoroughly, and allowed to stand for 60 minutes. That solution showing at least 50% haemolysis was arbitrarily taken as optimum. The molarity at this point was usually 0.045 M to 0.030 M.

The remainder of the unit of blood was then mixed with phosphate buffer (adjusted to optimum molarity) in the ratio 1 volume of cells to 4 volumes of buffer, and the bulk volume was split into 200 ml aliquots for ease of subsequent washing. After 60 minutes at room temperature the aliquots were spun at 1000 rev/min for 15 minutes at 20°C or room temperature using an MSE 6L centrifuge, the supernatant was removed, and fresh hypotonic buffer was added. This procedure was repeated until only a minimum of haemolysis was observed in the supernatant, and the remaining cells accounted for approximately 50% of those originally present. Wet film microscopy showed that the cells had lost their conventional biconcave morphology and become rounded. They were subsequently referred to as 'spherocytes'.

Fixation of Spherocytes
In a pilot experiment some spherocytes were divided into aliquots to which glutaraldehyde (Koch Light 25%) was added to give the following concentrations: 1 in 100, 1 in 200, 1 in 400, 1 in 800, 1 in 1600, 1 in 3200, and 1 in 6400. After thorough mixing, the cell aliquots containing fixative were left at room temperature overnight. The following morning cells from each aliquot were examined microscopically and subjected to a 'water-resistance' test, which...
involved adding 25 μl of 'settled' spherocytes to 2 ml of deionized water, when properly fixed cells resisted haemolysis. Dilutions of glutaraldehyde up to 1 in 1600 (0.06%) produced acceptable fixation, although dilutions up to 1 in 400 (0.25%) tended to distort the shape of the spherocytes, causing them to revert to biconcave discs. The remaining spherocytes (from the unit pack) were fixed in the appropriate concentration of glutaraldehyde (approximately 0.06%) as above and stored at 4°C in phosphate buffer containing 0.1% sodium azide. All solutions contained 0.1% sodium azide.

TANNING, SENSITIZATION, AND STABILIZATION OF FIXED SPHEROCYTES (for one million HAI tests)

Sixteen ml of packed glutaraldehyde-fixed spherocytes were washed twice with 500 ml volumes of physiological saline (MRC bottle spun at 1000 rev/min for 5 minutes in an MSE 6L centrifuge at room temperature). The deposited cells were resuspended in 500 ml of tannic acid (M & B at a 1 in 60000 dilution in phosphate buffer 0.15 M pH 7.2 containing 0.1% sodium azide and previously heated at 37°C) and left in a 37°C waterbath for 15 minutes with occasional mixing. The tanned cells were spun down as before, washed twice with 500 ml of phosphate buffered saline (PBS) pH 6.4 containing 0.1% sodium azide, and finally resuspended in 400 ml PBS pH 6.4.

Twenty millilitres HBsAg, prepared as previously described (Hopkins and Das, 1973) and containing both 'd' and 'y' antigenic determinants, was added, and the spherocyte-antigen suspension was mixed continuously at room temperature (see addendum). After 20 hours a 1 ml aliquot was removed, stabilized as previously described (Hopkins and Das, 1973), and used to titrate a known standard HBsAb containing serum. Sampling was repeated at approximately 20-hour intervals until a satisfactory sensitivity was achieved. The bulk reagent was removed from the mixer and the cells were recovered by centrifugation as before. The sensitized cells were then stabilized by resuspension and mixing for 4 hours at room temperature in 500 ml PBS pH 7.2 containing 2% normal human serum to give a cell suspension of approximately 3-2% (or 10 times working concentrations).

Storage of these cells overnight at 4°C was followed by a repetition of the stabilizing procedure. Thereafter, the 3-2% suspension of cells was dispersed into 50 ml aliquots and stored at 4°C ready for pasteurization.

PASTEURIZATION AND STORAGE OF HBsAg COATED SPHEROCYTES

Immediately before pasteurization the coated spherocytes were pelleted and resuspended to the same volume with PBS pH 7.2 containing 0.05% phenol. They were then left for 12-16 hours in a 60°C waterbath, allowed to cool, divided into smaller aliquots (for convenience), and stored at 4°C until required when each aliquot would be diluted 10-fold with PBS pH 7.2 to achieve working strength.

The coated cells could also be stored at −20°C or in the lyophilized state without appreciable loss of sensitivity.

Comment

Recent reports have highlighted the inadequacies of first and second generation methods of HBsAg testing (Ling and Overby, 1972; Cayzer et al, 1974; Hopkins et al, 1975). The American Red Cross appear to have opted for RIA as a means of blood donor screening, while transfusion centres in the United Kingdom seem to favour haemagglutination.

It is the purpose of this communication to emphasize that third generation testing efficiency is available at a fraction of the cost of purchasing commercial reagents. Preparation of sufficient HAI reagents for approximately one million tests is described in detail using a process which is readily reproducible (we have recently prepared a second batch of reagents sufficient for 3.5 million HAI tests) and requires relatively unsophisticated laboratory equipment. The cost of one million RPHA tests is £80 000, while a similar number of RIA tests bought commercially could cost £500 000.

Experience has shown that HAI reagents are extremely stable in that they will survive 4°C for over one year, repeated freezing and thawing, lyophilization and exposure to 2.5 mega rads of gamma irradiation from a cobalt source (Courtesy of Ethicon Ltd, Edinburgh), neither does transport over thousands of miles via air freight appear to have an adverse effect, even when cells are maintained in the liquid state.

The safety of laboratory staff handling hepatitis B testing reagents is of paramount importance. Until it is possible to evaluate the biological activity of HBsAg it is essential that every reasonable precaution should be taken to ensure the safety of such reagents. For this reason we have attempted to incorporate into reagent preparation accepted methods of virus inactivation, combining the principles of chemical and physical inactivation, namely, (a) betapropiolactone treatment and ultraviolet irradiation of raw serum (LoGioppo et al, 1971), (b) pasteurization of HBsAg coated glutaraldehyde fixed spherocytes, and, if required, (c) gamma irradiation of the lyophilized reagents.

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References


Addendum

We observed that cell sensitization was successful when prepared by mixing in an MRC 500 ml bottle by mechanical turntable but unsuccessful when performed in the same type of bottle using a magnetic mixer with plastic-coated follower. Adsorption of HBsAg onto the plastic follower has been eliminated but the electrochemical or electrophysical possibilities for the failure have yet to be investigated.

Letter to the Editor

Heparin in Intravenous Fluids

Okuno and Nelson (J. clin. Path., 28, 494, 1975) conclude that there is a substantial loss of anticoagulant activity when heparin is dissolved in intravenous solutions. We have been unable to demonstrate any progressive loss of activity using the anti-activated factor X assay of Denson and Bonnar after the addition of heparin to either 0-9% saline or 5% Dextrose.

We mixed 1 ml of Evans Pularin Heparin (5000 units per ml) with the intravenous solution to give a final concentration of 10 units of heparin per millilitre. This dilution was carried out immediately before assay, six hours before and 24 hours before, using the same vial of heparin. Just before assay each dilution of heparin was further diluted 1:100 into normal citrated plasma to give a final heparin concentration of 0-10 units per ml. In each experiment all the heparin assays were carried out at the same time, and the results were compared with a series of dilutions of the MRC Biological Standard Heparin.

There is no progressive loss of heparin activity and only a small loss of activity when heparin is added to 5% Dextrose.

The difference between our results and the previously published ones may be due to either the different brand of heparin used, the assay system used to detect the anticoagulant activity, or the fact that we carried out all the assays of a single experiment at the same time, so eliminating the drift that may occur when sequential assays are employed.

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Technical method

<table>
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<tr>
<th>Time</th>
<th>No. of Tests</th>
<th>Heparin Conc. (u/ml)</th>
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<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
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| Heparin in 0.9% Saline | 28 | 9-0 | 7.2-11.6 |
| 6h                     | 7  | 100 | 9.0-11.6 |
| 24h                    | 6  | 9-9 | 8.0-11.6 |

| Heparin in 5% Dextrose | 9  | 8-3 | 5.6-10.4 |
| 6h                     | 7  | 9-2 | 7.6-11.6 |
| 24h                    | 7  | 8-3 | 5.6-10.4 |

Although this book has 26 chapters written by different authors, there is a coherence about it not usually found in such multi-author works. One immediately obvious reason for this is the use of a common format for the many magnificent drawings and illustrations; the common format, which is also used for the text, is the one used by Scientific American. A deeper reason for the coherence of the book is the emergence in the last few years of the Singer-Nicholson model of the cell membrane; this model is repeatedly used by the various authors to explain the biochemical, physiological, and pathological properties of membranes. The articles originally appeared in Hospital Practice and were presumably written for clinicians, but the book