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Investigations and preparation of fibrinogen-coated tanned sheep red cells

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SYNOPSIS The preparation and investigations of tanned sheep red cells coated with human fibrinogen are presented in detail and noted variables are discussed. Both tanning and coating are quick processes. Higher concentrations of tannic acid improved the sensitivity of the cells but required an increased concentration of stabilizing serum to prevent autoagglutination. Fibrinogen concentration above 2 μg/ml had no significant effect, although a concentration above 100 μg/ml sometimes produced cells with incomplete patterns. The *p*H of the reaction media may be varied widely, although tanning and coating at a more alkaline *p*H yielded cells showing an indistinct pattern. Cells prepared by this method can be stored for at least six months.

The direct agglutination is one of the most sensitive means available for detecting an antibody to particulate antigens. The tanned red cell agglutination test, introduced by Boyden (1951), is a very useful modification for use with soluble antigens. In this method red cells are treated with tannic acid, coated with soluble protein antigens and subsequently used for the detection of a specific antibody. Boyden also demonstrated that his technique could be used for the detection of antigen by haemagglutination inhibition. In this way very small quantities of antigen have been detected (Stavitsky and Ingrahm, 1964).

A large number of workers have reported on the preparation of fresh tanned cells for a variety of antigens, including chorionic gonadotropin, human serum albumin and globulin, egg albumin, ovalbumin, insulin, and diphtheria antigen (Fullthorpe, 1959; Wide, 1962; George and Vaughan, 1962; Shioiri, 1964; Palvonini, 1966; Herbert, 1967a). They found that optima vary for different antigens and results found for one material are not necessarily applicable to others. Furthermore, the main disadvantages of the tanned cell technique are the short time for which the cells can be kept for use after preparation and the great variation in the sensitivity of cells prepared on different occasions.

Detection and quantitation of serum fibrinogen/fibrin degradation products by the haemagglutination inhibition method have been reported from this laboratory (Das, Allan, Woodfield, and Cash, 1967; Mackay, Das, Myerscough, and Cash, 1967; Woodfield, Cole, Allan, and Cash, 1968), for which the production of antigen-coated cells is an important stage of the assay system. The present paper deals with sensitization of sheep red cells by human fibrinogen and their subsequent storage.

Materials

FORMALDEHYDE SOLUTION[1]

A 3% solution (v/v) was made in normal saline. The *p*H was adjusted to 7·20-7·25 with 0·1 N NaOH.

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[1]BP (Evans Medical, Liverpool)

BUFFERS

Phosphate buffered saline

The solution was prepared by adding 35 ml of $Na_2H\ PO_4$ (0·15M) and 65 ml of KH_2PO_4 (0·15M) to 100 ml of NaCl (0·15M), and the *p*H was adjusted to 6·4. For those experiments in which the effect of variation in *p*H was studied, phosphate-buffered saline with different *p*H was prepared by adjusting the volumes of Na_2HPO_4 and KH_2PO_4 accordingly.

Citrate buffer

This was prepared similarly to phosphate-buffered saline except that NaCl was replaced by Na-citrate (0·15M). The buffers were stored at 4°C.

SODIUM AZIDE[1]

This was used as preservative, concentration of 100 mg/ml being added to the buffers so as to make a final concentration of 1 mg/ml.

Tannic acid[2]

Fresh stock solutions of tannic acid (Boyden, 1951) were made (10 mg/ml in distilled water) for each run, and further dilutions were made in buffers from the stock solution.

SHEEP RED BLOOD CELLS

These were obtained from the Burroughs Wellcome and Stayne Laboratories, England, and Royal Dick School of Veterinary Studies, Edinburgh. The blood was collected in Alsever's solution, stored as 25 ml aliquots in McCartney bottles at 4°C for three to five days before use.

STABILIZING SERUM

Normal sera from horse, rabbit, and bovine albumin which had a very low titre of natural agglutinin for sheep red cells were used. Residual agglutinins were absorbed by mixing 1 volume of packed normal sheep red cells with 4 volumes of stabilizing sera at 37°C for 30 minutes.

ANTIGEN: HUMAN FIBRINOGEN[3]

This had been prepared by the method of Blömback and Blömback (1956). A freshly prepared 0·02% (w/v) solution in distilled water was used as stock preparation. Further dilution was made in the buffer during the coating of the cells. No natural agglutinin against sheep red cells was present in the purified fibrinogen.

ANTISERUM

Rabbit antihuman fibrinogen serum[4] was stored at −20°C in small aliquots and a working antiserum was made on each day by thawing an aliquot and diluting it further as required.

Methods

FORMALIZATION OF SHEEP RED CELLS

This was carried out regularly by the method of Weinbach (1958) as described by Wide (1962), with slight modification. One volume of an 8% (v/v) sheep red cells was mixed with 1 volume of formaldehyde solution, agitated gently in a glass beaker at 37°C by a magnetic stirrer. After 20 to 24 hours the cells, which had turned a dark brown colour, were washed three times in 50 volumes of distilled water. Finally, a 10% (v/v) suspension was made in distilled water containing 1 mg/ml Na-azide, and stored at 4°C. In practice, despite the excellent capacity for allowing the cells to be stored for at least six months, for all the present studies the cells were normally tanned and coated within one to two weeks of formalization.

TANNING OF THE CELLS

The standard method was to take 1 volume of formalinized sheep red cells centrifuged at 1,000*g* for five minutes and washed three times in 50 volumes of the phosphate-buffered saline. The cells were then mixed with 1 volume of freshly prepared tannic acid solution (in phosphate-buffered saline), incubated in a water bath at 56°C, and stirred at 10-minute intervals.

COATING OF THE CELLS

After tanning the cells were washed three times with 50 volumes of phosphate-buffered saline, and finally suspended in citrate buffer and mixed with an equal volume of purified fibrinogen solution (in citrate buffer).

STORAGE OF THE SENSITIZED CELLS

After sensitization of the cells with fibrinogen the cells were washed four times in 50 volumes of citrate buffer, suspended as 2·5% (v/v) solution in the same buffer containing 0·4% horse serum as a stabilizer and 1 mg/ml Na-azide (as a diluting fluid), and stored at 4°C. After three to four days, the supernatant above the settled cells was decanted off and the same volume of fresh diluting fluid added. The concentration of the cells was rechecked, and 2·5 ml aliquots were distributed in plastic vials, left at 4°C. for the daily tests.

ANTIBODY TITRATION

Serial dilutions of antifibrinogen serum were placed in the wells of a microtitre plate[1]. To 1

[1]British Drug House Ltd, England
[2]May & Baker, England
[3]Kabi Pharmaceuticals Ltd, batch 83164, 97% clottable
[4]Behringwerke AG. Germany; batch 606 AC, and 1248 K

[1]V plate, Cook Engineering Co., USA

volume of antiserum, 1 volume of diluting fluid and 1 volume of 2·5% sensitized cell (1 vol ≡ 0·025 ml) was added. Controls, including 2 volumes of diluting fluid and 1 volume of sensitized cells, were incorporated in each plate. The plates were shaken and placed at 4°C overnight. A smooth mat of cells at the bottom of the wells was designated as positive (+) and a 'button' of cells in the centre of the wells was called negative (−); intermediate reactions were recorded as ±.

The end point of the antibody titre was taken to be the highest dilution of antiserum causing complete agglutination of the cells, and the titre of the antiserum was expressed as the reciprocal of the initial dilution of the antiserum. The results of different experiments are not strictly comparable unless specifically stated as several different batches of antiserum of different titres were used.

Results

Haemolysis and spontaneous agglutination occurred at the formalization stage only once in 80 batches of cells.

Three batches of cells were formalinized and stored at 4°C for various lengths of time, ranging from one week to six months, and portions of them were simultaneously tanned and coated by the standard method. Results of the titration of an antiserum are presented in Table I, and show that the age of the cells had no significant influence on the results obtained.

Age of Cells	*Titre Against Antifibrinogen Serum*		
	Cell Batch		
	Number P_2D	*Number P_5D*	*Number P_3D*
One week	64,000	96,000	32,000
One month	48,000	128,000	48,000
Two months	96,000	64,000	48,000
Four months	64,000	96,000	48,000
Six months	64,000	64,000	48,000

Table I *Titre of antifibrinogen serum given by three batches of formalinized cells, stored at 4°C, tanned and coated after different periods of time*

The results of the investigation of different variables at the tanning stage are summarized in Table II.

TANNIC ACID CONCENTRATION

When other variables were kept constant, an increased amount of tannic acid gave a somewhat greater final sensitivity. However, slight autoagglutination appeared in the controls at the higher tannic acid concentration (Table II). This could be due to an inadequate concentration of stabilizing agent in the final assay.

Tannic Acid Concentration (I)	pH	*Temperature (in Centigrade)*	*Time (in Minutes)*	*Cell Percentage (v/v) in Phosphate-buffered Saline*	*Titre of Antiserum*	*Control*[1]
Effects of tannic acid						
5,000	6·4	56	60	2	128,000	++
10,000	6·4	56	60	2	96,000	++
20,000	6·4	56	60	2	64,000	−
40,000	6·4	56	60	2	64,000	−
80,000	6·4	56	60	2	48,000	−
160,000	6·4	56	60	2	32,000	−
320,000	6·4	56	60	2	32,000	−
Effects of pH						
40,000	5·0	56	60	2	16,000	−
40,000	6·0	56	60	2	64,000	−
40,000	6·4	56	60	2	64,000	−
40,000	7·2	56	60	2	64,000	−
40,000	7·6	56	60	2	96,000	++
40,000	8·0	56	60	2	96,000	++
Effects of temperature						
40,000	6·4	22	60	2	32,000	−
40,000	6·4	37	60	2	64,000	−
40,000	6·4	56	60	2	64,000	−
Effects of time of incubation						
40,000	6·4	56	15	2	64,000	−
40,000	6·4	56	30	2	64,000	−
40,000	6·4	56	60	2	64,000	−
40,000	6·4	56	120	2	64,000	−
Effects of cell concentration						
40,000	6·4	56	60	2	64,000	−
40,000	6·4	56	60	4	32,000	−
40,000	6·4	56	60	8	16,000	−

Table II *Investigations at the tanning stage*

[1]2 volumes of diluting fluid to 1 volume of sensitized cells.
± signifies a moderate autoagglutination or occasional indistinct pattern, −, a firm end-point with distinct 'button'.

EFFECT OF *p*H

The results indicated that though a higher sensitivity was obtained at a more alkaline *p*H, the cells provided less distinct patterns (Table II); at an acid *p*H sensitivity was significantly impaired. A *p*H of either 6·4 or 7·2 was found to be equally satisfactory.

TEMPERATURE

Tanning was carried out at 22°C, 37°C or 56°C. It was found that both 37°C and 56°C were equally effective. A slightly lower titre was obtained at room temperature. Tanning at 56°C was therefore adopted in an attempt to adhere to Wide's (1962) basic method.

TIME OF INCUBATION

Tanning was carried out at 56°C for 15, 30, 60, and 120 minutes, and the results show that the process appears to be rapid (Table II); increasing incubation time has no effect on the sensitivity of the cells processed. An incubation time of 60 minutes was arbitrarily adopted for the routine tanning procedure described in this study.

CELL CONCENTRATION

This experiment was designed to investigate the proportion of cells necessary for optimal tanning and subsequent coating by human fibrinogen. Formalinized cells were washed and suspensions of 2%, 4%, and 8%, (v/v) were made. They were then tanned and coated by the standard method using the same quantities of reagents. Results (Table II) show that an increase in the quantity of cells caused a drop in titre: a 2% (v/v) suspension of cells was therefore adopted for the routine procedure.

EFFECT OF PHOSPHATE-BUFFERED SALINE AND CITRATE BUFFER

Five batches of cells were simultaneously coated in either of the two buffers. With no batch did titration against an antifibrinogen serum show any significant variation of titres between those coated using either buffer. In practice citrate buffer, *p*H 6·4, was routinely used as the diluting agent to prevent clot formation which might have occurred if phosphate-buffered saline had been used with plasma or fibrinogen.

EFFECT OF USING DIFFERENT CONCENTRATIONS OF ANTIGEN

Seven batches of cells were simultaneously tanned and then sensitized with a solution of fibrinogen containing between 0·1 and 100 μg/ml. It was evident that a fibrinogen concentration in excess of 2 μg/ml did not improve the sensitivity (Table III). Furthermore, occasionally an incomplete pattern emerged in the control when the fibrinogen

Fibrinogen Concentration (μg/ml)	*p*H	*Temperature* (in Centigrade)	*Time of Incubation* (in Minutes)	*Cell Percentage* (v/v) *in Citrate Buffer*	*Number of Cell Batch*	*Titre of Antiserum*	*Control*[1]
Effects of fibrinogen concentration							
0·1	6·4	37	60	4		4,000	−
0·5	6·4	37	60	4		48,000	−
1·0	6·4	37	60	4		96,000	−
2·0	6·4	37	60	4		128,000	−
10·0	6·4	37	60	4		192,000	−
50·0	6·4	37	60	4		128,000	−
100·0	6·4	37	60	4		128,000	+
Effects of p*H*							
2·0	5·0	37	60	4		12,000	−
2·0	6·0	37	60	4		32,000	−
2·0	6·4	37	60	4		96,000	−
2·0	7·2	37	60	4		96,000	−
2·0	7·6	37	60	4		96,000	−
2·0	8·0	37	60	4		96,000	+
Effects of time of incubation							
2·0	6·4	37	15	4		96,000	−
2·0	6·4	37	30	4		96,000	−
2·0	6·4	37	60	4		96,000	−
2·0	6·4	37	120	4		96,000	−
2·0	6·4	37	180	4		96,000	−
Effects of temperature							
2·0	6·4	22	60	4	F14	96,000	−
2·0	6·4	22	60	4	F15	128,000	−
2·0	6·4	37	60	4	F14	96,000	−
2·0	6·4	37	60	4	F15	128,000	−
Effects of cell concentration							
2·0	6·4	37	60	2		192,000	−
2·0	6·4	37	60	4		128,000	−
2·0	6·4	37	60	8		24,000	−

Table III *Investigations at the coating stage*

concentration was in the region of 100 μg/ml. There was also evidence that the sensitivity was considerably diminished if the fibrinogen concentration was below 2 μg/ml.

EFFECT OF VARYING THE *p*H

Six batches of cells were uniformly treated with tannic acid (1:40,000), and then suspended in phosphate-buffered saline of varying *p*H values between 5 and 8, and coated identically using fibrinogen solution in an appropriate buffer. When tested against an antifibrinogen serum it was found that there was no difference in sensitivity between *p*H 6·4 and 8, but a slight decrease in titre was noticed at low *p*H, and occasionally an incomplete pattern appeared in the control in the more alkaline solutions (Table III).

EFFECT OF VARYING THE INCUBATION TIME AND TEMPERATURE

Five batches of cells were tanned and coated by the standard method, except that the time during which each batch was left in 37°C water bath varied from 15 to 180 minutes. In a second set of experiments two batches of cells were sensitized for 60 minutes at 37°C and 22°C. Results are shown in Table III, and indicate that the cells were well sensitized by exposure to fibrinogen for 15 minutes; any longer periods of coating did not increase their reactivity. Furthermore, there was no consistent difference between the batches of cells sensitized at 37°C and at room temperature.

EFFECT OF CONCENTRATION OF CELLS

Cells were tanned in bulk, being treated with an equal volume of 1:40,000 tannic acid, and after washings and resuspension, these tanned cells were distributed as 2%, 4%, and 8% (v/v) suspension in three different containers. The cells in each container were then coated with fibrinogen by the standard technique, and finally a 2·5% (v/v) suspension was made in diluting fluid.

Table III shows that this resulted in a variation of the titre when these cells were tested with an antifibrinogen serum, so that larger quantities of cells produced a somewhat lower titre. A 2% cell suspension was therefore adopted for coating purposes.

STABILIZATION STAGE

During the present study experiments were performed on different stabilizers. Using 1:40,000 tannic acid as the standard tanning technique, fibrinogen-coated cells were found to be effectively stabilized by 0·4% horse serum, 0·2% normal rabbit serum and 0·1% bovine albumin, or it was also demonstrated that previously observed autoagglutination of cells when tanned with 1:5,000 tannic acid (Table II) could be prevented by increasing the concentration of stabilizing horse serum to 1%.

STORAGE OF SENSITIZED CELLS

Control tests upon batches of fibrinogen-coated cells were carried out on the same day as they had been prepared and again three days after preparation. Before the second control test the supernatant above the settled cells was removed and the same amount of diluting fluid added, and stored at 4°C. Table IV shows the results of such experiments. The titre was usually found higher in the second control test than in the first one. After the change of supernatant the fibrinogen-coated cells retained, for four to five months, an almost unchanged capacity to react in the test (Table V).

	Titre	
Cell Batch Number	*First Control Experiment*	*Second Control Experiment*
F20	64,000	96,000
F3	48,000	96,000
F4	64,000	128,000
F5	192,000	192,000
P2	32,000	64,000
M1	96,000	96,000
Ex 4	32,000	64,000
Ex 3	96,000	128,000

Table IV *Titre of an antifibrinogen serum against eight batches of cells*[1]

[1]The first control experiments were performed on the same day the cells were sensitized. The second control experiments were performed three days later.

	Titre		
Time of Storage (in Months)	*Cell Batch Number* MS_1	*Cell Batch Number* E_1	*Cell Batch Number* P_1
1	96,000	64,000	96,000
2	128,000	64,000	64,000
3	96,000	64,000	96,000
4	96,000	64,000	96,000
5	64,00	—	—

Table V *Titre of an antifibrinogen serum against three batches of sensitized cells stored at 4°C for varying periods of time*

Discussion

The present investigations suggests that formalinized and coated cells can be stored for at least five to six months, thus minimizing the technical disadvantages of the short storage time after preparation, and the great variation in separately prepared batches of cells (Read and Stone, 1958; Herbert, 1967b). The quantity of tannic acid, which is a hydrogen donor on biological membrane and interacts in solution with quaternar

nitrogen compounds (Allison, 1968; Nash, 1966) and thereby alters the stability of cell membranes, was of the first importance; the more tannic acid used the greater was the sensitivity. However, greater concentration of tannic acid (1:5,000) requires higher concentration of stabilizing serum (1%) to effect adequate stabilization and thus avoid the appearance of autoagglutinable cells (Herbert, 1967a). In the routine method, 1:40,000 tannic acid is used though lower concentrations have sometimes been found to be effective in other systems (Boyden and Sorkin, 1955; Heller, Jacobson, Kolodny, and Kammerer, 1954). On the other hand Fox, Wide, Killander, and Gemzell (1965) used 1:20,000 tannic acid for fibrinogen-coated cells. Some batches of cells may be abnormally sensitive or resistant to the action of tannic acid and it may therefore be worthwhile to carry out preliminary investigations with several different concentrations of tannic acid.

Tanning appears to be a rapid process and could be effectively done at 37°C and 56°C, which is in agreement with other workers (Boyden, 1951; Stavitsky, 1954; George and Vaughan, 1962; Heller *et al*, 1954; Wide, 1962; Herbert, 1967a). A slightly lower titre was obtained when the cells were tanned at room temperature (22°C) which was also claimed to be effective by Boyden and Sorkin (1955) during the tanning procedure.

The cells were found to be adequately coated by fibrinogen solution containing 2-100 μg/ml. This is similar to the findings of other authors (Linz, Lecocq, and Mandelbaum, 1961; Linz and Lecocq, 1962; Merskey, Kleiner, and Johnson, 1966; Herbert, 1967a) and is considered to be due to adsorption on the cells of only a very small part of the total protein present. However, some workers have reported optimum concentrations of antigen for sensitization of the cells (Boyden, 1951; Wide, 1962; Steffen and Rosak, 1963). Furthermore, George and Vaughan (1962) found a direct relationship between the quantity of antigen used to coat the cell and the titres obtained. Herbert (1967a), however, has found a relationship of this type only when fresh cells are employed but not with formalinized cells. Slight autoagglutination, noticed at fibrinogen concentrations in excess of 100 μg/ml, is similar to the findings of Ferreira, Silva, Murat, and Ferraz (1964) who reported that excess of fibrinogen produced large clumps of cells making them unsuitable for test purposes.

The length of time required to sensitize the cells was found to be short. The *p*H at which this can be done may be varied widely, though tanning and coating at more alkaline *p*H yielded cells showing an incomplete pattern and occasional moderate autoagglutination (Wide, 1962). This may be due to denaturation and precipitation of surface protein of the cells or of fibrinogen at alkaline *p*H. Lower titres, obtained when the cells were coated at *p*H 5, may be due to rapid digestion of fibrinogen at acid *p*H (Watt, 1968).

Cells sensitized by the standard technique gave excellent results. One of the reasons is perhaps that highly purified fibrinogen was used instead of plasma, since it is known that there are distinct advantages in using purified materials (Fisher, 1952; Linz and Lecocq, 1962; George and Vaughan, 1962). However, small batches of cells produced variable titres (1:16,000 to 1:256,000). This is possibly due to the antigen being denatured to a different extent on different occasions (Christian, 1958; George and Vaughan, 1962; Maurer, Gerulat, and Pinchuck, 1963; Epstein and Gross, 1964) and/or some batches of cells are abnormally reactive to tannic acid (Herbert, 1967a).

The cells stored at 4°C after the change of supernatant usually showed higher titres than the first control run. This is probably due to loss of antigen into the liquid as shown by Wide (1962). Although no free fibrinogen could be detected in the supernatant by haemagglutination inhibition, this does not preclude the possibility that the antigen present was too small to be detected. Lately we have been able to store the sensitized cells for nine to 10 months without any apparent loss of reaction.

The sensitized cells were successfully used in haemagglutination inhibition reaction for the detection of serum fibrinogen/fibrin degradation products (Das *et al*, 1967). The production of highly sensitive cells has distinct advantages, for the cells can be stored for longer periods and in haemagglutination inhibition reaction, more dilute antiserum can be used, which not only allows for economy but also improves the sensitivity of the test system (Das, 1968).

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