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Mixed Haemadsorption: A Mixed Antiglobulin Reaction Applied to Antigens on a Glass Surface

PREPARATION AND EVALUATION OF INDICATOR RED CELLS; SURVEY OF PRESENT APPLICATIONS

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Summary. Mixed haemadsorption should be regarded as an application of the mixed antiglobulin reaction to situations where the antigen is sessile on a glass surface. Antibody attached to the antigen when exposing the latter to an antiserum is traced by red cells carrying an antiglobulin layer which makes them adsorb to the antibody.

The indicator cells are prepared by coating them first with a layer of γ-globulin from the animal species, the antibody globulin of which they are intended to trace, and then with a layer of the corresponding antiglobulin. The most effective indicator cells were obtained by attaching antibody to natural receptors on the red cells to achieve their first coating of γ-globulin.

The preparation of indicator cells for tracing antibodies from a number of species, including human, is described.

The mixed haemadsorption technique is highly specific and has a sensitivity which is comparable to that of the most sensitive serological techniques.

Test procedures adapted for different purposes are outlined and a number of applications to experimental and clinical problems are reviewed.

INTRODUCTION

The principle of mixed agglutination was described by Wiener and Herman (1939). The general importance of this reaction has been emphasized by Coombs and co-workers in a number of publications since 1955 (e.g. Coombs and Bedford, 1955; Coombs, Bedford and Rouillard, 1956; Coombs, Daniel, Gurner and Kelus, 1961). Högman (1959) applied the method to monolayer cell cultures for the study of blood group antigens.

The mixed antiglobulin reaction was outlined by Coombs, Marks and Bedford (1956) and further elaborated by Chalmers, Coombs, Gurner and Dausset (1959). Independently of the latter authors Fagraeus and Espmark adapted the mixed antiglobulin reaction for the demonstration of antigens in monolayer tissue cultures and named this technique 'mixed haemadsorption'. The technique was initially used for tracing viral antigens (Fagraeus and Espmark, 1961), but was soon applied also for the analysis of cellular antigens (Espmark and Fagraeus, 1962) and for the titration of antibodies.

Principle of the technique

The mixed haemadsorption reaction is built up on a solid surface in a sandwich

arrangement analogous to that of the indirect fluorescent antibody technique of Coons (Weller and Coons, 1954).

The surface (glass or Perspex) is provided with an adherent layer of antigen (monolayer culture of cells, fixed molecular film of soluble antigen or sedimentation layer of particles).

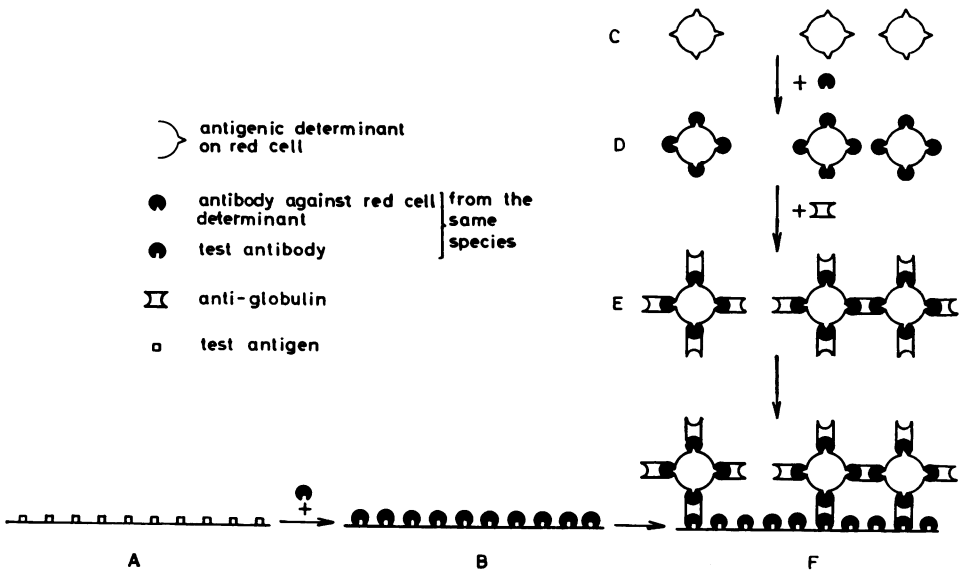

FIG. 1. Rationale of the mixed haemadsorption reaction. A, Test antigen fixed to a solid surface; B, antibody fixed to the test antigen; C, D, E, procedure for preparation of indicator red cells; F, adsorption of indicator cells to the antibody layer on the antigen.

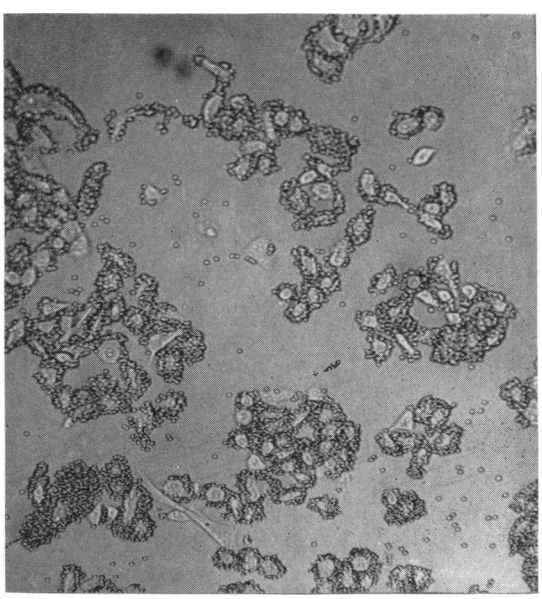

FIG. 2. Appearance of mixed haemadsorption (scored as + +) on HeLa cells in culture. Cells treated with anti-HeLa cell serum (from rabbit), diluted 1 : 10000 before addition of indicator red cells.

The reaction is then performed in two steps. In the first, which is the test reaction proper, the corresponding antibody is bound to the antigen layer. In the second step this antibody is traced by its antigenic character using anti-γ-globulin attached to erythrocytes which serve to visualize the reaction (see Fig. 1).

The anti-γ-globulin is linked to the erythrocytes over γ-globulin attached to the red cell surface. This γ-globulin must for obvious reasons be from the same species as the antibody fixed on the antigen surface in the test proper. The anti-γ-globulin coated erythrocytes are prepared separately in a procedure analogous to the indirect Coombs test (Coombs, Mourant and Race, 1945) and they are added as a preformed complex in the second step of the mixed haemadsorption reaction. Owing to the free reactive sites of the antiglobulin the red cells adsorb to the antibody globulin fixed in the test reaction. The final result can be characterized serologically as a mixed antiglobulin reaction.

If the test antibodies are fixed over a sufficiently large area the resulting haemadsorption is easily observed macroscopically.

When the test antibody is fixed in a very small area, e.g. on the surface of a single cell, the reaction is read microscopically and can then be related to morphological structures (Fig. 2).

The present report will deal chiefly with the preparation and evaluation of indicator red cells. To this will be added a review of the applications which have so far been tried by the authors.

MATERIAL AND METHODS

PREPARATION OF INDICATOR CELLS

I. Erythrocytes

Washed sheep red cells were used in all instances except for the indicator cells prepared with Rh antibody, for which human O Rh(+) red cells were necessary. Care was taken to obtain sheep red cells from the same animal and human O Rh(+) cells from the same Rh group.

II. Amboceptor coated indicator cells

a. *Anti-sheep red cell sera, 'amboceptors', for first coating.* Amboceptor sera were produced by injection of washed sheep erythrocytes twice weekly for 3 weeks. The animals were bled 7–10 days after the last injection.

The human amboceptor was kindly provided by Dr K. Aho, Helsinki (Aho, Harboe and Leikola, 1964).

The titres of the amboceptor sera were determined as the reciprocal of the last dilution giving a distinct bottom pattern of agglutination when testing against a 0·2 per cent suspension of sheep red cells. The titres of the different amboceptor sera varied between 320 and 10,000.

In agglutination experiments with varying concentrations of sheep erythrocytes it was found that an increase of the concentration of red cells from 0·2 per cent to 1 per cent resulted in a five-fold reduction of the haemagglutination titre. Since the titration was performed with a 0·2 per cent suspension and the first coating with a 1 per cent suspension the serum concentration for coating had to be adjusted with regard to this finding. The suitable concentration for the first coating was found to vary between one-half and two agglutinating units.

Procedure for first coating (with 'amboceptor'). Equal volumes of 2 per cent sheep red cells

suspended in PBS and the appropriate concentration of heat inactivated amboceptor serum were mixed and left at room temperature for 1 hour. The cells were sedimented by centrifugation at 450 *g* for 5 minutes and then washed twice with large volumes of PBS.* After the last centrifugation the erythrocytes were resuspended in the same buffer to make a 2 per cent suspension.

b. *Antiglobulin sera for second coating.* Antisera to serum globulins from rat, guinea-pig, mouse, calf, fowl, monkey and man were produced in rabbits by injection of purified γ-globulin preparations or, occasionally, whole serum.

Rabbits were injected once intramuscularly with γ-globulin incorporated in Freund's complete adjuvant. After 3 weeks they were given one to three booster injections intravenously and were bled after a further 7–10 days. Antiserum to rabbit γ-globulin was produced in sheep by injections of undiluted rabbit serum in Freund's complete adjuvant over a period of 3 months. A horse serum containing antibodies against rabbit γ-globulin was kindly provided by Dr B. Björklund (Björklund and Hellström, 1951).

Titration of the antiglobulin sera was performed in two-fold serial dilutions against a 0·2 per cent suspension (final concentration) of sheep red cells which were either tanned and coated with a purified γ-globulin preparation according to Boyden (1951) or coated with half an agglutinating unit of the relevant amboceptor serum.

The antiglobulin titre was determined after 6 or 18 hours as the last dilution giving a distinct bottom pattern of haemagglutination.

On immunoelectrophoresis all the antiglobulin sera gave heavy precipitation lines with γ_2 globulin of the sera of the corresponding species. The lines obtained with γ_{1M} and γ_{1A} globulin were weak or missing.

A suitable concentration of an antiglobulin serum for the second coating of the indicator cells was arrived at by comparing several cell lots, coated with varying dilutions of serum, in a haemadsorption test (see below under Results).

Procedure for second coating (with anti-γ-globulin serum). Equal volumes of amboceptor coated erythrocytes and the appropriate dilution of antiglobulin serum in PBS were mixed and left for 2 hours at room temperature. After centrifugation the cells were washed once in a large volume of PBS and then resuspended to a 2 per cent suspension.

III. First coating achieved in other ways

a. *Natural 'amboceptors'.* 1. Human non-agglutinating anti-Rh (anti-CD) serum coated on human O Rh(+) red cells ('Rh-system'): For the first coating human O Rh(+) red cells were incubated as a 2 per cent suspension with an appropriate dilution of anti-CD serum for 90 minutes at 37°; PBS was used as a diluent. After incubation the cells were washed three times and resuspended to 2 per cent. The second coating with rabbit anti-human globulin was performed as described above in the section II (b).

The anti-CD serum was titrated by coating O Rh(+) red cells with different dilutions of the serum in the same way as for the first coating of indicator cells and then adding cells from the different lots as a 0·2 per cent suspension to 0·5 ml of antiglobulin serum diluted 1/100 in 11 × 80 mm test tubes. A titre of 400 was obtained when noting the highest coating dilution of anti-CD serum giving agglutinable cells as judged from the bottom patterns in the tubes after 18–24 hours.

2. Human heterophile antibodies coated on sheep red cells: Serum from a case of mononucleosis was used. The titre determined by the Paul–Bunnell–Davidson test was

* When not otherwise indicated PBS means phosphate buffered saline, pH 7·4.

640. For the first coating of indicator cells a 4 per cent suspension of sheep red cells was incubated with an equal volume of serum diluted 1 : 50 according to the procedure described above for amboceptor coating.

b. *Tanned cells.* 1. Direct attachment of electrophoretically purified human γ-globulin, kindly supplied by AB Kabi, Stockholm, was achieved by incubating one volume of a 2 per cent suspension of tanned cells with five volumes of γ-globulin, 120 μg/ml in PBS, pH 6·4, for 10 minutes at room temperature. The cells were then washed three times in large volumes of PBS, pH 7·4 (Boyden, 1951).

2. Indirect attachment of human γ-globulin was achieved by first coating the tanned cells with staphylococcal toxoid, five units per ml, according to the procedure described for direct attachment. The sensitized cells were then exposed as a 1 per cent suspension to human immune globulin against staphylococcal toxoid for 30 minutes at room temperature and then washed three times. The immune globulin had a titre of 1 : 1600 in the Boyden test and was used in the dilution 1 : 100.

IV. Antiglobulin consumption tests (Steffen, 1955; Moulinier, 1955)

These tests were performed in order to evaluate the amount and quality of the antiglobulin picked up by erythrocytes coated with γ-globulin by different methods. Three aliquots of an anti-human γ-globulin serum diluted 1 : 400 in PBS, pH 7·6, were absorbed at room temperature for 2 hours with one-tenth volume of globulin-carrying erythrocytes (coated as described under sections II, III (a, 1) and III (b, 1) next above, respectively). The antiglobulin contents before and after absorption were estimated as agglutinating titres obtained with the same three types of human globulin coated erythrocytes.

MIXED HAEMADSORPTION TEST

I. Test in tissue culture tubes

Test antigen. Monolayer cell cultures were prepared in 13·5 × 100 mm tubes by common methods. Calf serum (5–15 per cent) was used in the growth medium. For the study of cell surface antigens the test was applied when the cell sheet had become nearly confluent.

Cultures inoculated with vaccinia virus were used for the control of indicator cells (Fagraeus and Espmark, 1961). The virus dilution chosen gave rise to ten to fifty discrete degeneration foci, 0·5–1 mm in diameter, within 2–3 days.

The cultures were washed once with serum-free medium before the test.

Anti-vaccinia sera were obtained in rabbits, rats, guinea-pigs, mice, calves, roosters and monkeys by inoculating vaccinia virus in the scarified skin. The animals were bled 15–20 days after the inoculation. A serum from a recently vaccinated person was used as the source of human antibody against vaccinia. Pre-immunization sera were used for negative controls.

All anti-vaccinia sera had significant titres in the haemagglutination-inhibition tests against vaccinia haemagglutinin.

Anti-human cell sera were prepared by injecting white rats and rabbits intraperitoneally with 5–50 millions of HeLa cells once weekly for 6–7 weeks. The animals were bled 7–10 days after the last injection. All sera were heated at 56° for 30 minutes prior to the test.

Performance of the test. The washed tube cultures were incubated with 0·3 ml of the appropriate test serum for 1 hour at room temperature. Then, after thorough draining and washing once with a large volume of serum free medium the cultures were covered

by 0·5 ml of a 0·2–0·4 per cent suspension of indicator cells. Reading was performed after ½–1 hour. Mostly the haemadsorption was read macroscopically, but for certain purposes a grading of the reaction in separate tubes was needed and was estimated microscopically (50 × magnification) as follows: + + + + test cells completely covered with indicator red cells, + + + cells partly covered, + + haemadsorption only at the margin of cells with a palissade-like arrangement in several rows, and + scattered indicator cells at the periphery of test cells. Haemadsorption scored as + + is shown in Fig. 2.

For quantitation of serum titres, dilution series (two-fold or four-fold) of serum were tested and the titre was expressed as the last dilution giving a distinct haemadsorption (+ + or more).

II. Test on agar-overlaid cell cultures in flasks

This is essentially the same test as described in section I, except that the application of test serum and reading of results are modified so as to improve quantitative estimations of the reactions. It has been mainly used for the study of cell surface antigens.

The cell cultures grown out in 200 ml flat-bottomed bottles were washed once and overlaid with a 3 mm deep layer of 0·75 per cent Difco agar in an isotonic, buffered solution. Sterilized filter paper* discs, 12 or 5 mm in diameter, soaked in test serum, were placed upon the solidified agar surface. As a rule several four-fold or ten-fold serum dilutions were applied. Eight to twelve discs could be placed in each bottle.

The stoppered flasks with serum discs were left for 2–3 days at room temperature. The agar layer with discs was then easily poured out without damage to the cells after 10–15 ml of saline had been added.

Ten ml of a 0·2–0·5 per cent suspension of indicator cells was added and readings were taken after ½–1 hour standing at room temperature.

Positive reactions appeared as round and well-demarcated red zones of haemadsorption, the diameters of which were directly related to the amount of antibody originally placed in the disc (Espmark and Fagraeus, 1962, 1965).

III. Test with soluble antigen

Human thyroglobulin extracted according to Derrien, Michel and Roche (1948) was used as an antigen. After dialysis it was further purified by gel filtration on a Sephadex G-200 column to remove contaminating human serum components.

The antithyroid serum used was from a patient suffering from chronic thyroiditis. This serum agglutinated thyroglobulin-coated tanned cells in the Boyden test up to the dilution 1 : 4 million. The antibodies were found by immunoelectrophoresis and by gel filtration on Sephadex G-200 to belong to the γ_2 variety exclusively. The serum was heated for 30 minutes at 56° before use.

Performance of the test. The procedure followed is described in detail in a separate paper (Jonsson, 1965a) and will be only briefly summarized here.

A glass slide was provided with a layer of thyroglobulin, 1 μg/cm², which was fixed with acetone. A Perspex template with holes serving as serum reservoirs and with a strip of tape at each margin was placed over the thyroglobulin layer. The space formed between the template and the slide was filled with 0·75 per cent agar. Series of two-fold dilutions of antithyroid serum and normal human serum were added to the holes of the template. After 40 hours the Perspex template and the agar were removed and the slide was rinsed

* Filter paper (quality, Munktell No. 5) purchased from AB Kebo, Stockholm.

and flooded with a suspension of indicator cells. The suspension was poured off after 10 minutes. The resulting haemadsorption zones were measured with a pair of calipers.

FLUORESCENT ANTIBODY TEST

The indirect fluorescent staining technique of Weller and Coons (1954) was applied to monolayer cultures of HeLa cells, human kidney cells (first passage) and monkey kidney cells (primary cultures) which were set up on cover slips in Leighton tubes. The same antisera to these cells were used as in the haemadsorption test and the cytotoxic test. The sera were heat-inactivated and serially diluted in four-fold steps with PBS, pH 7·0.

Anti-rabbit globulin serum produced in sheep as described above was conjugated with fluorescein isothiocyanate (Riggs, Seiwald, Burckhalter, Downs and Metcalf, 1958) and absorbed with calf liver powder. The serum was used in the dilution 1 : 5.

The preparations were examined in a Reichert Zetopan microscope. Positive sera gave bright fluorescense of the cytoplasm in comparison with that of the nucleus. The reaction was regarded as negative when no obvious difference in staining intensity was distinguished between cytoplasm and nucleus.

CYTOTOXIC TEST

For the cytotoxic test 0·5 ml of two-fold dilutions of anticellular sera and 0·5 ml of 10 per cent fresh guinea-pig serum were added to partially outgrown tube cultures of HeLa cells, human kidney and monkey kidney cells which were then placed with the cell sheet down in a 36° incubator.

Readings were taken microscopically after 4 and 24 hours. The cytotoxic titre was given as the last dilution causing ++ degeneration, i.e. cytotoxic damage involving 10–90 per cent of the cells. The titres in Table 5 refer to the reading after 4 hours. At reading after 24 hours' incubation no considerable increase of the cytotoxic titres was noted.

RESULTS

I. EVALUATION OF INDICATOR RED CELLS

(a) *Choice of concentrations of ingredients used for coating.* For testing the efficacy of an indicator cell system, tissue culture tubes with discrete vaccinia virus degeneration foci were used throughout. The advantage of this model was firstly that haemadsorption could be related to the morphologically characteristic vaccinia plaques, and secondly that specific anti-vaccinia sera could readily be produced in all animal species involved. Suitable concentrations of sera for coating were determined by testing several lots of indicator cells prepared with different concentrations of the sera. A range of three to five four-fold dilutions of amboceptor serum was combined in a chessboard fashion with similar dilution series of the anti-γ-globulin serum. Each cell lot was tested in a set of vaccinia virus infected tissue cultures treated with a dilution series of antivaccinia serum. The outcome of a typical test is shown in Table 1. In this example amboceptor serum 1 : 160 and anti-γ-globulin serum 1 : 25 or 1 : 100 gave the best results. With a higher concentration of amboceptor serum the indicator cells tended to form large aggregates. In the indicator cell systems tested the optimal concentration of amboceptor serum was found to vary from one-half to two agglutinating units. The suitable concentration of the anti-γ-globulin serum was found to be relatively high and had to be established by chessboard

tests as mentioned above. As exemplified in Table 1 the efficacy of indicator cells improved with increasing concentration of the anti-γ-globulin serum to a certain level (in this case 1 : 100), after which further increase of concentration did not seem to affect the quality of indicator cells. Table 2 shows the adopted coating concentrations of several anti-γ-globulin sera in relation to the titres obtained against γ-globulin coated tanned cells and amboceptor coated cells.

TABLE 1

TEST FOR SUITABLE CONCENTRATIONS OF REAGENT SERA USED FOR DOUBLE-COATING OF INDICATOR RED CELLS ('RAT SYSTEM')

Chess-board arrangement of dilutions of rat 'amboceptor' and anti-rat γ-globulin serum from rabbit. The twenty-five cell lots were tested in series of tissue cultures containing vaccinia virus degeneration foci and treated with serial dilutions of an anti-vaccinia serum from rat.

Final dilution of rat amboceptor (used for first coating)	Final dilution of anti-rat γ-globulin serum from rabbit (second coating)	Reaction (haemadsorption) in vaccinia infected culture tubes treated with: Rat anti-vaccinia serum diluted: 1 : 400	1 : 1600	1 : 6400	1 : 25600	1 : 102400	Normal rat serum 1 : 100	Comments
1 : 40	1 : 25	+++	+++	+++	++	−	(++)*	Large
	1 : 100	++++	++++	(++)	(++)	−	(++)	clumps of
	1 : 400	+++	+	+	(+)	−	−	indicator
	1 : 1600	+	−	−	−	−	−	cells
	1 : 6400	+	−	−	−	−	−	
1 : 160	1 : 25	++++	++++	++++	+++	(+)	(+)	Numerous
	1 : 100	++++	++	++++	+++	(+)	(+)	small
	1 : 400	+++	+	++	−	−	−	aggregates
	1 : 1600	++	+	−	−	−	−	
	1 : 6400	(+)	−	(+)	−	−	−	
1 : 640	1 : 25	++++	++++	++	++	−	(+)	Small
	1 : 100	++++	++++	+++	++	−	−	aggregates
	1 : 400	+++	++	++	−	−	−	
	1 : 1600	+++	+	−	−	−	−	
	1 : 6400	−	−	−	−	−	−	
1 : 2560	1 : 25	+++	+++	+++	++	−	−	Few
	1 : 100	++	++	++	+	−	−	small
	1 : 400	+	−	(+)	−	−	−	aggregates
	1 : 1600	++	−	−	−	−	−	
	1 : 6400	−	−	−	−	−	−	
1 : 10240	1 : 25	++	++	++	(+)	−	(+)	No
	1 : 100	++	+++	−	−	−	−	aggregates
	1 : 400	+	−	(+)	−	−	−	
	1 : 1600	−	−	−	−	−	−	
	1 : 6400	−	−	−	−	−	−	

* Parenthesis means that indicator cells are easily detached by gentle shaking of the culture tube.

(b) *Comparison of indicator cells prepared in different ways.* In order to find out whether different ways of providing the first coating of the indicator cells may affect their indicator capacity, the following five lots of cells representing five different modes of achieving the first coating were prepared.

1. Sheep red cells were treated with human amboceptor.
2. Human O Rh(+) erythrocytes were incubated with anti-CD serum.
3. Tanned sheep red cells were coated with staphylococcal toxoid and then exposed to human anti-staphylococcal immune γ-globulin.

4. Sheep red cells were treated with a serum from a patient with mononucleosis.
5. Tanned sheep red cells were coated with electrophoretically purified human γ-globulin (γ_2).

The second coating of all five lots was performed with one single concentration of an

TABLE 2

THE TITRES OF ANTIGLOBULIN SERA, PRODUCED IN RABBIT, SHEEP OR HORSE, GIVEN AS THE RECIPROCAL OF THE LAST DILUTION REACTING IN PASSIVE AGGLUTINATION (BOYDEN) AND WITH SHEEP RED CELLS COATED WITH ONE-HALF OF AN AGGLUTINATING UNIT OF THE PERTINENT 'AMBOCEPTOR' SERUM

For passive agglutination cells were coated with 100 μg of globulin, separated on Sephadex G-200 or through rivanol precipitation. The last column shows the dilution of antiserum which by chessboard titration was found the most adequate to use for the second step in the double coating.

Species to which antiglobulin serum was produced	Titre of the antiglobulin serum tested against		Total dilution of antiglobulin serum used for second coating
	Passively sensitized erythrocytes	Sheep red cells with half an agglutinating 'amboceptor' unit	
Human	128,000	12,800	1 : 400
Monkey	51,200	6,400	1 : 100
Rabbit:			
from sheep	327,680	40,960	1 : 100
from horse	81,920	40,960	1 : 200
Guinea-pig	204,800	12,800	1 : 400
Rat	102,400	51,200	1 : 100
Mouse	5,120	2,560	1 : 50
Fowl	40,960	25,600	1 : 200
Calf	25,600	5,120	1 : 100

TABLE 3

EFFICACY OF INDICATOR CELLS, IN WHICH THE FIRST COATING WAS ACHIEVED IN FIVE DIFFERENT WAYS, TO TRACE ANTIBODIES TO THYROGLOBULIN

All cell systems were tested simultaneously. The figures in the bottom row refer to indirect indication (see text).

Cell batch No.	Red cells	First coating	Second coating (dilution of anti-human globulin serum)	Titre of test serum (reciprocal of end-point dilution)	Quality of haemadsorption zones
1	Sheep erythrocytes	Human 'amboceptor' dil. 1 : 200	1 : 400	6400	Good density Regular and distinct periphery
2	Human O Rh(+) erythrocytes	Incomplete anti-Rh (anti-CD) serum, 1 : 100	1 : 400	6400	Good density Regular but somewhat indistinct periphery
3	Tanned sheep erythrocytes coated with staphylococcal toxoid, 1 : 6	Human immune globulin again staphylococci, 1 : 200	1 : 400	6400	Good density Regular and distinct periphery
4	Sheep erythrocytes	Human Paul-Bunnell positive serum, 1 : 100	1 : 400	1600	Very faint zones Regular periphery
5	Tanned sheep erythrocytes	Electrophoretically purified human γ_2-globulin (120 μg/ml)	1 : 400	1600	Good density Regular and distinct periphery
	Indicator cells indicative of rabbit γ-globulin, prepared in analogy with 1, used for indirect indication		1 : 400 used on the slide in the first step of indication	6400	Good density Somewhat serrated periphery

anti-human globulin serum (1 : 400). The different lots were evaluated by their capacity to trace human antithyroglobulin zones on replicate slides in an experiment set up as described under 'Mixed haemadsorption', III (see Materials and Methods).

As seen in Table 3 the cell lots differed in their indicator sensitivity depending on the nature of and mode of attaching the human antibody in the first coating. Antiglobulin consumption tests (Table 4) showed that these differences did not depend simply on the amount of human antibody globulin present on the cells. The 'Rh cells' were as efficient as the 'human amboceptor' cells in spite of the fact that the latter carried much more antiglobulin on their surface than the former. Also the tanned cells coated in the first step with human γ_2^- globulin were evidently less sensitive than the two other cell types although, according to the consumption test, they should carry about the same amount of antiglobulin on their surface as the 'amboceptor cells'. The consumption tests gave different results depending on the type of sensitized cells added to the tubes in the antiglobulin titrations. When the titrations were performed with γ_2^- globulin coated tanned cells the amount of antiglobulin consumed by adsorption with this same type of cells was found to be larger than the amount consumed by 'amboceptor' coated cells. When 'amboceptor' coated cells were used in the titrations the reverse was true. Thus it would seem that

TABLE 4

ANTIGLOBULIN CONSUMPTION BY RED CELLS COATED WITH HUMAN γ-GLOBULIN IN THREE DIFFERENT WAYS CORRESPONDING TO THE 'FIRST' COATING OF THREE TYPES OF INDICATOR CELLS USED IN THE EXPERIMENT ACCOUNTED FOR IN TABLE 3

Red cells coated in the same three ways were used as agglutinating cells in the antiglobulin titrations. Antiglobulin consumption is expressed as the reduction in the number of tubes showing agglutination in a two-fold dilution series.

Type of sensitized cells added to the tubes in the antiglobulin titrations	Reduction of antiglobulin titre after absorption with		
	'Rh cells'	'Amboceptor cells'	Tanned sheep red cells with human γ_2- globulin
'Rh cells'	<1	>10	>10
'Amboceptor cells'	1	4	2
Tanned sheep red cells with human γ_2- globulin	<1	3	6

different antiglobulin molecules with somewhat different specificities were picked up by the different types of cells and that this may to some extent have determined the different sensitivity observed with differently prepared indicator cells.

The first two methods of coating indicator cells (i.e. with human amboceptor serum and human anti-CD serum) are now being used when human antibodies are to be traced. In tests with fixed soluble antigens both types of cells act with about the same efficacy. When comparing results on tissue cultures, however, the former (coated with human amboceptor) has always been somewhat superior to the Rh system (sharper-edged haemadsorption zones and firmer sticking of indicator cells).

II. INDIRECT INDICATION

Results with this procedure are given at the bottom line of Table 3. Replicate slides from the experiment referred to above had their anti-thyroglobulin zones indicated by

first flooding the slides with diluted (1 : 100) rabbit anti-human globulin serum for 10 minutes. Then, after washing, the layer of rabbit antibody bound to the anti-thyroglobulin in this step was traced by indicator cells for rabbit globulin in the same manner as described for direct indication.

The advantage of indirect indication should be that only one type of indicator cell is needed, e.g. cells indicating rabbit γ-globulin could suffice for tracing antibodies from various species provided that all antiglobulin sera were produced in rabbits. The application of indirect indication to antibodies of various physical classes will be discussed later and in a following paper (Jonsson, to be published).

III. SENSITIVITY OF THE MIXED HAEMADSORPTION METHOD AS COMPARED TO THE CYTOTOXIC TEST AND THE FLUORESCENT ANTIBODY TECHNIQUE

Two experiments were set up to compare simultaneously the mixed haemadsorption reaction with the cytotoxic test and the fluorescent antibody technique as methods for

TABLE 5

THE SENSITIVITY OF THE MIXED HAEMADSORPTION REACTION COMPARED WITH THE FLUORESCENT ANTIBODY TECHNIQUE AND THE CYTOTOXIC TEST

Antisera produced in rabbits. Five per cent fresh guinea-pig serum added to serum for cytotoxic test.

Antiserum to	Test tissue	Reciprocal of titre (total serum dilution)		
		Mixed haemadsorption	Indirect fluorescence	Cytotoxic effect
HeLa	HeLa	81,920	N.D.	80
HeLa	Human kidney	81,920	80	640
Human kidney	HeLa	81,920	N.D.	10
Human kidney	Human kidney	81,920	20	160
Monkey kidney	Monkey kidney	50,000	20	40

detecting antibodies to cultured cells. In the first experiment antisera to HeLa cells and human kidney, produced in rabbits, were tested against homologous and heterologous tissue in culture. In the second experiment antiserum to monkey kidney was tested in the same manner against monkey kidney cells.

As will be seen from Table 5, the sensitivity of the mixed haemadsorption test surpassed that of the two other methods. The titres of the antisera were 100–1000 times higher when tested with mixed haemadsorption. The high titres with the latter method may not be entirely due to a higher degree of sensitivity, however, since it is possible that the three test methods measure different kinds of antibodies.

IV. SPECIFICITY OF INDICATOR CELLS

To a limited extent the species-specificity of the indicator cells was also tested. Anti-human cell sera produced in rats, rabbits and guinea-pigs were tested in bottle cultures of HeLa cells by the filter paper disc technique, described by Espmark and Fagraeus (1962). Homologous and heterologous indicator cells were then added. The results are shown in Table 6. All types of indicator cells reacted only with homologous antibody.

When, however, a similar cross-testing was performed with antisera of human and simian (Cynomolgus) origin to vaccinia, it was found that indicator cells prepared to trace human antibodies (human system cells) reacted strongly with antibodies from the monkey. The monkey system reacted only very slightly with the homologous serum and did not indicate human vaccinia antibody at all. A possible explanation of this unexpected result was the finding that the utilized anti-monkey globulin serum produced in the rabbit reacted predominantly with γ_{1M} globulin of both human and monkey serum while no confident reactions could be demonstrated with monkey or human γ_2-globulin, either in the immunoelectrophoresis or when tested against Rh coated human red cells. These findings could be utilized to prepare indicator cells reacting selectively to human γ_{1M} antibodies in the haemadsorption test by coating the cells first with human amboceptor (predominantly γ_{1M} globulin—Aho *et al.*, 1964) and then with the rabbit anti-monkey globulin. This work will be described in a separate report.

TABLE 6

RESULTS FROM A MIXED HAEMADSORPTION TEST (DISC TEST) ON HELA CELL CULTURES TO DEMONSTRATE THE DEGREE OF SPECIES SPECIFICITY OF THREE DIFFERENT INDICATOR CELL SYSTEMS

On each of replicate agar-overlaid bottle cultures of HeLa cells two discs with ten-fold dilutions of anti-human cell sera from rabbit, rat and guinea-pig were placed. Titres were expressed as the serum dilutions estimated to give haemadsorption zones of 10 mm diameter.

Test-sera	Serum titres with indicator cells prepared to trace γ-globulin from		
	Rabbit	Rat	Guinea-pig
Anti-HeLa (rabbit)	1 : 11400	<1 : 20	<1 : 20
Anti-HeLa (rat)	<1 : 20	1 : 5000	<1 : 20
Anti-human thyroid (guinea-pig)	<1 : 2	<1 : 2	1 : 7100

Controls: (a) Pre-immunization sera from rabbit, rat and guinea-pig gave no reactions with either indicator cell system; (b) untreated sheep red cells gave no reactions; (c) sheep red cells treated with anti-sheep red cell serum only (rabbit, rat, guinea-pig) gave no reactions; (d) sheep red cells treated only with anti-γ-globulin serum (anti-rabbit, anti-rat, anti-guinea-pig) gave no reactions.

DISCUSSION

Mixed haemadsorption should be regarded as an application of the mixed antiglobulin reaction to a situation where the antigen is sessile on a glass (or plastic) surface. The phenomenon actually observed as a positive outcome of the test is therefore not an agglutination but rather an adsorption of red cells to the surface on which the test antibody has been attached by reacting with the test antigen.

A further characteristic of the technique is that the antiglobulin is pre-attached to the indicator cells. The use of erythrocytes as indicator cells has several advantages. The reaction can be read macroscopically. Iron staining of the erythrocytes can be used to visualize the reaction more distinctly for photographic purposes. Furthermore, the red cells carry antigenic determinants on their surface. That this is an important advantage

appears from the experiments accounted for in Tables 3 and 4. They show that the efficiency of the indicator cells does not depend entirely on the amount of γ-globulin attached to them in the 'first' coating but rather on the qualities of that γ-globulin. The antibody globulin captured by the natural determinants of the red cells in the first coating apparently selected antiglobulins in the second coating which were more reactive than the antiglobulin selected by γ-globulin attached non-specifically to the surface of tanned red cells.

Other particles possessing natural antigenic determinants on their surface, i.e. bacteria, fungi, etc., may be more convenient to use for some special purposes.

So far only a few of the possible applications for the mixed haemadsorption technique have been investigated. There is a promising field of use for the study of antigenic properties of cell surfaces, notably those of cells grown in monolayer culture. The mixed haemadsorption technique was used as a test for species identity of cultured cells by Espmark and Fagraeus (1962, 1965).

Organ specific reactions were demonstrated with human sera from cases of thyroiditis and thyrotoxicosis when tested on monolayer cultures of human thyroid cells (Jonsson, Fagraeus and Espmark, 1964). The method has been used by others to trace antibodies to 'heterogenetic' antigens in mononucleosis and serum sickness (Kano and Milgrom, 1964) and for the study of antigens in tissue sections of brain and adrenal tissue (Tönder, Milgrom and Witebsky, 1964).

Certain viruses, multiplying in cell cultures will appear on the cell surface as mature particles or as partial viral antigens, and may accordingly be demonstrated by mixed haemadsorption. This was achieved with vaccinia virus, measles and canine distemper viruses (Fagraeus and Espmark, 1961), and later also with herpes simplex virus (Espmark, 1965) and respiratory syncytical virus. Barron, Milgrom, Karzon and Witebsky (1963) found the method sensitive for the titration of measles antibodies in infected tissue cultures. The mixed haemadsorption technique may be of practical value in the first place for viruses that do not spontaneously agglutinate any red cells and that exert a weak or no cytopathic action.

The test has been applied to several kinds of soluble antigens fixed on glass, as for instance PPD, various sera and γ-globulins as well as different cell extracts (Jonsson, 1965a). Different antinuclear factors were traced in LED sera using different nuclear components as antigens (Jonsson and Fagraeus, 1962) and antibodies in sera from patients with thyroid disease were traced using thyroglobulin and cytoplasmic extract from thyroid glands (Jonsson, unpublished).

In some instances it has been possible to distinguish between different antibody classes with the mixed haemadsorption test. By use of an anti-monkey globulin serum, indicator cells were obtained which traced γ_{1M} but not γ_2 antibodies. The same can be achieved by indirect indication, i.e. relabelling of fixed antibody with a γ_{1M} specific antiglobulin serum.

Antibodies of different molecular size diffusing through an agar layer give different slopes of the regression of zone diameter upon serum dilution. This may be exploited for antibody classification (Jonsson, 1965b).

The most important merits of the mixed haemadsorption technique are: high sensitivity, favourable conditions for quantitation and the possibility of reading the test macroscopically.

The sensitivity is much higher (100–1000 times) than for the ordinary fluorescent

antibody technique and cytotoxic test when used for the titration of antibody. In some experiments comparing the mixed haemadsorption (end-point titres) and Boyden's passive haemagglutination test equal serum titres were obtained (Jonsson, 1965a). Apparently the higher sensitivity may prove profitable for instance in tracing antibodies involved in autoimmune diseases.

Quantitation of serum titres may, as with other tests, be achieved through dilution assays. However, some subjectivity is inevitably involved in the determination of an extinction end-point. The use of serum reservoirs (discs, cups) resulting in measurable haemadsorption zones permits a more reliable quantitative estimation (Espmark and Fagraeus, 1965).

Only surface antigens are involved in the mixed haemadsorption test applied to monolayer cultures. This is an advantage when information is wanted on the location of the reacting antigen. On the other hand it precludes the use of this technique in the study of intracellular antigens. This obstacle may, however, often be partly overcome by using cellular extracts fixed on a glass surface.

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