Leucocyte grouping : a method and its application

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Chapter two

MATERIALS AND METHODS

Glassware and reagents

All glassware employed in this study was prepared from normal soda-glass. The following items were used.

Centrifuge tubes, 90 x 14 mm.
Round bottomed tubes, 50 x 7 mm, so-called "rhesus tubes".
Round bottomed tubes, 150 x 14 mm (type C).
Conical flasks, 50 ml.
Glass beads, diameter 4 mm.
Microscope slides, 25 x 75 mm and 50 x 300 mm.
Capillary pipettes.

Glassware was cleaned as follows. Immediately after use it was placed in a pail filled with tap-water in order to prevent the drying up of the blood or serum. At the end of the day most of the blood or serum still adhering was removed by rinsing under running tap-water. During the night the glassware was soaked in a 2.5% solution of the detergent Bosmanite AL-25 (Rogier Bosman, Rotterdam) in tap-water. If necessary the glassware was cleaned with a brush next morning. After another rinse in tap-water, the glassware was placed in distilled water for 15 minutes. Thereafter the glassware was dried in an oven at 250°C for 60 minutes. The prevention of the drying up of the blood or serum proved to be essential.

Glassware was siliconised as follows. It was filled with a solution of silicone oil 200/350 centistokes (Dow - Corning Corp., Midland, Mich., U.S.A.) 2% v/v in carbon tetrachloride and the silicone solution was immediately transferred to another tube or container. Glassware that had been in contact with the silicone was dried at 250°C for 60 minutes.

The following solutions were used.

A sterile, but not pyrogen-free solution of 0.15 M NaCl in distilled water buffered with 0.0013 M Na_2HPO_4-NaH_2PO_4 to pH 7.2. This solution will be referred to as saline.
Disodium ethylenediaminotetraacetate, 5% in unbuffered saline; for this solution the abbreviation EDTA will be used.
Sodium azide, 10% solution in saline.
Tween 80, 2% solution in saline.
6% Acetic acid.
A saturated solution of $(NH_4)_2SO_4$ in distilled water.
Dextran (Poviet, Amsterdam), mean molecular weight 200,000, 5% in saline.
Macrodex (Poviet, Amsterdam), a dextran, mean molecular weight 75,000, 6% in saline.

The following biological reagents were used:

Heparin (Vitrum, Stockholm), 1 ml = 5000 I.U.
Serum of a donor with blood group AB, who was apparently

healthy, had not been pregnant, and had never received a blood transfusion. This serum will be called AB serum.
A 20% solution of bovine albumin (Poviet, Amsterdam).
The other biological reagents will be described below.

To facilitate microscopical readings of the leucocyte agglutination reaction, the following device was constructed. From ordinary glass a large microscope slide, 50 x 300 mm, was made. Lines were cut in the glass with a glass-knife, dividing it into 20 equal rectangles. The ordinary object-holder of the microscope was removed and replaced by a light-metal strip, 130 x 70 mm, with a central open rectangle of 30 x 50 mm. Two springs made it possible to secure the large microscope slide, lightly clamped, on the metal strip. In this way the use of the mechanical stage was still possible, while 20 samples could be read without changing the slide (fig. 1).

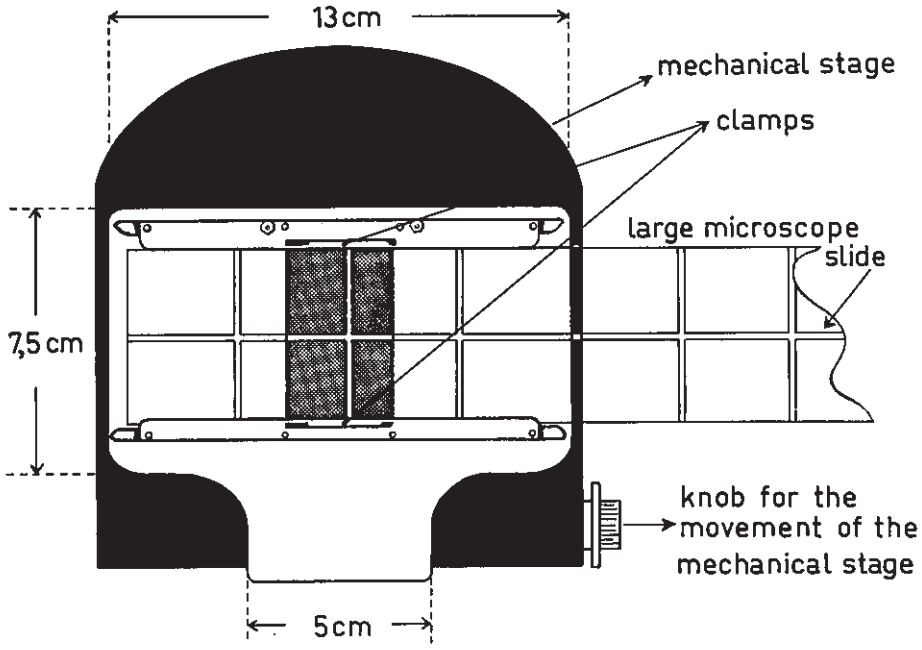

Figure 1
Bird's eye view of the device facilitating the microscopical reading

Sera

The sera from pregnant women were obtained from the Departments of Obstetrics of the University Hospital, Leyden (head, Prof. Dr. A. J. M. Holmer) and of the St. Elisabeth Hospital in Leyden (Dr. R. T. P. Niemer). In all more than 2000 sera were examined. Serial numbers

were given to the women whose serum proved to contain leucocyte agglutinins.

Sera from 520 patients - men, and women who had never been pregnant - were obtained from other departments of the University Hospital. This group is called the group of non-pregnant patients. Most of these sera were obtained from patients of the Department of Cardiology, who were eligible for open-heart surgery. A group of 68 sera from patients who had suffered from a transfusion reaction was also investigated.

In addition the sera from 545 donors of the Blood Transfusion Service were examined.

Blood was obtained by a smooth venepuncture and was collected in a dry clean tube or bottle. No special measures were taken to secure aseptic conditions. After incubation for at least one hour at 37°C the blood was centrifuged and the serum transferred to a dry clean tube. The sera, if not examined the same day, were stored at -20°C after addition of 10% sodium azide solution (one drop per 8 ml serum) and were inactivated at 56°C for 30 minutes before use. If the sera were not clear after storage or a manipulation e.g. absorption, they were centrifuged at 16,000 rpm (16,000 g) for 10 minutes at +4°C.

Leucocyte suspensions for the agglutination reaction

The blood for the agglutination and absorption reactions was in general obtained from the so-called panel, consisting of a group of 100 persons with bloodgroup O: nurses, doctors and technicians working in the University Hospital. They were numbered at random.

For the estimation of gene frequencies and the screening of sera for the presence of leucocyte agglutinins blood samples from donors of the Blood Transfusion Service were used.

Blood has also been obtained from women who had formed leucocyte agglutinins, their husbands and children. Finally the blood from 23 randomly selected families was collected.

The three cell suspensions to be described in the following paragraphs were used within two hours of the venepuncture.

Preparation of the leucocyte suspension from defibrinated blood

Blood was obtained by clean venepuncture and about 20 ml was collected in a siliconised 50 ml conical flask containing approximately 20 glass beads. The blood was defibrinated by an ellipsoid movement ($\pm$80 rotations per minute) of the bottle. Foaming was avoided as much as possible. The defibrinated blood was decanted into a siliconised tube type C and mixed with 2 ml dextran solution and 1 ml Tween 80 solution. After incubation of the tube at 37°C for about 30 minutes at an angle of 45° the upper four-fifths of the supernatant plasma was transferred to a siliconised centrifuge tube with a siliconised pipette. This part of the plasma contained 4000 $\pm$ 1000 leucocytes per mm^3 and was used in the agglutination reaction.

If necessary leucocyte suspensions were concentrated by centrifuging the suspension for 10 minutes at 1000 rpm (120 g) in siliconised centrifuge tubes. If a suspension of e.g. 40,000 leucocytes/mm^3 was

required and the original suspension contained 4000 leucocytes/mm^3; 9/10 of the supernatant was removed and the sediment was resuspended in the remaining plasma.

Preparation of the leucocyte suspension from EDTA blood

Blood was obtained by a clean venepuncture and about 8 ml was collected in a siliconised centrifuge tube containing 1 ml EDTA solution. After mixing the EDTA blood was poured into a siliconised tube type C containing 2.5 ml dextran solution. The tube was incubated at 37°C for 30 minutes at an angle of 45°. After incubation the upper four-fifths of the supernatant plasma was transferred with the aid of a siliconised pipette to a siliconised centrifuge tube. This part of the plasma contained 6000 $\pm$ 1500 leucocytes per mm^3 and was used for the agglutination reaction.

Leucocyte suspensions with a higher concentration were prepared as described in the preceding section.

Preparation of "pure" granulocyte and "pure" lymphocyte suspensions

Blood was obtained and a leucocyte suspension was prepared as described in the preceding section. The leucocyte suspension was centrifuged at 1000 rpm for 10 minutes. The platelet-rich plasma was removed and the sediment resuspended in three drops of the same plasma. This concentrated leucocyte suspension was cautiously and slowly squirted into 1 ml saline, which had been layered above 2.5 ml of bovine albumin in a siliconised centrifuge tube. This tube was centrifuged at 3000 rpm (1000 g) for 15 minutes. All erythrocytes and the greater part of the granulocytes were thereby concentrated in the sediment, while all the remaining platelets and the greater part of the lymphocytes occupied a layer between the albumin solution and the saline. The amount of contamination, i.e. granulocytes in the lymphocyte suspension and vice versa, varied from one sample to another. About 75% of the suspensions contained less than 5% contamination with the other component; only these suspensions were used.

Cell suspensions for the absorption experiments

Leucocyte suspensions for cross-absorption experiments

These suspensions were prepared as described under "Preparation of the leucocyte suspension from EDTA blood". "Packed" leucocytes were used for the absorption i.e. the leucocyte suspension was centrifuged for 30 minutes at 3000 rpm and the plasma was removed. The cells were not washed in saline. The cell suspensions contained not only leucocytes, but also erythrocytes and platelets. The erythrocytes do not contain the leucocyte iso-antigens under study, but the platelets do (see chapter four). As this antigen is present on the platelets only if it is also carried by the leucocytes, the contamination of the cell suspension with platelets did not interfere with the group specificity of the cell suspension. The average suspension contained 100 to 300 erythrocytes and 2000 to 10,000 platelets per 100 leucocytes. An average of 1.8×10^8 leucocytes could be isolated from 50 ml of blood.

"Pure" leucocyte suspensions

Blood was obtained by a clean venepuncture and collected in a bottle containing approximately 100 I. U. of heparin per 10 ml of blood. The heparinised blood was centrifuged for 15 minutes at 1000 rpm (120 g). The supernatant platelet-rich plasma was transferred to a second siliconised centrifuge tube (tube B) and the sediment of erythrocytes and leucocytes was put aside (tube A). The platelet-rich plasma (tube B) was centrifuged for 45 minutes at 3000 rpm. The sediment of erythrocytes and leucocytes (tube A) was resuspended in the platelet-poor plasma so obtained and the whole procedure was repeated. Three volumes of platelet-poor blood were mixed with one volume of Macrodex and the mixture was incubated for 45 minutes at 37°C. The resulting platelet-poor leucocyte suspension was centrifuged during 15 minutes at 1500 rpm, the supernatant plasma was discarded and the cellular sediment was resuspended in saline. Due to the paradoxical sedimentation (48) a leucocyte suspension was obtained with approximately one erythrocyte and two platelets per 100 leucocytes. Similar results can be obtained by differential centrifugation of the leucocyte suspension instead of the whole blood. Packed leucocytes were used for absorption.

Thrombocyte suspensions

These were obtained from EDTA blood by differential centrifugation as described above. The platelet sediments were washed twice in saline. They contained less than two erythrocytes and less than one leucocyte per 100 platelets.

The cell suspensions described above were used within four hours after the venepuncture.

Erythrocyte suspensions

The blood was obtained and processed as described under "Preparation of the leucocyte suspension from EDTA blood". To obtain a pure red-cell suspension the supernatant plasma was removed after dextran-sedimentation together with the upper 1/10 of the erythrocyte sediment. After the addition of 2.5 ml of dextran solution to the erythrocyte sediment the blood was once more incubated for 30 minutes at 37°C and the supernatant plasma with the upper 10% of the red cell sediment was again removed. The erythrocyte sediment contained not more than 4% of the leucocytes originally present. This sediment was washed three times in saline, the upper part of the "packed cells" being removed after every washing. The resultant concentration of leucocytes in the final erythrocyte suspension was less than 1% of the leucocytes originally present.

Preparation of placental and kidney tissue for absorption tests

Ten to forty grams of the (membrane-free) fresh tissue were cut into small blocks, washed free of blood in saline and homogenised in a Waring blender at 0°C ($2\frac{1}{2}$ minutes at full speed). The tissue was washed by centrifuging in saline until the washings were no longer pink. The resulting sediment was used for the absorption tests. From

an adjacent part of the tissue histological sections were prepared. The results of the absorption experiments were considered to be representative of the antigenic structure of the tissue under study only if the histological sections were free or practically free from leucocyte infiltration.

Agglutination reactions

With leucocytes from defibrinated blood (Dausset's test (20))

Two drops (equal to approximately 0.10 ml) of a leucocyte suspension were mixed in non-siliconised rhesus tubes with two drops of inactivated serum or serum dilution. The tubes were then incubated for 120 minutes at 37°C. After the incubation the supernatant plasma was removed and one drop of 6% acetic acid was added to the sediment to lyse the red cells. The sediment was transferred to a non-siliconised slide and read microscopically (magnification 50 - 100 x). When the serum and the blood used for the leucocyte suspension were incompatible with regard to the ABO groups, the anti-A or anti-B was absorbed out of the serum with leucocyte-poor A_1 or B packed erythrocytes before the serum was used in the leucocyte agglutination reaction.

Titration of the serum was performed by stepwise twofold dilution in normal AB serum. In comparative titrations of one serum against different leucocyte samples or antigen concentrations the serum under study was titrated in "bulk". This was done as follows: if eight identical titrations of a serum A were needed one ml of serum A was titrated by stepwise twofold dilution in AB serum. Hereafter two drops of leucocyte suspension were mixed with two drops of serum A or of the dilution of serum A obtained as mentioned above. In this way differences between the rows of dilutions are reduced to a minimum.

The following code was used in scoring the agglutination patterns: - (negative) =0, weak or dubious = 1, + = 2, ++ = 3, +++ = 4, ++++ = 5 (see fig. 2*). A cell suspension in which the leucocytes were evenly distributed was denoted negative. Macroscopically visible clumps with almost no free cells were graded as ++++. Between these two extremes, the reaction was scored according to the size of the clumps and the number of free cells. The sum of the numbers given to the results of a titration is the total score of the titration.

A serum was considered to contain leucocyte agglutinins only if leucocyte agglutinins could be demonstrated in more than one serum sample procured on different days and if these agglutinins gave a two plus reaction with at least one leucocyte sample, or if the serum was able to agglutinate the leucocytes of the husband in a titre of at least 2.

With leucocytes from EDTA blood (van Loghem's test (44))

This procedure was identical with the one described in the preceding section except for the difference that EDTA was used to make the blood incoagulable. Moreover the titrations were carried out in saline

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* Tables and figures marked with an asterisk are to be found at the end of this publication.

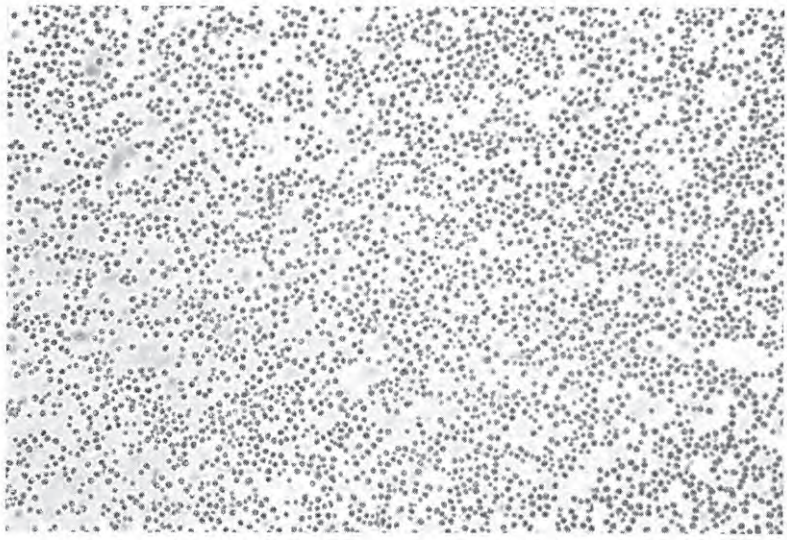

Figure 2a
Leucocyte agglutination test (80 x). Dausset's method.
Negative reaction

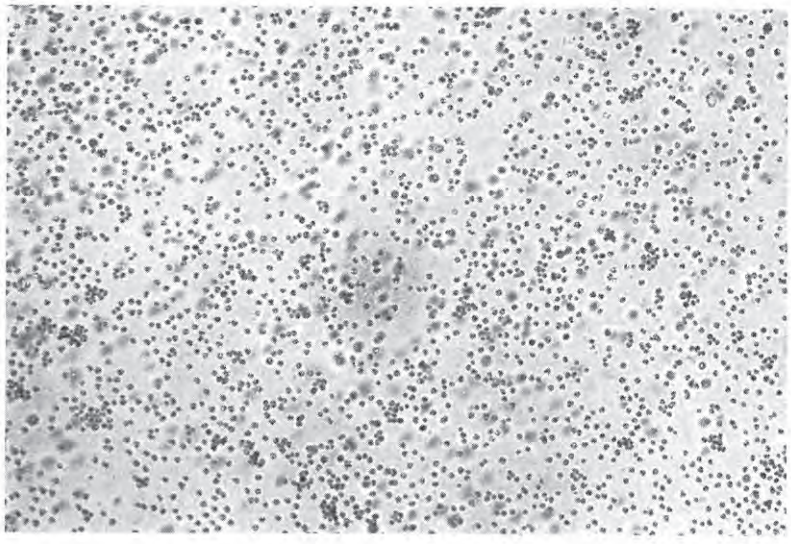

Figure 2b
Leucocyte agglutination test (80 x). Dausset's method.
Weakly positive reaction

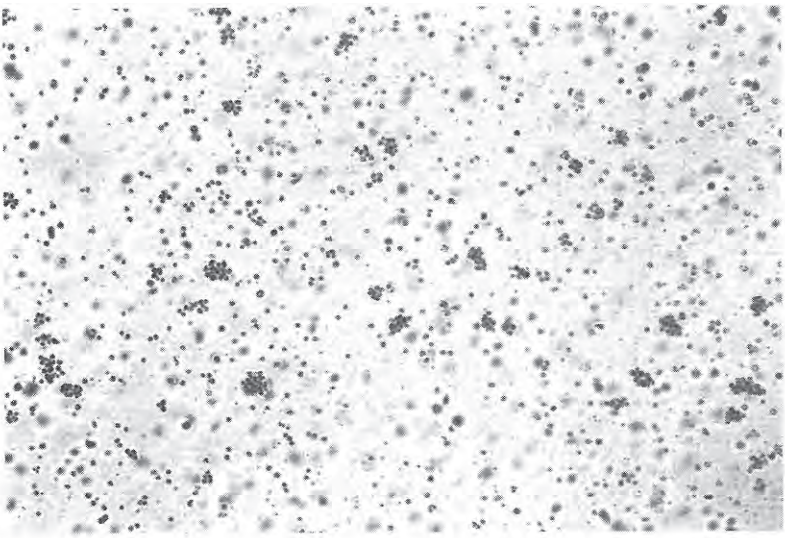

Figure 2c
Leucocyte agglutination test (80 x). Dausset's method.
+ positive reaction

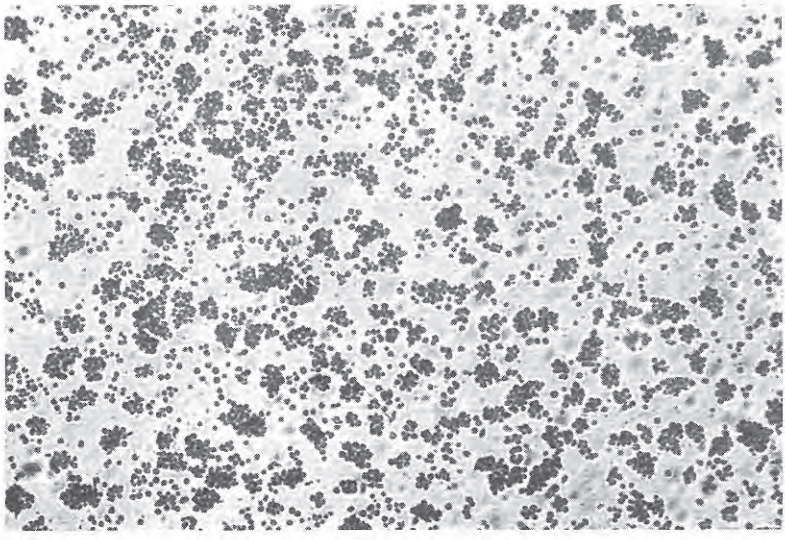

Figure 2d
Leucocyte agglutination test (80 x). Dausset's method.
++ positive reaction

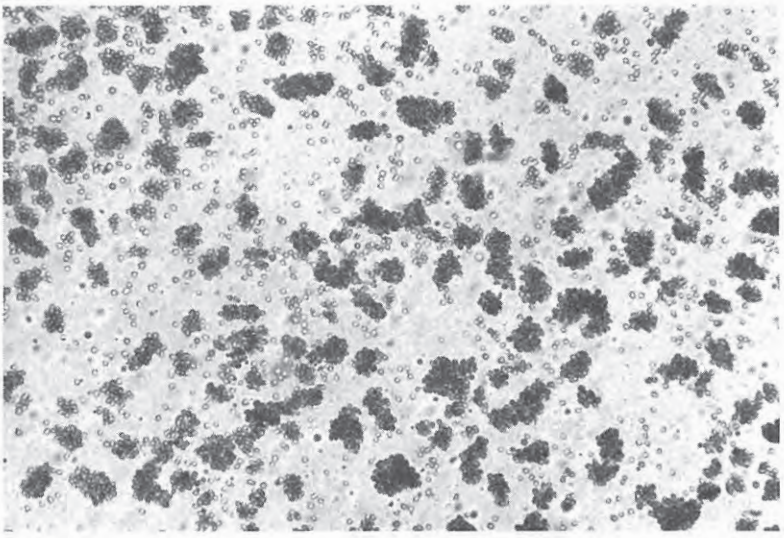

Figure 2e
Leucocyte agglutination test (80 x). Dausset's method.
+++ positive reaction

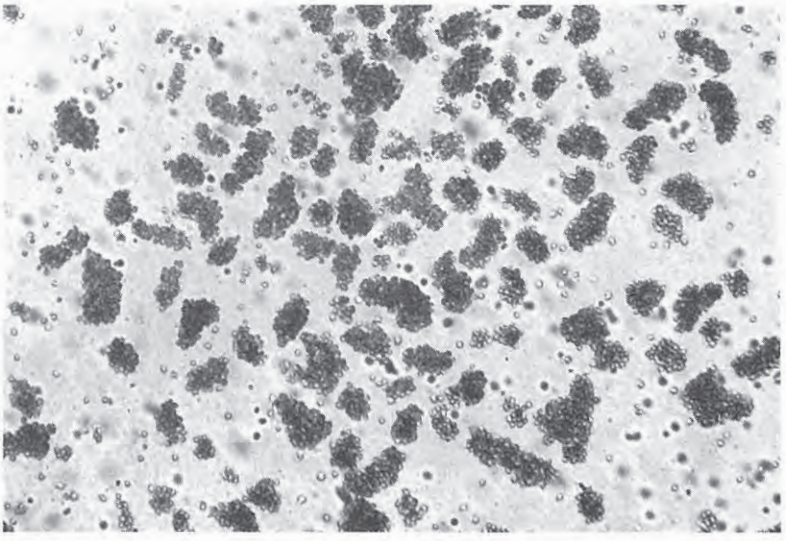

Figure 2f
Leucocyte agglutination test (80 x). Dausset's method.
++++ positive reaction

instead of AB serum. The different leucocyte agglutination patterns are shown in fig. 3*.

With both procedures the test was done in duplicate and recorded as the mean of the two observations. The reactions performed during the screening of the sera for the presence of leucocyte agglutinins were, however, carried out only once with each leucocyte sample.

Absorption tests

With leucocyte, thrombocyte and erythrocyte suspensions

Packed cells were incubated for 30 minutes at 37°C in various known concentrations with a known amount of inactivated serum containing antibodies against leucocytes. After centrifugation of the mixture at 3000 rpm for 45 minutes, two drops of the absorbed serum were titrated against the leucocytes of the donor of the cell suspension which had been used for the absorption. As a comparison the non-absorbed serum was also titrated against the same leucocyte suspension. In cross-absorption tests the absorbed serum was also tested with the leucocytes from other donors. To exclude the possibility that the absorption of the agglutinin was caused by a non-specific effect, the serum was absorbed not only with cells which carried the corresponding antigen, but also with cells which did not carry this antigen.

With placental and kidney tissue

Leucocytes from the patient whose tissue was under investigation were tested with about 12 sera, which contained agglutinins against leucocytes and which had been obtained from other patients. A serum which gave a strongly positive reaction with the leucocytes from the patient was used for the absorption tests (serum A). In order to exclude the possibility that the absorption was brought about by a non-specific factor, another serum with antibodies which reacted negatively with the leucocytes of the patient was also absorbed with the tissue under study (serum B).

The sera used for the absorption tests with placental tissue were selected after they had been incubated not only with the leucocytes of the mother but also with the leucocytes of the child, as it is impracticable to separate the maternal from the foetal part of the placenta. Serum A agglutinated the leucocytes from both the child and the mother, while serum B did not agglutinate either leucocyte sample.

The absorption was performed as follows: 0.8 ml "positive" serum A and 0.8 ml "negative" serum B were each incubated with approximately 0.7 gram (wet weight) of homogenised and washed tissue sediment. After carefully resuspending the sediments the two mixtures were placed in the waterbath at 37°C for 30 minutes. They were then centrifuged at 16,000 rpm (16,000 g) for 10 minutes at +4°C. The sera were tested before and after absorption with the leucocytes from the patient (or, in the case of placental tissue, the mother and the child) and those from a random donor who reacted positively with serum B.

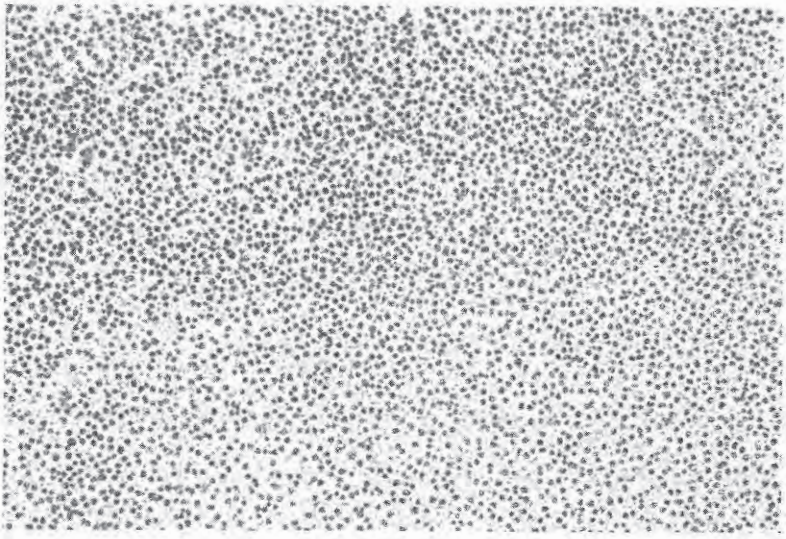

Figure 3a
Leucocyte agglutination test (80 x). van Loghem's method.
Negative reaction

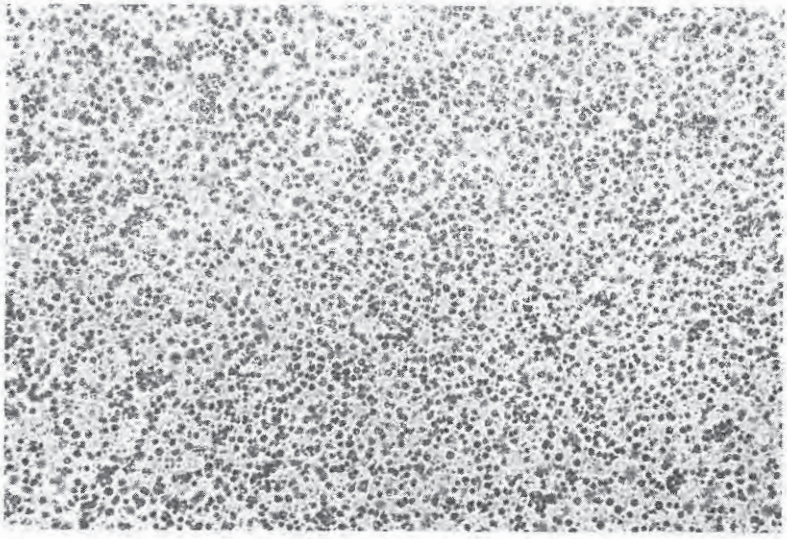

Figure 3b
Leucocyte agglutination test (80 x). van Loghem's method.
Weakly positive reaction

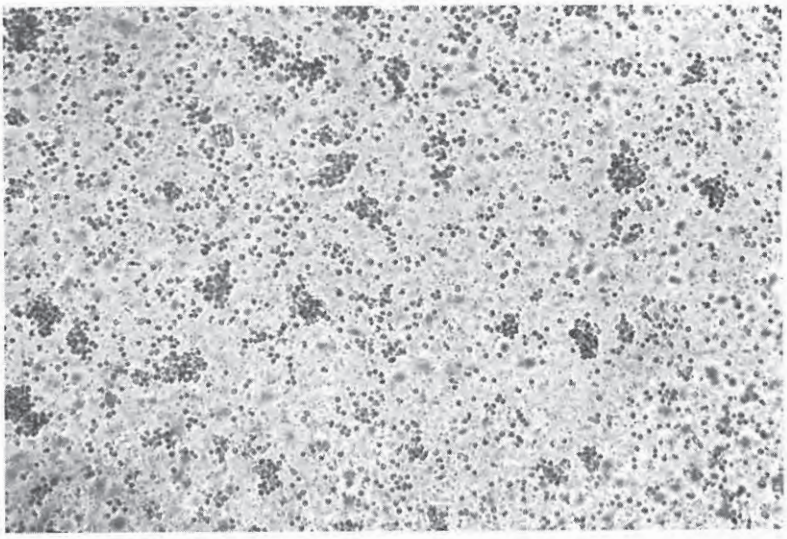

Figure 3c
Leucocyte agglutination test (80 x). van Loghem's method.
+ positive reaction

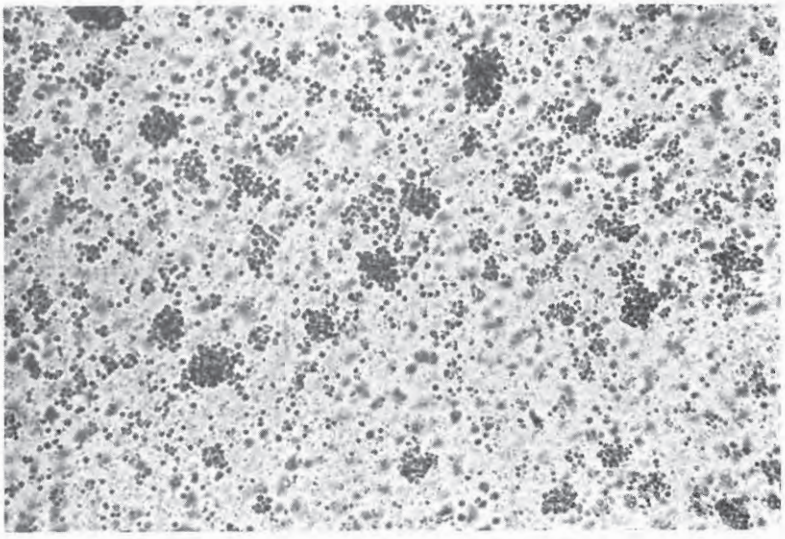

Figure 3d
Leucocyte agglutination test (80 x). van Loghem's method.
++ positive reaction

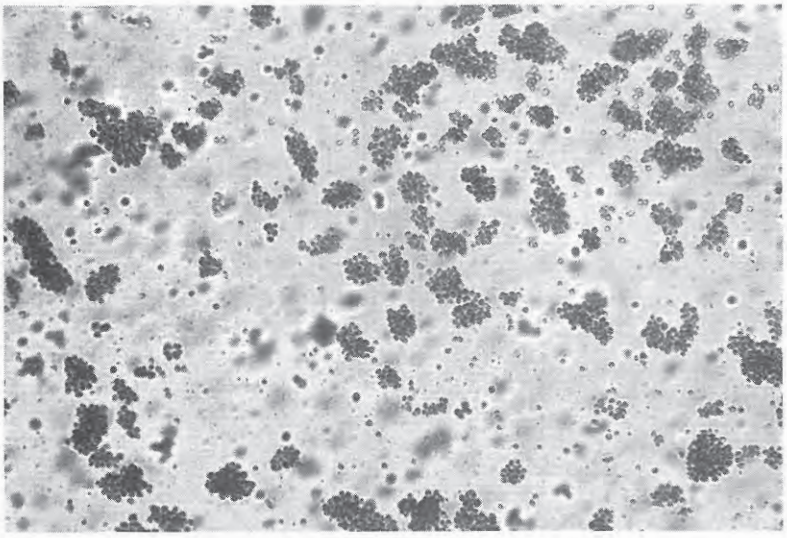

Figure 3e
Leucocyte agglutination test (80 x). van Loghem's method.
+++ positive reaction

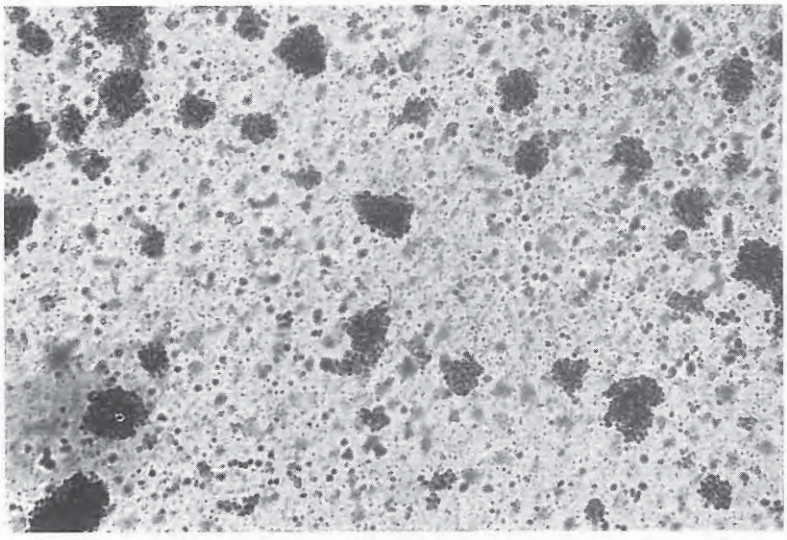

Figure 3f
Leucocyte agglutination test (80 x). van Loghem's method.
++++ positive reaction

Elution tests

Weiner's method was used (76): leucocytes which had been used for an absorption experiment (2.5×10^8 leucocytes with 1 ml serum) were washed thrice in saline. The leucocytes were lysed by freezing at -20°C after the supernatant saline had been removed.

Ethanol 50% v/v cooled to -20°C, a volume of 10 times the original volume of the packed leucocytes, was added to the lysed leucocytes after thawing. The cell ghosts were resuspended in the ethanol and the suspension was incubated at -20°C for at least one hour. After 5 minutes centrifugation at 3000 rpm the supernatant was removed and the sediment was resuspended in distilled water. After another centrifugation the sediment was resuspended in saline, and the tube was incubated at 37°C for 60 minutes. The supernatant obtained after a final centrifugation is the "eluate".

Isolation of γ-globulin

Kendall's method was used (33): 5 ml of a saturated $(NH_4)_2SO_4$ solution was added to 10 ml serum. The sediment was dissolved in 5 ml distilled water. Two and a half ml $(NH_4)_2SO_4$ solution was added with constant stirring to this solution. The procedure was repeated five times. The resulting euglobulin fraction was dialysed against saline until free of sulphate. The water-insoluble euglobulin fraction was precipitated by 1 - 2 hours' dialysis at +4°C against four changes of distilled water. The supernatant obtained after centrifugation contained the γ-globulins.

Calculations

To establish the correlation between two variables Fisher's 2 x 2 test was used (32).

As described in chapter four 66 sera were tested against the leucocytes from EDTA blood of a panel of 100 random donors (Table VI*). Each vertical row shows the results obtained with one serum. It was necessary to compare the results of each serum with those of all other sera. This implied that more than 2000 times two rows of results, each consisting of 100 agglutination tests, had to be compared. The results originally consisted of positive, negative and doubtful results. To facilitate the calculations the dubious results were changed in "positive" or "negative" according to the following rule: if serum incubated with leucocytes from EDTA blood gave a dubious reaction, the reaction was recorded as positive if the same serum leucocyte combination gave a positive reaction with leucocytes from defibrinated blood. If the serum-leucocyte combination gave a negative reaction with leucocytes from defibrinated blood the reaction was recorded as negative. To compare the two vertical rows of agglutination tests the leucocyte samples were divided into four groups:

group A consisted of those leucocyte samples which gave negative results with the two sera (the number of leucocyte samples belonging to group A is called a);

group B consisted of those leucocyte samples which gave a negative result with the first serum and a positive result with the second serum (= b);
group C consisted of those leucocyte samples which gave a positive result with the first serum and a negative result with the second serum (= c), while group D consisted of the leucocyte samples which were agglutinated by both sera (= d).

In view of the large number of comparisons to be made the statistical sorter IBM 101 was used for the classification of the results into the above-mentioned groups.

In order to investigate to what extent the distribution of the leucocyte samples in the above-mentioned groups could be due to chance Fisher's 2 x 2 test was used (32). The necessary calculations were made by the electronic calculator IBM 604 using the following formula:

$$\chi^2 = \frac{(ad - bc)^2 \ (a + b + c + d)}{(a + b) \ (c + d) \ (a + c) \ (b + d)}$$

The resulting χ^2 values were tabulated in two written records: in the first place in the order of the serum numbers used in the comparison e.g. serum no. 1 with sera nos. 2, 3, 4, 5 etc. (Record A) and in the second place in the order of decreasing magnitude of the χ^2 values (Record B).

The gene frequencies were calculated as follows (67): assume a theoretical leucocyte group Z has the phenotypes Z^a, Z^aZ^b, and Z^b. For Z^a the genotype frequency will be:

gene Z^a = frequency phenotype Z^a + $\frac{\text{frequency phenotype } Z^aZ^b}{2}$

and for

gene Z^b = frequency phenotype Z^b + $\frac{\text{frequency phenotype } Z^aZ^b}{2}$

After the estimation of the gene frequencies the expected frequencies of the phenotypes Z^a, Z^aZ^b and Z^b (in percentages) can be calculated as follows:

expected phenotype Z^a = (gene frequency $Z^a)^2$ x 100;
expected phenotype Z^aZ^b =
(gene frequency Z^a) (gene frequency Z^b) x 2 x 100;
expected phenotype Z^b = (gene frequency $Z^b)^2$ x 100.

A direct comparison of the expected and the observed distribution of the phenotypes will in many cases give an impression of consistency. To confirm this impression Fisher's χ^2 test for MN results can be used (67)

$$\chi^2 = \frac{((Z^aZ^b)^2 - (4 \times Z^a \times Z^b))^2}{(2 \times Z^a + Z^aZ^b)^2 \ (Z^aZ^b + 2 \times Z^b)^2}$$

in which Z^a, Z^aZ^b and Z^b are the observed numbers. This test can only be used if the genes are di-allelic.

Linkage

The presence of linkage was investigated with Smith's method (70) using Morton's tables (52). The procedure used can best be explained with the help of an example. In figure 4 the distribution of the hypothetical leucocyte group Z and the blood group MN in an imaginary family is depicted. We want to know what information this family can

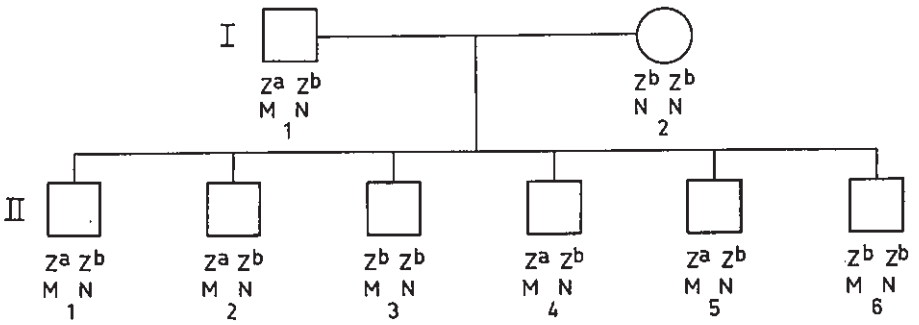

Figure 4

Pedigree of a hypothetical family

give on the existence of linkage between the leucocyte group Z and the blood group MN. The mother must inevitably give to all her children Z^b and N. The father's contributions, however, give evidence on the linkage (at some value θ) or non-linkage (independent assortment) of the Z and MN loci. This mating is tabulated in Morton's table 4 (52) as mating type 6. The value a from this table is in the family considered = 0, b = 2 (children nos. 3 and 6), c = 0 and d = 4 (children nos. 1, 2, 4 and 5), while s = 6.

The probability ratio for a particular θ when transformed to logarithms$_{10}$ is the value "lod" or "z" tabulated by Morton. In this case it is tabulated as z_1 (table 10 of Morton (52)) and this value assumes that the two alternative arrangements of the 4 relevant alleles in the father (coupling and repulsion) are equally likely. This family has s = 6, a + d = 4; b + c = 2 (or a + d = 2; b + c = 4 which gives the same result).

In the same manner the lod or z_1 scores for the other families can be found. By totalling the z_1 scores over all families the total z_1 scores for each θ are obtained. The antilogs of the lod scores are the relative probabilities. Λ is the average of the odds between $\theta = 0.0$ and $\theta = 0.5$ while Λ_2 is the average between $\theta = 0.0$ and $\theta = 0.2$. The final odds on linkage can be calculated by multiplying Λ by 1:21 which is the a priori odds that two genes are lying on the same chromosome. Using Ford's figure of 3000 centimorgans for the total chromosome length of man (27), the a priori chance of a locus being within 20% recombination fraction (say 27 centimorgans) of any other marker is $\frac{27 \times 2.}{3000}$ The factor 2 was introduced because the locus may be on either side of the marker. By combining these odds with Λ_2 the final odds on linkage closer than 20% can be calculated.