MEDICAL PRACTICE

Clinical Problems

Quantitation of Transplacental Haemorrhage

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Summary

On four occasions over a period of four years samples of adult blood to which known amounts of fetal blood had been added were distributed to 8-12 different laboratories taking part in clinical trials organized by an M.R.C. Working Party. Estimates were made of the proportion of fetal : adult red cells in the samples after preparing films by the acid-elution method. When the proportion of fetal : adult red cells was less than about 1 : 10,000, the highest and lowest estimates were separated by a factor of about 10. However, when the number of cells present was between about 1 : 100 and 1 : 1,000, most results were between half and twice the true number of cells present.

It is pointed out that since fetal red cells are approximately 30% larger than adult red cells, and since only about 90% of fetal cells stain darkly in the acid-elution method, estimates of the proportion of darkly-staining cells in a film underestimate the volume of fetal red cells present by about one-third. A simple formula is proposed which corrects for this factor and which gives an estimate of the total volume of fetal red cells present, deduced from the ratio of fetal : adult red cells and assuming a maternal red cell volume at term of 1,800 ml.

A method of screening blood films is suggested which, firstly, endeavours to standardize the density of adult red cells on films, and, secondly, takes into account the Poisson distribution. Thus limits are set for the number of fetal red cells which can be seen in scanning a given number of adult cells before the suspicion is aroused that

a transplacental haemorrhage exceeding a certain amount is present.

It is emphasized that the density of adult red cells on blood films varies very widely, and unless the cell density and the size of the low-power field are defined the practice of deducing the extent of transplacental haemorrhage from the number of fetal red cells seen per low-power field may lead to large errors.

Introduction

The work described here was undertaken on behalf of the M.R.C. Working Party on the Use of Anti-D Gamma Globulin for the Prevention of Iso-Immunization of Rh-Negative Women by Pregnancy. In the trials of treatment started by the Working Party in 1966 it was decided to make counts of fetal cells present in the mother's circulation at the time of delivery in all cases, using the acid-elution method of Kleihauer and Betke.¹ Estimates of the proportion of fetal: maternal red cells were made as described below. An attempt was made to assess the accuracy and precision of the method, as used at different centres taking part in the trials, by distributing samples of adult blood to which known amounts of fetal blood had been added.

The present paper records the results of these tests. In addition, it contains suggestions about the way in which acid-elution counts should be used, firstly, in screening samples from recently delivered Rh-negative women to detect cases in which there are more than a certain number of fetal red cells in the circulation, and, secondly, in making quantitative estimates of the absolute amount of fetal red cells present.

Methods

PREPARATION AND DISTRIBUTION OF BLOOD SAMPLES

Blood was freshly collected from an adult donor and mixed with A.C.D. (acid-citrate-dextrose); the haemoglobin of the diluted

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blood was estimated. Cord blood was mixed with A.C.D. and stored at 4°C for not more than 48 hours before being used; the red cells were then labelled with ^{51}Cr and the haemoglobin concentration of the suspension of the washed cells was determined. Carefully measured amounts of the suspension were added to known volumes of adult blood and the dilutions were checked by ^{51}Cr counting of the mixtures; only trivial errors, of the order of 1-2%, were found in the dilutions.

In all, five tests, here designated 1-5, were carried out; however, at the time of the first test some details of quantitation had not been settled so that only tests 2-5 are reported here. The proportions of fetal : adult blood, expressed in terms of haemoglobin concentration, were as follows in the various tests:

- 2 (May 1967) 1:20,000; 1:10,000; 1:20,000 1:2,000; 1:50,000
- 3 (March 1970) 1:5,800; 1:48,000; nil; 1:988; 1:48,000
- 4 (May 1970) nil; 1:500; 1:50,000; 1:50,000; nil
- 5 (July 1971) 1:248; 1:655; 1:812; 1:125; 1:500; 1:333

The number of laboratories testing the various samples was 8, 10, 12 and 11 in tests 2, 3, 4, and 5 respectively.

EXAMINATION OF BLOOD FOR RED CELLS

Preparation and Staining of Blood Films.—At some centres films were prepared from whole blood; at others, samples were diluted 1 in 3 in saline before being spread. Films were stained either by the original method of Kleihauer and Betke¹ or by some minor modification of it.

Measurements of Areas of High-power and Low-power Fields.—Areas were deduced from measurements of the diameters of the fields, determined by examining a micrometer scale set on the stage of the microscope.

Counting of Cells.—The average number of adult cells per high-power field was determined. No specific instructions were given about the number of fields to be examined; 8 out of 12 centres counted at least 2,000 cells but three centres counted fewer than 1,000. The number of low-power fields scanned was 50 or more at 10 out of 12 centres.

From the areas of the high-power and low-power fields and the numbers of adult cells per high-power field the number of adult cells per low-power field could be determined and thus the ratio of fetal : adult cells calculated for the various samples.

Supplementary Observations.—The percentage of darkly-staining cells in films of normal cord blood stained by the acid-elution method was determined in eight samples of normal cord blood; in seven samples the values were between 88% and 98% (average 94%), but in the eighth sample only 74% of the cells stained darkly. Eight samples of cord blood mixed with EDTA were examined in the Coulter "S" counter and found to have an average mean corpuscular haemoglobin of 39 pg compared with an average of 30 pg from blood samples from adults.

Results

DENSITY OF ADULT RED CELLS; AREAS OF MICROSCOPE FIELDS

The areas of the high-power fields used varied in different laboratories from 0.002 to 0.113 mm² and the density of adult red cells on the slides examined varied from 1,320 to 16,000/mm². The areas of the low-power fields varied from 0.785 to 2.20 mm². The number of adult red cells per low-power field varied from 1,040 to 18,500 (1,040 to 3,300 at four centres, from 3,750 to 9,550 at six centres, and from 15,600 and 18,500 at the remaining two centres).

Results in Tests 2, 3, and 4

These tests are considered together because almost all the samples contained relatively low concentrations of fetal red cells, only 2 out of 15 samples having a higher proportion than

1 : 2,000. As Fig. 1 shows, in tests 2 and 3 all estimates were below the expected values, but in test 4 samples were distributed more or less symmetrically around the average expected value.

Of the samples containing no fetal red cells, about one-third were reported as "positive," although only 2 out of 35 samples were reported as containing a higher ratio than 1 : 20,000. Of the samples containing 1 : 10,000 or less of fetal cells, only 2 out of about 100 were reported as containing 1 : 1,000 or more, and when the sample containing 1 : 500 was estimated all laboratories reported it as containing between 1 : 250 and 1 : 1,050.

FIG. 1—Results in tests 2 (■), 3 (●), and 4 (▲) expressed as a ratio of the number of darkly-stained (fetal) to unstained (adult) red cells in 15 different samples. The actual ratios of fetal : adult blood in the samples, in terms of haemoglobin concentration, are indicated by the horizontal lines.

In any particular test the results which were most in error tended to come from the same laboratories. For example, in test 2, on the two samples containing 1 : 20,000 fetal : adult cells, only two estimates were higher than 1 : 10,000 and both these came from the same laboratory. Similarly, in test 4 the two biggest overestimates (values of 1 : 2,300 and 1 : 15,600 on samples which in fact contained no fetal cells) came from the same laboratory, although it was not the same laboratory that recorded the overestimates in test 2.

Test 5

As mentioned above, at the time this test was carried out it had become clear that a major objective in carrying out acid-elution counts was to try to detect cases in which more than a certain amount of fetal red cells was present and that it would therefore be helpful to express results in terms of ml of fetal red cells.

The following assumptions were made: (a) that the maternal total circulating red cell volume at term is 1,800 ml (see Discussion); (b) that the number of darkly-staining cells underestimates the number of fetal cells present by about 10% (because, on the average, only about 90% of cord red cells stain darkly, as found in the present work and as previously reported by Kleihauer²); and (c) that the number of fetal red cells present under-represents the volume of fetal red cells because fetal red cells are about 30% larger than adult red cells. Combining factors (b) and (c), and as an approximation, the proportion of darkly-staining red cells in a film is multiplied by 4/3 to obtain the volume of fetal red cells present—that is, transplacental haemorrhage (TPH)—thus,

$$\text{TPH (ml red cells)} = \frac{1,800}{\text{number-ratio, unstained : darkly-stained red cells}} \times \frac{4}{3}$$

$$= \frac{2,400}{\text{number-ratio, unstained : darkly-stained red cells}}$$

Fig. 2 shows the results obtained in test 5, interpreted according to the above formula. In estimating the accuracy and variability of the results, for convenience, the values were trans-

formed into logarithms. When the transformed values were used the ratio of the log (actual dilution) to the average log (observed dilution), after allowing for the 4/3 factor, were: 99%, 101%, 100%, 101%, and 99% (average 100%), that is to say, the observed average values were virtually identical to the true values.

The overall standard deviation of log (observed dilution) was 0.1852. As an example of the implications of this S.D., when the ratio of fetal to adult red cells is 1 : 1,000 95% of estimates are expected to fall within the range 1 : 426 to 1 : 2,346. In fact, only 5 out of 66 estimates in test 5 fell outside the limits of half to twice the true values.

FIG. 2—Results in test 5. The observed ratios of darkly-stained to unstained cells have been interpreted as described in the text to give estimates of transplacental haemorrhage in terms of ml of red cells. The "correct" values for each sample are indicated by the horizontal lines.

Discussion

The results shown in Fig. 1 indicate that when the proportion of fetal red cells in the sample is less than about 1 in 10,000 the results must be expected to vary by a factor of about tenfold between different laboratories. Fig. 1 also indicates that when results are expressed as a proportion by number there is a marked tendency to underestimate the proportion present by haemoglobin concentration or by volume. As already discussed, the discrepancy is due mainly to the fact that the red cells of newborn infants are larger than those of adults. When this difference is allowed for, and a further small allowance is made for the fact that not all fetal red cells stain darkly, the systematic error disappears and mean values correspond very closely to predicted ones, as Fig. 2 shows. Fig. 2 also shows that the scatter of results is much smaller in samples containing relatively large amounts of fetal red cells.

METHODS OF QUANTITATING RESULTS

The results of counts of darkly-staining cells in blood films prepared by the acid-elution method can be interpreted quantitatively in two main ways: (1) they can be expressed in terms of the ratio of darkly-stained to unstained cells present, and (2) they can be expressed as the number of darkly-stained cells observed per low-power field of the microscope, having previously standardized the system in some way.

As described above, if ratios of darkly-stained to unstained cells are to be interpreted in terms of volumes of fetal cells in the maternal circulation, one must multiply the number of darkly-stained cells by a factor of about 4/3 to obtain an estimate of the volume of red cells present; in addition, some average value for the maternal red cell volume at term must be assumed.

There are relatively few estimates of the maternal red cell volume at term obtained by methods involving the use of labelled

red cells. The earliest and most thorough investigation was that reported by Caton *et al.*³ in which radio-iron-labelled red cells were used to estimate red cell volume at intervals during pregnancy in a group of 12 women; the average value at term was 1,780 ml. In a study in which ⁵¹Cr-labelled red cells were used, Berlin *et al.*⁴ found that the average value in nine women was 1,833 ml at the ninth month of pregnancy and in 18 different women was 1,657 ml at term. The figure of 1,800 ml may be somewhat too high for an average value for the red cell volume of pregnant women at term, but if it is in error it is on the safe side, since the finding of a given proportion of fetal red cells would then overestimate the true volume of cells present.

As has already been described, the above considerations lead to the formula:

$$\text{TPH (ml red cells)} = \frac{2,400}{\text{ratio unstained : darkly-stained cells}}$$

A formula based on similar principles was proposed by Grobelaar and Dunning⁵ but the assumptions they made were different in detail from those proposed here. Kleihauer² suggested the simple formula:

$$\text{TPH (ml blood)} = \text{ratio fetal : adult red cells as a percentage} \quad 50.$$

In fact, this formula gives very similar results to the one proposed above. As an example, suppose that the ratio of fetal to adult red cells (actually darkly-stained : unstained red cells) is 1 : 2,400. The formula proposed in the present paper leads to an estimate of a transplacental haemorrhage of 1 ml of red cells and that proposed by Kleihauer to an estimate of 2.5 ml of fetal blood—that is, approximately 1.25 ml of fetal red cells.

The other method of quantification which has been widely used has been to make a known mixture of fetal blood with adult blood, to prepare films from the mixture, and to discover the average number of darkly-stained cells per average low-power field when the films are scanned under the microscope. This method was used by Woodrow *et al.*,⁶ who injected known amounts of fetal blood into adult volunteers and concluded that with the method of spreading films which they used, 5 fetal red cells seen in 50 low-power fields correspond to the presence of 0.25 ml of fetal blood in the maternal circulation. A similar approach was used by Dr. Els Borst-Eilers (personal communication) who mixed 1 ml of fetal blood with 525 ml of adult blood in vitro and then followed the same procedure as that of Woodrow *et al.* Methods of this kind seem to be satisfactory, provided that films of similar density are spread on each occasion and, of course, that the same optical system is used. It is equally clear that the interpretation of the finding of a given number of darkly-stained red cells per low-power field will not apply when blood films are appreciably thinner or thicker than those which have been used in calibrating the original system.

As reported above, the number of adult cells per low-power field varied from 1,040 to 18,500 in the laboratories taking part in the present trials. As an illustration of the implication of this finding, suppose that the assumption was made that 50 fetal red cells in 50 low-power fields corresponded to a transplacental haemorrhage of 1 ml of red cells, and that this assumption had originally been based on a low-power field containing 5,000 adult cells. When the low-power field actually contained 1,000 cells, the finding of 1 cell per field would correspond to 5 ml of fetal red cells, but when it contained 18,500, to only about 0.3 ml.

PROPOSED SCREENING TEST

It is suggested that an attempt should be made to prepare films with a density of adult red cells of 5,000/mm². This objective could be achieved by preparing slides of various thicknesses, counting the red cells in unit area, and selecting a slide with the right cell density. This slide could be kept and used for reference purposes. In testing samples from recently delivered women

the object would then be to prepare slides with a density similar to that of the reference slide. Experience has shown that a slide which seemed to have a similar density to that of the reference slide would be unlikely to have a true density outside the limits 3,000-7,000/mm². Thus with a low-power field of area 0.8 mm² there would be 2,400-5,600 adult cells per low-power field.

If it is assumed that the dose of anti-Rh required for the suppression of Rh immunization is 25 µg/ml red cells⁷ then, when the routine dose of anti-Rh given to recently delivered women is 100 µg (as is the case at the present time in England and Wales), some screening test is needed to detect those women who have more than 4 ml of fetal red cells in their circulation. With the formula described above, a transplacental haemorrhage of 4 ml corresponds to a proportion of unstained: darkly-stained cells of 600:1.

With the use of appropriate tables giving the confidence limits for a Poisson variable,⁸ the maximum number of cells which could be seen without arousing the suspicion that the actual number present exceeded the above figures could be determined. For example, in three low-power fields the finding of as many as 4 cells would carry a 1 in 100 chance, and the finding of as many as 6 cells a 1 in 20 chance, that the average number present was in fact 12 in three low-power fields. If five fields were scanned, then the maximum number of cells which could be seen without arousing the suspicion that the average number present was 20 would be 9 at the P = 0.01 level and 12 at the 0.05 level.

In summary, with a low-power field of area 0.8 mm² and scanning films on which the density of adult red cells approximately matches a reference slide with a density of 5,000 cells/mm², five fields should be counted and the screening test regarded as negative when not more than 9 darkly-stained cells are seen in the five fields.

RELATIVELY LARGE TPH

When the total number of darkly-stained cells in five low-

power fields is 10 or more, a quantitative estimate of the number present should be made by counting the total number of darkly-staining cells in 50 low-power fields and the total number of unstained cells in five high-power fields. From these figures, and knowing the areas of the low-power and high-power fields, the number-ratio of adult: fetal cells in the slide can be deduced and the size of transplacental haemorrhage estimated by applying the formula already described. It is suggested that to allow for the possibility of underestimating by a factor of 2 the number of fetal cells actually present, the total dose of anti-Rh given should be 50 µg for every 1 ml of red cells estimated to be present. If, as a further check, the patient's circulation was examined for the presence of fetal red cells three to four days after the dose had been given and additional anti-Rh given if any fetal red cells were still present, very few cases indeed should be undertreated.

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Emotional Problems of Childhood and Adolescence

Antisocial Behaviour

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To understand antisocial behaviour in children it is helpful to consider how normal, socially acceptable behaviour develops. Such behaviour is largely a product of the child's family life and relationships. The school environment and the wider social setting also play a part.

The process may be looked at from various theoretical standpoints. It can be regarded as a series of learning experiences. Parents, and others, reinforce certain behaviour, perhaps by a smile or word of praise, and attempt to extinguish other behaviour by a frown, a harsh word, or some other punishment. Such a mechanistic approach, however, does not do full justice to the process, and a psychodynamic view is also helpful. The

effect of a smile or a frown will depend on the relationship that exists between the child and whoever is doing the smiling or frowning. Does the child want to please the person or to annoy him?

At the feeling level, identification is important. A warm, mutually loving relationship between parent and child will promote in the child the adoption of the parents' values and standards of behaviour as his own. The parents' values become internalized, a part of the process of conscience formation. So it is that certain actions or ideas come to awake in the individual feelings of guilt or anxiety. At the same time other things cause feelings of satisfaction or pleasure. The origins of these emotions are unconscious, so that the subject is not intellectually aware of them.

Social training of children thus depends on the presence of suitable figures with whom the child can identify, and also on the reasonably consistent communication of ideas and values from these people to the child. The latter may usefully be looked upon as a learning process.

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