THE DIFFERENTIAL DIAGNOSIS OF HAEMOLYTIC ANAEMIAS

By GEORGE DISCOMBE, M.D., B.Sc.

Pathologist, Central Middlesex Hospital, Park Royal, London, N.W.10

The differential diagnosis of haemolytic anaemias is difficult. It requires a wide clinical knowledge, complete command of a wide range of laboratory techniques and imagination. However, it is possible to base a system for differential diagnosis on no more than five basic tests. These are:—

1. Examination of a properly stained thin blood film, stained so that the cells are orange, but show polychromasia as a greyish tinge. This requires a buffered diluting fluid which has been carefully tested. Smears too thick, or stained dull grey, are quite useless, and it is usually much easier to obtain good staining with Leishman's stain (from British Drug Houses) than from any other brand, or from other Romanowsky stains such as Giemsa or Wright's stain (Discombe, 1950). A well-stained film may indicate spherocytosis, target cells, hypochromic cells, burr cells, or other odd forms. One should remember that severe carbon dioxide retention, or other acidosis, will make cells increase in diameter by as much as $1\ \mu$, while obstructive jaundice causes target cells to appear.

2. Examination of blood spread out between two coverslips coated with brilliant cresyl blue. In this the reticulocytes can be counted (and it is unusual for an anaemia to be due solely to haemolysis unless the reticulocytes exceed 2 per cent.), Heinz bodies searched for, Haemoglobin H precipitates seen, and some indication of the shape of the erythrocytes obtained. It is imperative that the blood be examined wet, after at least 20 min. incubation, and that the stain should be known to be effective in staining reticulocytes. This examination is so important that it should never be deputed entirely to a technician, unless he is exceptionally experienced. From this examination may be obtained the clues which lead to a diagnosis of several acute haemolytic episodes, such as those due to some poisons, drugs, favism and changes in the thickness of red cells.

3. Examination of urine for haemosiderin is easy, for the deposit is mixed with equal parts of 2 per cent. potassium ferrocyanide and N hydrochloric acid, and after 10 min. centrifuged again and the deposit examined wet. Profuse green granules, especially within cells or casts, indicate a chronic process in which haemoglobin has been

liberated into the blood stream and leaked into the urine; this is usually *paroxysmal nocturnal haemoglobinuria* (P.N.H.). Of course, stringent precautions must be taken against contamination, but contaminating iron is usually in irregular lumps, not the tiny spherical granules of haemosiderin.

4. The direct antiglobulin test should be performed on cells which have been allowed to cool to about 4°C . in contact with their serum, and the washed cells should be tested with antiglobulin serum at one, two, four and possibly eight times the concentration normally used for the demonstration of Rhesus antibodies. If positive, the test should be repeated using cells which have been taken in a warm syringe and at once washed with large volumes of saline previously warmed to 37°C . and with normal cells incubated in the patient's serum obtained from blood which has never been allowed to cool below 37°C ., so that the quality and quantity of the antibody may be further assessed.

5. The only common haemoglobinopathies are those causing sickling and thalassaemia. The former is quickly detected by mixing cells with 2 per cent. sodium metabisulphite, placing between slide and coverslip and examining after $\frac{1}{2}$ and 2 hours; the latter by dehaemoglobinization with a citrate-phosphate buffer of pH 3.4 of an air-dried film fixed for 5 min. in 80 per cent. ethyl alcohol; the foetal haemoglobin resists the buffer, while other haemoglobins are removed (Bethke and Kleinhauer, 1958). Positive tests should be confirmed by the alkali denaturation test. Occasionally one must proceed to electrophoresis or chromatography of the haemoglobin, but this is essential only if suspicion of a haemoglobinopathy is strong and these tests negative. If the Mean Corpuscular Haemoglobin concentration is below 25 per cent., it may safely be assumed that iron deficiency is present, either as a cause of the cell changes in the stained film, or as a complication of a haemoglobinopathy.

These five tests should suffice to indicate the most probable pathogenetic mechanism; but a final diagnosis will depend on knowledge of the natural history and of the geographical distribution of haemolytic disease, and on the securing of a complete and adequate history, in which special attention is given to family history and recent

exposure to drugs, household remedies and unusual foods. Further, in this field perhaps more than any other, memories are faulty, and assertions fallacious; patients and parents deny the use of any medicament, when in fact the whole household armamentarium (and the other doctor's) has been employed; and the parents of a child inflicted with an inherited disease will often have been perfectly healthy all their lives, though laboratory examination at once reveals the carrier state (Discombe, 1948; Young *et al.*, 1951). Biological false positive Wassermann reactions are the rule rather than the exception, and the disease process sometimes is made less active by injections of arsenical drugs (though not of penicillin)—a source of further confusion. Nevertheless, these tests should lead to the pathogenetic mechanism of about 19 cases out of 20, and with the aid of a few other tests enable one to identify the 'cause' should this concept be applicable. There are but four main groups of haemolytic anaemia, and the usual procedure of progressive dichotomy, using in the first place the simplest tests, will usually lead to a correct presumptive diagnosis within a few hours.

Fragility

It may be noted that the determination of osmotic fragility, which gives an index of cell

thickness, or rather, of thickness/diameter ratio, has not been included among the key investigations. It is, in fact, of little importance in differential diagnosis, though it is sometimes useful in confirming a diagnosis arrived at in other ways.

In normal blood the osmotic resistance, or fragility, of erythrocytes is normally distributed about a mean, and this is very clearly demonstrated by plotting the curve of salt concentration against proportion of haemoglobin liberated on arithmetic probability paper when it is found to be a straight line (Hunter, 1940; Discombe, 1948). Such a blood can be characterized by the median corpuscular fragility (M.C.F., normal 0.40-0.445 per cent. NaCl) and the standard deviation (normal 0.025 per cent. $\pm$ 0.006 per cent. NaCl). Among the haemoglobinopathies, published curves analysed in this way show a median corpuscular fragility in the region of 0.26-0.33 per cent. NaCl, and usually a standard deviation about twice the normal, thus suggesting that the erythrocytes constitute a homogeneous biological population which differs from normal cells only in that its members are all relatively thinner and more variable—thus accounting for the real microcytosis and apparent extreme hypochromia.

Congenital spherocytosis (acholuric jaundice or haemolytic anaemia of Chauffard-Minkowski) shows exactly the opposite change. The milder

DIFFERENTIAL DIAGNOSIS OF THE COMMONER FORMS OF HAEMOLYTIC ANAEMIA

PATHOGENETIC MECHANISM	Clues	Commoner Clinical Manifestations
A. Congenital Abnormalities of the Erythrocyte:		
(i) Haemoglobinopathies	Racial origins Morphological aberrations, especially target cells in stained film	Sickle cell anaemia (Africans) Thalassaemia (Mediterranean, S.E. Asia) Microdrepanocytic disease (Sicily, Caribbean, U.S.A.)
(ii) Lack of glucose-6-phosphate dehydrogenase	Acute episodes of haemolysis Heinz bodies. Racial origins	Favism; drug-induced haemolysis
(iii) Congenital spherocytosis	Spherocytosis	Often none, usually chronic anaemia with jaundice
B. Aberrations of the immunity mechanism		
(i) Auto-immune disease	Spherocytosis Positive anti-globulin test May be haemoglobinuria Raised E.S.R. (very high) may be suggestive	Often symptomatic—virus pneumonia, D.L.E. often idiopathic
(ii) Chronic haemoglobinurias	History Haemosiderin in urine	Haemoglobinuria at night, on exposure to cold, after violent exercise
(iii) Drug haptene disease	History Recurrence on exposure to drug	Usually severe; so far proven only for stibophen and quinidine, but probable mechanism of blackwater fever. To be differentiated from favism group
C. Poisoning	History Heinz bodies	Often cyanosis due to Heinz bodies, not to met/sulp/haemoglobin, though these pigments may be present
D. Symptomatic banal anaemias	Reticulocytes 2-3 % Cells large, often immature red and white cells present	Splenic enlargement usual and moderate. Often carcinoma, uraemia or hepatic cirrhosis present. If spleen very large, myelofibrosis

cases have a M.C.F. slightly greater than 0.45 and the standard deviation is usually about normal, so that we infer that the cells form a homogeneous population which is relatively thicker than normal. The cells in this disease show a marked increase in osmotic fragility when incubated for 24 hours, and this is sometimes a help in diagnosis. The clinically more severe cases usually give a fragility curve with one or two bends in it, thus suggesting the presence of two or more cell populations differing in their relative thickness. In mild cases with unimodal fragility curves and an almost normal M.C.F. it is quite common to find that the father of the child is a symptomless carrier of the abnormality (Discombe, 1948; Young *et al.*, 1951). However, it cannot be too clearly enunciated that increased fragility is a manifestation of increased cell thickness—or more correctly, of thickness/diameter ratio—and that such an increase occurs not only in acholuric jaundice but also in the autoimmune and other haemolytic anaemias, just as decreased fragility due to decreased cell thickness—or thickness/diameter ratio—occurs in iron-deficiency anaemia and in obstructive jaundice as well as in the haemoglobinopathies.

The Clinical Manifestations of Haemolysis

Whatever the mechanism, any increased destruction of erythrocytes is followed by:—

- (i) Increased bilirubin production;
- (ii) Increased stercobilin excretion;
- (iii) Reactive hyperplasia of the erythroid system.

If cell destruction is episodic, so will these changes be episodic, and if not fatal will produce few or no permanent changes. If, on the other hand, increased cell destruction is continuous, increased bilirubin production may lead to the production of pigment gall-stones and obstructive jaundice, while hyperplasia of the marrow may lead to dilatation of the bone marrow cavity, ultimately leading to disappearance of the outer table of the skull with the appearance of radiating spicules in the diploë, expansion of the zygoma to produce a Mongoloid facies, and if myeloid metaplasia of spleen and liver develops, a pot-belly (Astaldi, Tolentino and Sacchetti, 1951; Gasser, 1951; De Toni, Sansone and Tolentino, 1955). If haemoglobin is liberated into the blood-stream, occasional bouts of haemoglobinuria may occur, and if this is continued, haemosiderin appears in the urine, a possible cause of a complicating iron deficiency and a valuable diagnostic aid, far more convenient than the estimation of haptoglobin which is usually reduced or absent from the serum in such cases (Nosselin and Nyman, 1958).

Increased cell destruction can be proved only by a determination of cell survival by one of the

radioactive isotope methods, which are not yet universally available. Most hospitals have to rely on the Ashby technique which uses foreign cells, possibly not susceptible to some noxa or to the particular destructive process; and even this is too laborious for some routine laboratories, so that cell destruction must be inferred from jaundice, increased stercobilin excretion, reticulocytosis, or the rate of fall of haemoglobin—all indirect and therefore unreliable indices of cell destruction or marrow activity.

The Haemoglobinopathies (Jonxis and Huismans, 1958; Jonxis, 1958)

Abnormalities of haemoglobin synthesis depend on genetic factors which usually have partial expression in the heterozygote, and complete expression only in the homozygote. The homozygote is sometimes very much less viable than the heterozygote or the normal, so that these abnormalities can be regarded as an example of balanced polymorphism, wherein the loss of the less viable homozygotes is balanced by better survival by the heterozygote than by the normal; in the case of Hb S, the cause of sickle cell disease, the heterozygote is partially protected against heavy infection with *Plasmodium falciparum*. A similar advantage appears to be conferred by a single dose of Hb C, but the selective advantage conferred by the others is not known. Such a genetic system may be of local advantage only, so that in different areas there may be different polymorphic systems. This is so with the haemoglobins, for many are known. Nearly all lead to the appearance of small thin erythrocytes which look like target cells and arouse suspicion of iron deficiency, which is, of course, refuted by the failure of the patient to respond to treatment with iron. However, only two are of wide importance; that which causes the appearance of Hb S, common in Africa south of the Sahara and populations derived therefrom, but rare elsewhere except in a few scattered groups in Persia and India; and that which suppresses the appearance of Hb A, the normal haemoglobin of adult life. These, of course, produce respectively sickle cell disease and thalassaemia, and the double heterozygote who has inherited both factors in single dose develops microdrepanocytic disease, rare save in Sicily and the West Indies.

Haemoglobin S is the more interesting because the reduced form, being very insoluble, crystallizes out even within the erythrocyte envelope, and distorts this into sickle shapes ending in long filaments; these elongated cells tangle together and block small blood vessels. When this occurs, joint pains may be prominent, so much so that the first cases of sickle cell disease seen in my hospital were referred as cases of sub-acute rheumatism—

while, of course, high flying in unpressurized aircraft may lead to intra-abdominal thromboses.

Significant illness due to Hb S is thus common in all populations of Negro origin, such as are found in tropical Africa, the West Indies, U.S.A. and more recently in Britain, and in a few negroid groups in S. India and Persia; while thalassaemia occurs chiefly in those parts of the world to which the Ancient Greeks journeyed and microdrepanocytic disease is found where the two groups mixed, notably Sicily, the Caribbean Islands, and in the U.S.A. Hb C is quite common in West Africa, but the others are more commonly found in S.E. Asia.

Enzymic and Metabolic Defects of the Erythrocyte

Were any enzyme, co-enzyme, or essential metabolic process normally present in the erythrocyte, to be absent or to disappear completely, one would expect considerable changes in erythrocyte stability. In actual fact the abnormalities so far recognized have little effect. The absence of catalase increases the susceptibility to oral gangrene but has little other effect (Takahara, 1952). The absence of co-enzyme factor I decreases the rate of conversion of methaemoglobin to haemoglobin and so establishes a permanent cyanosis (Gibson, 1948); reduction in the level of glucose-6-phosphate dehydrogenase leads to a reduction in the stability of reduced glutathione and to no inevitable ill-consequences (Beutler, 1959; Sansone, Piga and Segni, 1959); and there are defects in glycolysis which are associated with congenital spherocytosis (Prankerd *et al.*, 1955) and with P.N.H. (Auditore *et al.*, 1959), but which are not necessarily responsible for the increased cell destruction in these diseases.

The level of glucose-6-phosphate dehydrogenase is determined genetically, and is inherited as a sex-linked or sex-affected dominant (Browne, 1957; Sansone *et al.*, 1959). A group of American workers, headed by Alving and Beutler, showed that the anaemia which followed the administration of primaquine in some 10 per cent. of American negroes was due to a low level of this enzyme (Carson *et al.*, 1956), and that subjects with low levels of this enzyme develop haemolytic attacks when challenged with other drugs, such as acetanilide and sulfoxone-sodium (Dern *et al.*, 1955). Another enzyme, glutathione reductase, is increased (Schrier *et al.*, 1957). Sansone and his co-workers (1958), and Szeinberg and his associates (1958) showed that the same peculiarity was present in patients with favism and in certain of their relatives, but that lack of the enzyme does not necessarily mean that the subject is a favist. The condition seems to be widely spread in the Middle

East, but is particularly common among persons of Sardinian origin (12 per cent.), in Sephardic Jews and in American Negroes (7.8 per cent.; Kimbro *et al.*, 1957), though extremely rare among Askenazi Jews or Northern Europeans. Since the onset of an attack of favism is determined by eating beans (fave) it has been suggested that the famous Pythagorean prohibition of beans was an early public health ordinance, but Solon Veras (1939) thinks this interpretation erroneous, though Arie (1959), drawing from the same authors, has come to the opposite conclusion. However, it remains true that persons of Mediterranean or Sephardic origin are more likely to be made ill by drugs, broad beans, peas (Carcassi *et al.*, 1951) or stinkwood (*Anagyris foetida*) (Carcassi personal communication) than are persons from Northern Europe. Presumably this is also an example of balanced polymorphism, though the advantage has not yet been discerned.

L. E. Young and his associates (Prankerd *et al.*, 1955) have shown that a defect of glycolysis is present in the cells of *congenital spherocytosis*; this may be responsible for the increase of fragility when standing outside the body. Rarer causes of similar haemolytic anaemias are elliptocytosis, in which the fragility is slightly increased, and at least two varieties of non-spherocytic anaemia which are associated with a defect in the chain of carbohydrate metabolism.

Auto-immune Haemolytic Disease

Some patients develop antibodies which attack their own red cells. These may have a detectable serological specificity (often anti-e or anti-hr⁺) or be non-specific. Further, they may be most active at 37°C., or attach themselves only at room temperature or below—'warm' and 'cold' antibodies respectively. Cells pretreated with trypsin or papain, or the cells of P.N.H., are often more sensitive indicators of the presence of these auto-antibodies, and undergo haemolysis when normal cells are merely agglutinated.

In general, cold agglutinins tend to appear in symptomatic or secondary haemolytic anaemias, such as those associated with virus pneumonia (Stewart and Friedlander, 1957), disseminated lupus erythematosus, and more rarely other virus infections such as influenza and glandular fever. As a rule, the antiglobulin test is not inhibited by normal gamma globulin, whereas it is in anaemias accompanied by warm antibodies. In some cases agglutination becomes obvious when an E.S.R. is set up because the blood agglutinates as it is chilled to room temperature; and occasionally haemolysis is exacerbated to produce haemoglobinuria if the patient is chilled. Occasionally such a patient complains of Raynaud's pheno-

menon or of haemoglobinuria (Dacie, 1957; Christenson and Dacie, 1957; Christenson, Dacie, Croucher and Charlwood, 1957).

The best-known of the cold haemoglobinurias is the original form in which Donath and Landsteiner showed that there was present an antibody which attached itself to erythrocytes at low temperatures (0-10°C.) and complement then lyses the cells when they are warmed to 37°C. In this condition syphilis seems often to have had some aetiological importance, but it is not certain that syphilis is always the cause, because biological false positive Wassermann reactions are very common in all forms of auto-immune disease.

One can rarely discover a cause for the appearance of 'warm' antibodies, which appear for no apparent reason, may cause neither haemolysis nor symptoms or may produce the most severe haemolysis—and then, for no apparent reason, disappear (Darnborough, 1958; Stratton and Tovey, 1959).

Drug Haptene Disease

A few drugs will cause the appearance, in very rare patients, of antibodies directed towards the erythrocyte; but these antibodies will destroy the erythrocyte only in presence of the drug. Thus on some subsequent occasion, administration of the drug will provoke a brisk haemolysis. So far the mechanism has been proved only for stibophen (Harris, 1956) and quinidine (Freedman *et al.*, 1956), but a similar mechanism probably operates in quinine haemoglobinurias such as blackwater fever.

Chemical Poisons

Many chemicals or drugs cause haemolysis. Some such as arsine or potassium chlorate have this effect in all patients, others such as primaquine, sulfonamides, naphthalene, vitamin K analogues only, or more easily, in those who lack glucose-6-phosphate dehydrogenase, the peculiar deficiency discussed above (Zinkham and Childs, 1957; Sansone, 1958). Nearly all these chemical haemolytic agents produce Heinz bodies in the erythrocytes, and if the patient is seen early in the attack the presence of Heinz bodies should direct attention to the possibility of poisoning or favism. In infants sensitivity to drugs may be unexpectedly great, and in particular naphthalene, the soluble vitamin K analogues (menaphthone, synkavit) and cryogenin (phenylsemicarbazide) seem particularly dangerous, and a substance probably *meta*-aminophenol formed from *para*-amino salicylic acid kept dissolved in sealed tubes is extremely toxic, to some, though not all children (Sansone and Zappa, 1955; Castanier *et al.*, 1956), though for some reason a mixture of P.A.S. and ascorbic acid, distributed in gelatin ampoules is often adminis-

tered to children by their mothers, especially in France and Algeria.

Other Haemolytic Processes

These are usually secondary to some other disease, usually a malignancy such as lymphadenoma, leukaemia, myelosclerosis, reticulosarcoma, or carcinomatosis when they form one component of the well-known leuco-erythroblastic anaemia; sometimes ovarian tumours, cirrhosis of the liver, or uraemia, when they may appear as an auto-immune process. Sometimes they are truly secondary, as in rheumatoid-arthritis (McCrea, 1957) and diagnosis must be based on radiochromium studies. There are no special diagnostic features except that spherocytosis and macrocytosis are common. They may be the presenting factor of the underlying disease, but are usually discovered as an incident in the downhill course of the patient.

In this group it may be convenient to place the haemolytic component of the megaloblastic anaemias, especially that of Addison and Biermer. Since loss of weight and appetite are prominent features in nearly half the sufferers, they may be easily misdiagnosed as 'carcinoma ventriculi' and left to die. Since marrow puncture can so easily be done on out-patients or in the patient's own bed, such errors of diagnosis should become fewer.

However, the most interesting in this group are thrombotic thrombocytopenic purpura, and *paroxysmal nocturnal haemoglobinuria*, the Marchiafava-Micheli syndrome. The latter is an insidious mild anaemia which varies in intensity and is, at some stage of the disease, associated with the appearance of haemoglobin in the urine secreted during the night. This is apparently due to a characteristic erythrocyte abnormality in which the patient's cells are lysed by serum to which acid has been added. It is usually easier, and very satisfactory as a presumptive test, to examine the urine for haemosiderin as described above. Thrombotic thrombocytopenic purpura is a rare disease in which the red cell life span is reduced to a few days and the platelet life span to a few hours, and arterioles and capillaries are occluded by material which looks like platelet thrombi—though no antibodies can be detected (Symmers, 1956).

Technique of Differential Diagnosis

This depends mainly on the history, which must be taken by someone with a wide knowledge of haemolytic syndromes—if necessary the clinical biologist. First, of course, the existence of a haemolytic syndrome must be demonstrated, and this usually demands a persistently raised reticulocyte count—but you must be cautious to avoid being misled by a response to sudden haemorrhage.

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or to the administration of liver to a patient with pernicious anaemia without your knowledge. In the absence of Heinz bodies or other clues, it is best to continue with an anti-globulin test, and then with an examination for haemosiderinuria—at least in a North European—though in a patient of African origin one would first of all consider the possibility of sickle cell disease, which should have been suggested by changes in the stained film. The most difficult diagnostic problem is when the haemolytic process is of low intensity, or when it fluctuates greatly; the earliest phase of the leucoerythroblastic anaemia of disseminated carcinoma is a barely detectable reticulocytosis and an equally slight macrocytosis. In such cases, 'De minimis curat medicus,' and clinical examination and careful history taking are outstandingly important.

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