HEREDITARY DIAPHORASE DEFICIENCY
METHAEMOGLOBINAEMIA IN MALTESE FAMILIES

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Summary

Hereditary Diaphorase Deficiency
Methaemoglobinemia was discovered in
seven persons belonging to four Maltese families. These are the first families of
diaphorase deficiency methaemoglobinemia to be recorded from the Maltese
Islands. It is suggested that the gene frequency for this type of congenital methae­
moglobinemia is high in the Maltese Islands and that wider systematic surveys
would reveal many more cases.

Methaemoglobin (ferrihaemoglobin) normally constitutes only 1% of the total
haemoglobin of the red cells. Its presence in a greater concentration is pathological
and may be due to a number of different causes, congenital or acquired. The con­
genital forms are rare and are of two types which are completely different from
one another. One type, inherited recessively, is due to an enzyme deficiency in
the red cell, (Gibson, 1948), while the other type, showing dominant inheritance,
is due to a defect in the globin moiety of the haemoglobin molecule (Horlein and

Seven affected individuals in four Maltese families with methaemoglobinemia secondary to deficiency of the enzyme diaphorase have been discovered in the
Maltese Islands and will be described in this paper.
Methods

**Methaemoglobin estimation** was carried out using the method of Evelyn and Malloy (1938) as described by Varley (1967).

**Diaphorase Activity** in the red cell was estimated by the method described by Scott (1960). Levels of diaphorase (measured as units of change in optical density per minute × 10⁴) below 100 are usually taken as characteristic of the methaemoglobininaemic homozygote, while levels between 100 and 300 represent the heterozygote. Levels in excess of 300 represent the normal state.

**Haemoglobin F Estimation:** The degree of denaturation of cyanmethaemoglobin was determined using the modification of Bethke, Marti and Schlicht.

**G-6 PD Estimation:** The Brilliant Cresyl Blue dye test (Motulsky and Kambell-Kraut, 1964) was used.

Haemoglobin estimation, blood smears and reticulocyte counts were performed by standard methods, PCV was determined using a microhematocrit technique.

Case Reports

**Family A.** None of our patients had been taking any analgesics or any other drugs. The father and the mother are apparently healthy, look normal and are not related to one another. They hail from an inland village in Gozo, the sister island of Malta. There are six offsprings from the marriage of whom two have been affected.

**Case 1.** The eldest sibling, a 16 year old male (J.V.) was noted to have had a cyanotic tinge since early childhood, and had been seen by various specialists who had not found any cardiac or pulmonary abnormalities. Intelligence is average and his only complaints have been occasional dizzy spells. When seen by one of us (L.V.), the patient had just undergone an emergency appendicectomy, and had caused some concern to the anaesthetist as the blood oozing at operation was unduly dark. Physical examination revealed no other abnormality. His Hb level was 13.6 g., PCV 40%, MCHC 34, reticulocyte count 0.5%, G6PD activity 50, and Hb F 0.32%. His methaemoglobin level was 10.5% (1.43Gm). His diaphorase level was 109. (Control 350).

**Case 2.** T.V., the youngest child of the marriage (d.o.b. 4.5.65) was noted to be cyanosed on the day of birth. Cyanosis persisted. There were otherwise no other abnormalities. At the age of nine months, he was seen by a specialist because of the worrying cyanosis, but no physical abnormalities were found. His lungs and heart, in particular, were thought to be normal. Appetite, developmental milestones, both mentally and physically appeared normal. At the age of 3, he developed peritonitis from a perforated appendix. He died a few days after an emergency laparatomy. During his terminal illness, intravenous ascorbic acid, 500 mg daily was given. Investigation results (taken when he was still healthy) were as follows: Hb level 13.2 g; PCV 44%; MCHC 30; reticulocyte count 1%; G6PD activity 50; Hb F 0.9%. His methaemoglobin level was 8.3% (1.07Gm). His diaphorase level was only 47. (Control 350).

**Family B.** The mother of this family is dead. The marriage is not consanguineous. There were eight sublings from this marriage, and two are affected.

**Case 3.** The eldest sibling (J.V.) a thirty year old male, was the most cyanosed member of the family. His only complaints have been dizzy spells especially after exertion. Cardiac and pulmonary disease have been excluded on medical examination. He works as a heavy manual labourer. He has emigrated to Australia, and investigations to prove the presence of methaemoglobininaemia secondary to diaphorase deficiency have unfortunately not been carried out. He has presumed diaphorase deficiency because the deficiency in his sister has been proved.

**Case 4.** (S.S., a 27 year old female, now married, is the third child of the marriage. She has always been noted to be moderately cyanotic, but less so than her elder brother (Case 3). Her health has always been good but she has been examined by specialists in the past, as her
parents were afraid there was serious underlying disease. The cyanosis had become especially marked when she was pregnant. The pregnancy was otherwise uneventful. The cyanosis diminished after pregnancy only to become more marked when she was again pregnant. She has now two children who appear normal. Her Hb level was 14.2g; PCV 43%; MCHC 33%; reticulocyte count 0.1%; G6PD activity 50; Hb F 1.4%. Her methaemoglobin level is 16.6% (2.35g). Her diaphorase level is 0. (Control over 400)

Family C. There are ten siblings from this union, which is not consanguineous. Only one child is affected.

Case 5. (R.V.), a fourteen year old girl is the fifth child of this family. She is of normal height and development. Her intelligence is average. She is symptomless. There are no physical abnormalities apart from the presence of a mild cyanotic or lavender hue. Her Hb level is 1218/g; PCV 41%; MCHC 31%; reticulocyte count 0.6%; G6PD activity 50; Hb F 0.7%. Her methaemoglobin level is 22.5% (2.82g). Her diaphorase level was 5. (Control 290).

Family D. The father and mother are unrelated though both hail from the same town in Malta. There are ten siblings in the family of whom two are affected.

Case 6. Y.B., is a 26 year old female. She is the eldest offspring. She had been noted by her parents to have had mild intermittent cyanosis when she was a child and again when she was pregnant. She is otherwise perfectly normal, and has a three year old son. Her methaemoglobin level is 6.2% (0.81g). Her diaphorase level was 0. (Control 290)

Case 7. The condition had been recognised in N.B. the fifth child now 19 years old, because of the colour of her blood at appendicectomy. Diaphorase levels were not estimated then. She had never complained of symptoms and was of normal intelligence and development. Her Hb level is 13.2g; PCV is 40%; MCHC 33%; G6PD activity 40; Hb F 0.6%. Her diaphorase level is 0. (Control over 400). She has since married and is now pregnant. The diaphorase of six healthy siblings of the above two cases (6 and 7) varied from 95 to 304.

The father's diaphorase level was 115 while the mother's was 90. (Control over 400).

Discussion

These are the first families of diaphorase deficiency methaemoglobinaemia to be recorded from the Maltese Islands. Though most of the cases so far described in the world literature have been in Europeans or in persons of European stock, interesting geographical concentrations of the disease have been described. Thus Scott and Hoskins (1958) found fifteen affected persons arising from 9 families of Alaskan Eskimos and Indians in four areas of Alaska. Four of these nine families were interrelated. A relatively high gene frequency has also been found among the Navajo Indians (Balsano et al., 1964). The condition is of world wide distribution as cases have been recorded among North Africans (Rousell et al., 1963), Chinese (Chang and Wu, 1954), Hindus (Raj et al., 1959) and Puerto Ricans (Jaffe et al., 1966). The incidence of this recessively inherited disease therefore is relatively high in the Maltese Islands whose total population is only one third of a million inhabitants.

The degree of methaemoglobinaemia has been noted to fluctuate from season to season and with age. Thus Scott and Hoskins (1958) found that methaemoglobin levels in their Alaskan patients were less in September than in December fifteen months later. They postulated an environmental factor such as ascorbic acid. The same workers also noted a tendency for the methaemoglobin levels to fail with age. They recorded a patient who had a level of 22 per cent methaemoglobin when eight years old, and 4.8 per cent when re-examined eight years later. It is interesting to note in this connection that Case 6 was described by her parents to have had obvious cyanosis in early childhood and that this had slowly disappeared with the years only to reappear when she became pregnant. The methaemoglobinaemia also
became more aggravated with pregnancy in Case 4. The cyanosis of methaemoglobinemia in pregnancy has been mistaken as being due to cardiac disease as has been pointed out by Vassallo and Cauchi, (1970). This aggravation by pregnancy has also been noted by other workers, and pregnancy may be the factor that leads to the diagnosis of methaemoglobinemia. (Pepper, Weinstein and Heller, 1961).

None of the patients had been taking any drugs. The recessively inherited form of congenital methaemoglobinemia appears to have been first described by Hitzenberger (1932) who described a mentally defective dwarf with a strong family history. Sievers and Ryan (1945) suggested that the defect lies in the reduction system of methaemoglobin. Gibson (1948) showed that the enzyme diaphorase was missing in methaemoglobinemic cells as methylene blue was extremely effective in reducing methaemoglobin. Diaphorase (diphosphopyridine reductase DPNH, NADPH, coenzyme factor 1) is an intermediate carrier which catalyses the reduction of methaemoglobin by reduced diphosphopyridine nucleotide (DPNH). Another far less important pathway for the reduction of methaemoglobin is mediated by Diaphorase 11 (reduced triphosphopyridine nucleotide, TPNH, NATPH). (Huennekers et al., 1957). Other unimportant systems known for the reduction of methaemoglobin in normal cells are ascorbic acid (Lian, Frumusan, and Sassier, 1939) and by means of reduced gluthathione (Scott, Duncan, Ekstrand, 1965). Lately another enzyme variant in a case of congenital methaemoglobinemia has been demonstrated (West et al., 1967).

Treatment of hereditary methaemoglobinemia secondary to diaphorase deficiency is not necessary except in unusual circumstances. Both ascorbic acid (Lian, Frumusan and Sassier, 1939) and methylene blue (Jaffe and Neuman, 1964) are both very efficacious and act by direct chemical reduction of methaemoglobin. Ascorbic acid was used intravenously in our Case 2 during his terminal illness as it was necessary for the oxygen transport system of haemoglobin to be utilised to the fullest extent and for the dead load of methaemoglobin to be minimised.

The discovery of these seven cases clinically would suggest that the relative gene frequency for this recessive type of congenital methaemoglobinemia is high in the Maltese Islands and that a future systematic survey for diaphorase deficiency among a suitable section of the population such as school children may reveal many more cases.

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References

THE INVESTIGATION OF AUTOIMMUNE DISORDERS

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Introduction
During the last few years our knowledge of autoimmune disease has increased considerably, and the techniques for detecting such disease have been improved and made simpler. The laboratory tests to detect humoral antibodies in patients suffering from an autoimmune disorder have become routine in a number of hospitals and the use of techniques, and, in particular immunofluorescence techniques, have assumed greater importance.

The purpose of the present communication is to outline some of the available immunopathological techniques and to illustrate how they are applied in the day to day investigation of patients in an acute general hospital.

Immunopathological Techniques

A) Immunofluorescent methods

The indirect immunofluorescence technique — also called the “sandwich” method — is used in most cases (Nairn 1969). Normal tissue is snap-frozen in a liquid nitrogen-isopentane mixture (temperature -180°C) and stored for future use at -70°C. When required, frozen sections are cut and the patient’s serum layered on top of the section. (Fig. 1). Antibodies present in the serum will combine with antigenic sites in the tissue section. Excess serum is then washed off, and a fluorescein-conjugated rabbit anti-human globulin is applied to the section.