THE DEVELOPMENT OF AN ACQUIRED HAEMOGLOBIN ABNORMALITY CAUSED BY THERMAL INJURY

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Alkali denaturation of the haemoglobin derived from burned patients at various intervals from the time of the injury was investigated. A delayed denaturation curve was always found.

The involvement of the transfused cells was established by a differential agglutination technique.

Red cell populations were fractionated into two portions, one of which was probably enriched in younger cells. This portion was preferentially affected.

A starch gel technique yielded a "slow" and a "fast" fraction of haemoglobin with differing denaturation rates, even in normals. The "slow" fraction appeared to be increased quantitatively and qualitatively, if compared with normal blood.

In 1951, Singer, Chernoff, and Singer had found that abnormal haemoglobins other than foetal haemoglobins showed a diminished alkali denaturation rate. Künzer (1955) described the occurrence of increased amounts of alkali-resistant haemoglobin, which he termed "foetal," in 47 children, all above 5 years of age and suffering from anaemia of varying aetiology. No quantitative relationship between the alkali-resistant fraction and the severity of the anaemia was found. Since these papers were published much chemical work has been done on the amino-acid composition of haemoglobin F, which was found to possess only 2.2 valine residues as compared with 4.6 in haemoglobin A (Huisman and Drinkwaard, 1955). The work of Perosa and Bini in 1954, however, showed that alkali-resistant haemoglobin was not necessarily identical with haemoglobin F.

The present study was undertaken in an effort to demonstrate a possible indirect thermal damage to the haemoglobin of burned patients.

Materials and Methods

Patients.—Sixteen patients were studied in detail; their ages ranged from 2 years to 70 years. Five of these were adults. The surface areas involved ranged from 16 to 90%.

Methods.—Heparinized blood was collected from the patients and immediately washed three times with 1% NaCl and the packed cells lysed by the addition of approximately 1 vol. of distilled water, followed by freezing and thawing. The stromata were removed

by 10 minutes' centrifugation in an angle centrifuge at 5,000 r.p.m. All specimens were stored at -15°C . before use and were not kept longer than one week before testing.

The haemoglobin solutions were examined for their rates of denaturation by dilute alkali. Oxygen was always bubbled through before testing. The rate of denaturation was studied by a suitable modification of the method described by Brinkman and Jonxis (1935). The haemoglobin solution was diluted to 0.15–0.3 g.% with 0.04% ammonia solution and 2.4 ml. of this solution was treated with 0.1 ml. N NaOH. The pH of this solution was 12.5.

The increase in optical density was noted at 10-second intervals in a Hilger "biochem" absorptiometer, which had been modified as follows. The galvanometer was replaced by a Cambridge spot galvanometer which was damped by a total resistance of 20k. A 5k resistor was placed across the photocell output. A portion of a "unicam" S.P.500 cell carriage was mounted in the light path and two precisely matched 10 mm. depth cells were used for the measurements. A Wratten 25 filter, which had 12.6% transmission at 590 m μ and 87.6% at 650 m μ , mounted between optical glass was used. Denaturation was assumed to be complete after 12 hours' incubation at 37° C. The method described made it possible to obtain accurate readings towards the completion of denaturation and yet utilize the high extinction coefficient of alkali haemochromogen at that wavelength as compared with 576 m μ . Fig. 1 shows the normal curve obtained using the blood of 12 young normal adults.

Some fractionation experiments were also carried out in order to obtain either a fraction enriched in old cells or young cells or transfused cells, the last having been exposed to the patient's circulation.

FIG. 1.—Alkali denaturation curve of normal blood (12 individuals).

Washed red cells from a burned patient were exposed to 0.4 g.% NaCl at 25° C. for 60 minutes. This yielded two fractions. The supernatant, somewhat enriched in older cells, was dialysed and concentrated by ultrafiltration. The other fraction, which was somewhat enriched in younger cells, was treated as usual. A similar scheme of age group fractionation has been described by Simon and Topper (1957). Gravitational fractionation was attempted on another sample. Thirty per cent. albumin was diluted to 20% with physiological saline. A Wintrobe tube was used for fractionation. This was filled to the 2 cm. mark with an approximately 10% cell suspension and to the 6 cm. mark with 20% albumin. The contents were then mixed gently with a fine pipette and the top 3 cm. layer was separated after standing for one hour at room temperature. This yielded a top fraction enriched in younger cells and a bottom fraction enriched in older cells. Several tubes were put up simultaneously and the appropriate fractions pooled.

The haemoglobin of transfused cells and the patient's own cells was obtained by the transfusion of M-negative blood and fractionating a sample obtained after a suitable time interval with anti-M serum. Films of the supernatant cells (transfused cells) showed less than 1% of the cells agglutinated after further treatment with anti-M serum.*

*This fractionation was kindly carried out by Dr. E. Topley.

Some haemoglobin solutions were fractionated by means of a starch gel electrophoretic technique as described by Baar (1958) using 0.05 M "tris" buffer as the electrolyte. After two hours' electrophoresis, separation into three zones could be observed. These, however, were not sharply demarcated. The complete width of the three zones was therefore divided into equal halves and the region nearest the cathode was termed "slow." Fig. 2 shows the curves obtained from normal blood by alkali denaturation of the isolated zone material.

Results

The patients studied were divided into four groups according to the area of the burn. The 30-second "alkali-resistant" fraction was chosen to represent graphically the quantitative variations of this fraction in relation to the time of the injury, the area involved in the burn, and the timing and the extent of the blood transfusion. Table I shows the ages, the body areas burned, the presence or absence of haemoglobinuria, and details of the primary excisions of the cases investigated. The denaturation curves of these cases are shown in

FIG. 2.—Starch gel fractionation of normal blood.

FIG. 3.—The 30-second alkali-resistant haemoglobin fraction of Cases 1-16.

TABLE I

Case No.	Age (yr.)	Estimated Area of Body Surface Burned (%)	Estimated Area of Full-thickness Skin Loss (%)	Haemoglobinuria	Primary Excision
1	11	16	13	0	0
2	2	35	10	L*	0
3	70	35	30	E*	0
4	1½	37	35	E	0
5	65	40	35	0	0
6	9	45	17	L (red cells)	0
7	3	45	40	0	0
8	7	45	20	E	+
9	8½	50	20	E, L (red cells)	+
10	4	50	45	E, L	+
11	36	55	50	0	0
12	8	55	50	—	+
13	20	65	35	0	+
14	45	80	30	L	0
15	10	85	80	E, L	0
16	5	90	90	E, L	0

* E=early (0–12 hours) and L=late (at least three haemoglobin-stained urines 12–24 hours).

Fig. 3. In all groups there is an increase in the alkali-resistant fraction and this abnormality is maintained for some time. Cases 6, 7, and 12 show secondary rises, which develop after the transfusion. In Group 1, Cases 1 and 3 did not receive any transfusion. The former only shows a minor development of the more resistant fraction, the maximum being reached within 24 hours and a return to normal levels within one week. Case 3, an adult, shows this maximum level about two days after injury. This is associated with a much slower return to normal. The other adult cases studied, namely, 5, 11, 13, and 14, do not show this delay. This fact may be due to the timing of the transfusion. Case 2 showed a severe haemolytic reaction with haemoglobinaemia and haemoglobinuria, both the transfused and the patient's blood being involved. Yet the level of the abnormal haemoglobin rose in spite of the dilution of the patient's blood by transfused cells and the increased rate of red cell destruction. Such a finding is highly suggestive for the abnormality developing not only in the patient's blood but also in the transfused cells. Case 4, a patient who was transfused after the initial blood sampling, shows that approximately 15% of the patient's cells may be involved in as little as eight hours after the injury. Case 7 in Group 2 and Cases 10 and 12 in Group 3, whose samples were obtained before transfusions, all confirm the relative rapidity of the change. Case 11, whose transfusion was only started after the third sampling, clearly demonstrates a qualitative increase in this abnormality. This could be due either to a larger number of cells becoming involved or to the already affected haemoglobin increasing in abnormality.

FIG. 4.—Starch gel fractionation of the blood of Case 12.

Starch gel fractionation on normal blood has shown that the slight tailing of the denaturation curve of normal blood (Baar and Hickmans, 1941) was due to a component of slower denaturation than the main portion, which also migrated more slowly on starch gel. This is shown in Fig. 2. Starch gel fractionation experiments on Case 12 are shown in Fig. 4. Two fractions were obtained, both with a delayed denaturation curve as compared with the normal. The "fast" fraction from the patient approximates the "slow" fraction from normal blood. Although both fractions appear to be affected to some extent, the "slow" fraction's denaturation is delayed about twice as much as the "fast" fraction. This delay in the denaturation of the "fast" fraction could reasonably be due to the incomplete method of separation.

Scrutiny of the curves obtained from the patient's blood in relation to the extent and timing of the transfusion reveals that transfusions involving about one blood volume or more

FIG. 5.—Alkali denaturation of whole (patient's) blood and transfused blood.

abolish the abnormality at least temporarily. The abnormality, however, reappears, except in Cases 9 and 10. The first of these patients received a very extensive blood transfusion lasting up to 10 hours after the injury and the second case was moderately and slowly transfused. The failure of the abnormality to reappear again in these two cases may be due to dilution of a plasma factor acting on the red cells—in Case 9 to a very low level and in Case 10 for a long period to a low level. Experiments *in vitro*, which will be reported elsewhere, have shown that plasma derived from heated blood can produce a similar change in normal red cells.

Some of the patients studied showed partial thickness loss of an appreciable portion of the burn, notably Cases 6 and 13. A comparison of the curves obtained from Cases 6 and 7 demonstrates the results of this difference fairly well.

As was stated above, the involvement of the transfused cells seemed likely. This supposition

was tested by Dr. Topley, who transfused M-negative blood to M-positive patients and separated the patient's cells from the transfused cells by a large-scale Ashby technique. Fig. 5 shows the results on Case 12, from which it is seen that the transfused cells are similarly affected.

A haemolysis technique was employed to obtain fractions enriched in older and younger cells. Using 0.4 g. % NaCl, approximately 90% of the cells remaining unlysed were 40 days old or younger (Marks *et al.*, 1958). The rates of alkali denaturation of these two fractions and those obtained by gravitational separation were tested. The results of both methods suggest that the younger age group of the cells may be preferentially affected.

Discussion

The temporary development of the abnormality indicates that the causative factor is most active during the first two days after injury. This is then followed by a gradual return to normal at a maximal rate of 5% (of the highest value) per day. As this exceeds the daily rate of red cell formation, it is probable that the affected cells are destroyed at least twice as rapidly as the normal cell population. The behaviour of Cases 6 and 7, which did not have a primary excision, but had very different areas of full-thickness skin loss, suggests that cells temporarily trapped in a partially damaged capillary bed, which would therefore suffer from transient anoxia, are more liable to be affected.

Fractionation experiments showed that the cells of low gravity and high saline resistance became more readily involved. It is well known that young cells are richer in enzymes than older cells, and, although Simon and Topper (1957) have shown that some young erythrocytes are also to be found among the most fragile cells, the method employed nevertheless yielded a fraction enriched in such cells. Levy, Walter, and Sass (1959) have shown that these young cells have the highest content of oxalacetic transaminase and lactic dehydrogenase. The conversion of glucose to lactate provides the major portion of the energy requirements of the cell. They are such that a high level of A.T.P. is required for the hexokinase reaction. Storage associated with oxygen lack causes a decrease in A.T.P. levels and hence a decrease in utilization of glucose. As the younger erythrocytes are metabolically the most active, at least part of the changes observed may be due to a premature ageing of some of the youngest erythrocytes.

Although the above hypothesis seems attractive, it must nevertheless not be overlooked that the method of fractionation would include cells which, although belonging to an older group, were rendered more saline resistant by exposure to unknown factors.

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