β -thalassaemia: increased chromosomal anomalies in lymphocyte cultures

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SUMMARY Lymphocyte cultures from homozygotes for the β^+ -thalassaemia gene were found to contain chromosomal gaps, breaks, and rearrangements more often than those from controls. Culture time seemed to have a determinant effect on the results. The possible influence of folic acid deficiency is discussed.

The chance finding of several chromosomal breaks and rearrangements in a β -thalassaemia heterozygote in our laboratory and in a homozygote in Birmingham (D. G. Harnden, 1977, personal communication) prompted us to look more closely at the frequency of such anomalies in other patients and their carrier relatives.

Material

Patients were selected at various stages of treatment. Peripheral blood was taken from two newly diagnosed children before any treatment, one 6-month-old baby who was given 5 mg folic acid every second day, two untransfused children who were given the same dose daily, three older patients who were not given any folic acid and were aged 11, 16, and 25 before their first transfusion 2 months after a splenectomy, and, finally, an 18-year-old girl who was given 5 mg folic acid daily, underwent monthly transfusions, and had had a splenectomy 13 years before. All were homozygotes for the β^+ -thalassaemia gene. The cells examined were lymphocytes; the contribution of dividing erythroblasts to our results was practically nil, as shown by the presence of an occasional single cell per slide after immediate harvesting on day 0. We also obtained blood from three asymptomatic heterozygotes chosen from among the patients' relatives.

Of the 8 controls, 5 were colleagues or laboratory technicians, while the 3 others were children sent to our unit for karyotyping because of mental retardation or slight malformations. None of them carried a thalassaemia gene. None of the patients, hetero-

zygotes, or controls had had a viral infection, vaccine, or x-ray for the last 6 months, and all had normal chromosome complements.

Methods

A few drops of whole blood were incubated at 37°C for 2, 3, or 4 days in 4 ml of a culture medium containing 1.0 mg folic acid per litre (Gibco Chromosome Medium 1A). Colcemid (Gibco, prepared in Hank's solution with phenol red) was then added to the cultures to a final concentration of 0.05 μ g/ml for 50 minutes. Conventional hypotonic shock and metaphase fixation consisted of treating the preparation with 0.075 M KCl for 15 minutes followed by three changes of 3:1 ethanol-acetic acid for a total of one hour. Slide preparation was monitored by phase contrast microscopy and the cell suspensions appropriately diluted to make the appearance of the control slides match that of the patients'. The slides were coded and mixed by one person and examined by another, so that the examiners never knew whose cells they were looking at. Scanning was done at low power and any apparently unbroken metaphase, where chromosomes were thought to have a clear morphology, was analysed at higher power, irrespective of chromosome length or overlapping. Those cells which were too difficult to analyse under the microscope were photographed and later analysed from prints. A maximum of 25 cells per slide was examined to prevent the recognition of a pattern of anomalies on a slide and consequent bias. Total chromosome number, chromatid and chromosome gaps, breaks, and exchanges were noted for each metaphase.

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Table 1 Clinical details and chromosomal anomalies seen in each individual

	Case	Age	Splenec- tomy	Trans- fusion	Folic acid tablets	Days in culture	Cells examined	Gaps and breaks	Trisomies and extra acentrics	Chromosome exchanges	Chromatid exchanges	Abnormal cells (%)	Anomalies per cell
β -thalassaemia homozygotes	1	18	+	+	+	2	318	18	0	0	0	4.1	0.06
	2	7	—	—	—	3	200	16	1	0	0	7.5	0.09
	3	1/2	—	—	+	3	100	14	1	0	0	13.0	0.15
	Subtotal					3	300	30	2	0	0	9.3	0.11
	4	3	—	—	—	4	200	55	1	1	5	20.5	0.31
	5	1	—	—	+	4	128	23	0	0	1	14.1	0.19
	6	2	—	—	+	4	161	19	0	2	0	12.4	0.13
	1	18	+	+	+	4	461	94	3	2	2	15.8	0.22
	Subtotal					4	950	191	4	5	8	16.0	0.22
	Total						1568	239	6	5	8	12.3	0.16
Hetero- zygotes	7	30	—	—	—	3	100	9	0	1*	0	9.0	0.10
	8	30	—	—	—	3	100	6	0	0	0	6.9	0.06
	9	20	—	—	—	3	100	10	1	0	0	10.0	0.11
	Total						300	25	1	1	0	8.3	0.09
Controls	10	26	—	—	—	2	188	3	2	0	0	2.7	0.03
	11	1/2	—	—	—	3	101	0	0	0	0	0.0	0.00
	12	1	—	—	—	3	200	15	0	0	0	6.5	0.08
	13	7	—	—	—	3	100	7	0	1†	0	7.0	0.08
	14	31	—	—	—	3	200	8	0	0	0	4.0	0.04
	15	31	—	—	—	3	100	7	1	0	0	8.0	0.08
	16	31	—	—	—	3	100	12	0	0	0	10.0	0.12
	Subtotal					3	801	49	1	1	0	6.4	0.06
	17	25	—	—	—	4	150	12	1	0	0	8.7	0.09
	10	26	—	—	—	4	176	7	2	0	0	5.1	0.05
	Subtotal					4	326	19	3	0	0	6.7	0.07
	Total						1315	71	6	1	0	5.6	0.06

*r (G).

†dic (Dp; Dq) without fragment.

Results

Basically, harvesting was very poor in the patients. The 3 untransfused cases who had had a splenectomy yielded so few metaphases which were of such poor quality that a blind study was not possible and they were abandoned. For the 6 others, there was usually nothing to harvest on the second day, and less than in the controls on the third day. Harvesting was more satisfactory on the fourth day.

Detailed results are shown in Table 1. The totals show significantly different incidence and severity of chromosomal anomalies between patients and controls. This difference is not uniform and seems to depend on the culture time. The increase of anomalies in patients over controls is two-fold on day 2 and becomes three-fold by day 4. In both controls and patients, the number of cells with 3 or more anoma-

lies is greater than the expected rates calculated from a Poisson distribution (Table 2). This effect is especially marked in the patients (Fig. 2), where metaphases were found that contained up to 10 anomalies. No control cell had more than 3 anomalies. Apart from the usual chromatid and chromosome gaps and breaks (Fig. 1a, b, c) and centric and acentric extra chromosomes, 13 more serious anomalies were noted in the patients (Table 1) and consisted of:

- 3 sister chromatid unions (Fig. 2), 2 of which were distally incomplete;
- 5 adjacent quadriradials (Fig. 1f, g), 3 of which were incomplete;
- 1 double chromosome break, probably in an Xp, producing an unrepaired interstitial deletion and a double minute (Fig. 1d);

Table 2 Expected and observed numbers of cells with 0, 1, 2, 3, or more anomalies. The expectations are those of the Poisson distribution: $e^{-\mu} \mu^x / x!$ where μ is the average number of anomalies per cell in each group, and $Y = 0, 1, 2, 3 \dots$ anomalies

Anomalies per cell	β -thalassaemia Expected	Observed	Heterozygotes Expected	Observed	Controls Expected	Observed
0	1330.11	1375	274.18	275	1239.27	1242
1	218.86	161	24.68	23	73.51	70
2	18.01	18	1.11	2	2.18	1
3 or more	1.03	14*	0.03	0	0.04	2†

*One of these 14 metaphases was pulverised.

†One cell each from controls nos. 12 and 16.

Fig. 1 Examples of chromosomal anomalies found in the patients. a, chromatid gap; b, chromosome gap; c, chromatid break; d, unrepaired interstitial chromosome deletion; e, dicentric; f, distally incomplete adjacent quadriradial with breakpoints in Cq and Cq; g, adjacent quadriradial with breakpoints in Cq and Gp; h, $t(2q; Dq)$; i, $t(8; 13)(q24; q14)$ with visible strangulation at the point of exchange.

- 1 similar chromatid interstitial deletion with a minute;
- 1 cell pulverisation;
- 1 dicentric (Cp;Cp) without acentric fragment (Fig. 1e); and
- 2 translocations (Fig. 1h, i), 1 of which displays an unusual strangulation at the point of exchange.

Control no. 16 (Table 1) was later found by chance to carry the gene for methaemoglobinaemia in another independent study where she was also used as a control. Even when her relatively high counts are not included in the totals, results from the β -thalassaemic heterozygotes are not statistically different from those of the controls on day 3, though their mean values fall between those of the homozygotes and the controls.

Discussion

Our results show a definite increase in the incidence and severity of chromosomal anomalies in patients homozygous for the β^+ -thalassaemia gene over heterozygotes and normal controls. Possible causes such as viral infections, vaccines, x-rays, and inherited diseases known to be associated with such anomalies were specifically excluded from this study. The excess of intranuclear free α -chains of haemoglobin that is postulated to have a marked effect on the disruption of DNA synthesis in erythroblasts (Wickramasinghe and Bush, 1975) is unlikely to be present in lymphocytes. Age, blood transfusions, splenectomy, and folic acid intake did not seem to affect the incidence of anomalies in any consistent way, though a definite conclusion with respect to these parameters must await a more detailed investigation. Only culture time showed a consistent

increasing effect on both the frequency and severity of the aberrations in patients and controls, but much more so in the patients. The concentration of anomalies in fewer cells than expected by chance is consistent with observations made in cases of Bloom syndrome, Fanconi pancytopenia, and sporadic quadriradials (Therman and Kuhn, 1976).

In theory, the important folic acid deficiency found in thalassaemia could account for the increased anomalies with which it is clearly associated in other anaemias (Heath, 1966; Krogh-Jensen and Friis-Møller, 1967; Bottura and Continho, 1968), and in individuals treated with folic acid antagonists (Ryan *et al.*, 1965; Herha and Obe, 1976). The exact degree of deficiency differs in thalassaemia according to the patient's age, spleen and liver malfunction, transfusions, and food intake. A more rapid folic acid depletion in the patients' test tubes than in the controls' could then explain both why the serious anomalies are not found in short term cultures—and presumably not *in vivo* either—and why thalassaemia in general is not known to be associated with leukaemia, since most patients take daily folic acid supplements. The fact that folic acid is a necessary co-factor in the incorporation of nucleotides into DNA, the incompleteness of a relatively high proportion of chromatid exchanges, and the unusual strangulation that we observed in a translocation are all compatible with the suggestion that what we saw was not the result of a higher rate of breakage but of a failure of the repair mechanism once breaks have occurred.

The 3 heterozygotes used in this study were asymptomatic. Though not statistically significant, their values were slightly more raised on average than those of the controls. It is open to question whether these intermediate values and those seen in the

Fig. 2 Several anomalies in a patient's metaphase. Among others, a sister chromatid union and a proximally incomplete adjacent quadriradial (arrows) can be seen.

heterozygote mentioned in the introduction, as well as in control no. 16 who is a heterozygote for another haematological disorder, methaemoglobinemia, are coincidental or not. Being asymptomatic, these people obviously did not take folic acid supplements. It would be of interest to study the chromosomes of anaemic heterozygotes in the future.

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