CHROMOSOME CHANGES CAUSED BY 6-AZAURIDINE DURING TREATMENT OF ACUTE MYELOBLASTIC LEUKAEMIA

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Changes produced in chromosomes in human tissue cultures have been described following the use of x-irradiation and radio-drugs (Puck *et al.*, 1957), after therapeutic irradiation (Tough *et al.*, 1960), radioiodine (Boyd *et al.*, 1961), and mustine hydrochloride (nitrogen mustard) (Conen and Lansky, 1961).

During the treatment of patients with acute myeloblastic leukaemia with 6-azauridine (6-AzUR) we were able to make serial observations of gross chromosome changes.

Case 1

An unmarried part-time cleaner aged 43 had been complaining of feeling unduly tired and short of energy for about two to three months, and for two months had had recurring blisters on the lower lip and a persistent cold. She was referred to us on October 6, 1961, as the symptoms had become worse and she had developed a rash over the anterior part of the chest.

There was no evidence of bruising or purpuric bleeding; the gums were normal; the lymphatic glands, spleen, and liver were not enlarged; and clinical examination did not show any abnormalities in the chest, heart, nervous system, and abdomen. Blood count showed: red cells, 1,659,000/c.mm.; haemoglobin, 4.7 g./100 ml. (32%); colour index, 1.0; white cells, 21,400/c.mm. (polymorphs 9.5%, lymphocytes 10.5%, blasts 39.5%, promyelocytes 19.5%, myelocytes 17%, metamyelocytes 4%); platelets, 26,000/c.mm. Sternal marrow biopsy showed a typical acute myeloblastic leukaemia. Skin biopsy from upper chest wall showed leukaemic infiltration.

Transfusion of 4 pints (2.3 litres) of packed red cells raised the haemoglobin to 8.8 g. (60%). On October 13 she was given, by intravenous drip, 10 g. of 6-AzUR daily.

Her white-cell count and blasts began to fall slowly from the fifth day (October 18), and on October 23 these were: white-cell count 15,300/c.mm. and blasts 20%, with platelets 10,000/c.mm. The 6-AzUR drip was continued, with occasional transfusions of packed red cells (1 pint, 570 ml.) as required on account of a certain amount of persistent vaginal bleeding; this was thought to be due to the thrombocytopenia, which fell to 3,000/c.mm. on December 15, so the 6-AzUR was stopped temporarily and fluoxymesterone 5 mg. twice daily and prednisolone 75 mg. daily was given.

Some improvement was noted, the rash had gone, vaginal bleeding was very slight, and she was allowed to go home on December 22 for Christmas. She returned to hospital on December 29, the vaginal bleeding having now ceased; the platelets were 11,000/c.mm., but the white-cell count had increased to 169,000/c.mm. and the blasts to 72%. There was some haematuria for a few days.

A further course of 6-AzUR (10 g. daily) was given by continuous intravenous infusion until January 12, 1962, when her white-cell count was 8,000/c.mm., blasts 10%, and platelets 3,000.

She was sent home on February 6 on prednisolone 25 mg. daily. Her blood count was: red cells, 4,336,000/c.mm.; haemoglobin, 12.3 g. (84%); white cells, 6,600/c.mm. (polymorphs 70.5%, lymphocytes 14.5%, monocytes 2.5%, eosinophils 1%, basophils 1%, blasts 0.5%, metamyelocytes 3.5%, myelocytes 5.5%, promyelocytes 0.5%, plasma cells 0.5%); platelets, 143,000/c.mm.

On May 11 she was readmitted to hospital because of excessive vaginal haemorrhage, extensive purpura in the skin, and some vomiting. In spite of further transfusions of fresh packed cells, and treatment with prednisolone (75 mg. daily) and fluoxymesterone (5 mg. three times a day), she did not respond and died on May 15. Necropsy disclosed a generalized acute haemorrhagic condition throughout the body.

Case 2

A man aged 31 was referred to us from another hospital on August 14, 1962. During the preceding fortnight he had become increasingly tired and listless. Seven weeks previously he developed a dental abscess, for which three teeth were removed, and he was put on penicillin; three weeks later he had another abscess and this time four teeth were removed. He now noticed glands in the left side of the neck, which were soon followed by glands on the other side.

On examination, besides the cervical lymphadenopathy there were enlarged discrete glands in both the axillae and the groins. Liver and spleen were two finger-breadths below the costal margin. Apart from a small retinal haemorrhage in the left eye there was no evidence of bruising or purpuric bleeding. Blood count showed: red cells, 2,515,000/c.mm.; haemoglobin, 7.2 g./100 ml. (49%); colour index, 0.98; white cells, 40,000/c.mm. (polymorphs 2%, lymphocytes 6%, blasts 91%, metamyelocytes 0.5%, myelocytes 0.5%); platelets, 29,000/c.mm. Sternal marrow consisted chiefly of primitive stem cells; myeloblasts, promyelocytes, and normoblasts were also present, and mitoses were seen in granulocytes and normoblasts. The marrow picture was consistent with the diagnosis of acute myeloblastic leukaemia.

Three pints (1.7 litres) of packed red cells were given over a period of three weeks. On August 15 therapy with 6-AzUR was started. 10 g. a day intravenously by a continuous infusion. The patient showed a dramatic subjective response, generalized aches and pains disappeared within 24 hours of the start of the treatment, and he became cheerful. However, the peripheral blood count and the marrow did not show any response to the therapy. On August 29 a course of chloramphenicol 250 mg. q.i.d. was given to potentiate the antileukaemic effect of 6-AzUR. Once again no haematological response was discerned. On September 2 he became pyrexial and developed signs of chest infection. Sputum culture yielded a heavy growth of coliforms, which were sensitive to chloramphenicol.

The illness ran a progressively downhill course and he died on September 5. His blood count on that day was: haemoglobin, 5.8 g. (40%); white cells, 83,000/c.mm. (polymorphs 0.5%, lymphocytes 6%, metamyelocytes 0.5%, and blasts 93%); platelets, 4,000/c.mm.

Post-mortem examination showed acute leukaemic involvement of bone-marrow, liver, spleen, and lymph nodes; hypostatic pneumonia; and generalized haemorrhage.

Case 3

A married woman aged 60 was first seen in July, 1962. For two months she had been aware of increasing lethargy and tiredness; and she became dyspnoeic on climbing stairs.

On examination she appeared of average colour. There was no superficial lymphadenopathy. Spleen and liver were not palpable. There was no evidence of purpura or spontaneous bruising. Blood picture showed: red cells, 1,951,000/c.mm.; haemoglobin, 5.8 g./100 ml. (40%); colour index, 1.02; white cells, 2,800/c.mm. (polymorphs 3%, lymphocytes 25.5%, monocytes 2%, eosinophils 0.5%, neutrophil metamyelocytes 2%, neutrophil myelocytes 6.5%, basophil myelocytes 0.5%, and blasts 60%). Bone-marrow showed increased cellularity; normoblasts and megakaryocytes were scanty. Leucopoiesis was active; the majority of the cells were atypical myeloblasts. The marrow appearance was compatible with acute myeloblastic leukaemia.

She was given 10 g. of 6-AzUR daily for three weeks by a continuous intravenous infusion and 1 pint (570 ml.) of packed blood cells per week. Her blasts began to fall slowly and the platelets began to rise. On the 11th day of treatment her blood count was: white cells 1,400/c.mm.; blasts 36%; and platelets 39,000/c.mm. At the end of three weeks' therapy, although there was considerable subjective improvement and sense of well-being felt by the patient, the peripheral blood count remained relatively unchanged. The white-cell count was 650/c.mm. and the blasts were 51%. A repeat sternal marrow showed a relatively acellular marrow, most of the cells being atypical blasts. Consequently 6-AzUR therapy was stopped and treatment with oral prednisolone 75 mg. daily was begun. The blood count showed a prompt response to the steroid therapy. On her discharge, a week later, the blood count was: white cells, 6,150/c.mm.; blasts, 11%; and platelets 43,000/c.mm.

She was sent home on prednisolone 75 mg. daily, which was gradually tapered off to 25 mg. daily. Her latest count (September, 1962) was white blood cells 11,500/c.mm. and blasts 92%.

Chromosome Studies

Chromosome preparations were made using a modification of the method of Moorhead *et al.* (1960) in all three cases. Serial cultures were set up from Case 1 immediately prior to the beginning of treatment, on three separate days during the course of treatment, and on other days after the period of treatment. Cultures were set up from Cases 2 and 3 after 15 and 27 days respectively. The results obtained are summarized in the Table.

In Case 1 the pretreatment preparations showed an abnormally high degree of aneuploidy, which, of course, is to be expected in this type of leukaemia, and there was no consistent karyotype abnormality (see Fig. 1), fragmentation and chromosome loss accounting for most of the non-modal cells. After 52 days of treatment the cultures showed a further increase in the amount of aneuploidy with fragments in some of the cells, dicentrics in two cells, but again no consistent karyotype abnormality. In these cultures, however, a new feature was seen in that some of the cells contained lightly staining bodies, apparently chromosomes, which tended to adhere to one another with formation of bridges, giving the impression of stickiness. Another feature seen for the first time in this culture was the pre-

sence of a few cells containing globule-like bodies which, although looking different from normal chromosomes, did have shapes which suggested a chromosomal origin (see Figs. 2 A and B). In the cultures from Cases 2 and 3 the same type of cells was seen. In no other cultures of either leukaemic or non-leukaemic patients have we seen anything resembling these cells, and as the fixation of all the other cells on these slides was excellent these changes do not appear to be artifacts, but to have been produced under the influence of 6-AzUR.

In the further cultures set up at later stages in the treatment of Case 1, no normal mitoses were seen, but the "globule" cells were present in greater numbers than in the previous sample. In cultures set up 44 days after the termination of the treatment no mitotic activity was observed, and very few globule cells were seen. In the last culture, five days later, a few rather compact mitoses were seen which could neither be counted nor analysed, and there were now very few "globule cells."

Chromosome Counts

Date of Culture	44	44	45	46	47	48	48	% Modal Cells	Total Cells Counted	Dicentrics
Case 1: Pretreatment	2	2	2	23	3	0	2	68	34	0
52 days ..	3	1	2	12	3	2	2	48	25	2
60 " ..										—
76 " ..										—
120 " ..										—
125 " ..										—
Case 2: 15 days past treatment										—
Case 3: 27 days past treatment										—

Discussion

6-AzUR is a pyrimidine analogue which interrupts the main pathway in the *de novo* synthesis of pyrimidine

FIG. 1.—Pretreatment karyotypes showing an additional group 6-12 chromosome.

FIG. 2.—A, "Globule" cell. B, "Globule chromosomes" arranged in groups corresponding to the normal karyotype.

nucleotides, and so eventually causes defective nucleic acid synthesis.

In the body it is converted to 6-azauridine-5-phosphate (6-AZUMP), this phosphorylation being dependent upon the presence of the enzyme uridine kinase. The 6-AZUMP is thus a competitive inhibitor of the enzyme orotidylic decarboxylase. The efficacy of the treatment, therefore, might be determined by the estimation of orotic acid and orotidine excreted in the urine as a result of this enzyme block. These pathways are summarized in Fig. 3.

FIG. 3.—Pathway of *de novo* synthesis of pyrimidine nucleotides and its inhibition by 6-azauridine.

6-AzUR will interfere directly with nucleic acid synthesis, and it is not surprising to find the increase in aneuploidy which we have observed and the gross and bizarre changes in the appearances of the chromosomes of the affected cells. It is interesting to note the change in the morphology of the leukaemic blast cells seen in the peripheral blood of all three cases during therapy, which affected the nuclei to a great extent and may be

a result of the interference with nucleic acid synthesis (see Fig. 4).

It seems probable that the 6-AzUR was the causal agent of the chromosome aberrations seen, which by their very nature would probably make the cells so affected incapable of dividing to produce viable progeny. The total white-cell count did not in fact show any significant increase during the period of treatment. Relatively normal-looking mitoses and the decrease in the number of "globule cells" seen later on in Case 1 after the end of the treatment suggested a recovery of some of the leukaemic cells or the proliferation of a cell line which was resistant to the drug. Active proliferation of leukaemic cells did in fact occur soon after this culture, and by two months the white-cell count had once more risen to a high value.

Summary

Three patients with acute myeloblastic leukaemia were treated with 6-azauridine (6-AzUR), and the effect of this therapy upon the chromosomes of peripheral leucocytes was studied.

In one case in which serial observations were made a marked increase in the degree of aneuploidy was found after 52 days of treatment. At this time there appeared a number of cells containing globule-like bodies which bore a resemblance to chromosomes, and they were thought to be consistent with nuclear damage. After 60 days no normal mitoses were observed but the globule cells were still present. This state of affairs persisted until the cessation of treatment, and 125 days after the commencement of the therapy a few mitoses were

FIG. 4.—A, Leukaemic blast cell in peripheral blood before treatment. B, Binucleate and other abnormal blast cells in peripheral blood after 52 days' treatment. C, Abnormal blast cells in peripheral blood after 76 days' treatment.

•evident. In the two other cases globule cells were seen after 15 and 27 days of treatment respectively.

It is suggested that these abnormal structures were a result of faulty synthesis of nucleic acids due to 6-AzUR. No similar structures have been seen in other leukaemic or normal subjects.

These studies were done during the tenure by one of us (M. W. E.) of a Medical Research Council Scholarship.

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DIABETES MELLITUS AND PERNICIOUS ANAEMIA

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The possibility of an association between diabetes mellitus and pernicious anaemia has been discussed for a number of years. Root (1931) reviewed the literature and found 48 patients with both diseases. Thirty-one of these patients had developed diabetes mellitus first, four were known to have pernicious anaemia when the symptoms of diabetes mellitus appeared, while the diseases were diagnosed simultaneously in the remaining 13. Subsequent observations on large groups of patients with diabetes mellitus have provided further evidence for the association (Table I). The incidence of pernicious anaemia in these patients varied between 2 per 1,000 (Hagen, 1956) and 10.5 per 1,000 (Watson, 1933), whereas the incidence of pernicious anaemia in

TABLE I.—Recorded Frequency of Pernicious Anaemia in Patients with Diabetes Mellitus

Author	No. of Patients with Diabetes Mellitus	No. who had Pernicious Anaemia	Incidence per 1,000
Goudsmit (1930) ..	1,650	5	3.3
Murphy and Maxon (1931) ..	827	3	3.6
Watson (1933) ..	949	10	10.5
Beckert (1940) ..	900	9	10.0
Hagen (1956) ..	1,000	2	2.0
Dotevall (1959) ..	1,218	12	9.8
Panzram (1960) ..	7,129	16	2.2

the general population has been found to be 1.27 per 1,000 in Great Britain (Scott, 1960), 1 per 1,000 in Denmark (Mosbech, 1951), and 1.2 to 1.5 per 1,000 in Sweden (Waldenström, 1942).

Recent studies of the incidence of diabetes mellitus in patients with pernicious anaemia have likewise shown that it is higher than in the general population, where

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it has been found to vary between 1.3 per 100 (Diabetes Working Party, 1962) and 1.7 per 100 (Wilkerson and Krall, 1947). However, it must be admitted that some of the earlier surveys gave a normal incidence (Table II). A criticism of all these observations is that they

TABLE II.—Recorded Frequency of Diabetes Mellitus in Patients with Pernicious Anaemia

Author	No. of Patients with Pernicious Anaemia	No. who had Diabetes Mellitus	Incidence per 100
Goudsmit (1930) ..	379	5	1.3
Wilkinson (1937) ..	1,000	13	1.3
Panzram (1960) ..	385	15	3.9
Sundberg and Grönberg (1960) ..	314	21	6.7

were based on retrospective surveys of hospital and clinic records. Panzram (1960) collected his data from 12 German diabetic clinics, Dotevall (1959) studied the patients in two hospitals in Sweden, and the other observers looked through the records of large diabetic clinics, searching for patients who also had pernicious anaemia. Both diabetes mellitus and pernicious anaemia may be present in a subclinical form and may easily be missed if they are not actively looked for.

Assuming that there is an association between diabetes mellitus and pernicious anaemia, the surveys confirm the observation of Root (1931) that either diabetes mellitus or pernicious anaemia may be the first diagnosis and that the two diseases may present together. In Wilkinson's (1937) group six patients developed pernicious anaemia before diabetes mellitus, four were known to have diabetes mellitus when pernicious anaemia was diagnosed, and in the other three both diseases were diagnosed at the same time. Sundberg and Grönberg (1960) noted that among their 21 patients with both diseases, pernicious anaemia occurred before diabetes mellitus in 12 cases.

Present Study

For a number of years members of this department have been interested in methods of screening groups of patients for achlorhydria and pernicious anaemia (Aitken, Spray, and Walters, 1954; Callender and Denborough, 1957; Denborough, Retief, and Wits, 1958; Bock and Wits, 1961), and it seemed interesting to study diabetes mellitus from this point of view. Diabetics are mainly treated as out-patients, and we felt that it would be difficult to obtain the accurately timed samples of urine which are necessary for the "diagnex" test of gastric acid secretion and the estimation of urinary pepsinogen from a random sample of diabetics, many of whom are elderly. We have therefore used the method of taking a single blood sample, on which are estimated the haemoglobin concentration, the serum vitamin B₁₂, and serum pepsinogen. We have previously shown that the serum-pepsinogen level provides a reliable indication of severe atrophic gastritis or gastric atrophy (Bock, Arapakis, Wits, and Richards, 1963).

Methods

Estimation of Serum Pepsinogen.—The method was a modification of that described by Edwards, Jepson, and Wood (1960). Serum was added to a substrate (dehydrated human plasma), the acidity adjusted to pH 1.5–2, and the mixture incubated for 24 hours at 37° C. The reaction was stopped by the addition of trichloroacetic acid, and after centrifugation the amount of