Leucocyte Ultrastructure and Folate Metabolism in Down's Syndrome

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SUMMARY

Electron microscopical and haematological investigation of peripheral blood has shown a higher percentage of leukaemia-like nuclear ultrastructural abnormalities in the leucocytes of 30 individuals with Down's syndrome (mean 6,3%) than in normal controls (mean <1%). Most of these aberrations consisted of nuclear membrane abnormalities. Red cell folate values were very low in the group with Down's syndrome. Although mean serum folate and vitamin B_{12} levels were normal in this group, these individuals displayed increasing macrocytosis and decreasing serum folate levels with age. The whole group with Down's syndrome showed an increased mean corpuscular volume (MCV). The percentage of ultrastructural abnormalities did not correlate with folate levels when they were analysed individually. The existence of nuclear membrane abnormalities and folate deficiency, both of which may be associated with increased chromosome breakage, may be partly responsible for the increased leukaemia risk in patients with Down's syndrome.

S. Afr. med. J., **51**, 369 (1977).

Various abnormalities of the haemopoietic system and an increased risk of leukaemia have been found to exist in association with Down's syndrome.[1] The factors which cause leukaemia in this condition have been investigated by different methods including ultrastructural studies of leucocytes in disease-free persons with Down's syndrome[2] and of blast cells in leukaemic patients with Down's syndrome.[3] It should be stressed that nearly 2 decades of electron microscopic research have revealed no specific and universally present ultrastructural features in tumour cells. Characteristic lesions which are frequently encountered have however been described. Djaldetti *et al.*[2] recently reported ultrastructural abnormalities in the nuclei of granulocytes, similar to those described for some leukaemias, in patients with Down's syndrome. According to Bessis[4] nuclear projections have not been encountered in trisomy 21, so that the findings of Djaldetti *et al.*[2] contradicted views held in 1973. Secondly, increased leucocyte protein synthesis, increased erythrocyte production and erythrocyte macrocytosis have previously been found in patients with Down's syndrome.[5] We wanted to investigate the possibility of folate depletion in this situation of increased haemopoietic activity. Careful scrutiny of the literature has shown that folate metabolism in Down's syndrome has not been fully investigated. Folate depletion is associated with increased *in vivo* chromosome breakage. It has been shown that the effect is usually transient and ceases when folate intake is restored to normal.[6] A sustained folate deficiency in rapidly proliferating leucocytes in Down's syndrome may be important in view of the raised leukaemia risk ascribed to increased chromosome breakage. The complex interaction of factors which may cause leukaemia in Down's syndrome has been discussed elsewhere.[7] The aims of the present study were to confirm the occurrence of increased ultrastructural abnormalities of leucocytes in Down's syndrome, to detect possible abnormalities in folate metabolism and to determine whether the ultrastructural abnormalities could be correlated with folate depletion.

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Date received: 2 September 1976.

SUBJECTS AND METHODS

Thirty White patients with Down's syndrome in an institution for mentally deficient patients were studied. In 29 patients the chromosomal abnormality was a standard trisomy 21 and 1 had a 46,XX/47,XX, + 21 mosaic defect All the subjects were healthy, showed no stigmata of malabsorption and received no drugs at the time of the investigation. The subjects were cared for by individual foster mothers and were fed on a balanced diet. Control subjects consisted of 7 healthy volunteers for the investigation of granulocyte ultrastructure and 30 other healthy individuals for the investigation of full blood counts, blood cell morphology, serum folate, red cell folate and vitamin B_{12} levels. Five mentally retarded persons from the same institution were included in the control group for haematological studies.

Electron Microscopy

Ten millilitres of citrated blood was centrifuged and the bulk of the plasma was discarded. The residue was then re-centrifuged in a tube smaller than a Wassermann tube to obtain a buffy layer as thick as possible. The plasma was drawn off until very little covered the cell layer. Leucocytes were fixed by carefully layering 2,5% phosphate-buffered glutaraldehyde, pH 7,4, on top of the buffy layer. After approximately 7 minutes the buffy layer was removed and fixed for a further 12 hours in the above-mentioned fixative. Blocks of about 1 mm^3 were cut from

the fixed buffy layer and post-fixed in Caulfield's osmium tetroxide,[8] prestained in 1% uranyl acetate, dehydrated through graded alcohols and embedded in Spurr's resin.[9] After 18 hours of polymerization at 60°C, 70 - 90-nm sections were cut on LKB Ultratome III and examined with a Zeiss 9S2 electron microscope. Samples from controls and from subjects with Down's syndrome were examined in duplicate and differential counts were performed each time on at least 200 cells.

Haematological Studies

Blood counts were done with a Coulter Model S electronic counter. Serum and red cell folate levels were assayed by means of *Lactobacillus casei* growth.[10] Serum vitamin B_{12} estimations were done by means of a commercial radio-immunoassay (Phadebas B_{12} Test; Pharmacia Diagnostics), supplied in kit form. Special attention was paid to leucocyte morphology as revealed by light microscopy.

RESULTS

The results of electron microscopical and haematological studies are shown in Table I.

Ultrastructure

Ultrastructural changes were identical to those found by Djaldetti *et al.*[2] and are shown in Figs 1 - 4. These include nuclear bridges, appendages and pockets, swollen mito-

TABLE I. RESULTS OF ULTRASTRUCTURAL AND HAEMATOLOGICAL FINDINGS IN 30 PATIENTS WITH DOWN'S SYNDROME

Patient No.	Sex	Age	Ultrastructural abnormalities (%)	Serum folate (μg/l)	Red blood cell folate (μg/l)	Serum vit. B_{12} (ng/l)	MCV (fl)	Haemoglobin (g/100 ml)
1	M	4	16	7,8	65	90	84	13,1
2	M	5	5	6,5	105	810	87	12,2
3	M	6	6	8,5	94	1 300	80	10,3
4	M	9	6,	6,4	61	760	80	11,9
5	M	4	5	7,2	67	1 100	77	11,0
6	M	6	9	4,3	62	1 000	86	13,8
7	M	11	12	7,3	100	1 600	83	13,3
8	M	16	11,5	4,7	129	1 300	91	13,9
9	M	15	14	3,0	45	620	92	13,2
10	M	23	8	3,5	46	510	99	14,0
11	M	18	11	4,9	103	800	93	12,9
12	M	20	12	2,8	49	1 300	97	14,7
13	F	9	3	5,3	107	1 300	92	13,5
14	F	11	2	5,1	41	840	87	12,7
15	F	16	6	3,5	105	700	92	14,2
16	F	15	5	5,7	79	1 200	86	13,0
17	F	17	9,5	4,3	61	1 600	81	11,4
18	F	59	3	3,5	39	510	93	13,9
19	F	45	4	2,8	79	870	98	12,3
20	F	29	4,5	3,0	62	1 600	106	14,0
21	F	50	2	4,6	92	760	101	13,8
22	F	11	6	5,3	52	700	88	13,1
23	F	22	2	3,2	43	1 000	98	15,4
24	F	24	12	3,7	145	1 600	88	13,8
25	F	19	1	8,5	109	1 300	98	12,7
26	F	16	5	11,3	65	1 300	87	13,7
27	F	18	4,5	2,8	30	620	96	13,2
28	F	14	1	8,1	61	1 000	76	12,7
29	F	11	2	5,3	121	520	89	13,7
30	F	19	2	3,2	40	1 000	96	15,0
Mean		17,5	6,3	5,2	75,2	987	88	13,1
SEM		2,36	0,76	0,4	5,5	70,0	2,4	0,22
Males only								
Mean		11,4	9,6	5,6	77,2	932,5	87,4	12,8
SEM		1,9	1,06	,56	8,01	121,0	2,03	0,37
Females only								
Mean		22,5	4,1	4,96	73,94	1 023,3	91,8	13,45
SEM		3,38	0,68	0,54	7,74	86,2	1,74	0,22

chondria and eosinophilic crystalloid inclusion granules. The percentage of abnormalities in the granulocytes of the individuals with Down's syndrome (mean 6,3%) differed significantly from that of the 7 normal control patients (mean <1%). In addition, abnormalities occurred in a mean of 9,6% of male cells as opposed to a mean of 4,1% of female cells. The boys were younger as a group but the percentage of abnormalities did not show a correlation with age. It can be seen from Table I that patient 1 displayed the highest percentage of ultrastructural abnormalities. This patient also showed numerous eosinophilic crystalloid granules in the cytoplasm (Fig. 4) not seen in any of the other subjects. Patient 10 displayed intracytoplasmic pseudovirus particles approximately 200 nm in diameter in 24 out of 200 cells (12%) (Fig. 5). The significance of the damaged nuclear membrane in this photograph will be mentioned in the discussion.

Phagocytosis was observed in 1 - 5% of cells of 3 of the subjects with Down's syndrome. Although increased phagocytosis can be characteristic of cancer, the chance finding of this phenomenon in cells of an individual with Down's syndrome is unknown. Fibrillar bodies[2] were not seen in our specimens.

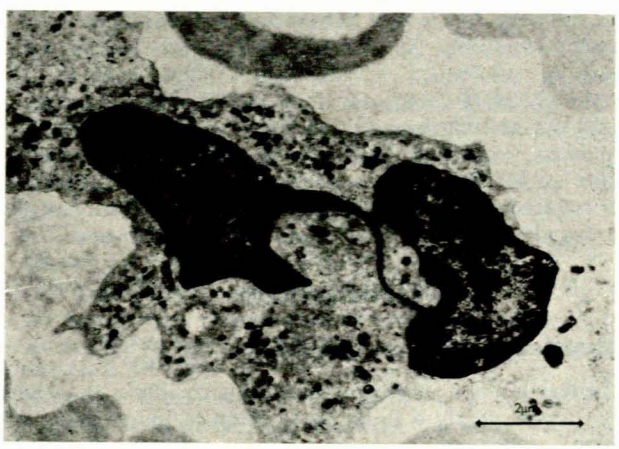

Fig. 1. Neutrophil with a nuclear bridge (× 17 600).

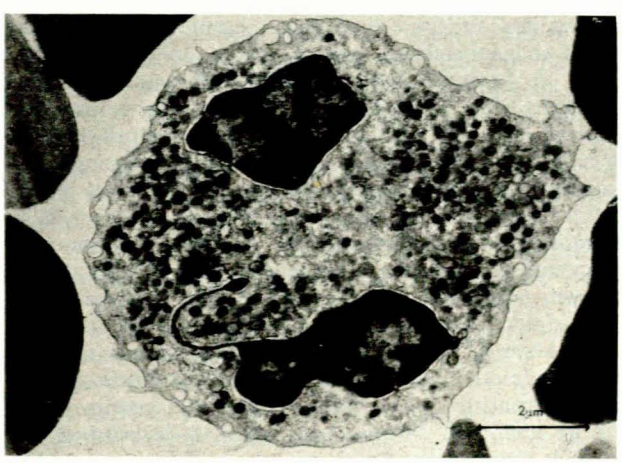

Fig. 2. Neutrophil with a nuclear appendage (× 18 150).

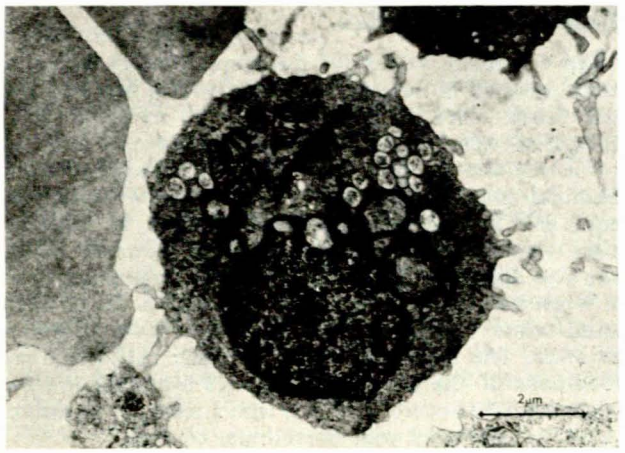

Fig. 3. Granulocyte with nuclear pockets, irregular nuclear outline, and swollen mitochondria (× 17 600).

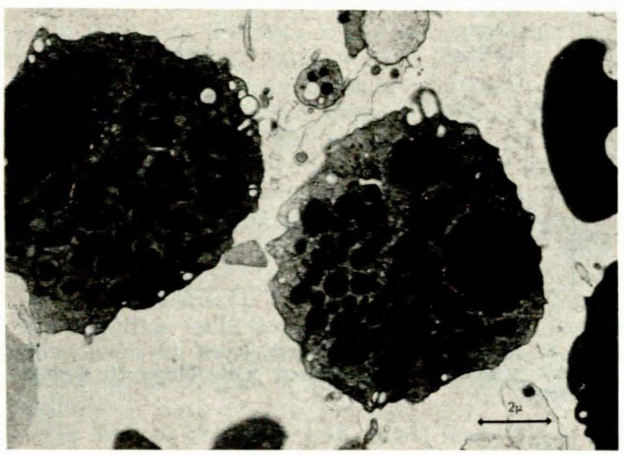

Fig. 4. Eosinophil of patient 1 of Down's syndrome group with numerous cytoplasmic crystalloid granules (× 11 800).

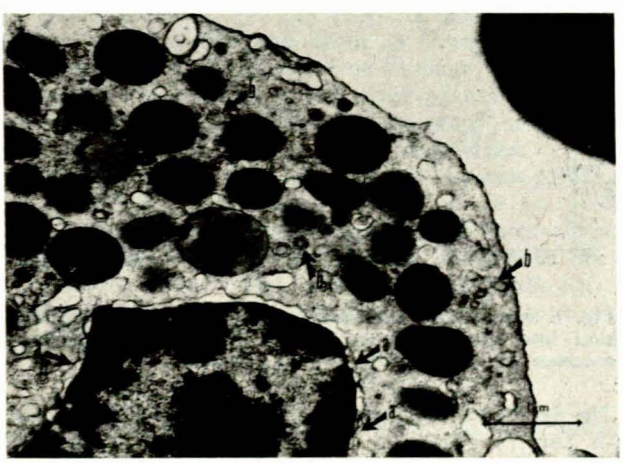

Fig. 5. Granulocyte of patient 10 of Down's syndrome group with pseudovirus particles 200 nm in size. Note damaged nuclear membrane with focal dilatation (a) and possible pinocytotic vesicle (b) (× 32 500).

Haematological Studies

Red cell folate values for the group with Down's syndrome were all in the deficient range, with a mean of 75,2 μg/l (SEM 5,56) as compared with a mean of 196,2 μg/l (SEM 9,04) in 30 normal control patients (Fig. 6). No correlation between individual percentage of ultrastructural abnormalities and red cell folate levels was found in Down's syndrome ($r = -0,01$). In addition, males showed an increasing tendency towards serum folic acid deficiency and macrocytosis with age (Figs 7 and 8). When the effect of age is not taken into account, mean serum folate values did not differ significantly between the sexes. The mean corpuscular volume (MCV) of red blood cells of the group with Down's syndrome showed a significant difference ($P<0,05$) with a tendency to macrocytosis, as compared with our normal controls.

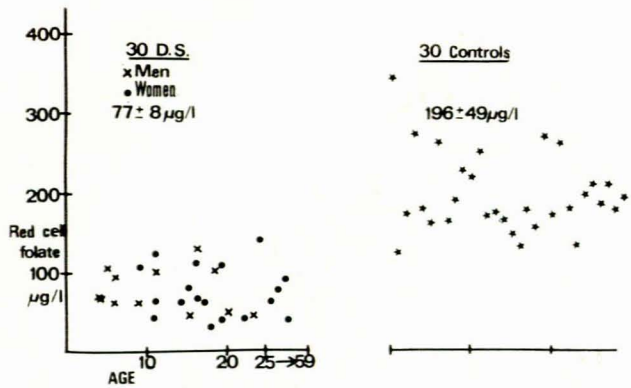

Fig. 6. Red cell folate-deficient levels in group with Down's syndrome as compared with those of normal subjects.

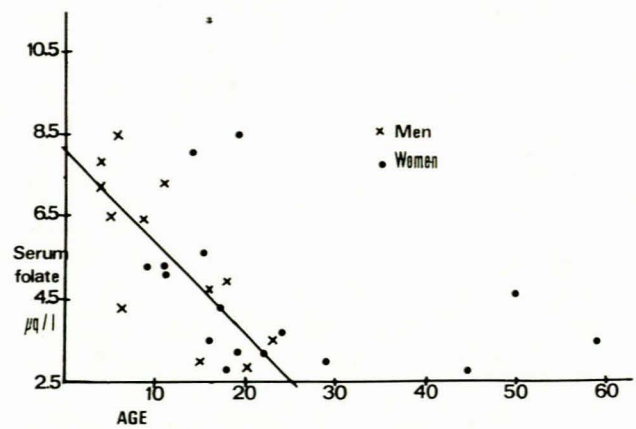

Fig. 7. Serum folic acid decreasing with age. The regression line was drawn to indicate the more prominent decrease for males with Down's syndrome.

The difference was even more significant for the females with Down's syndrome (Mann-Whitney test). However, the mean age of the females was higher and therefore the expected mean MCV would be higher. Mean serum folate and vitamin B_{12} levels were within normal limits except for patient 1, who displayed a lowered vitamin B_{12} level.

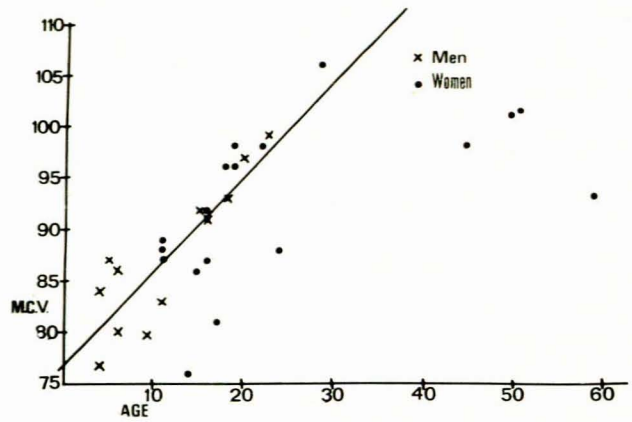

Fig. 8. Macrocytosis increasing with age. The regression line shown the male increase which was more prominent than the scattered female increase.

There was a negative correlation between serum folate and age in the group with Down's syndrome. The following results were obtained by using Spearman's rank correlation test: $r_s = -0,709$ ($P<0,01$) for males and $r_s = -0,524$ ($P<0,05$) for females. There also was a negative correlation between MCV and serum folate in the group with Down's syndrome. Spearman's rank correlation gave the following results: $r_s = -0,774$ ($P<0,01$) for males and $r_s = -0,524$ ($P<0,05$) for females. This last correlation could not entirely be accounted for by the effects of age on serum folate, although the partial correlation coefficients of MCV on serum folate, when age is controlled, are reduced to $r_s = -0,556$ for males, and $r_s = -0,286$ for females, which was not significant in either sex.

DISCUSSION

Ultrastructural Abnormalities

Leukaemia-like ultrastructural abnormalities in nuclei were found nearly 6 times more often in individuals with Down's syndrome than in normal controls. According to Bernhard,[11] nuclear membrane infolding or cytoplasmic projections of nuclear membrane reflect malformations and possibly facilitate nucleocytoplasmic exchange. The bridges and appendages seen in Figs 1 - 3, where separate masses of chromatin have formed, could represent one of the mechanisms by which the chromosome complement is altered after cell division. No visible structural differences have previously been described for the chromosomes themselves in such cellular alterations.[12] Changes in the nuclear membrane are important because of the role of chromosomes in organizing its function. Chromosome replication is a membrane-associated phenomenon, and it has been postulated that the deleted No. 22 chromosome (Philadelphia chromosome), associated with chronic myeloid leukaemia, may cause a fatal interference with the proper formation and functioning of the nuclear membrane.[12] Breakage is the phenomenon that occurs before such an abnormal chromosome forms. It is believed that the single chromosome break might provide evidence to link viral and somatic mutation theories of carcinogenesis.[13]

The single chromosome break associated with virus infection was first described by Hampar and Ellison in 1961.[14] It has subsequently been shown that viruses need not be actively replicating to induce chromosomal abnormalities. The pulverization phenomenon (chromosomal disintegration) can be produced by non-infectious virus fractions.[15] Increased susceptibility of cells to transformation by oncogenic SV40 virus in patients with Down's syndrome has been documented. Saksela and Moorhead,[16] and Moorhead and Weinstein,[17] demonstrated chromatid breaks to be one of the multiple effects of transformation, so that cells from patients with Down's syndrome may point to a link between viral and somatic mutation theories of carcinogenesis.

Higurashi *et al.*[18] recently found that patients with Down's syndrome showed more chromosomal breakage after virus infection than did normal subjects. The authors stated that this may be a factor contributing to malignancy in childhood. It may well be that the nuclear membrane abnormalities provide the explanation for increased chromosomal breakage in Down's syndrome. These changes may be aided by a virus or viruses which persist due to antiviral immunodeficiency associated with Down's syndrome.[19]

A damaged nuclear envelope in association with 'virus-like' particles has been noted in the blast cells of a leukaemic patient with Down's syndrome.[3] Cells from patient 10 in our study showed nuclear membrane damage in association with double-core cytoplasmic inclusions 200 nm in diameter. These particles resemble viruses closely but no evidence was found of assembly sites or budding. It is difficult to establish a viral origin for these cellular inclusions with certainty,[20] and the example shown in Fig. 6 may even represent pinocytotic vesicles. Nevertheless, this remains an important area for future studies.

It is difficult to account for the difference in the incidence of nuclear abnormalities between males and females. No sex difference in mortality in Down's syndrome has been noted.[21] The rate of development of the immune system as reflected in certain diseases in normal individuals seems to be different between the sexes.[22] According to Taylor and Ounsted,[23] delay in development affords greater opportunity to interact in a less mature way with the environment. It has been shown that males delay their development in a wide variety of systems. Such a delayed characteristic suggests that the variance will be greater for males, where more of them will be represented at the extreme of the spread. On the other hand, the male preponderance may reflect easier chromosome breakage, which may aid in studying the association between nuclear membrane abnormalities and chromosome breakage in patients with Down's syndrome when taking sex into account.

A direct correlation between red cell folate levels and the percentage of ultrastructural abnormalities was not found when patients were analysed individually. Furthermore, as mentioned above, the percentage of ultrastructural abnormalities differed between the sexes but mean red cell folate did not. These results seem to indicate that folate deficiency is not directly correlated to the spectrum of ultrastructural abnormalities displayed by our patients, but does not exclude the possibility that *some* of these abnormalities are due to a folate deficiency.

Haematological Studies

It can be deduced from the results obtained in this study that although mean serum folate was normal in the group with Down's syndrome, an age-related decrease occurred which was more prominent in the male group. This did not correlate with length of stay in the institution. The importance of these age-related changes and their association with leukaemia in Down's syndrome is unknown, since leukaemia in Down's syndrome usually appears in childhood. Serum vitamin B_{12} was high normal in most patients except no. 1, who had a deficient level. The most prominent change was shown by the red cell folate levels, which were deficient in the group with Down's syndrome. This group also showed a tendency to macrocytosis as compared with the control group. The reason for deficient red cell folate in the face of normal mean serum folate and vitamin B_{12} levels is unknown. At this stage we do not know whether tissue coenzymes are generally affected or whether low levels concern only the red cells. No other cause for folate deficiency could be incriminated for the patients in this study. Samples taken from 5 non-trisomic mentally retarded patients from the same institution showed normal values for all parameters.

Future research should include evaluation of folate polyglutamate in Down's syndrome. Folate in cells appears in two forms: as a monoglutamate and as a polyglutamate containing up to 6 glutamic acid residues. Polyglutamates have been shown to be the active intracellular form. In 1974 Chanarin *et al.*[24] found that the biochemical lesion in vitamin B_{12} deficiency is due to failure of polyglutamate synthesis. This leads to impaired entry of monoglutamate forms of folate into cells. Plasma folate subsequently increases so that normal or slightly raised serum levels are found.

The low red cell folate and normal serum folate found in Down's syndrome may point to a similar situation. The high normal vitamin B_{12} levels in this context are unexplained.

Deficiency of active intracellular folate coenzyme necessary for single carbon unit transfers affecting RNA and DNA synthesis *may* thus occur in Down's syndrome. It is necessary to investigate these findings for a probable correlation with many of the observed haemopoietic abnormalities ascribed to this trisomy. Many of these were previously explained by a gene dosage effect.

In the presence of sustained increased haemopoiesis and folate consumption, chromosome breakage may occur. Transient increased chromosome breakage with abnormal DNA metabolism has been shown in direct bone marrow preparations from folic acid- and vitamin B_{12}-deficient patients, demonstrating a definite *in vivo* effect.[6]

CONCLUSION

Much remains to be done in order to elucidate the exact relationship between the increased risk of leukaemia in Down's syndrome and factors such as viruses, nuclear membrane pathology, folic acid depletion and chromosome breakage. We feel that such an approach could be profitable in terms of clarification of the leukaemia risk

in Down's syndrome. Although care must be exercised in translating findings to non-trisomic individuals, the elucidation of definite aetiological factors in Down's syndrome could lead to the establishment of guidelines for research in normal children.

We wish to thank Prof. J. J. van der Walt of the Department of Anatomical Pathology for facilities and assistance with the ultrastructural studies, Mr M. V. Kayser of the same Department for expert technical assistance, Prof. W. B. Becker of the Department of Medical Virology for guidance in connection with the pseudovirus particles, Dr A. B. Daneel, Superintendent of the Alexandra Institute, Maitland, Cape, for his assistance and permission to publish patient data, Prof. J. G. Steytler and the Department of Haematology, Tygerberg Hospital for haematological investigations, Mr T. Fraser and Miss J. van der Hout for the folate and vitamin B_{12} studies and Mr G. P. Y. Clarke, of the University of Rhodesia and staff of the MRC Institute for Biostatistics for aid in the statistical analysis of our data.

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Bacteriuria in Black Diabetics

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SUMMARY

Urine of 198 diabetics and 147 non-diabetics was examined for bacteriuria by means of the Uricult dip slide method. Prevalence of bacteriuria in diabetics (18,7%) was significantly higher than in the control group (7,6%). This increased prevalence in diabetics was due primarily to an exceedingly high prevalence in diabetic women (27%). There was no relation between bacteriuria and age, duration of diabetes, treatment for diabetes, quality of control of diabetes, symptoms of urinary tract infection or hypertension.

S. Afr. med. J., **51**, 374 (1977).

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It has long been believed that diabetics are prone to urinary tract infection, but the evidence for this belief has been questioned. Thomsen[1] concluded that there was no concordant view of the incidence of urinary tract infection in diabetics. Studies during life have not helped to resolve this problem. Vejlsgaard[2] found an increase in the incidence of urinary tract infection in diabetics while several other studies[3-5] showed no increase. In more recent years, investigators who re-examined this problem found an increased incidence of urinary tract infection among elderly diabetic women.[6-8] The prevalence of urinary tract infection in Black diabetics does not appear to have been assessed recently. This article describes a study of this problem.

PATIENTS AND METHODS

Diabetic patients were selected at random from outpatients who regularly attended the Diabetic Clinic at Baragwanath Hospital. Control patients were similarly selected from among those who regularly attended the medical outpatient department at the same hospital for conditions other than