Mycoplasma and Leukaemia in Childhood: A Brief Report

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The recovery of organisms of the genus *Mycoplasma* from leukaemic bone marrow has been reported on a number of occasions (*Brit. med. J.*, 1965). In view of these findings, which suggested a new approach to the aetiology of leukaemia, a sizable group of children with this condition, together with appropriate control children, were examined for the presence of mycoplasma. The results of this investigation are given in the following brief report.

Selection of Patients and Specimens

Patients and Controls.—All children diagnosed as suffering from leukaemia and seen in the Hospital for Sick Children between 23 April 1965 and 21 March 1966 were included in the study.¹ Their ages ranged from a few weeks to 16 years; the majority were between 4 and 8 years. Control specimens from non-leukaemic patients were examined whenever marrow puncture was considered essential for diagnosis. These marrow aspirates were not ideal control specimens, but they were the only ones available.

Bone-marrow Specimens.—Most of the children with leukaemia were participants in a trial of treatment, organized by the Medical Research Council and administered by the Department of Haematology. In this trial bone-marrow examinations were carried out on each child at eight-weekly intervals and at other times as indicated. On almost all these occasions marrow aspirates were obtained for the cultivation of mycoplasma.

Blood Specimens.—Specimens of venous blood were taken and cultivated for mycoplasma whenever more than about 500 blast cells/cu. mm. were present.

Other Specimens.—Cerebrospinal fluid, pleural fluid, throat swabs, and other specimens were taken on occasion; they were examined in the same way as the marrow and blood specimens.

Cultivation of Mycoplasma

Human Embryo Lung Tissue Cultures.—Tissue cultures were prepared from human embryos by a modification of the method of Tyrrell *et al.* (1960). All experiments were carried out in cultures derived from three pairs of lungs. Cultures were grown to confluence in Eagle's basal medium with 10% tryptose phosphate broth, 10% calf serum, 0.088% sodium bicarbonate, and added antibiotics. They were then washed with Eagle's basal medium and maintained on Eagle's basal medium with 10% tryptose phosphate broth, 2-5% foetal calf serum, and 0.17% sodium bicarbonate, with added antibiotics.

Mycoplasma Medium (Augmented Pleuropneumonia-like organisms (P.P.L.O.) Medium).—This consisted of P.P.L.O. broth or agar (Difco, without crystal violet) 70 ml., horse serum (not inactivated) 10 ml., yeast extract 10 ml., boiled blood extract 10 ml., deoxyribonucleic acid 0.4%, thallium acetate (1% in distilled water) 2.5 ml., penicillin (100,000 units/ml.) 0.1 ml. The components of the medium were stored in small

quantities: fresh plates and bottles were prepared as required and used on the same day. Plates were incubated in a moist 5% CO₂ atmosphere at 37° C.

Positive Control Mycoplasma.—A pool of nine strains of *Mycoplasma* derived from patients with leukaemia (Negroni, 1964) was used as a positive control specimen. It grew luxuriantly on direct inoculation into the augmented P.P.L.O. broth and agar in two to four days at 37° C. The sensitivity of the medium to wild strains of *Mycoplasma* was shown by direct inoculation of throat swabs from children with respiratory and other illness and from apparently healthy adults. Twenty-four specimens were inoculated on to augmented P.P.L.O. agar plates immediately after they had been taken. One isolation of mycoplasma was made, and three further specimens yielded characteristic mycoplasma colonies which failed to grow on subculture.

Precautions Against Contamination.—A particular, rather isolated, laboratory was allocated for this work. No continuous cell-culture lines were brought into the laboratory. Once inoculated, agar plates and broth were incubated and observed elsewhere to obviate possible contamination of tissue cultures by positive recoveries of mycoplasma. The positive control specimens were not acquired until the close of the study and were handled with stringent precautions against accidental contamination of those plates and broth which had been inoculated with leukaemic material.

Methods Employed in Examinations of Leukaemic Material

Direct Method.—About 0.1-0.2 ml. of marrow, or 1-1.5 ml. of blood, was inoculated immediately after withdrawal from the patient into 4 ml. of augmented P.P.L.O. broth. These cultures were incubated for five to seven days at 37° C., and 1-2 ml. inocula were subcultured on to augmented P.P.L.O. agar plates, which were observed every other day for 10 to 14 days. Subculture of suspicious areas readily dispelled confusion between the microscopic colonies of mycoplasma and artifacts on the surface of the agar plate.

Indirect Method.—About 0.1-0.2 ml. of marrow, or 0.2 ml. of plasma or cerebrospinal fluid, was inoculated immediately after withdrawal from the patient into four to six human embryo lung tissue cultures, the same number of culture tubes being left uninoculated as controls. All cultures were examined daily for cytopathic effects for as long as practicable (usually 19 to 27 days). At 5 to 16 days after inoculation half the cultures were examined for evidence of growth of non-cytopathic agents by an interference method (Sutton and Tyrrell, 1961). The remaining cultures were harvested (cells and tissue-culture fluid) at from 19 to 27 days and one to three further passages were given before being discarded as negative. Cultures were examined at each passage for evidence of interfering agents. At the second passage in tissue culture 1-2 ml. of cells and fluid was inoculated on to augmented P.P.L.O. agar plates, which were subsequently observed as in the direct method.

Uninoculated tissue-culture cells and fluid were passaged in exactly the same way and were inoculated on to augmented P.P.L.O. agar plates.

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¹ One child was seen at St. Thomas's Hospital through the kindness of Dr. D. G. Cottom.

Owing to the small amounts of tissue available, and to the demands of other experimental and diagnostic procedures, not all the marrow specimens were inoculated into tissue cultures.

Results

Of the 67 children included in the survey, 43 were diagnosed as cases of leukaemia (39 of these being acute lymphoblastic leukaemia and one each being acute myelomonoblastic, acute myeloid, chronic myeloid, and acute lymphoblastic leukaemia with lymphosarcoma). The remaining 24 suffered from a variety of malignant and non-malignant conditions. One hundred and ten specimens of bone marrow or peripheral blood were obtained from the children with leukaemia and examined for mycoplasma; 27 similar specimens taken from the remaining children were examined in the same way. The results of these investigations are given in the Table. In addition, three specimens of cerebrospinal fluid from three children with leukaemic involvement of their central nervous system, two specimens of pleural fluid from a child with a leukaemic pleural exudate, and garglings from the child with acute myeloid leukaemia were examined for mycoplasma. A chance specimen of breast milk from a mother whose previous child was suffering from acute lymphoblastic leukaemia was also investigated. None of these specimens yielded mycoplasma. Two strains of *Mycoplasma*, so far unidentified, were recovered by the indirect method from throat swabs and faeces of an infant who developed acute lymphoblastic leukaemia during the first few weeks of life; no recoveries were made from nose swabs, urine, or marrow from this child, nor from garglings and faeces from the mother.

Discussion

During the past five years a number of workers (Berg and Rosenthal, 1961; Ang *et al.*, 1962; Benyesh-Melnick *et al.*, 1963) have failed to recover mycoplasma from the bone marrow of patients with leukaemia. More recently, however, such recoveries have been reported on several occasions. Thus Negroni (1964) recovered "viruses" from leukaemic bone marrow in 10 out of 25 cases. This material was later (Grist and Fallon, 1964; Girardi *et al.*, 1965) shown to contain strains of a mycoplasma closely related to *Mycoplasma pulmonis*, a species normally found in rodents (Fallon *et al.*, 1965). Hayflick and Koprowski (1965) recovered by direct inoculation of cell-free medium one strain of *Mycoplasma* from bone marrow taken from three patients with acute lympho-

blastic leukaemia. This was identified as *Mycoplasma orale* type 1 (Hayflick and Chanock, 1965). Murphy *et al.* (1965) recovered mycoplasma, also by direct inoculation, from 14 children and three adults with leukaemia. These findings are supported by the report of mycoplasma-neutralizing antibodies in 50% of sera from leukaemic patients, as opposed to 10% of sera from control individuals (Imperial Cancer Research Fund, 1966), and speculation about the possible aetiological role of mycoplasma in leukaemia has been further provoked by their ability to produce chromosomal aberrations in tissue culture (Fogh and Fogh, 1965).

There is no doubt about the authenticity of the recoveries of mycoplasma from these few cases of leukaemia; Fallon *et al.* (1965) have answered criticism on the grounds of possible laboratory contamination in the case of some (Negroni, 1964) of the recoveries in tissue culture, while the validity of those instances of direct recovery in cell-free media (Hayflick and Koprowski, 1965; Murphy *et al.*, 1965) is almost indisputable.

The results described in the present report agree with those of earlier workers and with more recent investigators, such as Phillips *et al.* (1965), who have failed to recover mycoplasma from large numbers of patients with leukaemia. They suggest with some strength that mycoplasma cannot be recovered with any ease or regularity from such patients. They serve to cast a considerable amount of doubt upon the notion that infection with mycoplasma can bear a causal relation to leukaemia in man. Mycoplasma have been recovered in only a minute proportion of those cases investigated and there is no evidence from animal experiments that this class of micro-organisms is oncogenic. It seems more likely that the recoveries of mycoplasma from leukaemic patients which have been recorded represent coincidental infections associated with this disease by virtue of its debilitating effect, rather than the continuing presence of a causal agent.

Summary

One hundred and ten specimens of marrow and blood from children and 27 similar specimens from children with non-leukaemic disease were examined for the presence of mycoplasma. No isolations were made.

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Attempts at Recovery of Mycoplasmas from Children with Leukaemia and with other Illnesses

Haematological State	No. of Patients	No. of Specimens Tested		No. of Specimens Tested by:						Recoveries of Mycoplasma	
				Direct Method		Indirect Method		Direct + Indirect Method			
		Marrow	Blood	Marrow	Blood	Marrow	Blood	Marrow	Blood		
Leukaemia											
Acute lymphoblastic (total):	39*	88	15	81	8	56	9	47	2	0	
Untreated	15	16	4	13	3	5	2	3	0	0	
Relapse	19	16	11	15	5	7	7	6	2	0	
Remission	29	56	0	53	0	44	0	38	0	0	
Acute lymphoblastic + lymphosarcoma: remission	1	1	0	1	0	1	0	1	0	0	
Acute myeloid: untreated	1	0	4	0	3	0	3	0	2	0	
Acute myelomonoblastic: untreated	1	1	0	1	0	1	0	1	0	0	
Chronic myeloid: remission	1	1	0	1	0	0	0	0	0	0	
Other Illness											
Malignancy or reticulosis	5	7	1	7	0	5	1	5	0	0	
Idiopathic thrombocytopenic purpura	5	5	0	5	0	4	0	4	0	0	
Other diagnoses†	14	14	0	14	0	8	0	8	0	0	
All leukaemia	43	91	19	84	11	58	12	49	4	0	
		(110)		(95)		(70)		(53)			
All other illnesses	24	26	1	26	0	17	1	17	0	0	
		(27)		(26)		(18)		(17)			

* Specimens were obtained from most children during all phases of disease, hence the apparent disparity between the figures for patients in untreated, relapse, and remission phases and the total figure.

† These were as follows: pyrexia of unknown origin (3 cases), hypoplastic anaemia (2 cases), histiocytosis X (2 cases), and single cases of Hodgkin's disease, hyperglycaemia, Pyle's disease, sickle-cell anaemia, thalassaemia, toxocariasis, and tuberous sclerosis.

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Preliminary Communications

An Enzymatic Defect in a Group of Obese Patients*

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The possibility has been raised that a metabolic defect underlies the development of some forms of obesity in man (Mayer, 1957; Tepperman, 1958; Astwood, 1962). However, attempts to define such a lesion in the metabolism of human adipose tissue in vitro has so far proved unsuccessful. Reports have confirmed that lipolysis in adipose tissue of obese patients is unimpaired when stimulated by adrenaline in vivo (Orth and Williams, 1960) or in vitro (Mosinger *et al.*, 1965; Björntorp and Hood, 1966; Galton and Bray, 1966), and therefore makes a block in the mobilization of fatty acids from adipose tissue an unlikely basis for the excessive accumulation of triglycerides in this disease.

A study of lipogenesis in adipose tissue of obese and normal-weight patients has not revealed, so far, any differences in the activity of glucose-6-phosphate dehydrogenase and malic enzyme, which generate essential cofactors for the synthesis of fatty acids (Shrago *et al.*, 1966; and citrate-cleavage enzyme, which is thought to supply an important part of the acetyl coenzyme A necessary for fatty-acid synthesis (Bhaduri and Shre, 1963), is essentially absent in human adipose tissue (Shrago *et al.*, 1966). This suggests that the synthesis of fatty acids in human adipose tissue may not be an important factor in the development of obesity.

Another essential precursor for lipogenesis is α -glycerol phosphate, which is formed from dihydroxyacetone phosphate during glycolysis by the action of a cytoplasmic α -glycerophosphate dehydrogenase. The experiments reported here were designed to examine the source and disposal by oxidation of α -glycerol phosphate in human adipose tissue obtained from obese and normal-weight patients, and to see whether there was an increase in the availability of α -glycerol phosphate for synthesis of triglycerides in adipose tissue of the former. It was also possible to study two overweight patients in whom a hypothalamic lesion was very likely responsible for the obesity, and the metabolic behaviour of their adipose tissue was compared with tissue of obese patients with no other disorder.

MATERIALS AND METHODS

Source of Tissue.—Human subcutaneous adipose tissue was obtained during abdominal operations from 25 patients (9 males,

16 females) whose ages ranged from 15 to 69 years and whose mean body weight was 152 lb. (68.9 kg.). Patients with diabetes and jaundice were excluded. Preoperative treatment involved a fast of eight hours before the procedure, premedication with pentobarbitone sodium and atropine, and general anaesthesia induced by thiopentone and maintained with either cyclopropane with oxygen or halothane with nitrous oxide and oxygen. Adipose tissue from obese patients was removed from the anterior abdominal wall under local anaesthesia (approximately 6 ml. of 1% xylocaine injected intradermally). A summary of the clinical details of these patients is given in Table I. After removal the adipose tissue was washed and either transferred to 3 ml. of albumin-bicarbonate buffer for preparation of isolated adipose cells by a modification of the procedure of Rodbell (1964), or homogenized for approximately one minute in 0.25 M sucrose (1:4) in a glass mortar surrounded by chipped ice with a motor-driven teflon pestle. It was frequently necessary to complete homogenization in a hand mortar-and-pestle when the adipose tissue was fibrous.

TABLE I.—Summary of the Clinical Details of the Obese Patients

No. of patients	..	..	..	11 (2 male, 9 female)
Age range	..	..	..	15–40 years
Mean weight (range)	..	..	..	340 (280–430 lb.); 154.2 (127–195) kg.;
Height range	..	..	..	5 ft. 2 in.–5 ft. 9 in.; 157–175 cm.
Onset of obesity	..	..	..	Early teens, except for one in late twenties

Mitochondria were prepared from the homogenate by a method similar to that of Schneider (1951) by means of a Lourdes high-speed centrifuge (motor head: 16RA) in a cold room (4° C.) or in a refrigerated International Centrifuge (motor head: 856). The supernatant after the first centrifugation at 17,000 $\times g$ was kept for assay of the soluble cytoplasmic enzymes. After washing, the mitochondrial pellet was suspended in 0.03 M phosphate buffer (4:1) and used for the assay of mitochondrial glycerophosphate and succinic dehydrogenases.

Assay Procedures.—Oxygen consumption was measured in conventional Warburg vessels, as previously described (Galton, 1966). Assays of nicotinamide-adenine dinucleotide (NAD)-linked malate dehydrogenase and glycerophosphate dehydrogenase were performed in the cytoplasmic fraction, with the use of an incubation system, as described elsewhere (Galton and Fain, 1966), except that enzyme activity was measured by the change in absorption of nicotinamide nucleotides in a recording spectrophotometer (Bausch and Lomb, spectronic 505) at a wavelength of 340 m μ . The pH of the sample was measured after each assay, and was 9.4 ± 0.5 . The temperature of the cuvette holder was $28 \pm 1^\circ$ C., and the activity of the enzyme was expressed as m μ moles of NADH formed/min./mg. of protein homogenate.

Assay of the NAD-independent glycerophosphate dehydrogenase was performed on a suspension of the mitochondrial fraction according to the method of Ringler and Singer (1962).

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