of diverticulosis originate in other parts of the gut which also have not adapted to a low-residue diet and whose motility is altered by this diet?

The concept that the whole of the alimentary tract has to struggle with a low-residue diet explains not only why "upper intestinal" symptoms accompany diverticulosis but also how the bran diet relieves symptoms which appear to arise at different levels of the gastrointestinal tract.

It is a pleasure to thank Surgeon Captain Cleave for his advice on the use of unprocessed bran, and Mr. Denis Burkitt, Sir Richard Doll, and Sir Clifford Naunton-Morgan for their helpful advice. We are indebted to the records department of Manor House Hospital and to my secretary, Miss Joan Inglis, for their assistance. Messrs. Allinsons donated the bran, and advised us of its composition.

ADDENDUM.—Since this follow-up was completed in March

1971 we know of no patients who relapsed on this diet. One died from other causes.

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PRELIMINARY COMMUNICATIONS

Unbalanced Deoxyribonucleotide Synthesis Caused by Methotrexate

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Summary

The effect of methotrexate on the free intracellular pools of thymidylate triphosphate (dTTP) and deoxyadenosine triphosphate (dATP) in normal human phytohaemagglutinin-transformed lymphocytes has been studied. Methotrexate caused a fall in the dTTP pool ranging from 38% to 88% and a rise in the dATP pool ranging from 24% to 185%.

A rise in the free intracellular pool of dATP is thought to inhibit both rubonucleotide reduction and polynucleotide ligase, an enzyme concerned in DNA synthesis and repair. The hypothesis is suggested here that folate deficiency per se, as well as a functional folate deficiency induced by methotrexate may cause reduced DNA synthesis, megaloblastic changes, and chromosome abnormalities by producing a rise in the free intracellular pool of dATP as well as by causing a fall in free intracellular dTTP.

Introduction

It is now well accepted that megaloblastic anaemia arises because of a disturbance in DNA synthesis in haemopoietic cells. Folate deficiency is thought to cause this anaemia by reducing synthesis of thymidylate triphosphate (dTTP) (one of the two pyrimidine nucleotides incorporated into DNA), since a folate coenzyme, 5,10-methylenetetrahydrofolate, is needed for synthesis of thymidylate from its immediate precursor deoxyuridylylate (Wahba and Friedkin, 1962; Metz *et al.*, 1968; Das and Hoffbrand, 1970) (see Fig.). Vitamin B₁₂ is not required directly

for the reaction but its deficiency is thought indirectly to cause depletion of the folate coenzyme involved in thymidylate synthesis and thus secondarily to reduce synthesis of dTTP (Herbert and Zalusky, 1962; Waters and Mollin, 1963). The base composition of the DNA formed in megaloblastic anaemia is normal (Hoffbrand and Pegg, 1972) but it is still not clear, however, whether lack of thymidylate alone is the cause of the abnormal morphological and chromosomal appearance seen in megaloblastic anaemia.

Normal synthesis of DNA from purine and pyrimidine nucleotides.

The purpose of the present communication is to report a second disturbance in nucleotide synthesis that may be brought about by folate deficiency in human haemopoietic cells and which may be relevant to the disturbance in DNA synthesis that occurs in megaloblastic anaemia and in patients receiving the folate antagonist methotrexate—namely, a rise in the intracellular pool of one of the two purine nucleotides incorporated into DNA, deoxyadenosine triphosphate (dATP) (see Fig.). An increased intracellular level of this compound has been shown to cause a number of disturbances of the synthesis of DNA and its precursors in bacteria and other mammalian cells, and we suggest here that a rise in the intracellular pool of dATP as well as a fall in the intracellular pool of dTTP may be responsible for the effect of folate deficiency on proliferating (DNA-synthesizing) cells. In these experiments methotrexate, a folate antagonist, was used to produce folate deficiency in

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vitro, and phytohaemagglutinin-stimulated lymphocytes were used as a source of proliferating human haemopoietic cells.

Materials and Methods

Cultures of phytohaemagglutinin-transformed lymphocytes obtained from healthy human volunteers were set up as described by Das and Hoffbrand (1970). Methotrexate (in concentrations of 10^{-3} , 10^{-4} , and 10^{-5} M) was added at three days and the cells were incubated for a further hour at 37°C . The cells were then harvested by washing three times in cold (4°C) phosphate-buffered isotonic saline (pH 7.4). The nucleotides dATP and dTTP were extracted and the pools of each of these nucleotides estimated by the method of Solter and Handschumacher (1969) as modified by Lindberg and Skoog (1970). The micrococcus DNA polymerase used was obtained from P.L. Biochemicals, poly dA-T primer from Miles Laboratories, and the radioactive isotopes from the Radiochemical Centre, Amersham.

Results

The results of three experiments are shown in the Table. The intracellular free pool of dTTP in 10^6 phytohaemagglutinin-transformed lymphocytes ranged from 5.8 to 12.1 (mean 8.8) pmol. Incubation with methotrexate at a concentration of 10^{-5} M for one hour caused a substantial fall in the free intracellular pool of dTTP ranging from 38% to 85%. In experiment 1 methotrexate at 10^{-3} M caused a fall of 88% in the intracellular pool of dTTP, and in experiment 3 methotrexate at 10^{-4} M caused a fall of 50% in the intracellular pool of dTTP.

Effect of Incubation for One Hour with Methotrexate on Free Intracellular Concentrations of Thymidylate Triphosphate (dTTP) and Deoxyadenosine Triphosphate (dATP) in 10^6 Phytohaemagglutinin-transformed Normal Human Lymphocytes. Each Result is the Mean of Triplicate Assays and is Expressed as pmol/ 10^6 Cells

Experiment No.	dTTP			dATP		
	Alone	+ Methotrexate (Concentration)	% Fall	Alone	+ Methotrexate (Concentration)	% Rise
1	12.1	$\begin{cases} 1.8 (10^{-3}\text{M}) \\ 1.4 (10^{-3}\text{M}) \end{cases}$	$\begin{cases} 85 \\ 88 \end{cases}$	3.3	$\begin{cases} 9.1 (10^{-5}\text{M}) \\ 9.4 (10^{-5}\text{M}) \end{cases}$	$\begin{cases} 176 \\ 185 \end{cases}$
2	5.8	$3.6 (10^{-5}\text{M})$	38	2.8	$3.5 (10^{-5}\text{M})$	24
3	8.6	$4.3 (10^{-4}\text{M})$	50	2.9	$4.3 (10^{-4}\text{M})$	48

In contrast, in each experiment methotrexate caused a pronounced rise in the free intracellular pool of dATP. The concentrations of dATP in 10^6 phytohaemagglutinin-transformed lymphocytes ranged from 2.8 to 3.3 (mean 3.0) pmol. Methotrexate at 10^{-5} M caused a rise of dATP of between 24% and 176% of the control. In experiment 1 methotrexate at 10^{-3} M caused a rise of dATP of 185%, and in experiment 3 methotrexate at 10^{-4} M caused a rise of dATP of 48%.

Discussion

These preliminary results show that folate deficiency induced in vitro by methotrexate causes a fall in the free intracellular pool of dTTP and a rise in dATP in human phytohaemagglutinin-transformed lymphocytes. The finding that methotrexate causes a fall in thymidylate levels is to be expected in view of the known action of methotrexate (and folate deficiency generally) on thymidylate synthesis in mammalian cells, though so far as we are aware this fall has not previously been directly shown. The observation of a rise in intracellular dATP levels with methotrexate is also new but parallels the findings of Neuhaard (1966), who showed that removal of thymine from the growth medium of thymine-requiring mutants of *Escherichia coli* caused an immediate rise in dATP content, reaching a maximum at 60

minutes. The explanation for this increase in dATP concentration is not certain. Neuhaard, however, suggested that it arose because dTTP normally inhibited intracellular reduction of adenosine diphosphate (ADP) to deoxyadenosine diphosphate (dADP). Lack of dTTP would therefore increase the intracellular levels of dADP and dATP at the expense of ADP. At any event it is likely that the rise in dATP in methotrexate-treated cells is in some way a consequence of the fall in dTTP levels rather than a direct effect of methotrexate on dATP synthesis. Indeed methotrexate might directly inhibit the early stages of purine synthesis which require folate coenzymes.

A rise in the free intracellular pool of dATP has been found to have at least two major effects on DNA synthesis in both bacterial and mammalian proliferating cells. Firstly, a rise in free dATP has been shown to inhibit reduction of ribonucleotide diphosphates to the corresponding deoxyribonucleotide derivatives (Reichard *et al.*, 1961; Klenow, 1962; Morris *et al.*, 1963). A rise in free dATP might therefore cause a considerable, rapid overall reduction in synthesis of DNA and a low cell DNA/RNA ratio, a feature of megaloblastic erythropoiesis (Wickramasinghe, 1972). Secondly, Neuhaard and Munch-Peterson (1966), Weiss *et al.* (1968), Freifelder (1969), and Pritchard and Zaritsky (1970) suggested that a rise of the intracellular pool of dATP produces inhibition of polynucleotide ligase, an enzyme thought to be essential for normal DNA synthesis and repair. Inhibition of polynucleotide ligase could be responsible for random single strand chromosome breaks in bacteria starved of thymine (Freifelder, 1969) and, by analogy, could be responsible for the random chromosome breaks as well as the uncoiling of chromosomes observed in the cells of patients with severe folate deficiency and receiving methotrexate therapy.

The hypothesis that the rise in dATP levels as well as a fall in dTTP levels is the cause of megaloblastic changes whether secondary to folate deficiency per se or of functional folate deficiency induced by methotrexate is in keeping with the observation that drugs which inhibit pyrimidine synthesis—for example, methotrexate and cytosine arabinoside—cause gross megaloblastic changes, whereas purine antagonists—for example, mercaptopurine—characteristically do not, even though they inhibit DNA synthesis, since the purine antagonists would be expected to cause a fall rather than a rise in dATP levels. Moreover, megaloblastic anaemia is characteristic of the inherited disorder of pyrimidine synthesis, orotic aciduria (Smith *et al.*, 1966), but is not typically seen in the inherited disorder of purine synthesis, the Lesch-Nyhan syndrome Kelley *et al.*, 1969).

If these preliminary results are confirmed they demonstrate a new important mechanism by which methotrexate may kill cells. They also point to a new mechanism by which folate deficiency may affect DNA synthesis, and by which megaloblastosis, in general, arises. The hypothesis is tentatively proposed that megaloblastosis arises when there is an imbalance in intracellular cell pools of dATP and dTTP with a rise in dATP as well as a fall in dTTP.

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Protection of Rat Bronchial Epithelium against Tobacco Smoke

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Summary

Addition to tobacco of phenylmethyloxadiazole (PMO) protects rats against some of the adverse effects of exposure to cigarette smoke. Two groups of 15 rats were exposed to 25 cigarettes a day for 24 days; the group whose cigarette included PMO showed less immediate distress after exposure, a smaller tracheal goblet cell count, less thickening of the tracheal epithelium, and less cells in mitosis than those exposed to ordinary tobacco.

Introduction

Human epidemiological studies have shown that tobacco smoking causes hypersecretion of mucus which is typical of chronic bronchitis and that it induces bronchial cancer. The fundamental structural changes seem to be hypertrophy of the bronchial submucosal mucous glands and an increase in the number of goblet cells and their extension into peripheral small airways that are normally free of them. Such changes can be produced experimentally in animals by tobacco smoke and other irritants such as sulphur dioxide (Lamb and Reid, 1968, 1969; Mawdesley-Thomas, Healey, and Barry, 1971) and nitrous oxide (Freeman and Haydon, 1964). The similarity to the morphological changes in man provides a satisfactory animal model for the study of chronic bronchitis (Reid, 1970).

In animals the mitotic rate also is increased during exposure to an irritant. This increase may, in part, reflect the repair of ulcerative damage seen in the first weeks after exposure to sulphur dioxide (Lamb and Reid, 1968), but cell division does not always seem proportional to such damage. For example, in one series of experiments, though two types of tobacco produced a similar increase in goblet cells flue-cured tobacco produced many more mitoses than air-cured tobacco (Lamb and Reid, 1969). In rats these changes have not been seen to develop into bronchial cancer, but a high mitotic count offers evidence of disturbance of cell division and presents features of bronchial damage additional to the increase in goblet cells. Detailed mitotic counts after exposure to tobacco smoke (Lamb and Reid, 1969) were not given in the previous report and so are reported here for the first time. Furthermore the higher mitotic count after exposure to flue-cured or barn-cured tobacco rather than to air-cured or Burley tobacco accords with the higher prevalence of human bronchial cancer after exposure to the

former type of tobacco (Passey, Blackmore, Warbrick-Smith, and Jones, 1971).

Because of the relevance to human disease of the changes produced experimentally by tobacco smoke—increase in goblet cells and cells in mitosis—each count offers a suitable test for screening tobacco substitutes or additives.

Dalhamn (1966) and Dalhamn and Rylander (1971) have shown that phenylmethyloxadiazole (PMO), a substance first used as an antitussive agent, offers protection against the ciliostatic effect of tobacco smoke. At their suggestion we undertook the experiments reported here to test the effect of this additive on the increase in goblet cells and on the nature of the distribution of acid glycoprotein they contain, on the cells in mitosis, and on the epithelial thickness. The histochemical study of the intracellular mucus is reported separately (Jones, Bolduc, and Reid, 1972).

Material and Methods

The effect of two types of cigarette was investigated. Each was made with the same tobacco, but to the tobacco for one type 2% by weight of PMO was added.

In the experiments described here 41 Sprague-Dawley rats (Carworth Europe, CFY strain) from specific pathogen-free stock were used. The animals weighed about 150 g at the start of the experiment. Lungs from additional animals of the same batch were examined microscopically to ensure that lymphocytic infiltration was minimal (Reid, 1970).

The rats were divided into three groups—(1) tobacco, 15 animals exposed to cigarette without any additive; (2) tobacco and PMO, 15 animals exposed to cigarettes to which the anti-inflammatory agent PMO had been added; and (3) control, (a) 6 rats given a "sham" exposure in that they spent time in an exposure chamber as groups 1 and 2 but were subjected only to airflow and not to added tobacco smoke, and (b) 5 animals kept in cages. Since statistical analysis showed that groups 3a and 3b were indistinguishable for any of the features studied here, in this paper the results are presented with groups 3a and 3b added as the control group.

Groups 1 and 2 were exposed to 25 cigarettes a day. The animals were placed in an aluminium chamber and the cigarettes were burnt in a Wright Autosmoker (Wright, 1972). The details of exposure are similar to those reported by Lamb and Reid (1969). The animals were exposed four days a week for six weeks. The mean values of humidity and temperature for each chamber used for tobacco exposure were similar. The difference between the cages used for tobacco and sham exposures was greatest with respect to humidity, doubtless due in part to increased water loss from the animals induced either by the greater rise in temperature or by a nicotine effect, and perhaps because of the water vapour in the tobacco smoke. It has been shown that neither extremes of temperature nor humidity influence the number of goblet cells (Jones, Baetjer, and Reid, 1971).

During the four and a half hours or so of exposure no animal received food or water and the control animals were removed from the room. Apart from this period all animals spent their time in the same type of cage, in the same room, and with as much food and drink as required. Each day the chambers and the Autosmoker were cleaned.

The two types of cigarette supplied to us were identified by a code number whose key was not known to any one of us; it was not broken until it had been decided which cigarette had most effect on the animals.

The animals on whom the mitotic studies were to be made were left quietly in the same room for 18 hours before they were

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