The Inhibition of Nucleic Acid Synthesis by Mycophenolic Acid

By T. J. FRANKLIN AND JENNIFER M. COOK
*Imperial Chemical Industries Ltd., Pharmaceuticals Division,
 Alderley Park, Macclesfield, Cheshire*

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1. Mycophenolic acid, an antibiotic of some antiquity that more recently has been found to have marked activity against a range of tumours in mice and rats, strongly inhibits DNA synthesis in the L strain of fibroblasts *in vitro*. 2. The extent of the inhibition of DNA synthesis is markedly increased by preincubation of the cells with mycophenolic acid before the addition of [^{14}C]thymidine. 3. The inhibition of DNA synthesis by mycophenolic acid in L cells *in vitro* is reversed by guanine in a non-competitive manner, but not by hypoxanthine, xanthine or adenine. 4. The reversal of inhibition by guanine can be suppressed by hypoxanthine, 6-mercaptopurine and adenine. 5. Mycophenolic acid does not inhibit the incorporation of [^{14}C]thymidine into DNA in suspensions of Landschütz and Yoshida ascites cells *in vitro*. 6. Mycophenolic acid inhibits the conversion of [^{14}C]hypoxanthine into cold-acid-soluble and -insoluble guanine nucleotides in Landschütz and Yoshida ascites cells and also in L cells *in vitro*. There is some increase in the radioactivity of the adenine fraction in the presence of the antibiotic. 7. Mycophenolic acid inhibits the conversion of [^{14}C]hypoxanthine into xanthine and guanine fractions in a cell-free system from Landschütz cells capable of converting hypoxanthine into IMP, XMP and GMP. 8. Preparations of IMP dehydrogenase from Landschütz ascites cells, calf thymus and LS cells are strongly inhibited by mycophenolic acid. The inhibition showed mixed type kinetics with K_i values of between 3.03×10^{-8} and 4.5×10^{-8} M. 9. Evidence was also obtained for a partial, possibly indirect, inhibition by mycophenolic acid of an early stage of biosynthesis of purine nucleotides as indicated by a decrease in the accumulation of formylglycine amide ribonucleotide induced by the antibiotic azaserine in suspensions of Landschütz and Yoshida ascites cells and L cells *in vitro*.

Mycophenolic acid (I) has been found in these laboratories (Carter, 1966; Carter *et al.* 1969), and also independently by Williams *et al.* (1968), to have marked activity against a range of experimental tumours in rats and mice. In view of the potential importance of these findings an investigation into the biochemical activity of mycophenolic acid has been carried out. Gosio (1896) was apparently the first to isolate mycophenolic acid from a *Penicillium* culture. This work was confirmed by Alsberg & Black (1913), who gave the substance its name. Structural studies were carried out by Raistrick and his co-workers, culminating in the publication of the structure illustrated in (I) (Birkinshaw, Raistrick & Ross, 1952). Mycophenolic acid was found to have limited antibacterial and antifungal activity by a number of workers including Abraham (1945), Florey, Gilliver & Jennings (1946) and Gilliver (1946).

The demonstration in these laboratories that

mycophenolic acid has a potent antimitotic effect against mammalian cells and that it is an effective antitumour agent (Carter, 1966) led us to make a study of the biochemical activity of the antibiotic on mammalian cells. Mycophenolic acid is shown to be a potent inhibitor of nucleic acid synthesis largely by its ability to inhibit the enzyme IMP dehydrogenase (IMP-NAD oxidoreductase, EC 1.2.1.14) (Franklin, 1968). A preliminary account of this work has been given (Carter *et al.* 1969).

MATERIALS AND METHODS

Chemicals. [2-¹⁴C]Thymidine (35.7 mc/m-mole), [8-¹⁴C]-adenine (35 mc/m-mole), [8-¹⁴C]guanine (36.1 mc/m-mole), sodium [¹⁴C]formate (10.8 mc/m-mole) and L-[U-¹⁴C]leucine (8 mc/m-mole) were all obtained from The Radiochemical Centre, Amersham, Bucks. Other chemicals were obtained from the following sources: the monosodium salt of mycophenolic acid, isolated from the culture filtrate from *Penicillium stoloniferum* Thom, was provided by Mr E. G. Jefferys; IMP (disodium salt) and XMP (disodium salt) from Koch-Light Laboratories Ltd., Colnbrook, Bucks.; ATP (disodium salt), phosphoenolpyruvic acid (cyclohexylammonium salt) and NAD⁺ from C. F. Boehringer and Soehne G.m.b.H., Mannheim, Germany; adenine, guanine, hypoxanthine and xanthine from British Drug Houses Ltd., Poole, Dorset; glutamine from Mann Research Laboratories Inc., New York, N.Y., U.S.A.; azaserine from the Sigma Chemical Co., St Louis, Mo., U.S.A.; ribose 5'-phosphate from Calbiochem, Los Angeles, Calif., U.S.A.

Enzymes. Crystalline pancreatic ribonuclease was obtained from British Drug Houses Ltd. and ribonuclease-free deoxyribonuclease from the Sigma Chemical Co. Solutions of ribonuclease were heated at 80° for 10 min. before use to destroy residual deoxyribonuclease activity. Trypsin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. (trypsin 1-300).

Cells. (1) L strain mouse fibroblasts were grown on glass in Eagle's medium (Parker, 1961) supplemented with 8% (v/v) filtered calf serum (Eagle's growth medium). These cells were used in the exponential phase of growth for the study of the effects of mycophenolic acid. The medium covering the cells (1 ml.) was replaced with fresh medium containing various additives (see the Results section) immediately before the start of an experiment.

(2) LS strain fibroblasts were supplied to us by the Microbiological Research Establishment, Porton Down, Wilts.

(3) Ascites cells. Landschütz and Yoshida ascites-tumour cells were obtained respectively from white mice 7-10 days, and from albino rats 5-6 days, after inoculation of the tumours. Either the cells were immediately sedimented at 600g for 5 min. at 0-1° and then resuspended in one of the buffers described in the Results section or the ascitic fluid was diluted directly with one of these buffers. To obtain the most reproducible results it was necessary to start the incubations within 20 min. of the removal of the tumour cells from the animals.

Uptake of radioactive precursors. The uptake of labelled precursors into the nucleic acid of cells *in vitro* was determined as described by Franklin & Higginson (1967). The distribution of [¹⁴C]adenine between the RNA and DNA of L cells was determined by incubating the filter-paper strips impregnated with washed precipitated protein and nucleic acid in a medium containing 50 mM-tris-HCl buffer, pH 7.2, 5 mM-MgCl₂ and 20 µg. of either ribonuclease or deoxyribonuclease/ml. After incubation for 1 hr. the strips were washed successively in ice-cold 5% (w/v) trichloroacetic acid, ethanol-diethyl ether (3:1, v/v) and finally diethyl ether. The strips were dried and the radioactivity was measured as described by Franklin (1963).

In experiments where the uptake of labelled hypoxanthine into purine bases was studied, the acid-soluble and -insoluble nucleotides were isolated as follows. When intact cells were

used the cells were washed by suspension and centrifugation in ice-cold 0.14M-NaCl solution. The cell pellet was then extracted with 5% (w/v) trichloroacetic acid at 0° for 30 min. followed by centrifugation at 10000g for 10 min. The pellet was washed with ice-cold 5% (w/v) trichloroacetic acid and centrifuged and the supernatant fractions were combined (acid-soluble fraction). The pellet was then extracted with 5% (w/v) trichloroacetic acid at 90° for 5 min. to hydrolyse the nucleic acids. After centrifugation the pellet was washed with 5% (w/v) trichloroacetic acid and the supernatant fractions were combined (acid-insoluble fraction). Both acid-soluble and -insoluble fractions were extracted three times with diethyl ether to remove trichloroacetic acid. After the aqueous layers had been evaporated to dryness the residues were dissolved in 2M-HCl, and the solutions were sealed in hard-glass tubes and heated at 100° for 60 min. The purine bases were then separated by high-voltage electrophoresis on Whatman no. 1 paper in a medium containing equal volumes of 1.5M-formic acid and 2M-acetic acid, pH 2.0. After electrophoresis for 30-45 min. at 6 kv, the sheets were dried and the areas corresponding to purine marker spots were cut out and the radioactivity was measured as described by Franklin (1963).

In experiments involving cell-free preparations the reaction was terminated with an equal volume of ice-cold 10% (w/v) trichloroacetic acid. After centrifugation the purine bases in the supernatant were isolated as described above.

The incorporation of [¹⁴C]formate into FGAR* was determined as follows. After incubation with the labelled precursor as described in the Results section, the cells were separated by centrifugation and washed by resuspension in ice-cold 0.14M-NaCl solution. The cell pellet was then extracted at 0° with 0.2M-HClO₄ and centrifuged. The supernatant fraction was neutralized with 1M-NaHCO₃. FGAR was isolated from the neutral supernatant by using a column (3 cm. x 1 cm.) of Dowex 1 (formate form) as described by Henderson (1962). The authenticity of the FGAR isolated by this procedure was checked by using the ion-exchange method of Moore & LePage (1957). The FGAR fraction was evaporated to dryness and then redissolved in water. This solution (2.5 ml.) was mixed with the scintillator solution (17.5 ml.) described by Scales (1967) and the radioactivity measured in a Packard Tri-Carb model 3003 liquid-scintillation spectrometer. The efficiency of counting, determined by the addition of an internal radioactive standard, was about 60%.

Cell-free extracts. (1) Simple ultrasonic extracts of Landschütz cells were prepared as follows. The cells were removed from mice 8-10 days after tumour inoculation. The cells were harvested by centrifugation at 0-1°, washed twice with 0.36% NaCl to lyse erythrocytes and then disrupted in water by a 20 sec. period of ultrasound delivered by an MSE 60 w ultrasonic oscillator at 0-1°. An equal volume of a medium containing 50 mM-tris-HCl buffer, pH 7.8, and 10 mM-MgCl₂ was added to the disrupted cells and the preparation was centrifuged at 21000g for 15 min. at 0-1°. The sediment was discarded and the supernatant fraction (containing 12 mg. of protein/ml.) retained.

(2) A preparation of IMP dehydrogenase was obtained from calf thymus as follows. The tissue, which had been

* Abbreviation: FGAR, formylglycine amide ribonucleotide.

stored for several days at -20° , was thawed, minced and homogenized with ice-cold 0.14M -NaCl (155 g. of tissue with 465 ml. of solution). The homogenate was centrifuged at $21000g$ for 10 min. The sediment was discarded and 2% (w/v) protamine sulphate added to the supernatant (1 ml. of protamine sulphate solution/300 mg. of supernatant protein) and the mixture was left at 0° for 10 min. The precipitated nucleic acids were removed by centrifugation at $21000g$ for 15 min. The supernatant fraction was brought to 60% saturation with $(\text{NH}_4)_2\text{SO}_4$ and left at 0° for 30 min. The precipitate was removed by centrifugation at $21000g$ for 15 min. and dissolved in 15 mM-potassium phosphate buffer, pH 7.4. A fraction precipitated from this solution between 25% and 45% saturation with $(\text{NH}_4)_2\text{SO}_4$ was redissolved in 15 mM-potassium phosphate buffer, pH 7.4 (final concn., 5 mg. of protein/ml.). This fraction contained most of the IMP dehydrogenase activity and had little xanthine oxidase activity.

IMP dehydrogenase was obtained from Landschütz ascites cells and LS cells by suspension of the cells in a buffer containing 15 mM-potassium phosphate, pH 7.4 at $0-1^{\circ}$. With ascites cells, erythrocytes were first removed as described above. The cells were treated in the ultrasonic oscillator for 20 sec. and then centrifuged at $21000g$ for 30 min. The supernatant fraction was mixed with protamine sulphate solution as described above. After 10 min. at 2° , the precipitate was removed by centrifugation and discarded. The supernatant was adjusted to 25% saturation with $(\text{NH}_4)_2\text{SO}_4$ and the precipitate removed by centrifugation. The fraction precipitated between 25% and 45% saturation with $(\text{NH}_4)_2\text{SO}_4$ was dissolved in 15 mM-potassium phosphate buffer, pH 7.4 (final concn., 5 mg. of protein/ml.). Most of the IMP dehydrogenase activity was again found in this fraction, which was stored at -20° . Usually a small amount of precipitate was found on thawing and this was removed by centrifugation and discarded. The xanthine oxidase activity of IMP dehydrogenase preparations from Landschütz ascites cells and LS cells was negligible. IMP dehydrogenase activity was assayed by the method of Atkinson, Morton & Murray (1963).

Determination of nucleic acids. Nucleic acid was extracted from cells with 0.5M -HClO₄ at 70° . The DNA was determined by the method of Burton (1956) and RNA by the method of Ceriotti (1955).

Determination of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used, with crystalline bovine plasma albumin as a standard.

RESULTS

Effects of mycophenolic acid on nucleic acid synthesis in L cells. L cells, growing on the inner surface of glass tubes (about 10^6 cells/tube), were incubated in Eagle's growth medium (1 ml./tube) with or without mycophenolic acid and $0.1\text{ }\mu\text{C}$ of ^{14}C -labelled precursor/ml. At the end of the incubation period the tubes were chilled and the cells scraped off the glass into ice-cold 0.14M -sodium chloride solution. The uptake of labelled precursor into nucleic acid was then determined as described in the Materials and Methods section. The incorporation of precursors

Fig. 1. Effect of mycophenolic acid on the incorporation of ^{14}C thymidine into DNA by L cells. ○, Control cells; △, ● and □ indicate the incorporation in the presence of 0.1, 1.0 and 10.0 μg . of mycophenolic acid/ml. respectively.

in the absence of mycophenolic acid was linear for at least 6 hr.

Fig. 1 shows that the antibiotic markedly inhibited DNA synthesis. A lag that was observed before the inhibitory effect of mycophenolic acid appeared decreased as the concentration of the antibiotic was increased. However, even at a concentration of mycophenolic acid as low as $0.1\text{ }\mu\text{g}$./ml. DNA synthesis ceased after 4 hr. incubation with the drug.

The results of preincubation of the cells with mycophenolic acid before the addition of ^{14}C -thymidine are shown in Fig. 2. After preincubation with $10\text{ }\mu\text{g}$. of antibiotic/ml., the label was added and incubation continued for 2 hr. The inhibition of DNA synthesis by mycophenolic acid was markedly increased by preincubation.

To determine the effect of mycophenolic acid on RNA synthesis, L cells were preincubated for 1 hr. with or without $10\text{ }\mu\text{g}$. of mycophenolic acid/ml. ^{14}C Adenine ($0.1\text{ }\mu\text{C}$ /ml.) was then added and incubation continued for 2 hr. The distribution of the radioactivity between RNA and DNA was then determined as described in the Materials and Methods section. The effects of treatment with

Fig. 2. Effect of preincubation of L cells with mycophenolic acid on the inhibition of [^{14}C]thymidine incorporation into DNA. The cells were preincubated with mycophenolic acid ($10\text{ }\mu\text{g./ml.}$) for the indicated periods. [^{14}C]Thymidine was then added and the incubation continued for 2 hr. The inhibition of DNA synthesis was determined at the end of this period.

Table 1. *Effect of mycophenolic acid on the incorporation of [^{14}C]thymidine into DNA, and [^{14}C]adenine into RNA and DNA, by L cells*

The cells (in 1 ml. of Eagle's growth medium/tube) were preincubated for 1 hr. with or without mycophenolic acid ($10\text{ }\mu\text{g./ml.}$). The incorporation of thymidine and adenine (both $0.1\text{ }\mu\text{Ci/ml.}$) into RNA and DNA was subsequently determined as described in the Materials and Methods section.

Additions to cells	Radioactivity incorporated (c.p.m./ $\mu\text{g.}$)		Inhibition by mycophenolic acid of incorporation of radioactivity (%)	
	RNA	DNA	RNA	DNA
[^{14}C]Thymidine	0	404	—	—
[^{14}C]Thymidine + mycophenolic acid	0	103	—	74
[^{14}C]Adenine	1106	163	—	—
[^{14}C]Adenine + mycophenolic acid	790	59	29	64

ribonuclease and deoxyribonuclease on the radioactivity of the cold-acid-insoluble material of cells labelled with [^{14}C]thymidine were also measured. Less than 5% of this radioactivity was removed by ribonuclease whereas more than 95% was removed by deoxyribonuclease. The results in Table 1 show that the incorporation of [^{14}C]adenine into RNA was much less sensitive to mycophenolic acid than

was the incorporation of the precursor into DNA. The antibiotic had approximately similar effects on the incorporation of [^{14}C]adenine and [^{14}C]thymidine into DNA.

Effect of mycophenolic acid on DNA synthesis in ascites cells in vitro. The growth of the Landschütz ascites tumour *in vivo* is insensitive to mycophenolic acid whereas growth of both the solid and ascitic forms of the Yoshida tumour is strongly inhibited by the antibiotic (Carter *et al.* 1969). The effect of mycophenolic acid on DNA synthesis in suspensions of cells from these two ascitic tumours was therefore studied. The incorporation of [^{14}C]thymidine *in vitro* by the ascites cells was often linear for only 90 min. or less. A number of different suspension media were tried including (1) Eagle's growth medium, (2) Krebs-Ringer phosphate medium (Umbreit, Burris & Stauffer, 1964) supplemented with glucose (2 mg./ml.) and in some cases with either calf serum (8%, v/v) or crystalline bovine plasma albumin (0.2%) and (3) the medium used by Wu & Racker (1959) for metabolic studies with ascites cells. Centrifugation and washing of the cells was found to result in significantly decreased incorporation of thymidine and a diminished period of linear incorporation. Eventually the following procedure was found to give linear incorporation of thymidine into DNA for 90–120 min.: the ascitic fluid was removed from the host animals (time of removal given in the Materials and Methods section) and diluted directly to give 10^7 cells/ml. with Krebs-Ringer phosphate medium (Ca^{2+} -free), pH 7.4, containing glucose (2 mg./ml.). The cell suspension (2 ml.) was then preincubated with gentle shaking for 30 min. with or without mycophenolic acid and the uptake of [^{14}C]thymidine ($0.1\text{ }\mu\text{Ci/ml.}$) into cold-acid-insoluble material over the next 60 min. was measured. Mycophenolic acid at concentrations up to $50\text{ }\mu\text{g./ml.}$ did not inhibit incorporation of thymidine by either Landschütz or Yoshida ascites cells *in vitro*.

Effect of purine compounds on the inhibition of DNA synthesis in L cells by mycophenolic acid. The phthalane ring system of mycophenolic acid is shown by model-building to be sterically similar to the purine ring system. It was therefore conceivable that mycophenolic acid inhibited DNA synthesis by interfering in some way with the supply of purine nucleotides. The effect of the addition of a number of purines on the inhibition of DNA synthesis by mycophenolic acid in L cells was therefore tested. The cells were preincubated for 1 hr. with $10\text{ }\mu\text{g.}$ of mycophenolic acid/ml. and graded concentrations of various purines. [^{14}C]Thymidine ($0.1\text{ }\mu\text{Ci/ml.}$) was added and the incubation continued for 2 hr. Table 2 shows clearly that guanine was very effective in reversing the inhibitory effect of mycophenolic acid whereas

Table 2. *Effects of purine compounds on the inhibition of DNA synthesis in L cells by mycophenolic acid*

The cells (in 1 ml. of medium/tube) were preincubated with mycophenolic acid (10 $\mu\text{g./ml.}$) for 1 hr. before the addition of the purine compounds and [^{14}C]thymidine (0.1 $\mu\text{C/ml.}$). Incubation was continued for 2 hr. and DNA synthesis assessed at the end by measuring the uptake of radioactivity into cold-acid-insoluble material as described in the Materials and Methods section. Guanine alone, at concentrations greater than 0.1 $\mu\text{g./ml.}$, stimulated DNA synthesis by about 10%. The other bases alone had little or no effect on DNA synthesis.

Purine compound added	Amount ($\mu\text{g./ml.}$)	Inhibition of DNA synthesis by mycophenolic acid (%)
None		81
Guanine	0.05	84
	0.1	82
	0.5	25
	1.0	2
	10.0	3
Adenine	1.0	86
	10.0	90
	100.0	78
Hypoxanthine	1.0	88
	10.0	76
	100.0	84
Xanthine	1.0	90
	10.0	92
	100.0	81

Table 3. *Inability of mycophenolic acid to overcome the reversal by guanine of the inhibition of DNA synthesis in L cells*

The cells (in 1 ml. of medium/tube) were preincubated for 1 hr. with the indicated additions. [^{14}C]Thymidine (0.1 $\mu\text{C/ml.}$) was then added and the incubation continued for 2 hr., after which the uptake of radioactivity into cold-acid-insoluble material was measured as described in the Materials and Methods section. The concentration of guanine was 0.5 $\mu\text{g./ml.}$

Additions to cells	Radioactivity incorporated (c.p.m./ $\mu\text{g.}$ of DNA)
None	235
Guanine	266
Mycophenolic acid (10 $\mu\text{g./ml.}$)	42
Guanine + mycophenolic acid (10 $\mu\text{g./ml.}$)	284
Guanine + mycophenolic acid (25 $\mu\text{g./ml.}$)	193
Guanine + mycophenolic acid (100 $\mu\text{g./ml.}$)	274

adenine, hypoxanthine and xanthine had no effect. Guanine, adenine and hypoxanthine were all readily incorporated into the nucleic acids of L cells in the absence of mycophenolic acid. The action of guanine in reversing the inhibition caused by myco-

Table 4. *Effects of purine compounds on the reversal of mycophenolic acid-induced inhibition of DNA synthesis by guanine in L cells*

The cells (in 1 ml. of medium/tube) were preincubated with the indicated additions for 1 hr. [^{14}C]Thymidine (0.1 $\mu\text{C/ml.}$) was then added and the incubation continued for 2 hr., after which the uptake of radioactivity into cold-acid-insoluble material was measured as described in the Materials and Methods section. The concentration of mycophenolic acid was 10 $\mu\text{g./ml.}$

Additions to cells	Inhibition of DNA synthesis (%)
Mycophenolic acid	81
Mycophenolic acid + guanine (1 $\mu\text{g./ml.}$)	0
Mycophenolic acid + guanine (1 $\mu\text{g./ml.}$) + hypoxanthine (10 $\mu\text{g./ml.}$)	80
Mycophenolic acid + guanine (10 $\mu\text{g./ml.}$) + hypoxanthine (10 $\mu\text{g./ml.}$)	6
Mycophenolic acid + guanine (1 $\mu\text{g./ml.}$) + 6-mercaptopurine (10 $\mu\text{g./ml.}$)	78
Mycophenolic acid + guanine (10 $\mu\text{g./ml.}$) + 6-mercaptopurine (10 $\mu\text{g./ml.}$)	25
Mycophenolic acid + guanine (1 $\mu\text{g./ml.}$) + adenine (10 $\mu\text{g./ml.}$)	67
Mycophenolic acid + guanine (10 $\mu\text{g./ml.}$) + adenine (10 $\mu\text{g./ml.}$)	41
Mycophenolic acid + guanine (1 $\mu\text{g./ml.}$) + xanthine (10 $\mu\text{g./ml.}$)	5
Hypoxanthine (10 $\mu\text{g./ml.}$)	0
6-Mercaptopurine (10 $\mu\text{g./ml.}$)	50
Adenine (10 $\mu\text{g./ml.}$)	0

phenolic acid was apparently not a competitive effect, since Table 3 shows that, when the minimum effective concentration of guanine was used, it was not possible to overcome its reversal of mycophenolic acid inhibition with ten times the usual inhibitory concentration of mycophenolic acid.

Guanine is directly converted into its corresponding ribonucleotide by the enzyme hypoxanthine-guanine phosphoribosyltransferase, i.e. IMP-pyrophosphate phosphoribosyltransferase (EC 2.4.2.8). This conversion is competitively inhibited by hypoxanthine and by 6-mercaptopurine, which are also substrates for the enzyme (Atkinson & Murray, 1965). Thus it was considered that these two substances might prevent the reversal by guanine of the inhibition of DNA synthesis by mycophenolic acid if the action of guanine depended on its prior conversion into GMP. The effects of hypoxanthine and 6-mercaptopurine are shown in Table 4. Although the action of 6-mercaptopurine may be questionable because of the intrinsic inhibitory action of this compound on DNA synthesis, the action of hypoxanthine in suppressing the reversal by guanine is particularly striking, since hypoxanthine itself did not inhibit DNA synthesis. When

the ratio of guanine to hypoxanthine or of guanine to 6-mercaptopurine was increased from 0.1 to 1.0, the reversal of the inhibition by mycophenolic acid was restored. Adenine, which is not a substrate for hypoxanthine-guanine phosphoribosyltransferase (Atkinson & Murray, 1965), rather surprisingly suppressed the reversal by guanine. In this case an increase in the guanine/adenine ratio from 0.1 to 1.0 was much less effective in restoring the reversing action of guanine. Xanthine did not suppress the reversing effect of guanine on the inhibition by mycophenolic acid.

The effects of the various purines on the inhibition of DNA synthesis in L cells by mycophenolic acid strongly suggested that the effect of the antibiotic was due to an interruption in the supply of guanine nucleotides. The failure of hypoxanthine to reverse the action of mycophenolic acid, despite the fact that hypoxanthine is readily converted into guanine nucleotides in the absence of the antibiotic (see below), indicated that the site of inhibition by mycophenolic acid lay in the two-step conversion of IMP to GMP via XMP. More direct evidence for this possibility was therefore sought.

Effect of mycophenolic acid on the incorporation of [¹⁴C]hypoxanthine into the purine nucleotides of mammalian cells in vitro. (1) Landschütz and Yoshida ascites cells. The ascitic fluid was diluted to 10⁷ cells/ml. with Ca²⁺-free Krebs-Ringer phosphate medium, pH 7.4, containing 5.5 mM-glucose, 1 mM-glutamine and 2.3 mM-glycine. The cell suspension (8 ml.) was preincubated at 37° with or without 50 µg. of mycophenolic acid/ml. for 30 min. followed by the addition of 0.06 µC of [¹⁴C]hypoxanthine/ml. for 1 hr. At the end of this

period the distribution of radioactivity among cold-acid-soluble and -insoluble fractions of the cells was determined as described in the Materials and Methods section. The results in Table 5 show that mycophenolic acid strongly inhibited the conversion of hypoxanthine into the guanine of the acid-soluble and -insoluble fractions of both types of ascites cell. It is worth noting that there was some increase in the total radioactivity of the acid-soluble adenine fractions in the presence of mycophenolic acid, although specific-radioactivity measurements were not possible.

(2) L cells were grown in flat-sided bottles for this experiment (approx. 6 × 10⁷ cells/bottle). The medium in each bottle was replaced by 40 ml. of fresh Eagle's growth medium at 37° with or without 10 µg. of mycophenolic acid/ml. and the bottles were incubated for 1 hr. After the addition to each bottle of 4 µC of [¹⁴C]hypoxanthine the incubation was continued for 2 hr. The incorporation of label into cold-acid-soluble and -insoluble fractions was then determined. Table 5 shows that mycophenolic acid produced a striking decrease in the radioactivity of the acid-soluble guanine fraction. As expected from the inhibitory action of mycophenolic acid on nucleic acid synthesis in L cells, the radioactivity of both adenine and guanine of the acid-insoluble fraction was lower in the antibiotic-treated cells, although the decline in the radioactivity of the guanine fraction was the most marked.

Effect of mycophenolic acid on the fate of [¹⁴C]hypoxanthine in an ultrasonic extract from Landschütz ascites cells. In the presence of the appropriate cofactors, crude cell-free extracts from ascites cells

Table 5. *Effects of mycophenolic acid on the incorporation of [¹⁴C]hypoxanthine into the acid-soluble and acid-insoluble fractions of ascites-tumour cells and L cells in vitro*

The ascites cells (10⁷ cells/ml.) were preincubated with or without mycophenolic acid (50 µg./ml.) for 30 min. The incorporation of [¹⁴C]hypoxanthine into the purine bases of the acid-soluble and acid-insoluble fractions was determined after 60 min. further incubation. L cells, growing on the inner surface of flat-sided bottles, were preincubated with or without mycophenolic acid (10 µg./ml.). [¹⁴C]Hypoxanthine (0.06 µC/ml.) was added and the incorporation of radioactivity into the purine bases determined after 2 hr. further incubation. Full experimental details are given in the Materials and Methods section. The radioactivity results refer for the ascites cells to material derived from 8 × 10⁷ cells and for L cells to that derived from 3 mg. of cell protein.

		Total radioactivity incorporated (c.p.m.)			
Cell type	System	Acid-soluble fraction		Acid-insoluble fraction	
		Guanine	Adenine	Guanine	Adenine
Yoshida ascites cells	Control	19500	37600	23100	14890
	Mycophenolic acid	240	76800	2000	18800
Landschütz ascites cells	Control	11200	80000	8450	14210
	Mycophenolic acid	900	91000	640	14400
L cells	Control	44000	135000	176000	109000
	Mycophenolic acid	10380	157000	10290	57000

Table 6. *Effect of mycophenolic acid on the fate of [¹⁴C]hypoxanthine in a cell-free extract from Landschütz ascites cells*

An ultrasonic extract prepared from Landschütz ascites cells as described in the Materials and Methods section was incubated at 37° for 15 min. in the following reaction mixture: extract (0.5 ml., 6 mg. of protein), 27 mM-ATP, 42 mM-NAD⁺, 10 mM-glutamine, 82 mM-phosphoenolpyruvate, 270 mM-nicotinamide, 140 mM-ribose 5-phosphate, 0.1 mM-[¹⁴C]hypoxanthine, 12.5 mM-tris-HCl buffer, pH 7.8, 2.5 mM-MgCl₂; total volume 1 ml. The concentration of mycophenolic acid was 50 µg./ml.

System	10 ⁻³ × Total radioactivity of purine bases (c.p.m.)		
	Hypoxanthine	Xanthine	Guanine
Complete	302.0	91.7	89.6
Complete + mycophenolic acid	291.0	20.3	4.9
Glutamine omitted	314.0	191.0	12.5
NAD ⁺ omitted	473.0	26.2	6.5

convert hypoxanthine into IMP, AMP, XMP and GMP (Shigeura & Gordon, 1962; Atkinson *et al.* 1963). The effect of mycophenolic acid on the conversion of [¹⁴C]hypoxanthine into xanthine and guanine nucleotides by an ultrasonic extract from Landschütz ascites cells was therefore studied by using a reaction mixture based on that described by Shigeura & Gordon (1962). To diminish the conversion of IMP into AMP, the necessary cofactors, GTP and aspartate, were omitted. After the incubation period the cold-acid-soluble fraction was isolated and the nucleotides were converted into the free bases. The distribution of radioactivity among the purine bases was determined as described in the Materials and Methods section.

Table 6 shows that mycophenolic acid strongly inhibited the incorporation of label into the xanthine and guanine fractions. This suggested that mycophenolic acid interfered with the formation of guanine nucleotides by inhibiting the oxidation of IMP to XMP, although the result of this experiment is open to the interpretation that the antibiotic might have inhibited the direct oxidation of hypoxanthine to xanthine by xanthine oxidase or alternatively the conversion of hypoxanthine into IMP. The next experiment, however, illustrates clearly that mycophenolic acid does in fact strongly inhibit the conversion of IMP into XMP.

Inhibition of preparations of IMP dehydrogenase by mycophenolic acid. (1) Preliminary experiments with preparations of IMP dehydrogenase from calf thymus indicated that mycophenolic acid is an extremely potent inhibitor of this enzyme. A kinetic analysis of the inhibition indicated that it was of the mixed type (Dixon & Webb, 1964). The

Fig. 3. Inhibition of IMP dehydrogenase by mycophenolic acid. The enzyme was prepared from LS cells. Each point represents the mean of three determinations. The lines were fitted to the points by the method of least squares; O and ● indicate the activity of the enzyme in the absence and presence of mycophenolic acid respectively. The concentration of mycophenolic acid was 26.6 nM.

value for K_i calculated by the method of Dixon & Webb (1964) was 4.5×10^{-8} M. Similar results were also obtained with a preparation of IMP dehydrogenase from Landschütz ascites cells. A preparation from *Escherichia coli*, however, was insensitive to mycophenolic acid (T. J. Franklin & J. M. Cook, unpublished work).

(2) Kinetic studies were also carried out with a preparation of IMP dehydrogenase from LS cells. The K_m for this preparation and the K_i for mycophenolic acid were calculated from the plot illustrated in Fig. 3 and gave values of 3.3×10^{-5} M for K_m for IMP and 3.03×10^{-8} M for K_i . The kinetics of inhibition again appeared to be of the mixed type.

Effect of mycophenolic acid on the accumulation of FGAR in suspensions of Landschütz and Yoshida ascites cells and L cells induced by azaserine. A number of workers have shown that purine nucleotides, but not the corresponding free bases or nucleosides, exert feedback inhibition on the biosynthesis of phosphoribosylamine from phosphoribosyl pyrophosphate and glutamine (Wyngaarden & Ashton, 1959; Henderson, 1963). Since the addition of mycophenolic acid to ascites cells and L cells *in vitro* resulted in some increase of the radioactivity of the adenine nucleotide fraction it was decided to determine whether the antibiotic had

Table 7. *Effect of mycophenolic acid on the incorporation of [¹⁴C]formate into FGAR in the presence of azaserine in vitro*

The cell suspensions were incubated at 37° for 30 min. with or without inhibitors before the addition of sodium [¹⁴C]formate (1.0 μ C/ml.). The incubation was continued for 30 min. and the uptake of radioactivity into FGAR was then determined. Full experimental details are given in the Materials and Methods section.

	System	Radioactivity incorporated into FGAR (d.p.m./10 ⁷ cells)
Yoshida ascites cells	Control	2620
	Azaserine (2 μ g./ml.)	34800
	Azaserine + mycophenolic acid (10 μ g./ml.)	28300
	Azaserine + mycophenolic acid (50 μ g./ml.)	20400
	Mycophenolic acid (10 μ g./ml.)	3700
	Mycophenolic acid (50 μ g./ml.)	2000
	Azaserine (2 μ g./ml.). Glutamine and glycine omitted	3260
Landschütz ascites cells	Control	9900
	Azaserine (2 μ g./ml.)	78200
	Azaserine + mycophenolic acid (10 μ g./ml.)	56100
	Azaserine + mycophenolic acid (50 μ g./ml.)	38900
	Mycophenolic acid (10 μ g./ml.)	6600
	Mycophenolic acid (50 μ g./ml.)	8000
	Azaserine (2 μ g./ml.). Glutamine and glycine omitted	4600
L cells	Control	3170
	Azaserine (2 μ g./ml.)	124900
	Azaserine + mycophenolic acid (10 μ g./ml.)	76200
	Azaserine + mycophenolic acid (50 μ g./ml.)	65300
	Mycophenolic acid (50 μ g./ml.)	2890

any effect on the early stages of the biosynthesis of purine nucleotides. The antibiotic azaserine, which is an antagonist of glutamine, strongly inhibits the amination of FGAR to give formylglycine amidine ribonucleotide, and FGAR therefore accumulates in cells treated with azaserine (Moore & LePage, 1957). Thus the extent of the accumulation of FGAR under these conditions may be used to investigate any possible inhibition of the reaction sequence leading to the formation of FGAR.

(1) Landschütz and Yoshida ascites cells. The ascitic fluid was diluted with Ca²⁺-free Krebs-Ringer phosphate medium, pH 7.4, containing 5.5 mM-glucose, 1 mM-glutamine and 2.3 mM-glycine. The suspensions (1 ml.) were preincubated for 30 min. with or without azaserine (2 μ g./ml.) or mycophenolic acid (10 or 50 μ g./ml.). Sodium [¹⁴C]formate (1 μ C/ml.) was then added and the mixture incubated for 30 min. and the uptake of radioactivity into FGAR was determined as described in the Materials and Methods section. Table 7 shows that azaserine alone produced the expected marked increase in the radioactivity of the FGAR fraction. Mycophenolic acid in the presence of azaserine caused a small decrease in the radioactivity of the FGAR. Mycophenolic acid alone, however, had no significant effect.

(2) L cells. The cells were grown in flat-sided bottles and then removed from the glass by incubation for 2 min. at 37° in a solution containing: 10 mM-potassium phosphate buffer, pH 7.3, 0.15 M-sodium chloride, 1.6 mM-EDTA (disodium salt) and 0.5 mg. of trypsin/ml. The cells were removed by centrifugation at 600g for 5 min. and resuspended in Eagle's growth medium containing 2.3 mM-glycine (final concn., 8×10^6 cells/ml.), and the effect of azaserine and mycophenolic acid on the uptake of [¹⁴C]formate into FGAR was determined as described for the ascites cells. Again, mycophenolic acid depressed the uptake of radioactivity into the FGAR accumulated in the presence of azaserine (Table 7).

DISCUSSION

The results described in this paper indicate that mycophenolic acid is a potent inhibitor of DNA synthesis in L cells *in vitro*. The uptake of thymidine and adenine into DNA is considerably more sensitive to the antibiotic than is the incorporation of adenine into RNA. Conceivably, when the concentration of guanine nucleotides is depressed by mycophenolic acid, the competition for GDP for reduction to deoxy-GDP on the one hand, and for

phosphorylation to GTP on the other, might be weighted in favour of the latter pathway. A precise explanation, however, for the apparently greater sensitivity of DNA synthesis to mycophenolic acid must await further work. The possibility that mycophenolic acid has an additional, more specific, action on DNA synthesis is excluded by the completeness of the reversal of the inhibition of DNA synthesis by guanine.

Evidence has been presented showing that mycophenolic acid is a powerful inhibitor of IMP dehydrogenase. The kinetic analysis of the inhibition suggests that it is of the mixed type (Dixon & Webb, 1964); no evidence could be obtained of any competition with IMP despite the steric similarity between the phthalane ring of mycophenolic acid and the purine ring system. A study of the kinetics of inhibition by mycophenolic acid in relation to the second substrate, NAD^+ , may be useful. A detailed kinetic study of the IMP dehydrogenase of sarcoma 180 cells (Anderson & Sartorelli, 1968) has indicated that the enzymic mechanism involves an ordered sequential addition of IMP, NAD^+ and K^+ to the enzyme to form active enzyme-substrate complexes. It is conceivable that mycophenolic acid could interfere in some way with this orderly process or alternatively with the subsequent dissociations.

It is impossible to decide from the present results whether the decrease in the radioactivity of the FGAR accumulated in the presence of azaserine is a direct or indirect effect of mycophenolic acid. Whatever the explanation, a partial inhibition of an early stage of purine nucleotide synthesis induced by mycophenolic acid is likely to be of only minor importance in accounting for the inhibition of DNA synthesis by the antibiotic. With L cells, guanine is not converted into adenine nucleotides (T. J. Franklin & J. M. Cook, unpublished work). Thus the complete reversal of mycophenolic acid-induced inhibition of DNA synthesis by guanine alone provides convincing evidence that the primary action of the antibiotic is to inhibit the conversion of IMP into XMP. Presumably the failure of xanthine to reverse the inhibition of DNA synthesis was due to the inability of L cells to convert this purine into its corresponding nucleotide (T. J. Franklin & J. M. Cook, unpublished work).

It remains to be considered whether the effects of mycophenolic acid on biosynthesis of guanine nucleotide can explain the action of the antibiotic on cell division *in vitro* and *in vivo*. With L cells the reversal of the antimitotic effect of mycophenolic acid by guanine again strongly suggests a primary effect of the antibiotic on biosynthesis of guanine nucleotides (Carter *et al.* 1969).

Since mycophenolic acid evidently inhibited the conversion of IMP into XMP in Landschütz and

Yoshida ascites cells *in vitro* two observations with these cells require explanation: (1) the failure of the antibiotic to inhibit thymidine incorporation into DNA in suspensions of ascites cells *in vitro*; (2) the failure of mycophenolic acid to depress the growth of the Landschütz tumour *in vivo* on the one hand and the great sensitivity of the Yoshida tumour to the antibiotic *in vivo* on the other (Carter *et al.* 1969).

The difficulties that were encountered in obtaining a sustained linear incorporation of [^{14}C]-thymidine into the DNA of ascites cells *in vitro* suggest that these cells probably undergo rapid degeneration under the conditions used in this investigation. It therefore seems reasonable to suppose that the guanine nucleotide requirements for limited DNA synthesis may be met by the increased catabolism of macromolecules that would be expected in degenerating cells. Thus, although mycophenolic acid inhibits the oxidation of IMP under these conditions, this would not be reflected in an inhibition of incorporation of thymidine into DNA.

A consideration of the differential effects of mycophenolic acid *in vivo* on the growth of the Landschütz and Yoshida ascites tumours is of considerable practical importance. The influence of the species difference in the host animal is probably not of major significance, since a range of tumours in the mouse has been found to be sensitive to mycophenolic acid (Carter *et al.* 1969; Williams *et al.* 1968). Leaving aside pharmacological considerations such as the distribution and penetration of mycophenolic acid into the tumours *in vivo* and assuming that the conversion of IMP into XMP is inhibited by the antibiotic *in vivo* (unpublished work indicates that this is so), the response of a tumour to mycophenolic acid will depend in part on its ability to meet its requirements for guanine nucleotides by the conversion of guanine into GMP by hypoxanthine-guanine phosphoribosyltransferase.

Thus a high rate of conversion of guanine into GMP could result in resistance to mycophenolic acid. Since 6-mercaptopurine is an effective competitive inhibitor of hypoxanthine-guanine phosphoribosyltransferase, combination of this agent with mycophenolic acid might be expected to result in a synergistic antitumour action. A dramatic synergistic effect of 6-mercaptopurine and mycophenolic acid has been demonstrated against the Ehrlich ascites tumour in mice (Carter *et al.* 1969) although neither agent alone has significant growth-depressing activity against this tumour. These results may have significance in relation to the clinical use of mycophenolic acid.

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