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In Vitro Antiviral Activity of Mycophenolic Acid and Its Reversal by Guanine-Type Compounds

J. C. CLINE, JANET D. NELSON, K. GERZON, R. H. WILLIAMS, AND D. C. DELONG
The Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46206

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With the agar diffusion test and BS-C-1 cells, mycophenolic acid was found to give a straight-line dose-response activity in inhibiting the cytopathic effects of vaccinia, herpes simplex, and measles viruses. Plaque tests have shown 100% reduction of virus plaques by mycophenolic acid over drug ranges of 10 to 50 μg/ml and virus input as high as 6,000 plaque-forming units (PFU) per flask. Back titration studies with measles virus inhibited by mycophenolic acid have indicated that extracellular virus titers were reduced by approximately 3 $\log_{10}$ and total virus was reduced by 1 $\log_{10}$. The agar diffusion test system lends itself readily to drug reversal studies. Mycophenolic acid incorporated into agar at 10 μg/ml gave 100% protection to virus-infected cells. Filter paper discs impregnated with selected chemical agents at concentrations of 1,000 μg/ml (20 μg per filter paper disc) were placed on the agar surface. Reversal of the antiviral activity of mycophenolic acid was indicated by virus breakthrough in those cells in close proximity to the filter paper disc. Chemicals showing the best reversal of the antiviral activity of mycophenolic acid were guanine, guanosine, guanylic acid, deoxyguanylic acid, and 2,6-diaminopurine. The reversal of antiviral activity was confirmed by titrations of virus produced with various amounts of both mycophenolic acid and guanine present and by isotope tracer methods with uptakes of labeled uridine, guanine, leucine, and thymidine in treated and nontreated, infected and noninfected cells as parameters. All antiviral effects of mycophenolic acid at 10 μg/ml could be reversed to the range shown by untreated controls by the addition of 10 μg/ml of those chemicals exhibiting reversal activity.

The isolation and the potential use of mycophenolic acid as an antitumor agent has been described previously (10; R. M. Williams et al., Intersci. Conf. Antimicrob. Agents Chemother., 8th, New York, N.Y., 21-23 October 1968). In this paper, we present data which pertain to the in vitro antiviral effects produced by mycophenolic acid. The data show some of the cytochemical effects contributing to the antiviral activity of mycophenolic acid. The ability of guanine-type compounds to reverse or block the antiviral activity of mycophenolic acid may give some insight into the possible mode of action of this drug.

MATERIALS AND METHODS

BS-C-1 cells were used in all experiments described. Vaccinia virus, strain V-1, was acquired from J. Lindenman of the University of Florida (6). Measles virus was acquired by plaque selection from Lilly Laboratory vaccine stocks. Difco purified agar was used in all agar diffusion experiments. Medium 199 with 5% calf serum and 150 units of penicillin plus 150 μg of streptomycin per ml was used in all experiments. Chemicals used in drug reversal studies were obtained from Nutritional Biochemicals Corp. Guanine-8-^{14}C, uridine-5-^{3}H, thymidine-6-^{3}H, and ^{14}C-L-leucine were obtained from Schwarz BioResearch, Inc. All plaque counts were made in 25-cm^2 flasks (Falcon Plastic).

Agar diffusion. The procedure for detection of active antiviral compounds is essentially the agar diffusion test described by Herrmann et al. (4) and Siminoff (9). In this test, a monolayer of BS-C-1 cells was grown to confluency on a flat plate [7.5 by 15 by 1.25 inches (19 by 38 by 3.2 cm)] fabricated from double-strength plate glass previously described (3). The cells were then infected with the desired virus. The infected cell sheet was overlaid with a thin layer of agar, and 0.25-inch (0.64 cm) filter paper discs (no. 740E; Carl Schleicher & Shuell Co.), impregnated with samples, were applied to the agar surface. The plate was then incubated for 72 hr at 37 C. The virus was inactivated, and the surviving cells were fixed to the glass by the addition of 10% Formalin-2% sodium acetate. The plates were stained with a polychrome

stain such as Wright's and zones of protection, indicated by dark-stained fixed cells, were measured if present. Microscopic examination of the area of protection showed regions of typical viral cytopathology. Zone size in this type of agar diffusion test is only a reflection of the ability of a chemical to diffuse through agar. A grading system was developed to distinguish chemicals showing incomplete protection from those showing complete protection. A grade of 4+ indicated complete cell protection within the measured zone of inhibition; a grade of 3+ indicated isolated areas of typical virus cytopathology within the measured zone; a grade of 2+ indicated a more general distribution of viral cytopathology, with the appearance of gross areas of cytopathology (microplaques); a grade of 1+ indicated an extreme distribution of microplaques with the appearance of several well-developed plaques within the measured areas. A chemical without antiviral activity was graded as minus, there being no area of stained cells and only cellular debris found in close proximity to the filter paper disc. This debris was identical to the debris found on areas of the plate to which no filter paper discs had been placed.

Some chemicals demonstrated cytotoxicity associated with antiviral activity. This type of activity was expressed in the agar diffusion test as follows: a nonstained area appeared in close proximity to the filter paper disc surrounded by a ring of stained cells showing protection to some degree from virus damage. A chemical in this class had a cytolytic effect at high concentrations but at lower concentrations interfered only with viral replication. An example of a compound demonstrating this type of protection is gliotoxin (8).

Plaque reduction. Cells grown in Falcon flasks were infected with virus in various concentrations and then overlaid with agar containing different concentrations of the chemical to be tested. Plaques were counted, and counts of treated cultures were compared with nontreated cultures.

Mycophenolic acid was added to three series of cultures, containing approximately 6,000 plaque-forming units (PFU), 600 PFU, and 60 PFU of virus per flask, respectively. The levels of mycophenolic acid ranged from 25 to 0.07 μg/ml. The minimal inhibitory effect was indicated by the appearance of microplaques.

Virus multiplication. Monolayers of BS-C-1 cells in Falcon flasks were infected with measles virus at approximately 10^5 PFU/flask, and then 50% of the cultures were treated with mycophenolic acid at 50 μg/ml. Cultures, with and without drug, were removed from the test at intervals of 24, 39, 64, and 88 hr. Viral titrations were performed on the supernatant fluids and on disrupted cells.

Cellular competence. BS-C-1 cells were grown to confluency in Falcon flasks. The flasks were treated for 48 hr with mycophenolic acid at the following levels: 50, 25, 12, 6, and 3 μg/ml. The cell monolayers were then washed extensively. One-half of the flask cultures were infected with poliovirus, a virus against which mycophenolic acid shows no effect, and one-half were infected with vaccinia virus against which mycophenolic acid usually shows very good activity.

Blood levels. Mice were treated, both orally and intraperitoneally, with mycophenolic acid at 300 mg/kg. Blood samples were removed by orbital sinus bleeding at 2 and 4 hr. The samples were centrifuged in an International hematocrit centrifuge, and 20 μliters of sera was placed on filter paper discs. The discs were placed on the surface of an agar diffusion assay plate previously infected with vaccinia virus.

Reversal studies. Plates were prepared as described above except that mycophenolic acid was incorporated near its minimal inhibitory concentration (10 μg/ml) in the agar overlay. Filter paper discs were dipped in various solutions of single metabolites or their analogues at a concentration of 1,000 μg/ml and then placed on the agar surface. Reversal of mycophenolic acid treatment was indicated by zones of virus breakthrough, i.e., plaques in close proximity to a filter paper disc. The approximately 300 agents used included amino acids, peptides, vitamins, nucleic acids, metabolic analogues, and growth factors. This method has been previously described for use with other systems (5).

Reversal studies (confirming). In these studies, mycophenolic acid was tested with guanosine in solutions containing: (i) guanosine in progressive $\log_2$ decrements from an initial concentration of 500 μg/ml (guanosine control); (ii) a mixture with mycophenolic acid at a constant concentration of 500 μg/ml and guanosine in $\log_2$ decrements; (iii) mycophenolic acid in $\log_2$ decrements from an initial concentration of 500 μg/ml (mycophenolic acid control); and (iv) a mixture with guanosine at a constant concentration of 500 μg/ml and mycophenolic acid in $\log_2$ decrements. Filter paper discs were dipped in the solutions and then placed on a plate previously infected with vaccinia virus.

Isotope procedures. Replicate cultures of BS-C-1 cells (3 ml) of approximately 10^5 cells/ml were planted directly into standard scintillation vials capped with white rubber stoppers. The cells were allowed to attach to the bottom surface of the vials and to grow to confluency. Treatment schedules were performed in quadruplicate with 2-ml samples of media containing drugs or isotopes, or both, per scintillation vial. Normal cellular metabolites during the transitional stages of drug control could and did, in the case of mycophenolic acid, mask the drug effect. Therefore, we pretreated cultures approximately 18 hr before the addition of isotope. We were unable to distinguish by tracer methods any gross differences with virus present during mycophenolic acid studies and therefore carried out our isotope studies in nonvirus-infected cultures. We found that the maximum detectable and reproducible effect of mycophenolic acid occurred 48 hr after the addition of the isotope. Samples of labeled input media were taken so that per cent uptake could be computed on each treatment medium.

After the 48-hr treatment period, the media were removed by suction and the cell sheets were washed three times with tris(hydroxylmethyl)aminomethane (Tris)-buffered saline (*p*H 7.4) cooled to 2 to 4 C. The cells were digested in 2 ml of a tissue solubilizer containing toluene and strong base (NCS^T; Nuclear-Chicago Corp.) and in 15 ml of scintillation fluid containing 4 g of 2,5-diphenyloxazole per liter and 100 mg

of 1,4-bis-(2,5 phenyloxazolyl)-benzine qer liter in toluene. Counts were made in a Packard Tri-Carb liquid scintillation spectrometer model 3375. Internal standards were used and disintegrations per minute (dpm) were computed. Raw counts, because of varying counting efficiencies, did not always reflect true isotope content of samples. Counts of these samples reflected total cell uptake. The trichloroacetic acid fractions were obtained as follows. The samples were washed in Tris-saline as above, and cold 5% trichloroacetic acid was added to the flasks fixing the cells to bottom of the flask. We found that cells so treated had uptakes identical to cells which were washed with Tris-saline; in other words, no trichloroacetic acid-soluble fractions could be demonstrated. We found that these fixed cells could be removed from the glass and disrupted to a smooth precipitate by a 15-sec dip into a sonic water bath found in most laboratory glassware preparation rooms. The one we used was manufactured by Ultrasonic Laboratories, Plainview, N.Y. We found that the addition of 0.1 ml of a 3% bovine albumin carrier was advisable. The scintillation vials containing the precipitate were centrifuged at 2,000 rev/min in an International refrigerated centrifuge for 10 min. By using head (no. 276) and cups (no. 353), we were able to accomodate 32 vials at a time. The supernatant fluid was decanted by suction, and the procedure was repeated two more times without additional albumin carrier. The precipitates were dissolved in NCS^T, and scintillation fluid was added and counts were made which reflected the trichloroacetic acid-precipitable fraction. The trichloroacetic acid-soluble fraction was the difference between the trichloroacetic acid precipitate and total uptake.

RESULTS

Mycophenolic acid has shown striking activity against vaccinia, herpes simplex, and measles viruses in the agar diffusion assay. Figure 1 shows

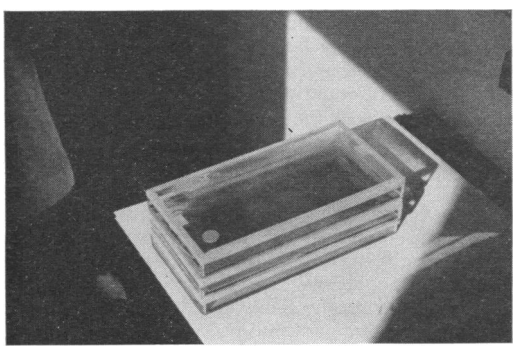

FIG. 1. *These are the 7.5 by 15 by 1.25 inch plates used in our in vitro virus screening program. A glass lid with a drilled port is held in place by polyethylene tape. Media removal and addition is made through the port which is sealed with Scotch tape. After the plates have been overlaid with agar, the entire top is removed and filter paper discs impregnated with sample are positioned by the use of a numbered grid under the plate.*

assay plates during the cell growth phase, and Fig. 2 shows the activity obtained when filter paper discs containing mycophenolic acid were placed on a vaccinia-infected plate. The antiviral activity was first observed with crude fermentation broth samples. Mycophenolic acid was later isolated and identified as the active component of the fermentation broth. No gross cytopathology resulting from drug action was seen with cells present in the agar diffusion test; cleared zones normally appear within the stained zones of antiviral activity when direct drug cytopathology is operative in conjunction with an active antiviral compound.

The results of simple plaque reduction tests appear in Table 1. Cell protection was afforded by

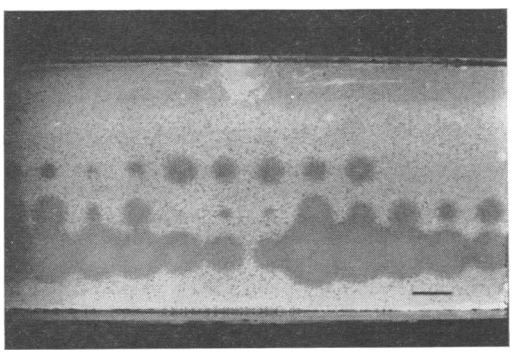

FIG. 2. *Assay plate (7.5 by 15 inches) of BS-C-1 cells infected with vaccinia virus photographed by transmitted light. The dark areas are intact cells which have taken up the stain. The light background contains the debris of virus-destroyed cells. The drug assayed on this plate is mycophenolic acid. The larger zones indicate higher drug concentration. The marker represents 30 mm.*

TABLE 1. *Reduction of vaccinia virus plaque count in BS-C-1 cells by mycophenolic acid*

Amt of mycophenolic acid (μg/ml)	Virus dilutions		
	10^{-1}	10^{-2}	10^{-3}
25.0	0	0	0
12.0	0	0	0
6.0	0	0	0
3.0	0	0	0
1.5	0	0	0
0.7	Micro plaques	Micro plaques	Micro plaques
0.35	Micro plaques	Micro plaques	Micro plaques
0.15	TNC[a]	TNC	53
0.07	TNC	599	59
0	TNC	622	65

[a] Too numerous to count.

mycophenolic acid in the presence of massive virus inoculum.

The effect of mycophenolic acid at 50 μg/ml on measles virus multiplication is shown in Table 2. Mycophenolic acid was added to cultures infected with a high multiplicity of measles virus. This level of mycophenolic acid prevented the induction of pathological change in the virus-infected cells. Total and extracellular virus were titrated. Total virus production was delayed slightly, and, after 88 hr of treatment, total virus was reduced over 1 log. The appearance of extracellular virus was delayed nearly 64 hr, and, at 88 hr, titers were reduced nearly 3 logs.

An effort was made to establish that mycophenolic acid does not permanently decrease the ability of the cell to support virus growth. There was no effect on cell competence when the cells were infected either with poliovirus type I, a virus which normally is not inhibited by mycophenolic acid, or with vaccinia virus, which is inhibited when mycophenolic acid is present (Table 3). Both viruses produced plaques which were normal in both number and configuration when compared to cells not pretreated with mycophenolic acid. Virus growth indicates that

TABLE 2. *Virus multiplication studies: reduction of measles virus titer by mycophenolic acid at 50 μg/ml*[a]

Virus	Reduction in titer at			
	24 hr	39 hr	64 hr	88 hr
Total[b]				
Nontreated	1.3[c]	4.1	5.9	6.3
Treated	0	1.9	4.3	5.0
Extracellular[d]				
Nontreated	0	1.0	2.3	4.1
Treated	0	0	0	1.4

[a] No cytopathic damage occurred for the duration of the test.

[b] Titrations were made from cells disrupted in growth media.

[c] Virus titer reported as $\log_{10}$ PFU.

[d] Titrations were made from cell-free growth media.

TABLE 3. *Competence of BS-C-1 cells pretreated for 48 hr with mycophenolic acid and then infected with poliovirus and vaccinia virus*[a]

Amt of mycophenolic acid (μg/ml)	Poliovirus type I	Vaccinia
50	30[b]	218[b]
25	34	248
12	27	234
6	38	204
3	36	218
No drug	23	224

[a] Mycophenolic acid was removed before virus infection.

[b] Expressed as PFU.

TABLE 4. *Antiviral activity detected in the sera of mice treated with mycophenolic acid at 300 mg/kg by use of agar diffusion techniques*[a]

Mouse	At 2 hr		At 4 hr	
	Zone[b]	Activity	Zone	Activity
1[c]	10	3+	17	3+
2	15	4+	10	3+
3			7	+
4	10	2+	Died	
5	7	+	7	+
1[d]	25	4+	20	4+
2	18	4+	13	4+
3	18	4+	17	4+
4	13	4+	13	4+
5	18	4+	18	4+

[a] Blood was taken from the orbital sinus. A 20-μliter amount of serum was placed on a filter paper disc which was placed on a plate previously infected with vaccinia virus.

[b] Zone measured in milliliters.

[c] For animals in this group, mycophenolic acid was administered by oral route.

[d] For animals in this group, mycophenolic acid was administered by intraperitoneal route.

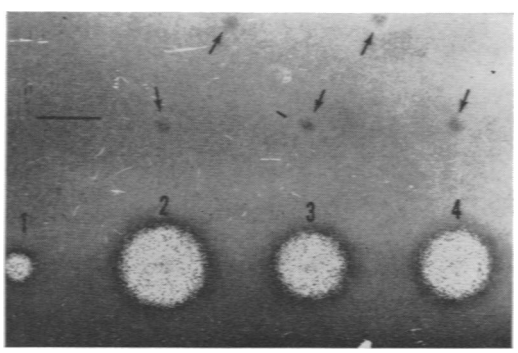

FIG. 3. *Plate (7.5 by 15 inches) of BS-C-1 cells infected with vaccinia virus photographed by transmitted light. Mycophenolic acid was incorporated in the agar overlay. The appearance of this plate is just the opposite of the plate shown in Fig. 2. The cell sheet is protected in all areas except where drug reversal is operative and in those areas where typical virus damage occurs. The chemicals showing drug reversal are (1) guanine, (2) guanosine, (3) guanylic acid, and (4) deoxyguanylic acid. The arrows point to areas where filter paper discs impregnated with other chemical agents not showing drug reversal were placed. Marker represents 25 mm.*

the metabolic processes of a cell are functioning. When such processes are not functioning, both plaque number and size are altered.

With the use of the agar diffusion test, serum levels of drug were detected after both oral and intraperitoneal administration of mycophenolic acid in mice (Table 4). The oral levels were somewhat irregular possibly because of diet or injury, or both, during administration by gavage, but the intraperitoneal levels obtained were uniform. The zone sizes and activities were evaluated in terms of similar zones produced by filter paper discs dipped in solutions of mycophenolic acid and were found to be equivalent to drug concentrations ranging between 7 and 15 μg/ml.

Initially, drug reversal experiments were performed with mycophenolic acid at concentrations of 10 μg/ml in the agar overlay, and it was found that guanine, guanosine, guanylic acid, and deoxyguanylic acid fully reversed the cytoprotective effect of mycophenolic acid. This reversal is shown in Fig. 3 and 4. Figure 3 represents a plate infected with vaccinia virus, and Fig. 4 represents a plate infected with measles virus. The zones of drug reversal, from left to right, refer to guanine, guanosine, guanylic acid, and deoxyguanylic acid. Other agents placed on these plates but not showing cytoprotective reversal were adenosine, thymidine, cytosine, inosine, xanthosine, and uridine, as well as their corresponding nucleoside-5'-monophosphates. When the level of mycophenolic acid was lowered to 5 μg/ml, 2,6-diaminopurine also showed an indication of cytoprotective reversal. A closer view of the zone of drug reversal produced by guanylic acid is shown in Fig. 5. The staining properties of the cell sheet on the glass plates were very uniform in noninfected cells and in cell sheets treated with mycophenolic acid. However, when filter paper discs impregnated with guanine-like compounds were placed on the surface of an agar-overlaid plate that contained mycophenolic acid but was not infected, dark staining areas comparable to the dark staining areas shown on the measles infected plate in Fig. 4 appeared. Microscopic examination of this area indicated an increased cell density; the cells in the dark staining areas had multiplied. BS-C-1 cells have a generation time of less than 24 hr when grown under normal cell culture conditions, but, if mycophenolic acid was added at any time during the growth period, multiplication ceased although the cells were not killed. After 120 hr in the presence of mycophenolic acid at 10 μg/ml, there was no reduction of total cells, and, if mycophenolic acid was removed or a guanine-like compound was added, normal cell multiplication continued. This demonstrated that mycophenolic acid was not cytocidal or cytotoxic but cytostatic.

Results of secondary studies confirming drug reversal are shown in Fig. 6. Note the decreasing zones of reversal within the zones of viral inhibition in column 2 and the absence of antiviral activity in column 4. In column 1, a guanosine control shows no visible viral inhibition or enhancement. In column 3, a mycophenolic acid control, a good dose response is indicated by the decreasing size of the zones of viral inhibition with decreasing amounts of mycophenolic acid.

An attempt to determine the fate of exogenously

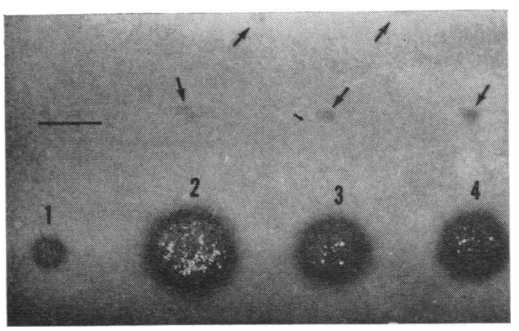

FIG. 4. *This plate was prepared identically to the plate shown in Fig. 2, except that it was infected with measles virus. Again, it is photographed with transmitted light. The cell sheet has been stained, and the areas where drug reversal is operative stain darker than the background stain indicating an increase in cellular density. Typical measles plaques are sprinkled through the high cell density areas. The chemicals showing reversal are (1) guanine, (2) guanosine, (3) guanylic acid, and (4) deoxyguanylic acid. The arrows point to areas where filter paper discs impregnated with other chemical agents not showing drug reversal were placed. The marker represents 25 mm.*

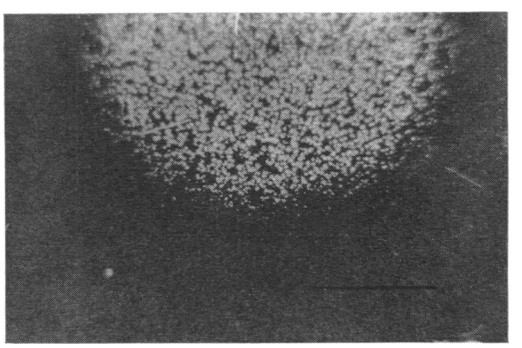

FIG. 5. *This is a closer view of the zone of the reversal produced by a filter paper disc impregnated with guanylic acid. The zone shows typical vaccinia plaques. Marker represents 10 mm.*

supplied guanine by tracer methods is shown in Table 5. Mycophenolic acid, without added cold guanine, eliminated the 5% trichloroacetic acid-soluble fraction and reduced the total isotope uptake by an equivalent amount. The absence of label in the soluble pool indicates a shortage of guanine-like compounds. When excess guanine was added, the shortage was apparently overcome, even in the presence of mycophenolic acid. We also found that medium 199 was either limiting in the amount of guanine present (3 μg/ml) or that the addition of (10 μg/ml) of guanine induced increased cellular uptake of guanine, a marked increase when isotope dilution is considered. Total uridine and thymidine uptakes in tracer amounts (medium 199 contains neither uridine nor thymidine) were drastically reduced (90%), and leucine uptake was reduced 20%. When additional amino acids, in the form of 2% Trypticase Soy Broth (BBL), were added to medium 199, an expected isotope dilution of leucine occurred.

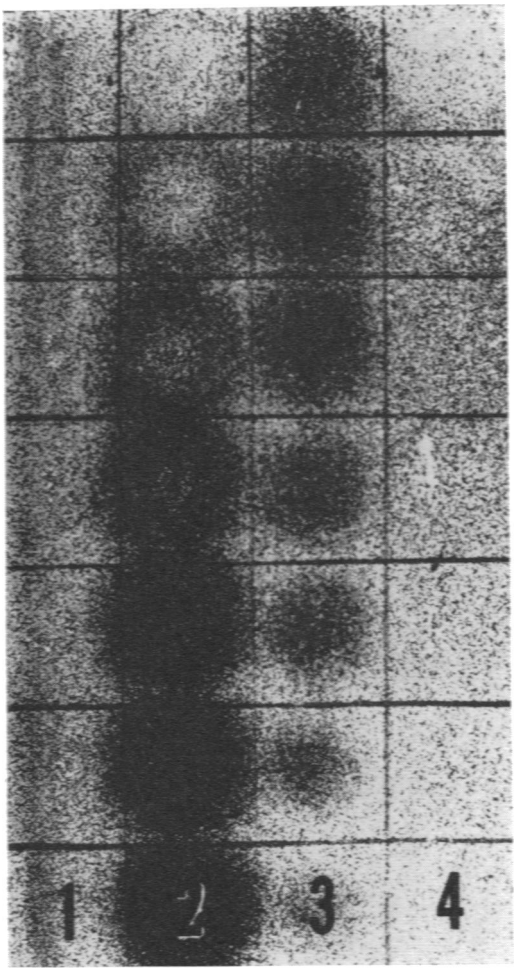

FIG. 6. *This plate, which was photographed with transmitted light, confirms the reversal of the cytoprotective effect of mycophenolic acid in the presence of vaccinia virus. (1) Guanosine in progressive $\log_2$ decrements from an initial concentration of 500 μg/ml. (2) Mycophenolic acid constant at 500 μg/ml and guanosine in progressive $\log_2$ decrements from 500 μg/ml. (3) Mycophenolic acid in progressive $\log_2$ decrements from an initial concentration of 500 μg/ml. (4) Guanosine constant at 500 μg/ml and mycophenolic acid in progressive $\log_2$ decrements from an initial concentration of 500 μg/ml.*

TABLE 5. *Guanine-8-^{14}C uptake in replicate BS-C-1 cultures during 48-hr treatment with mycophenolic acid (MA) or guanine, or both*

Treatment (10 μg/ml)	Total	Trichloroacetic acid 5% fraction soluble	Trichloroacetic acid 5% ppt
	14.61[a]	2.37	12.24
MA	12.28	0	12.99
Guanine[b]	24.10	4.80	17.68
MA + guanine[b]	24.40	5.80	18.76

[a] Per cent uptake of labeled guanine.
[b] Added guanine induced a marked increase in total guanine uptake in both treated and non-treated cultures.

DISCUSSION

The above studies show that mycophenolic acid effectively blocks the unique hyperplastic events associated with and possibly required for replication of the viruses described. Hyperplasia, as a prerequisite for viral replication, has been reported with a poxvirus in vivo (2), and multinucleated giant cells have been found associated with measles virus infections both in vitro and in vivo (1). The extent of these virus-induced hyperplastic events which lead to tissue destruction in vivo determines the prognosis of clinical disease.

The data also suggest that cells, which require proliferation for their survival or for support of multiplication of certain viral agents, require an increased supply of compounds in the guanine series. The question remains whether mycophenolic acid prevents the endogenous production of guanine or limits the entry of exogenous guanine. The first possibility appears plausible in view of the differential effects of the acid on various virus infections in the same cell strain. If the action of mycophenolic acid is indeed an intracellular one, the reversal of its activity by guanine suggests that mycophenolic acid affects an intracellular guanosine metabolic process re-

quired for the multiplication of the sensitive viruses. This observation raises the intriguing notion of a molecular resemblance of the acid (as the anion) to a guanine-related nucleotide. Such resemblance is not immediately apparent from conventional organic chemical formulas but is quite conceivable in terms of a similarity of substrate-enzyme fits. An analogous suggestion of molecular resemblance has been made for the not obviously related drug-metabolite pair of griseofulvin and adenosine (7). Further experimentation will be needed to identify the actual enzyme system(s) of the affected guanosine pathway and to establish the nature of the observed inhibition (competitive?). Parallel studies dealing with the sensitive site(s) involved in the inhibition of certain tumor cells are in progress.

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