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Effect of Cyclophosphamide In Vitro and on Vaccinia Virus Replication in Tissue Culture

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The effect of cyclophosphamide on the growth of Vero, BSC-1, and HeLa cells in monolayer cultures was studied. By using hemocytometer counts and tritiated thymidine uptake as indicators of growth, it was found that cyclophosphamide significantly interfered with the metabolism of Vero and BSC-1 cells when sustained in Leibovitz medium. Vero cells and HeLa cells grown in Eagle medium were not affected by exposure to cyclophosphamide. Vaccinia virus replication in Vero cell monolayer cultures incubated with cyclophosphamide was markedly augmented, and this enhanced growth was reflected by virus quantitation techniques and metabolic studies using tritiated thymidine uptake. No difference in the distribution of infectious particles was found when cyclophosphamide-treated and control infected cultures were compared. Pathways other than through hepatic enzymes appear available to activate cyclophosphamide in vitro. These effects are dependent on both the cell type and the medium in which the cells are grown. Cyclophosphamide can facilitate vaccinia virus replication in vitro through metabolic interactions at the cellular level. The precise mechanisms underlying this effect require further study.

Cyclophosphamide is a potent immunosuppressive agent that exerts its effect through metabolic degradation products (1, 3). Previous experimental studies indicate that cyclophosphamide is a "transport" structure that becomes biologically active only upon appropriate biochemical change. Such "activation," sufficient to interfere with cell growth as judged by in vitro assays, is accomplished primarily by liver enzymes (2). Without such activation, cyclophosphamide has been reported to be inactive in mammalian cell cultures although exhibiting antitumor activity in experimental animals and man (13).

Recent work with vaccinia (13-15) and other viruses (7) suggests that cyclophosphamide may potentiate viral infections in vivo. A compromised immune host capability is the usual explanation offered for this potentiation phenomenon; however, few attempts have been made to ascertain the effect of cyclophosphamide on viral infections in tissue culture—a situation without cellular and humoral immune factors. Studies in mice pretreated with cyclophosphamide and then inoculated intracerebrally with vaccinia (A. H. Ginsberg and K. P. Johnson, unpublished data) showed increased mortality that could not be attributed to suppression of humoral antibody response. Therefore, we investigated the effect of cyclophosphamide on vaccinia virus infection in vitro to learn whether cyclophosphamide could potentiate or retard vaccinia virus replication in the absence of host defenses.

MATERIALS AND METHODS

Materials. The IHD strain of vaccinia virus, obtained from the American Type Culture Collection, was passaged 51 times in mouse brain, four times in LLC-MK_2 tissue culture, and one time in chicken embryo tissue culture. Stock virus, prepared from the supernatant fluid of infected BSC-1 tissue culture (Grivet monkey kidney), contained $10^{6.3}$ 50% tissue culture infective doses per 0.1 ml when titrated in monolayer cultures of Vero cells (green monkey kidney).

Cyclophosphamide (Cytoxan, Mead Johnson Laboratories, Evansville, Ind.) was diluted with sterile water to 1.25 mg/ml, and its effect on growth of Vero, BSC-1, and HeLa Cells (Microbiological Associates, Los Angeles, Calif.) was studied. Eagle (Microbiological Associates) and Leibovitz (L15; Media Facility, University of California, San Francisco) media were used to support Vero cell growth, whereas L15 medium was used for the other cell lines.

Tritiated thymidine (^{3}T) (2 Ci/mmol) was obtained from New England Nuclear, Boston, Mass., and used to indicate the total turnover of DNA. No attempt was made to differentiate viral from cellular DNA.

Effect of cyclophosphamide on growth of cell cultures. Vero, BSC-1, and HeLa cells were trypsin-

ized with trypsin-EDTA and suspended in L15 medium with 5% fetal calf serum (FCS). Vero cells were also grown in Eagle medium with 5% FCS and incubated in 10% CO_2. Approximately 2×10^6 cells were added in 1 ml of medium to 25-cm^2 duplicate flasks (Falcon Plastics, Oxnard, Calif.) for subsequent harvesting at 24, 48, 72, 96, and 120 h. To each flask was added 1 ml of sterile water containing 1.25 mg of cyclophosphamide. The balance was made up by adding 4 ml of medium with 5% FCS. Control flasks contained the same constituents without cyclophosphamide. All cultures were incubated at 37°C. When a confluent monolayer had formed (usually by 24 h), the medium was replaced with fresh medium containing 5% FCS. Thus, the cells were exposed to cyclophosphamide only for 24 h. At 24, 48, 72, 96, and 120 h, cells from two flasks of both the cyclophosphamide treated and control sets were trypsinized with equal volumes of trypsin-EDTA and counted in a hemocytometer.

Effect of cyclophosphamide on replication of vaccinia virus in Vero cell cultures. Vero cells were prepared as described above. After 24 h of exposure to cyclophosphamide, the treated and control flasks were simultaneously infected with 10 50% tissue culture infective doses of vaccinia virus in 0.1 ml of Hanks solution. At 24, 48, 72, 96, and 120 h, two flasks from each set were collected, and the cells were disrupted by freezing at −70°C and thawing. The supernatant after centrifugation at 1,500 rpm for 10 min was titered in duplicate 2-cm^2 well plates (Falcon Plastics) containing Vero cell monolayers. Cultures were overlaid with 50% 2× L15 plaquing medium and 50% agarose. When plaques were apparent, additional agarose medium containing 1:10,000 neutral red was added. The plates were maintained in a dark CO_2 incubator, and the plaques were counted and averaged for each time period at 3, 5, and 7 days after titration inoculation. Results were expressed as PFU per milliliter of inoculum.

Effect of cyclophosphamide on quantitative measurement of intracellular and cell-free virus. To determine whether cyclophosphamide might affect the distribution of replicating virus, duplicate flasks were prepared and infected as above for sampling at 120 h, the time when maximal differences in viral replication between treated and control cultures were apparent. Supernatants were titered for virus content in the manner described above. To sample intracellular virus, the supernatant was decanted and the cell layers were washed three times with Hanks solution. The cells were then trypsinized with trypsin-EDTA and counted in a hemocytometer. Since cyclophosphamide-treated cultures always contained fewer cells, the control cell suspension was diluted to contain an equal number of cells. Comparable portions were then frozen at −70°C and thawed. Cell debris was removed by centrifugation at 1,500 rpm for 10 min, and the supernatant was titered in the manner described above. Results were expressed as percentage of total PFU for cyclophosphamide-treated and control intracellular and extracellular samples.

Effect of cyclophosphamide on total DNA metabolism in uninfected and in infected cultures using ^{3}T as indicator. Nucleic acid synthesis was assayed by ^{3}T incorporation. Uninfected and vaccinia-infected cell cultures with or without cyclophosphamide were assayed 24, 48, 72, 96, and 120 h postinfection as follows. At 16 h prior to harvesting, 1 μCi of ^{3}T in a 0.1-ml volume was added to each flask. After incubation, the flasks were washed with trypsin-EDTA and incubated for 5 min with 1 ml of residual trypsin-EDTA. Normal saline (1.5 ml) was added to each culture, and the suspended cells were pipetted into culture test tubes (13 by 100 mm). The 1.5-ml saline wash was repeated to transfer any residual cells. These suspended cells were spun at 2,000 rpm for 10 min. The pelleted cells were then washed and centrifuged twice with saline and once with 5% trichloroacetic acid. The pelleted nucleic acids were dissolved in 6 ml of Aquasol II scintillation cocktail (New England Nuclear Corp.), placed in scintillation vials, and counted on a Packard beta counter for 1 min.

RESULTS

Effect of cyclophosphamide on growth of cell cultures. The addition of 1.25 mg of cyclophosphamide to de novo Vero cell monolayers for 24 h resulted in a marked inhibition of cell division as documented by hemocytometer counts illustrated in Fig. 1. A similar depression in cell counts was noted for BSC-1 cells. Some recovery was noted after 48 h; however, the difference between treated and control cultures was still evident at 120 h. Control cells showed an increase in counts between 72 and 96 h that was not apparent in the treated cultures. Marked morphological changes such as paucity of cells, enlarged and occasionally pycnotic nuclei, and scanty cytoplasm occurred in the cyclophosphamide-treated cell sheets (Fig. 2). This effect of cyclophosphamide was not observed in HeLa cells grown in L15 medium or in Vero cells grown in Eagle medium.

Effect of cyclophosphamide on replication of vaccinia virus in Vero cell cultures. Little gross morphological change was noted in either control or cyclophosphamide-treated cultures

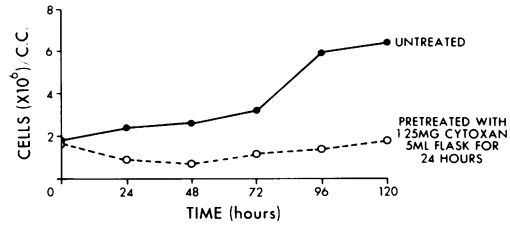

FIG. 1. *Effect of cyclophosphamide on Vero cell growth, monitored by hemocytometer count: $P < 0.05$, cyclophosphamide treated versus control.*

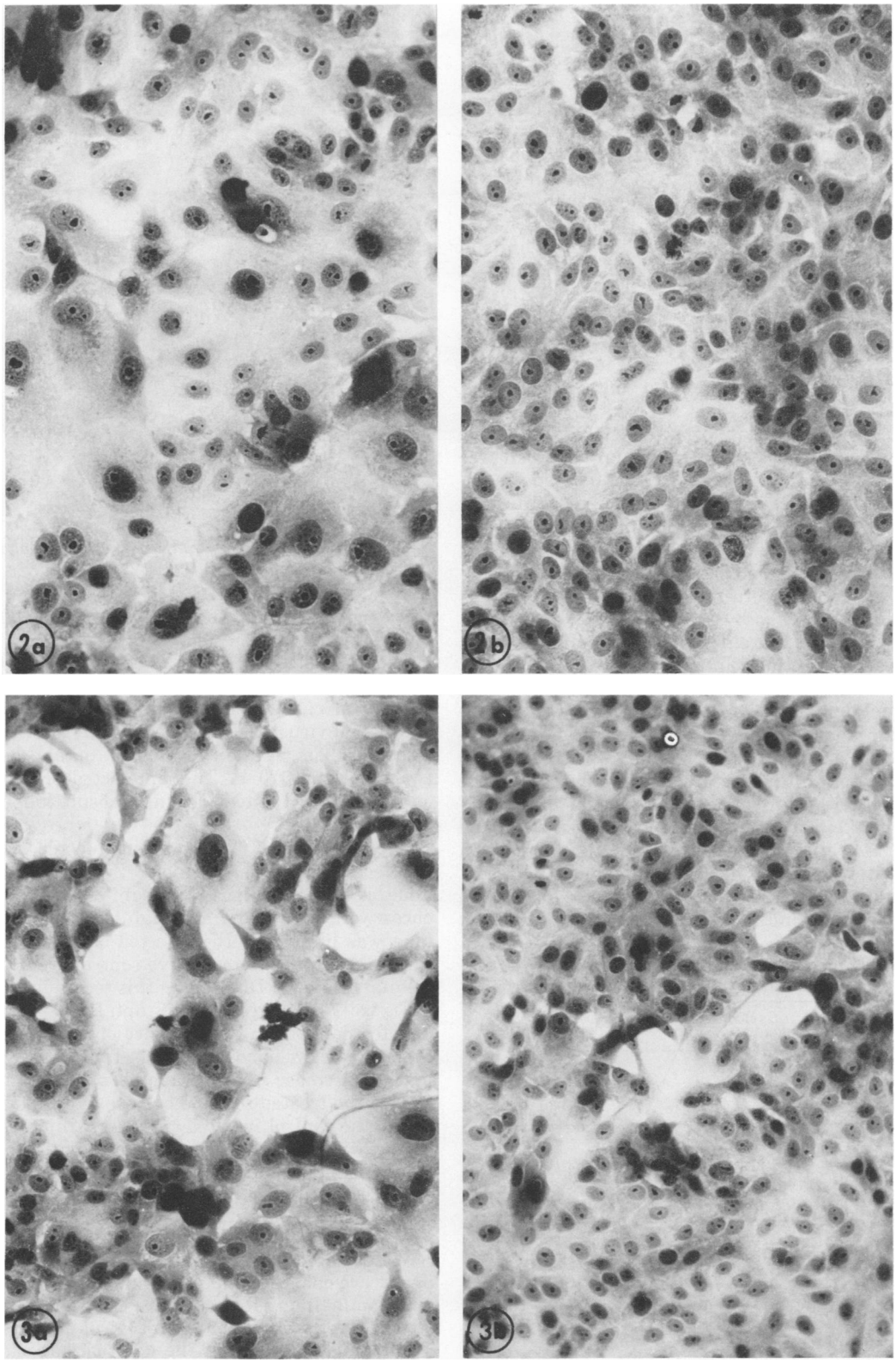

FIG. 2. *Morphological changes in (a) uninfected, cyclophosphamide-treated Vero cells and (b) uninfected controls (hematoxylin and eosin, ×270).*

FIG. 3. *Cytopathic changes in (a) infected, cyclophosphamide-treated Vero cells and (b) infected controls (hematoxylin and eosin, ×170).*

infected with identical amounts of vaccinia until 72 h. Then, progressive cytopathic effect (CPE) was noted in the treated cultures, with almost complete destruction by 120 h, whereas CPE, although present in control cultures, was focal and progressed much more slowly (Fig. 3). Recovery and quantitation of total virus revealed a marked increase in infectious units in cyclophosphamide-treated cultures from 72 through 120 h despite the significantly decreased number of cells, described previously. This increase is expressed in infectious units per cell in Table 1. No difference in PFU/cell was noted between treated and control cultures at 48 h.

Effect of cyclophosphamide on quantitative measurements of intracellular and cell-free virus. Quantitation of extracellular and intracellular virus in treated and control cultures at 120 h postinfection is noted in Table 2. Despite the greater total number of PFU seen in treated cultures, the distribution of infectious units expressed as a percentage of the total PFU was the same for both treated and control cultures. It is also apparent that the majority of virus was intracellular in both situations.

Effect of cyclophosphamide on total DNA metabolism in uninfected and infected cultures using ^{3}T as an indicator. The difference in ^{3}T uptake between uninfected treated and control cultures is illustrated in Fig. 4. The control cultures showed an initial drop in ^{3}T uptake, with a subsequent rise between 48 and 120 h, that seemed to parallel the increase in mitotic activity after establishment of a confluent monolayer (Fig. 1). Treated cells showed a similar initial fall in uptake; however, no recovery was noted, and by 120 h almost all metabolic activity had ceased.

TABLE 1. *PFU/cell in cyclophosphamide-treated and control cultures 72, 96, and 120 h postinfection*

Time (h)	Cyclophosphamide treated	Control
72	15	1.25
96	20	0.51
120	20.5	1.17

TABLE 2. *Distribution of vaccinia virus in Vero cell cultures, cyclophosphamide-treated versus control (untreated), at 120 h postinfection*

Source	PFU × 10^6/ml	Total PFU. (%)
Cyclophosphamide-treated intracellular	36	92
Control intracellular	7	87.5
Cyclophosphamide-treated extracellular	3	8
Control extracellular	1	12.5

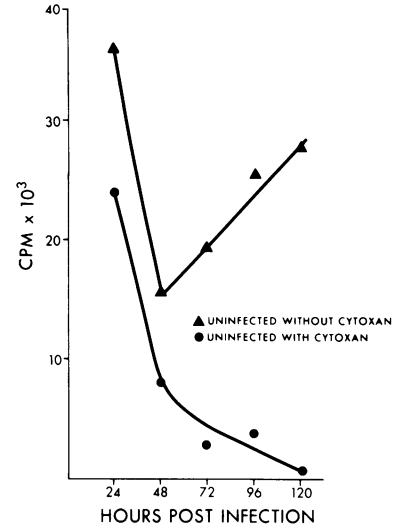

FIG. 4. *^{3}T uptake in uninfected cyclophosphamide-treated and control cultures.*

The difference in ^{3}T uptake between treated and uninfected cell cultures and treated infected cell cultures is shown in Fig. 5. The addition of virus resulted in a marked increase in metabolism as indicated by ^{3}T uptake between 72 and 120 h. The uninfected treated cultures showed a steep decline and subsequent metabolic death.

A comparison of ^{3}T uptake in infected and uninfected cultures, without cyclophosphamide, is noted in Fig. 6. No significant differences were noted, and although the 120-h uptake was higher for the infected culture, one cannot separate the respective contributions of cellular and viral DNA using this technique.

Figure 7 compares the ^{3}T uptake between infected cultures in the presence of and in the absence of cyclophosphamide. There was a marked decrease in ^{3}T uptake between 24 and 72 h in the treated infected culture in comparison to the control infected culture. A rapid recovery was seen between 72 and 120 h in the treated infected cultures with equal end points at 120 h.

DISCUSSION

We explored the in vitro effect of a cytotoxic agent, cyclophosphamide, on growth of continuous mammalian cell culture lines, as well as its effect on replication of vaccinia virus in vitro. The results showed that the addition of cyclophosphamide to Vero and BSC-1 cells for 24 h after plating out affected their morphology and division rate, resulting in degeneration of the

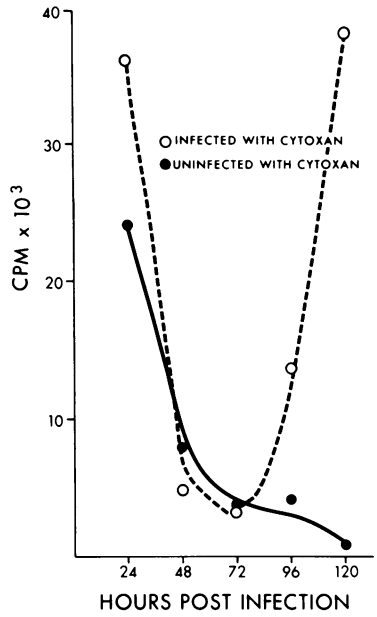

FIG. 5. *³T uptake in infected and uninfected cyclophosphamide-treated cultures.*

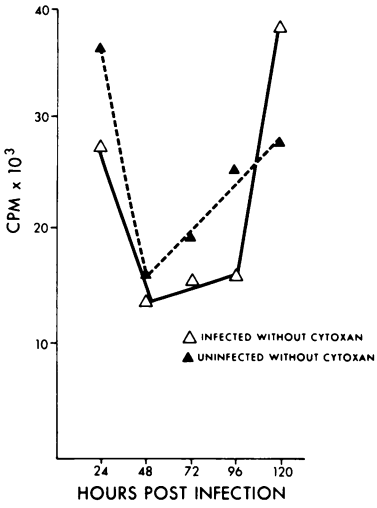

FIG. 6. *³T uptake in untreated, infected and uninfected cultures.*

culture 120 h after initiation. This was confirmed by cell counting, histopathology, and ³T uptake.

The failure of cyclophosphamide to inhibit the growth of HeLa cells and Vero cells sustained in Eagle medium requires explanation. These observations suggest that the antimetabolic effect of cyclophosphamide in vitro depends on both cell susceptibility and basic nutrients used in sustaining cell growth. Previous work has suggested that cyclophosphamide is inert in vitro (11) because of lack of activation by hepatic enzymes. The results in this study show that cyclophosphamide can inhibit cell growth in vitro, suggesting that a nonhepatic activation mechanism may be present in certain cells and may require some cofactor provided by certain media.

The addition of vaccinia virus to the cyclophosphamide-treated cultures resulted in widespread CPE, in an increased amount of vaccinia infectious units as measured by plaque-forming technique at 72, 96, and 120 h, and in raised metabolic activity compared with the uninfected treated cultures that degenerate.

To better define the mode of action of cyclophosphamide in potentiating vaccinia virus replication, distribution of the virus was studied. No apparent differences in proportions of cell-free or cell-associated virus were found at a time when the total output of vaccinia virus was significantly greater in the treated cultures.

The metabolic studies confirmed the histological and virus quantitation observations. The addition of virus to a treated culture resulted in a marked increase in ³T utilization (Fig. 5). This increase reflects viral DNA since cyclophosphamide destroys the uninfected cell culture and results in an almost total decline in ³T uptake (Fig. 4). The addition of virus to a control culture also results in some augmentation in ³T uptake; however, the rise is almost paral-

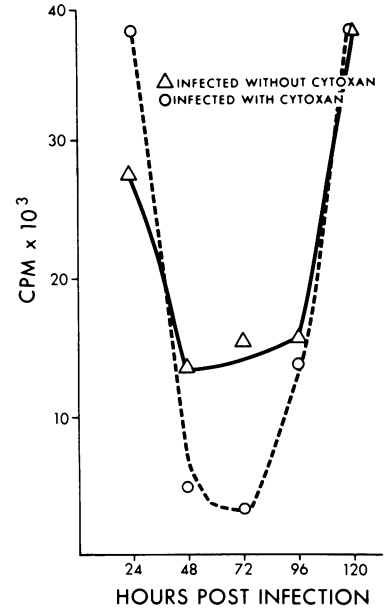

FIG. 7. *³T uptake in infected cyclophosphamide-treated and control cultures.*

lel to the rise seen in uninfected control cultures, presumably on the basis of host cell DNA metabolism (Fig. 6). A comparison of ^{3}T uptake in infected cultures in the presence and absence of cyclophosphamide provides a clue to the nature of the potentiation of viral replication in the treated culture (Fig. 7). A significantly greater decline in host cell metabolism occurs in the treated infected culture over 48 h. Although the 120-h end point ^{3}T uptake is similar for both control and treated infected cultures, one can hypothesize that the mechanism for potentiation of viral replication involves first a shutdown in host cell DNA metabolism. Increased virion synthesis, through augmented utilization of host cell products, follows. This shift in competitive balance in favor of viral synthesis results in an increased infectious virion output and an increased CPE. How cyclophosphamide shifts this competitive balance is not defined.

Previous studies have suggested that cyclophosphamide must be activated to a cytotoxic metabolite, primarily by mixed-function oxidase action of the hepatic endoplasmic reticulum, resulting in the formation of the active principle, aldophosphamide (10). Cyclophosphamide was without effect on growth of HEp_2 and of KB cells in tissue culture (12), or in other mammalian cell lines (3). This study shows that without any attempt at activation, cyclophosphamide inhibited Vero cell metabolism and potentiated vaccinia virus replication in vitro.

Other cytotoxic agents including methotrexate, 5-fluorouracil, and mitomycin C have been noted to inhibit cell replication and increase production of vaccinia virus in cells of human origin 100 to 1,000 times (6). It was hypothesized that cessation of cell division might lead to an accumulation of defective particles in the transformed cells. The remaining intact virions might then initiate a new cycle of infection, resulting in an increased production of complete particles. Such cyclic activity has been reported when vesicular stomatitis virus was successively passaged in high multiplicity in Chinese hamster ovary cells. A burst of standard virus synthesis was noted by the fourth successive undiluted passage (5). Enhanced replication of vaccinia virus in vitro has also been reported when cells were grown in the presence of 5'-iododeoxyuridine for 48 h prior to the addition of virus (4). Studies using actinomycin D have suggested that vaccinia virus-mediated inhibition of host protein synthesis may be due to competitive inhibition of host mRNA by inactive viral RNA formation induced by the drug (9). This finding might explain the synergistic effect of virus plus drug by the shift in availability of metabolic substrates in favor of the virus.

Since most of the cytotoxic agents in some way interfere with DNA metabolism or protein synthesis, it is interesting to speculate on a similar mode of action by cyclophosphamide in potentiating vaccinia virus replication.

It is precarious to relate these in vitro observations to those in the in vivo setting with a relatively static cell population and with humoral and cell-mediated immune factors. But the increased incidence of viral infections in hosts receiving cytotoxic agents for a variety of reasons (8) suggests the possibility of direct potentiation of these agents at the cellular level rather than suppression of immune-mediated factors. Further studies should define more precisely the mechanism of the in vitro effect.

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