INDUCTION OF MAREK'S DISEASE VIRUS ANTIGENS IN A LYMPHOBLASTOID CELL LINE

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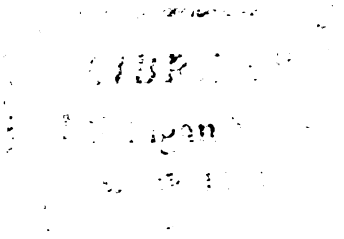

ABSTRACT

INDUCTION OF MAREK'S DISEASE VIRUS ANTIGENS IN A LYMPHOBLASTOID CELL LINE

By

Kathi Dunn

Expression of Marek's disease virus (MDV) genome in a lymphoblastoid cell line, MSB-1, was studied. Spontaneous activation of the virus genome and synthesis of virus specific antigens, as detected by immunofluorescence, occurs in a low percentage of the population. In the presence of 5-iodo-2'deoxyuridine (IUDR), the MDV genome is further activated and a significant increase in the number of cells positive for MDV specific antigens is observed. Activation of the MDV genome appears to require incorporation of IUDR into cellular DNA, and occurs during the first 12 hours of culture. Expression of the activated genome requires de novo protein synthesis, and occurs during the next 12 hours.

In the presence of 20 µg/ml or more of IUDR, MSB-1 cells show no increase in the number of cells producing virus particles, when examined by electron microscopy. However, upon removal of IUDR, there is an increase in

virus antigens followed by virus assembly as detected by immunoflourescence and electron microscopy.

MSB-1 cells were found to vary in the number spontaneously producing virus antigens. The MDV genome of high producer cultures could be activated with low concentrations of IUDR as well as with cytosine arabinoside (ara-c). In contrast, low producer cultures were activated only with higher concentrations of IUDR and to a lesser degree. Activation of the MDV genome may be dependent on inherent properties of the cell line which may vary from time to time.

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INTRODUCTION

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by a cell-associated herpesvirus (47,65). MD shows many similarities to Burkitt's lymphoma (BL). In contrast to tumors of MD, however, continuous cell cultures derived from tumors of BL are easy to establish (7,8). The lack of such cell lines from MD tumors had prevented a comparison of the virus-host relationship between these two systems. Recently, however, MD tumor-derived cell lines were established by Akiyama and Kato in 1973 (2) and Powell et al. in 1974.

The MSB-1 cell line, derived from tumors of chickens infected with BC-1 strain of Marek's disease virus (MDV), has been the most extensively studied. The cell line has been shown to carry 60-90 genome equivalents of MDV (44) and have T cell surface markers (45,56). More recently, MSB-1 cells have been shown to be activated to produce MDV specific antigens in the presence of IUDR or BUDR (43).

This study has further examined the effects of IUDR on MSB-1 cells. Inhibitors of DNA and protein synthesis and their effects on activation and expression of the MDV genome have also been examined.

LITERATURE REVIEW

Properties of MSB-1

Only in the past few years have Marek's disease (MD) tumor derived cell lines become available for study. Akiyama and Kato in 1973 established the first of such cell lines (2). Two lines were originated from ovarian or splenic tumors of chickens infected with BC-1 strain of Marek's disease virus (MDV) and called MOB-1 and MSB-1 respectively. This was followed by Powell et al., in 1974, with the development of an additional two lines from tumors of chickens infected with HPRS-16 strain of MDV (56). Of these lines, MSB-1 has been extensively studied.

Growth

MSB-1 grows best at 41^{0}C and apparently requires this higher temperature for establishment and long term maintainance. It is a fast growing culture with a doubling time of 10-12 hours, and requires subcultures every 48 hours (48).

Virus-Host Interactions

Immunofluorscence with MD antisera and electron microscopy has identified virus antigens and particles

in a low percentage of the MSB-1 population. The virus antigens are detected from cell preparations on acetone fixed slides. Approximately 1-5% of the population are positive for virus antigens and these populations also contain virus particles. The particles, usually naked and in the nucleus, are similar to nucleocapsids of known herpesviruses (40).

Although enveloped virions are occasionally seen, cell free infectious virus is not found in tissue culture fluid or from cellular lysates. However, infectious virus is readily recovered by co-cultivation of MSB-1 cells with avian embryo fibroblasts (48). As is true with other strains of MDV (6), long term passage of MSB-1 in productive cultures results in attenuation of the virus (45).

A loss of pathogenicity, however, did not occur with continuous passage of MSB-1 cells for 300 days (45). Therefore, it has been suggested that in lymphoblastoid cells the MDV genome is in the state of a provirus (45). Furthermore, cRNA hybridization has demonstrated in MSB-1 cells the presence of 60-90 MDV genome equivalents (44). All colonies established from MSB-1 cultures consistently produce virus antigens and particles in a small number of cells. This provides further evidence for the continued presence of the MDV genome in all cells (2).

The MSB-1 cells are free from replicating particles or group specific antigens of avian leukosis virus (ALV) (2). Attempts to isolate ALV from MSB-1 by phenotypic mixing (51) have been unsuccessful. Transformation of the MSB-1 cells is most likely caused by the herpesvirus of MD, although transformation of normal lymphoid cells has yet to be shown (48,35).

Viral Antigens

Chubb and Churchill used the immunodiffusion technique to identify antigens of MDV (5). These antigens have not been associated with those seen in MSB-1 cells, probably due to the insensitivity of the immunodiffusion technique and the low concentration of virus antigens in MSB-1 cells (45).

Powell et al., (56) and Witter et al., (69) have described antigen on the surface of MD tumor cells and lymphoblastoid cells derived from MD tumors. The antigen does not appear to be related to the infectious cycle of MDV as it is not present on the surface of cells productively infected. This antigen has been designated Marek's associated tumor specific antigen (MATSA) and does not seem to be related to embryonic antigens. It is present on the surface of nearly all MSB-1 cells, and of a large number of MD tumor cells. Its role, if any, in transformation has not been elucidated (69).

In addition, MSB-1 cells react with antisera specific for thymus cell surface markers (46,56).

The Epstein Barr Virus

Unlike Marek's disease tumors which only rarely yield continuous cell lines in culture (56), Epstein-Barr virus (EBV) carrying lymphoblastoid lines are readily established from a variety of sources (7,8). All cell lines examined show the presence of EBV genome (3,48,70). However, the virus-host relationships in these established lines do not appear uniform as evidenced by a large margin of experimental variation. At least two of these lines, Raji and P3HR-1, have been of particular interest. P3HR-1 is a producer line, releasing quantities of infectious virus in the culture media (39). This producer cell line has allowed for a concentration of infectious virions and has made EBV superinfection of other lines possible (37). The Raji cell line is a non producer line which, with the exception of a nuclear complement fixation antigen, shows little or no trace of virus associated antigens (59). This cell line has been used extensively in delineating the events in virus activation (23). Studies on different EBV transformed lines have provided information on a number of virus induced antigens.

EBV Virus Induced Antigens

Four groups of EBV associated antigens have been serologically identified in EBV carrying cell lines. Through the use of various metabolic inhibitors, the order of appearance of virus antigens during the virus cycle has been determined.

1. EBV determined nuclear antigen.--Of the antigens so far identified, only the EBV determined nuclear antigen (EBNA) is present in all cell lines (36). It is associated with the presence of a detectable EBV genome (40,58), and is not dependent on virus production (36). Based on immunofluorescence and immunoelectron studies, it has been suggested that EBNA is a chromosomal protein, induced or changed by the virus (58). The disappearance of EBNA has been associated with the loss of EBV genome from somatic cell hybrids (38). EBNA is only detected by anticomplement immunofluorescence and may increase after IUDR treatment (36).

2. Membrane antigen.--While EBNA is present in most cells carrying EBV genome, a membrane antigen is detected with irregularity. Two serologically unrelated membrane antigens have been identified (9,62). The early membrane antigen is present on cells not actively producing virus and is uninfluenced by the presence of inhibitors of DNA synthesis. A late membrane antigen is

found on the surface of cells producing virus and is believed to be associated with the viral envelope. The late membrane antigen is sensitive to inhibitors of DNA synthesis.

3. Early Antigen.--Henle et al., (28) discovered the early antigen complex (EA) when they observed differential activity of antisera from various patients. Serum from Burkitt's lymphoma, nasopharyngeal carcinoma, and infectious mononucleosis that stained the normal number of cells in producer lines was found to also stain a proportion of EBV superinfected Raji cells. This was in contrast to EBV positive healthy donor serum which stained only cells of producer lines. Therefore, superinfection of Raji cells with EBV allowed the expression of an antigen(s) only detected by some antisera, particularly from donors with an active EBV related disease (28). The early antigen was so named because of its appearance in the presence of inhibitors of DNA synthesis, without an accompanying production of virus particles (14,19,37).

Henle et al., (27) have determined the EA complex to consist of two components. A diffuse (D) component is present in both the nucleus and the cytoplasm and a restricted (R) is limited to the cytoplasm. D and R differ from each other in antigenic specificity and susceptibility to various solvents, such as methanol. Although the

chemical nature of EA is unknown, it was suggested to be a glycoprotein and highly immunogenic (36). Further evidence indicated that it may be associated with intracellular membranes (36).

4. Viral capsid antigen.--Viral capsid antigen (VCA) can be detected in a small number of cells within producer lines. All sera that contain antibodies to early antigen also are positive against VCA (28). The appearance of VCA is dependent on viral DNA synthesis and is associated with the production of EBV particles (14,29).

Induction of Virus Antigens

With the establishment of EBV lymphoblastoid cell lines, it was found that some of these lines were producers of virus, while others showed no evidence of virus production and were non producers (11). DNA-cRNA hybridization revealed that eight non producer lines all carried the EBV genome (48,71). The availability of a "virus free" cell line has allowed both the use of metabolic inhibitors and superinfection with EBV concentrates as means to explore a virus-host relationship of neoplastic origin.

The non producer line most widely used is the Raji line developed from a tumor of Burkitt's lymphoma (57). Treatment with inhibitors of DNA synthesis has allowed the expression of virus associated antigens within this cell line. Short term treatment with IUDR or BUDR resulted in

an increase and accumulation of EA, but not VCA (10,11, 12,22,23,41,68). Hybridization studies showed no increase of EBV DNA in the presence of 60 μg/ml of IUDR (15). Superinfection of Raji cells with EBV concentrates allowed the expression of EA only. However, unlike induction studies, superinfection led to an increase in EBV genome content, with little if any VCA (50). Long term treatment with BUDR, then subsequent replacement with normal medium, allowed the expression of EA, VCA, and virus particles (10). This demonstrated that a complete viral genome was present in these cells. When Raji cells were fused with D-98 mouse fibroblasts, the resulting hybrid could be induced with IUDR to synthesize EA. Upon removal of IUDR, the hybrids synthesized EBV DNA and virus particles (15). Raji cells cannot be induced to express virus antigens by a mere inhibition of DNA synthesis, such as with excess thymidine, cytosine arabinoside (ara-c), or hydroxyurea. However, such inhibitors have been successful in activation of Raji cells presensitized with BUDR.

Similar studies have been performed on spontaneously producing cell lines with a different pattern of results. One such cell line, P3HR-1, could be induced with IUDR or BUDR to produce virus antigens. The induction was to a much greater extent than that observed in non producers (67), and was more effective with IUDR

than BUDR (14,37). In the presence of 25 µg/ml or less of IUDR, EA was detected initially, followed by the appearance of VCA (14,67). This is contrary to the Raji cell line which can be induced to only produce EA, even after the removal of IUDR (11,20). It is significant that these concentrations of IUDR do not completely inhibit viral DNA synthesis. In P3HR-1 cells made resistant to BUDR, 55.9 ng/ml of BUDR for up to five days did not inhibit viral replication (19,21) whereas replication was completely blocked by concentrations of 100 µg/ml. Hampar et al., reported that concentrations of IUDR greater than 20 µg/ml will inhibit VCA detection in EB-3 producer cells (20). That IUDR can inhibit cellular DNA synthesis is indirectly evident by a sharp decrease in cell growth. Studies have shown that 100 µg/ml of IUDR will inhibit incorporation of ^{3}H-thymidine into acid insoluble material (12). No reference, however, was made acknowledging that IUDR will compete with the labeled thymidine for incorporation into DNA (20). Regardless of the concentration, after removal of IUDR, producer lines show production of virus DNA and virus capsid antigens whereas non producer Raji lines do not (20,41).

There is a lack of information regarding the viral genome content of producer lines in the presence of lower concentrations of IUDR. Furthermore, cells activated by IUDR are often not examined by electron microscopy.

Untreated cell lines, positive for VCA, showed the presence of virus particles (26). However, the actual assembly of virus particles in the presence of, or after the removal of IUDR is not always clearly established.

Unlike non producer lines, the induction of EA in producer lines can be accomplished by treatment with inhibitors of DNA synthesis, such as excess thymidine, ara-c, or hydroxyurea (14,24,67,91).

Mechanism of Activation

The mechanism of IUDR activation is not known. However, some of the parameters affecting such activation have been studied, largely through the use of synchronized cell cultures.

In all instances, IUDR or BUDR activation required DNA synthesis (37), as induction was inhibited by ara-c or hydroxyurea (10,19,21,22). This indicated that incorporation of the analogue is a necessary prerequisite for activation. The relationship between activation of the viral genome and the events of the cell cycle was investigated. Hampar et al. (20) established a critical period of the S phase (S-1) for activation of EBV producer and non producer cell lines by IUDR. This critical 60 minute period begins approximately one hour into the S phase, and does not correspond to the greatest amount of ^{3}H-IUDR incorporation. Therefore, it was suggested that DNA synthesized during this critical period contains

unique sequences which control the activation of the EBV genome (20). Further studies revealed that the replication of the resident repressed EBV genome in synchronized non producer Raji cells also occurred during the early S-1 phase (25). These findings suggest that viral activation occurs at or near the site of the resident viral genome within cellular DNA, and that replication of the genome is under cellular control.

This same group of investigators has proposed that the analogue substituted DNA may result in the absence or malformation of a cellular repressor protein, responsible for repression of the viral genome (20). It is also possible that stereochemical changes produced by the analogue alter the binding capacity of such a repressor. In either case, the mechanism would involve enhancing those events involved in spontaneous production. Whatever the mechanism, IUDR incorporation does not alter those controls which determine how far the activated genome can proceed through the virus cycle (20).

Since a short term treatment with IUDR (60 minutes) is sufficient to induce activation, it is unlikely that a genetic mutation is responsible (20). This is in contrast to activation of Raji cells made resistant to BUDR. An inheritable mutation has probably occurred, allowing the expression of VCA in addition to EA (10).

Events of Activation

Since the number of lymphoblastoid cells activated to produce virus associated products is very low, it is not feasible to biochemically study macromolecular synthesis in activated cells. Such limitations have been overcome, to some extent, with the combined use of immunofluorescence and autoradiography. Gerely et al. (13) showed inhibition of DNA, RNA, and protein synthesis in EA positive cells. In contrast, RNA and protein synthesis appeared unaffected in MA positive, EA negative cells.

The procedure was extended to synchronized cell cultures to study the events of activation. Hampar et al. (23) activated producer EB-3 cells and non producer Raji cells with IUDR during the S-1 period of the cell cycle. They demonstrated that EA appears at a time corresponding to the G-2 phase of the cell cycle, and is independent of both further DNA synthesis and of the cells' S phase. Activated Raji cells, which only synthesize EA (11), showed incorporation of deoxycytidine and in some cases proceeded to mitosis. Therefore, the presence of EA does not necessarily result in immediate cell death (23).

VCA appeared in producer cells approximately 3-4 hours after the synthesis of EA. VCA was found dependent on further DNA synthesis (23).

Comparison of EBV Carrying Lines

The ease with which EBV carrying lines can be established has resulted in a number of cell lines with different virus-host interactions. Comparison studies on these cell lines have allowed predictions as to the control mechanisms regulating the repressed and activated virus genome. Klein et al. (37) compared producer and non producer lines in three aspects: (1) absorption of EBV concentrates; (2) EBV superinfection; and (3) inducibility with IUDR. Their results can be summarized as follows:

1. Producer and non producer cells, receptor positive, showed a positive relationship between superinfection and activation. If such a cell line could be superinfected, it could also be activated with IUDR. The reverse was also found to be true.

2. For the most part, receptor negative lines were limited to the producer category. As would be expected, these lines, in general, are insensitive to superinfection. There was not, however, a relationship to IUDR activation. Some lines were highly inducible, and others were not.

This information led Klein and his associates (37) to postulate a negative control that could recognize the superinfecting and activated viral genomes in receptor positive lines. They found it interesting that

spontaneously producing cell lines could not always be activated. This suggested that the spontaneously activated genome is not subject to the same control mechanisms as the superinfecting or chemically activated virus. Such a theory is in contrast to that of Hampar et al. (20), who proposed that IUDR merely enhanced an already probable event of spontaneous activation.

Producer P3J-HR1 (16,17) and non producer Raji cells (15) have been fused with human cells to form somatic hybrids. The resulting hybrids synthesized EA in the presence of IUDR (60 µg/ml) but viral DNA or VCA were not detected until after removal of the drug. This is especially interesting with Raji cells,which normally cannot be induced to synthesize viral DNA (15) or make VCA (10,22,23). This was interpretated as cellular control over expression of the virus (15).

Effects of IUDR on Other Herpesviruses

The effect of thymidine analogues on the lytic cycle and transformed state of other virus systems has been studied. Although 100 µg/ml prevented any increase of plaque forming herpes simplex virus (HSV), it did allow the accumulation of viral DNA, viral components and partially assembled non enveloped particles. The particles formed were non infectious (64,63). A similar situation was found for psuedorabies virus (PRV))31,32,33). This information indicated that, while IUDR allowed viral

DNA synthesis and the formation of viral proteins, it apparently blocked the assembly process (32,63). Kaplan et al. (34) found that IUDR substituted DNA could replicate to give normal DNA, which could then code for functional proteins. Furthermore, human nonpermissive cells pretreated with IUDR, allowed the replication and formation of infectious cytomegalovirus (CMV) (59,60). It was then suggested that the action of IUDR allowed the derepression or repression of a cellular product which could then facilitate replication of the virus (60).

It has also been shown that AKR mouse embryo cells can be induced to form murine leukemia virus (MLV) in the presence of 20 μg/ml of IUDR (1,42). Such activation could be overcome with equimolar concentrations of thymidine and was enhanced by 5-fluorodeoxyuridine (42).

Molecular Aspects of IUDR

5-iodo-2'deoxyuridine (IUDR) is an analogue of thymidine, differing only in the replacement of the methyl group with an iodine atom on the 5 position of the pyrimidine ring. While the iodine atom does not affect hydrogen bonding of the helix, it does change the electron configuration of the pyrimidine group. The net result is that IUDR has a Pk_a of 8.25, while thymidine has a Pk_a of 9.8. This means that a greater proportion of IUDR is in an ionized form than that of thymidine.

Such changes affect helical stability, as well as DNA replication and transcription (52).

Hermann in 1961 (30) made the original observation that IUDR inhibited replication of several DNA viruses in vitro, especially vaccinia and herpes simplex viruses. Studies with this analogue were also conducted by Kaplan and Ben-Porat using PRV (32). In all instances, the titer of infectious virus was reduced (53). It was hypothesized that IUDR may selectively affect viral induced enzymes, in particular thymidine kinase (TK) or DNA polymerase. Extensive studies, however, showed no difference between the activities of viral induced TK in the presence of IUDR and that of normal cellular TK. The same situation was found true when DNA polymerase was examined from non-infected and HSV infected cells (61). The nucelotide of IUDR, IdUTP, was found to be a more effective inhibitor of TK than thymidine triphosphate (55). However, these enzymatic sites are of a competitive nature, or are readily reversible. It was therefore proposed that the main effects of IUDR were due to its incorporation into DNA as an analogue of thymidine (54).

IUDR substituted DNA has been shown to (1) increase the rate of mutation, (2) increase errors in protein synthesis, (3) inhibit replication of DNA, and (4) increase sensitivity to X, ultraviolet, and near visible

radiation (53). IUDR allows DNA synthesis of HSV and PRV, but prevents the assembly or formation of infectious particles. Therefore, halogenated DNA may not be able to direct the synthesis of functional proteins necessary for assembly and maturation (52).

Because a greater proportion of IUDR is in the anionic form, there is an increased opportunity to pair with guanine rather than adenine. Even when paired correctly with adenine, it may allow the misplacement of a guanine during transcription. An error in transcription could subsequently lead to the formation of a protein unable to function properly (52).

Goz and Prusof (18) co-cultivated T_4 amber mutants and IUDR substituted T_4 on a non permissive host to test for possible differential incorporation of IUDR. A decrease in the titer of infectious virus would indicate that IUDR T_4 was unable to supply the missing function of the amber mutant. Interestingly, they observed the function of only some genes to be appreciably affected by the presence of IUDR. Further studies, however, revealed that such selection did not appear to be related to the function of the gene. It was suggested that thymidine rich sequences may influence selective incorporation, but experimental proof is not yet available (18).

Such selective inhibition has also been observed in mammalian cell systems (66).

MATERIALS AND METHODS

Cells and Virus

Lymphoblastoid cell line MSB-1 was provided by Dr. S. Kato of Osaka University, Japan. The cell line was grown at 41°C in 5% CO_2 humidified atmosphere. MSB-1 was seeded in Falcon plastic petri dishes at an initial concentration of 5×10^5 cells/ml. RPMI-1640 medium was obtained from Flow laboratories and supplemented with 10% bovine fetal calf serum (BFS) and penn-strep. Subcultures were made every 2-3 days. The number of viable cells were determined by trypan blue exclusion.

Reagents

Aminopterin was obtained from ICS Pharmaceuticals, Inc. Thymidine was from Cal Biochem.; 5-iodo- 2' deoxyuridine (IUDR) and cytosine arabinoside (ara-c) were from Sigma Chem Co. Hypoxanthine and cycloheximide were obtained from the Aldrich Chem Co., Inc. Concentrated stock solutions were prepared and stored at 4°C.

Antiserum

The antiserum used in this study was kindly provided by Dr. R.L. Witter. It was prepared in line 7

chickens innoculated intraperitoneally with GA strain of MDV.

IUDR-Activation

MSB-1 cells were subcultured as usual and various concentrations of IUDR were added to the medium and maintained for 24-48 hours. With some cultures, cells were pellated, washed with PBS, and resuspended in normal medium for an additional 48 hours. At the end of the specified time, cells were harvested and prepared for immunofluorescence or electron microscopy.

Immunofluorescence

Light smears of MSB-1 cells were prepared on glass slides, air dried, and fixed in acetone at -20^{0}C for 24 hours. Slides were removed from acetone, dried under forced air, and washed in phosphate buffered saline (PBS). The cells were incubated in a 1:40 dilution of anti MD chicken serum (kindly provided by Dr. R.L. Witter) for 30 minutes, washed for an additional 30 minutes, and further incubated for 20 minutes in a 1:20 dilution of fluorescanisothiocyanate (FTTC) conjugated anti-chicken globulin, obtained from Roboz Surgical Instruments Co. Inc. After a final washing for 15 minutes, the slides were counter stained with 0.04% Evans blue in distilled water. All samples were examined by a UV microscope equipped with a vertical illuminator. At least 300 cells were

counted from each sample and care was taken not to include non specifically stained dead cells.

Electron Microscopy

MSB-1 cells were pelleted and fixed in 1% osmium tetroxide for 90 minutes. After washing in a buffer, specimens were dehydrated in ethyl alcohol (ETOH); 15 minutes sequentially in 30, 50, and 70%. Samples were further dehydrated in 95% ETOH for 15 minutes, and three times in 100% ETOH. Samples were incubated for 15 minutes in propylene oxide, and then again twice for 30 minutes. Samples were stored overnight in a 50:50 mixture of epon 812 (Fisher) and propylene oxide, after which they were embedded in epon 812 and baked for three days. Epon blocks were sectioned with a MT-2 Porter-Blum ultramicrotome. Sections were mounted on copper grids, and stained 30 minutes in uranyl acetate and 15 minutes in lead citrate. After carbon coating, preparations were examined with a Siemans 1A electron microscope.

Standard Deviation

Calculation of standard deviation (S) was based on the following equation:

$$\sqrt{\frac{\Sigma\ (X_i - \bar{X})^2}{n - 1}} = S$$

where X = experimental value

$\overline{X}$ = mean of experimental values

N = number of trials

RESULTS

Properties of MSB-1 Cells

The normal doubling time for MSB-1 cells was 10-14 hours. After 48 hours in culture, 85-90 percent of the cells were viable.

Immunofluorescence (IF) showed the presence of cells positive for MDV antigens. However, the percentage of cells positive for virus antigens was not constant. Some cultures spontaneously produced virus products at a low level (less than 1%) and are called low producers. Other cultures, with 2-5% positive for MDV antigens, are high producers. Low producer populations were characteristic of high passaged cultures (greater than 80 passages in this laboratory), while high producers were found restricted to lower passage levels (less than 50).

Effect of IUDR on Growth of MSB-1

Concentrations of IUDR ranging from 1 to 25 μg/ml inhibited MSB-1 growth to various degrees. A concentration of 1 μg/ml allowed the most growth, while concentrations greater than 10 μg/ml were extremely toxic and resulted in the death of 70% of the cells.

The growth rate of MSB-1 cells was monitored in the presence of 1 μg/ml IUDR (Figure 1). While IUDR allowed some cell growth the population doubling time appeared to be delayed. MSB-1 cells treated with IUDR doubled at least one time before viable cell count declined.

Activation of MDV in MSB-1 Cells

MSB-1 cells, when incubated with IUDR (1 to 25 μg/ml) for 24 hours, showed an increase in cells positive for MDV antigens as detected by IF (Figure 2b). If the incubation period was extended for an additional 24 hours, there was a further increase in IF positive cells (Figure 2c). When cultures treated with 20 μg/ml of IUDR were examined by electron microscopy, there was no increase in the number of cells producing virus. Therefore, IUDR allowed an increase in virus antigens detected by IF, without an accompanying increase in virus production. However, MSB-1 cells grown in the presence of 1 μg/ml of IUDR had some virus producing cells.

If IUDR was removed after 24 hours and replaced with normal medium for an additional 48 hours, there was again a further increase in the percentage of antigen producing cells (Figure 2d). Electron microscopy revealed these cultures to have an increase also in the number of virus producing cells (Figure 3). Therefore the presence

of 20 μg/ml of IUDR appeared to have prevented at least the assembly of virus particles.

There are two ways in which to enumerate the percentage of antigen positive cells; either based on the viable cell count, or based on the total cell count. While all concentrations of IUDR (1 to 25 μg/ml) were capable of inducing MDV antigens, higher concentrations (greater than 10 μg/ml) gave a greater increase when percentages were calculated with viable cell count. However, if percentages were calculated based on the total cell count, a lower concentration of IUDR (1 μg/ml) gave the most effective increase in antigen positive cells (Table 1). Because higher concentrations of IUDR are more toxic and result in an accumulation of dead cells, large increases in IF positive cells based on viable cell count may be misleading.

To determine the time required for activation of MDV, MSB-1 cells were incubated with IUDR (1 μg/ml), harvested at various times, and prepared for IF. From Figure 4, it is seen that IUDR had no immediate effect on antigen expression during the first 12 hours of incubation. However, by 24 hours many antigen positive cells were found. This shows a time requirement for interaction of IUDR with MSB-1 cells before activation can take place. The small level in induction seen at 24 hours was further increased by 48 hours.

To test the effect of other inhibitors of DNA synthesis, MSB-1 cells were treated with excess thymidine or ara-c. Thymidine concentrations from 25-100 µg/ml had no effect on antigen induction. Concentrations of ara-c from 1 to 5 µg/ml were extremely toxic. Surviving cells showed no increase in antigen positive cells. However, a concentration of .25 µg/ml (added 12 hours after subculture) did allow some growth as well as an increase in IF positive cells. As seen in Table 2, incubation of MSB-1 with ara-c resulted in an increase in IF positive cells, up to 14% in treated cultures. This level of antigen production, while higher than untreated cultures, was lower than similar treatment with IUDR. Furthermore, IUDR activation, in the presence of ara-c was substantially reduced. This is a further indication that incorporation of IUDR during the first stages of DNA synthesis is a necessary prerequisite for MDV activation. In addition, activation of MDV is not unique to IUDR treatment, and may be partially the result of general effects due to inhibition of DNA synthesis.

Finally, to test the procedure of Long et al., (41), MSB-1 cells were incubated with IUDR (10 µg/ml), hypoxanthine (7 µg/ml) and aminopterin (.2 µg/ml) for 24 hours, followed by removal and replacement with normal medium and thymidine (10 µg/ml) for an additional 24 hours. No significant increase in antigen positive cells

over the IUDR treatment alone was evident. However, the presence of the added drugs did not appreciably alter cell viability. Further adjustments of drug concentrations may cause an increase not observed in these experiments.

Effect of IUDR on High and Low Producer MSB-1 Cells

During the course of these experiments, it was noticed that different cultures of MSB-1 cells did not behave similarly. High producer MSB-1 cells could be activated to a greater extent than low producer cells (Tables 1 and 3). In addition, a low concentration of 1 μg/ml was relatively ineffective in activating low producer cells when compared to the activity of high producer cultures under similar conditions (Figures 2c and 2f). However, the effect of IUDR on cell viability was essentially the same in high and low producer cultures (Tables 4 and 5). Therefore, it would appear that the level of MDV induction depends on an inherent property of the cell population.

Effects of Cycloheximide on IUDR Activation

Because MSB-1 is a producer cell line, it could not be ruled out that IUDR was merely selecting for those cells in the population already producing virus antigens. To show that the increase in antigen expression required de

novo protein synthesis, cycloheximide was used in conjunction with IUDR. Control and IUDR treated cultures were treated with 10 µg/ml of cycloheximide at various intervals during a 48 hour period. All cultures were harvested at 48 hours and examined for IF antigens (Figure 2e). As shown in Figure 5, the addition of cycloheximide fully inhibited any increase in virus antigens until sometime later than 24 hours. Such results show that expression of MDV activation required additional protein synthesis. Cycloheximide had the same qualitative effect on spontaneously producing cells in MSB-1 untreated cultures.

It would appear that the process of antigen induction occurs in at least two sequences in MSB-1 cells. First, IUDR interacts with MSB-1 cells during the first 12 hours of incubation, which most likely results in activation of the MDV genome. Secondly, virus antigens are synthesized during the next 12 hours, and these antigens are sensitive to inhibitors of protein synthesis. The entire process requires at least 24 hours before a significant increase in antigen positive cells is observed. This same general course of events was observed in MSB-1 cells regardless of the level of spontaneously producing cells.

MSB-1 cells were incubated with 25 µg/ml of IUDR for 24 hours and replaced with medium containing

cycloheximide (25 μg/ml) for an additional 24 hours (Table 6). The presence of cycloheximide after removal of IUDR decreased the percentage of antigen positive cells. The inhibition of antigen expression was not complete. Virus proteins synthesized during the first 24 hours in the presence of IUDR could not be inhibited by the addition of cycloheximide at a later time.

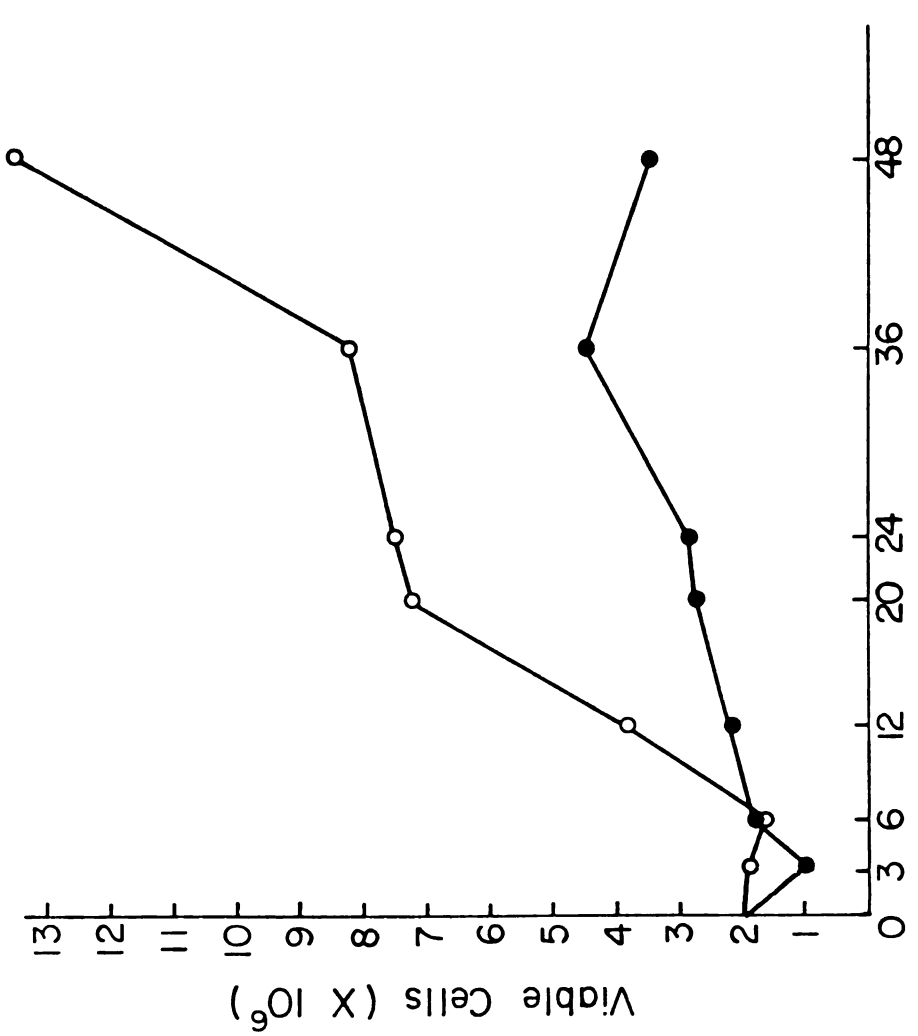


Figure 1.--Growth curve of MSB-1 cells in the presence (●—●) and absence (o—o) of 1 µg/ml IUDR.

Figure 2.--Immunofluorescence of MDV antigens in MSB-1 cells. a-e are high producer cultures.

a. Untreated cells at 48 hours

b. Treated with 1 µg/ml IUDR for 24 hours

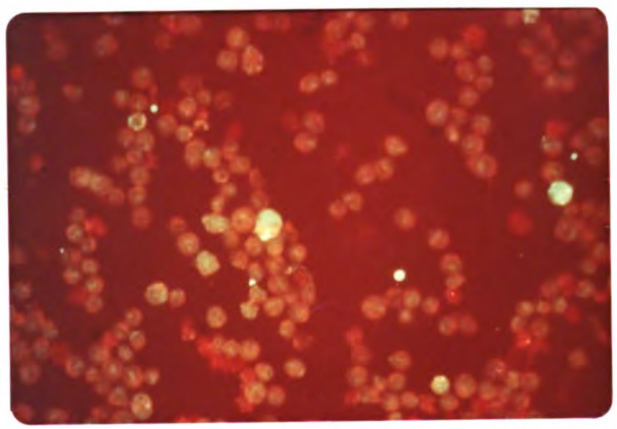
c. Treated with 1 µg/ml IUDR for 48 hours

d. Treated with 1.µg/ml IUDR for 24 hours, followed by removal and incubation in normal medium for an additional 48 hours

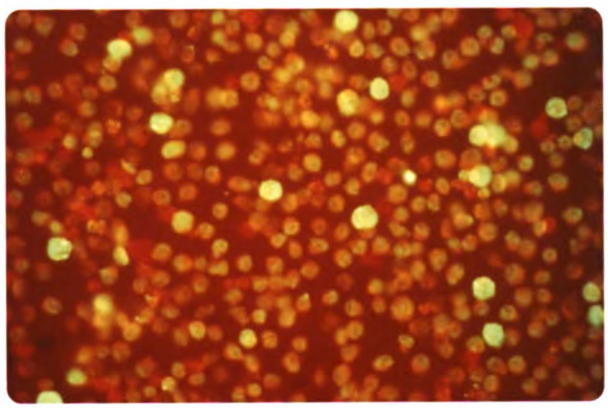
e. Treated with 1 µg/ml IUDR and 6.5 µg/ml cycloheximide for 48 hours

f. Low producer MSB-1 in presence of 1 µg/ml IUDR for 72 hours

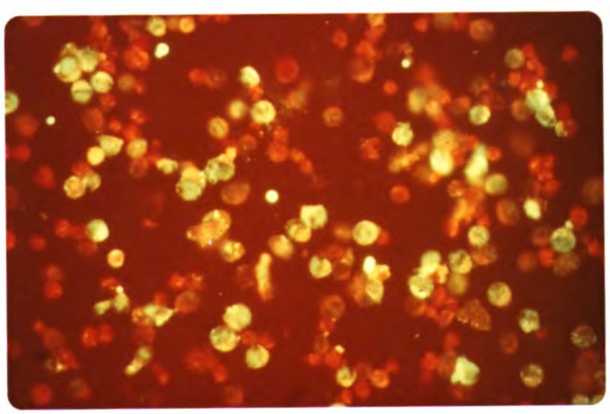
2a



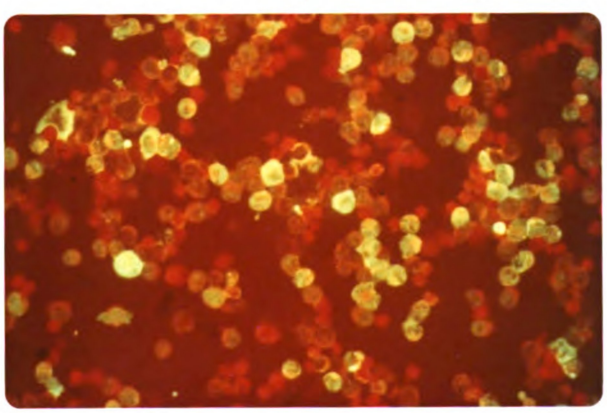
2b



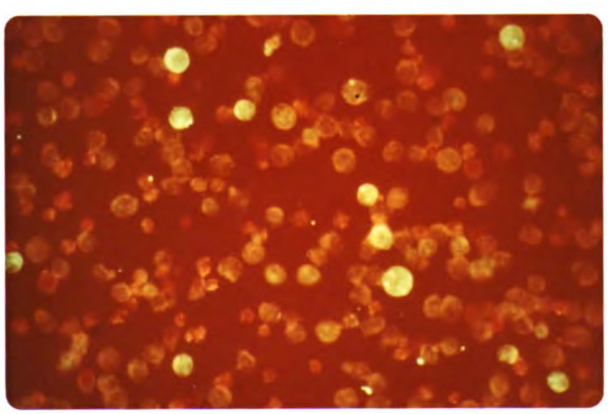
2c



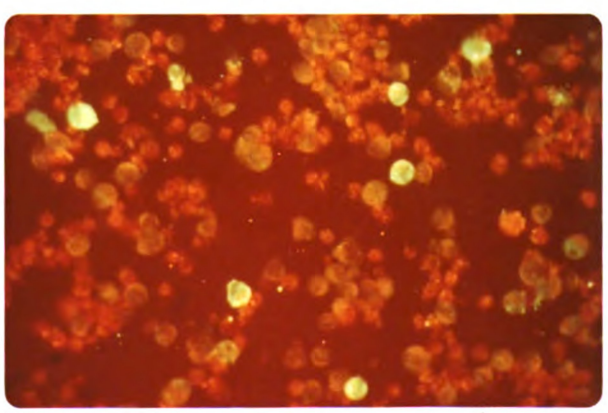
2d



2e



2f



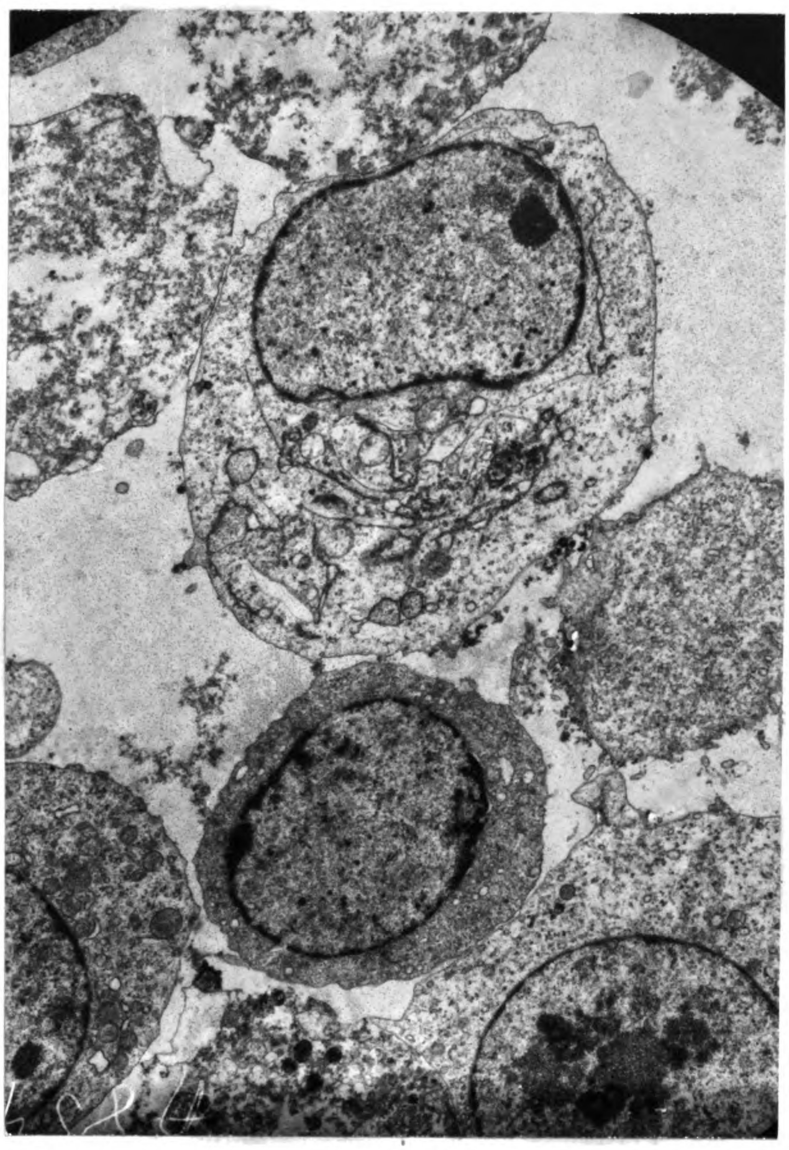


Figure 3.--Electron micrograph of MSB-1 cells treated with 20 µg/ml IUDR for 24 hours, followed by removal and incubation in normal medium for 48 hours. Two cells shown with enlarged nuclear membranes were found producing herpesvirus particles. Mag. X 12,000.

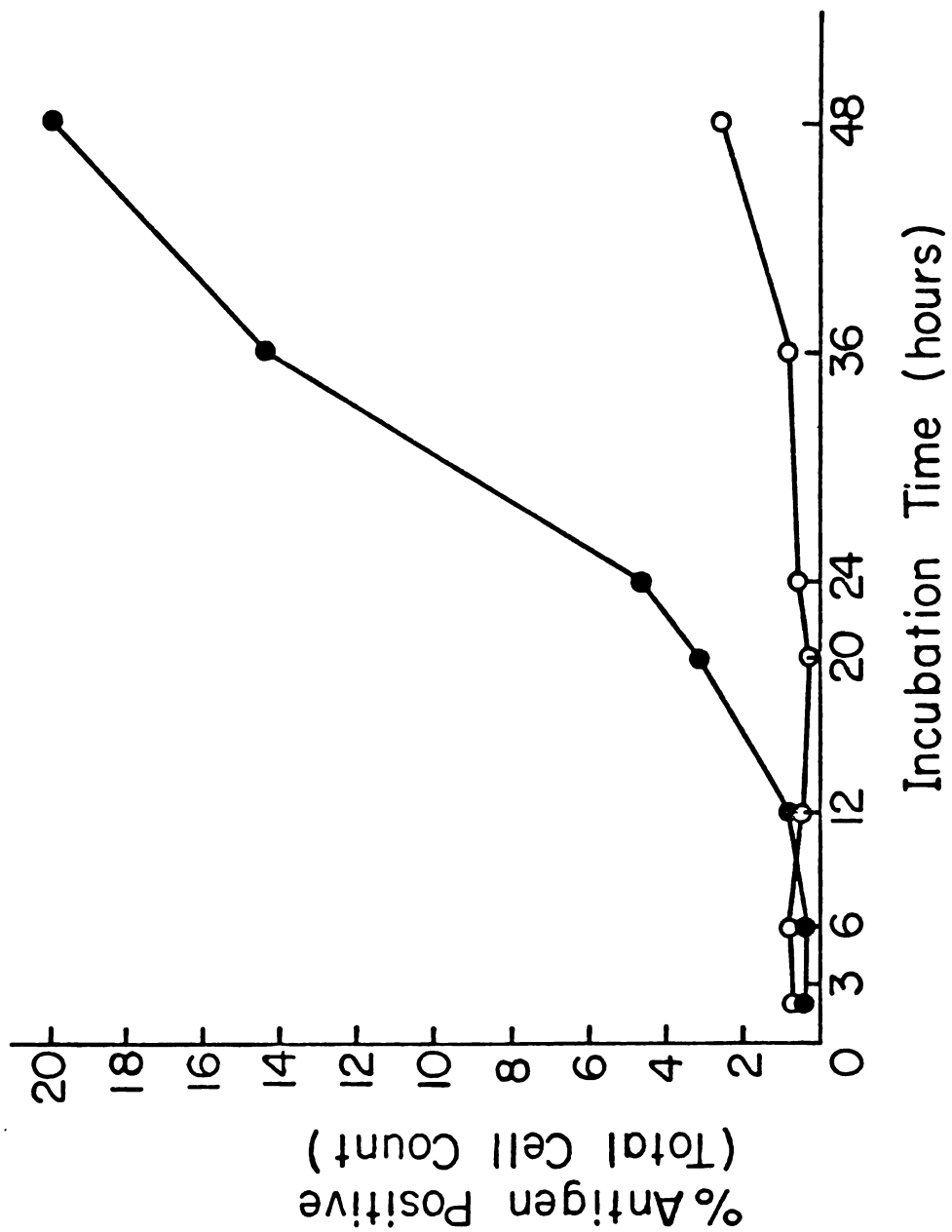


Figure 4.--The effect of IUDR on % antigen positive MSB-1 cells as observed by immunofluorescence. o—o untreated; ●—● treated with 1 μg/ml IUDR.

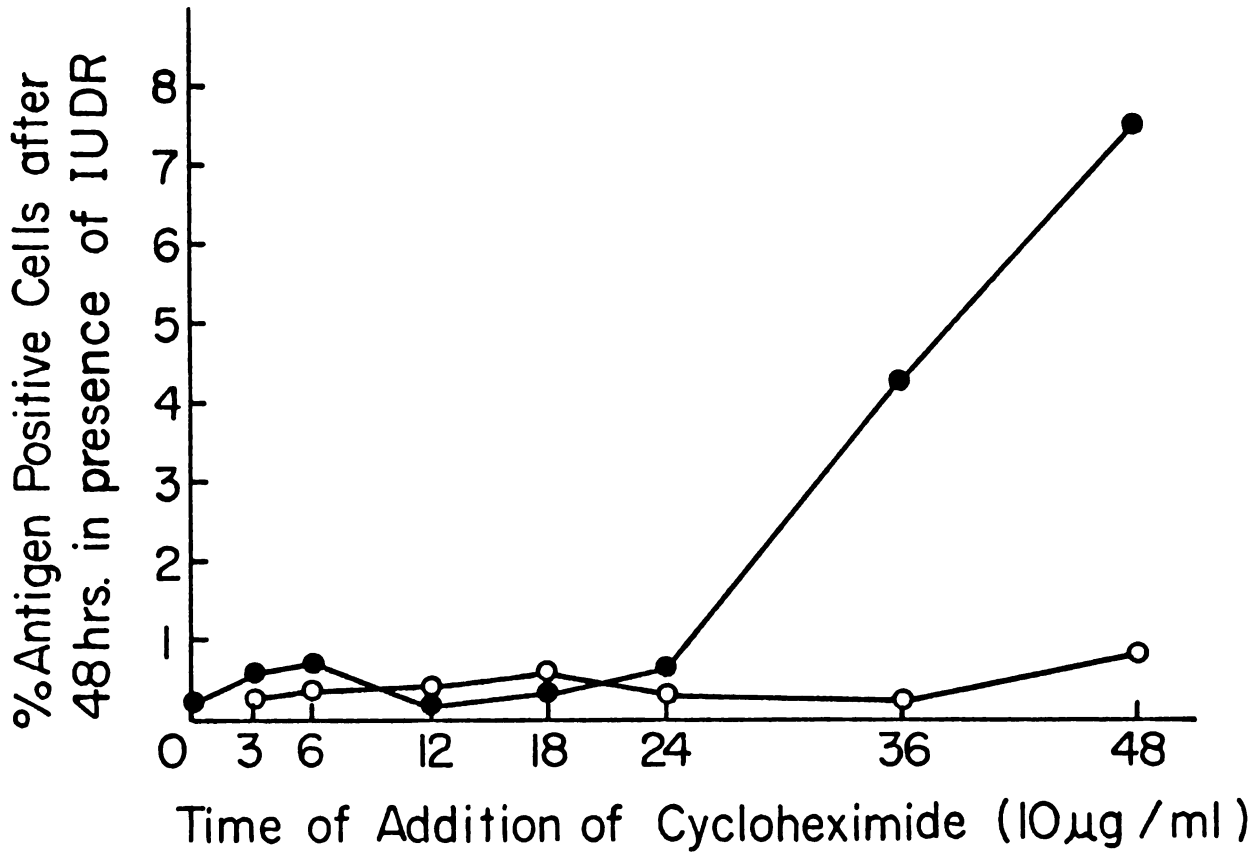


Figure 5.--The effect of cycloheximide on induction of MDV antigens. MSB-1 cells were grown in presence (•—•) and absence (○—○) of 1 µg/ml IUDR. Beginning with time zero, cycloheximide was added at various times to MSB-1 cells. Cultures were harvested after 48 hours and prepared for immunofluorescence.

TABLE 1.--Effect of IUDR on % MDV antigen positive cells in high producer MSB-1.[a,b]

Incubation Time (Hours)		Concentration of IUDR							
		1 µg/ml		10 µg/ml		20 µg/ml		Control	
With IUDR	Without IUDR	Viable Cells	Total Cells	Viable Cells	Total Cells	Viable Cells	Total Cells	Viable Cells	Total Cells
24	none	$4.9^{2} \pm 0.1$	$4.0^{2} \pm 0.5$	5.6^{1}	3.8^{1}	ND[c]	ND	$2.0^{2} \pm 0.7$	$1.6^{2} \pm 0.6$
24	48	$26.5^{5} \pm 9.2$	$16.5^{4} \pm 3.8$	$34.5^{3} \pm 5.6$	$11.4^{2} \pm 2.0$	50.3^{1}	8.9^{1}	$4.3^{6} \pm 0.9$	$3.0^{4} \pm 1.0$
48	none	$29.8^{4} \pm 10.0$	$19.5^{3} \pm 1.3$	$21.2^{2} \pm 5.8$	6.6^{1}	$40.0^{2} \pm 9.1$	$8.0^{2} \pm 1.7$	$2.8^{6} \pm 0.72$	$2.6^{4} \pm 0.7$

[a] Superscript denotes number of trials

[b] ± one standard deviation

[c] Not done

TABLE 2.--Percentage of MDV antigen positive cells in MSB-1 cultures treated with IUDR (1 μg/ml) and/or ara-c (0.25 μg/ml) for 48 hours.

Treatment	% Positive[a,b]	% Positive[c]
Untreated	3.2 ± 3.0	2.9 ± 2.24
IUDR	38.1 ± 8.72	15.37 ±
Ara-c and IUDR	15.0 ± 9.5	2.26 ± 2.02
Ara-c[d]	13.0 ± 2.6	4.6 ± 2.56

[a]Average of two experiments.

[b]Percentages based on viable cell count ± one standard deviation.

[c]Percentages based on total cell count ± one standard deviation.

[d]Ara-c added to MSB-1 cells 12 hours after sub-culture.

TABLE 3.--Effect of IUDR on % MDV antigen positive cells in low producer MSB-1.[a,b]

Incubation Time (Hours)		Concentration of IUDR							
		1 µg/ml		10 µg/ml		20 µg/ml		Control	
With IUDR	Without IUDR	Viable Cells	Total Cells	Viable Cells	Total Cells	Viable Cells	Total Cells	Viable Cells	Total Cells
24	none	0.66^{1}	0.62^{1}	$0.96^{2} \pm 1.0$	$0.89^{2} \pm 1.0$	0.2^{1}	0.11^{1}	$0.14^{2} \pm 0.3$	$0.1^{2} \pm 0.3$
24	48	$2.3^{3} \pm 1.7$	$1.2^{3} \pm 0.9$	$5.8^{5} \pm 3.6$	$1.7^{3} \pm 1.2$	$12.1^{7} \pm 6.9$	$1.2^{3} \pm 1.1$	$0.12^{5} \pm 0.6$	0.5^{1}
48	none	$2.4^{3} \pm 2.0$	$1.2^{3} \pm 1.1$	$4.1^{3} \pm 3.3$	$0.61^{3} \pm 0.8$	$5.0^{4} \pm 1.2$	$0.5^{3} \pm 0.3$	$0.31^{4} \pm 0.2$	$0.5^{2} \pm 0.3$

[a] Superscript denotes number of trials

[b] ± one standard deviation

TABLE 4.--Viability (%) of high producer MSB-1 cells in the presence and absence of IUDR.[b,c]

Incubation Time (Hours)		Concentration of IUDR			
With IUDR	Without IUDR	1 µg/ml	10 µg/ml	20 µg/ml	Control
24	none	$82^{2} \pm 1.4$	$67^{2} \pm 5.6$	ND[a]	$84^{2} \pm 1.4$
24	48	$59^{4} \pm 6.0$	$30^{3} \pm 11.3$	17^{1}	$73^{4} \pm 3.9$
48	none	$55^{6} \pm 5.7$	$28^{2} \pm 1.4$	19^{1}	$82^{6} \pm 12.8$

[a]Not done.

[b]Superscript denotes number of trials.

[c]± one standard deviation.

TABLE 5.--Viability (%) of low producer MSB-1 in the presence and absence of IUDR.[a,b]

Incubation Time (Hours)		Concentration of IUDR			
With IUDR	Without IUDR	1 µg/ml	10 µg/ml	20 µg/ml	Control
24	none	95^{1}	$82^{2} \pm 22.0$	56^{1}	$93^{2} \pm 7.7$
24	48	$54^{3} \pm 4.7$	$28.2^{4} \pm 6.0$	$11^{3} \pm 9.4$	$78^{4} \pm 4.5$
48	none	$49.6^{3} \pm 6.7$	$27^{3} \pm 7.6$	$16^{4} \pm 9.0$	$86^{4} \pm 4.5$

[a]Superscript denotes number of trials.

[b]± one standard deviation.

TABLE 6.--Effect of cycloheximide (25 µg/ml) on MDV antigen production in MSB-1 cells after removal of IUDR (25 µg/ml).

Treatment		% Positive[a,b]	% Positive[c]
24 hours	48 hours		
IUDR	none	12.07^{7} ± 6.9	1.2^{3} ± 1.1
IUDR	cycloheximide	2.4^{2} ± 0.14	$.03^{2}$ ± .014
none	none	0.12^{5} ± 0.6	0.5^{1} ±

[a]Percentage of positive cells based on viable cell count ± one standard deviation.

[b]Superscript denotes number of trials.

[c]Percentage of positive cells based on viable cell count ± one standard deviation.

DISCUSSION

Human lymphoblastoid cell lines carrying EBV genome have been activated by IUDR to produce EBV associated antigens (12,68,23,41). Virus producer lines have been induced to synthesize EA and VCA, while non producer lines may only synthesize EA (23,37). Concentrations of IUDR greater than 20 μg/ml led to an accumulation of EA in producer and non producer lines. However, upon removal of IUDR, producer lines synthesized VCA, whereas non producer lines did not.

Initial studies have shown that the chicken lymphoblastoid cell line, MSB-1, is sensitive to IUDR and BUDR. Treatment with either drug resulted in an increase of cells positive for MDV antigens (43). Electron microscopy, however, showed no increase in cells producing virus particles. Because these results were similar to studies with EBV induction, the induced antigens were considered as early antigens of MDV (43). Although the work presented here confirms and extends the previous MDV study, it is not possible, at this time, to clarify the nature of the induced antigens.

MSB-1 cells appear to be more sensitive to IUDR than EBV transformed human lymphoblastoid cell lines.

Other cell cultures have also shown a wide variation in their sensitivity to IUDR. A likely explanation is the difference in cellular metabolism. In their studies with SV40, Buettner and Werchau (4) showed that the ratio of IUDR to thymidine determined to what extent IUDR would effect cellular activities. Therefore, cells which have a high level of endogenous thymidine synthesis or are grown in thymidine rich medium may be able to resist higher concentrations of IUDR. This may not be the case with MSB-1 cells because after 48 hours in 20 μg/ml of IUDR, only 20% of the cells remain viable.

It was not determined at what point of the cell cycle production of MDV antigens begins. However, several lines of evidence indicate the effects of IUDR are observed during the G-2 phase. First, IUDR does not influence MDV antigen production during the first twelve hours of incubation (Figure 4). Secondly, a population of MSB-1 cells in the presence of IUDR doubles at a slower rate. The increased doubling time is most likely a characteristic of the population and not of the individual cells. Thirdly, activation of the MDV genome signals entry of the virus into its lytic cycle. Activated cells, under normal circumstances, will not divide again. Remaining inactivated cells should continue on in the growth cycle until the toxicity of IUDR causes death.

Because of the existence of human sera specific to EBV EA and VCA, it was possible to distinguish between EA and VCA in human lymphoblastoid cell lines induced by IUDR (28). The anti MD serum used in this study is believed to be positive for early and late antigens associated with productive infection. Therefore, the antigens induced by IUDR in MSB-1 cells can be specified only in relation to their appearance during the virus cycle, and not to their specificity to the corresponding antibody. It is possible that the increase in antigen positive cells represented an accumulation of EA, provided the concentration of IUDR was sufficient to inhibit viral DNA synthesis. This would be further substantiated by the lack of virus particles in the presence of IUDR and the subsequent appearance of these particles after removal of IUDR. However, the intracellular effects of IUDR are not easily understood. IUDR may allow viral DNA synthesis, translation of virus proteins, but prevent the assembly of virus particles. Such effects of IUDR have been observed in the lytic cycle of other herpesviruses (32,63). In this latter case, the increase of antigen positive cells could represent a number of undetermined virus antigens. In addition, higher concentrations of IUDR are quite toxic to MSB-1 cells and result in the death of a majority of cells. Although lower concentrations of IUDR were used to reduce the toxicity of the drug, it would be even less

likely that these concentrations of IUDR would inhibit viral DNA synthesis. Further, it has been shown that some EBV producer human lymphoblastoid cell lines synthesize both EA and VCA in the presence of low concentrations of IUDR (10 μg/ml) (67,14). Therefore, even though IUDR can induce virus antigens in MSB-1 cells, the nature of these antigens remains to be clarified.

Evidence presented here suggests that IUDR must be incorporated into cellular DNA before the MDV genome can be activated. First, since IUDR is an analogue of thymidine, it may be implied, although not proved, that IUDR becomes incorporated into cellular DNA. Secondly, a minimum of 12 hours incubation time with IUDR is necessary before any expression of the MDV genome is observed. Thirdly, in the presence of ara-c, a potent inhibitor of DNA synthesis, activation by IUDR is substantially reduced.

The mechanism of activation is not known. It has been suggested by other investigators that incorporation of IUDR into DNA results in the repression or derepression of a cellular product (24). The presence or absence of such a product allows the expression of the viral genome. It is also possible that the steric hinderance due to the larger iodine atom may prevent attachment of a normal repressor product. In addition, activation of the MDV genome may be the result of inhibition of DNA synthesis. Hampar et al., showed that IUDR could reversibly inhibit

cellular DNA synthesis by 20% (24). Ara-c, also an activator of MDV, inhibits DNA synthesis by interfering with enzyme activity, and does not interact with the DNA to any substantial extent. Therefore, the increase of antigen positive cells in the presence of IUDR may be the combined result of two independent events. The general effects of inhibition of DNA synthesis and the molecular interaction of IUDR with cellular DNA may both result in the activation of the MDV genome.

Since cyclohexomide can inhibit expression of the MDV genome, the observed increase in IF positive cells is not due to an accumulation of cells already positive for MDV specific antigens. However, activation of the viral genome may involve several changes which cannot be resolved with the present techniques. A number of cells within the population may have already undergone changes which will eventually result in the expression of the virus genome. IUDR may cause a selection process for those cells already capable of spontaneously producing the antigens.

A number of EBV carrying human lymphoblastoid cell lines, producer and non producer, have been compared (37). The interaction of the virus with its host is not a uniform relationship, evidenced by the variation of responses to IUDR induction and EBV superinfection. Although MSB-1 is a producer line, it varies in the number of cells producing virus. High producer cultures are

easily activated by IUDR. Low producer cultures are activated to a lesser degree and only at higher concentrations of IUDR. It is possible that the level of spontaneously producing cells is a indication of the state of the virus within the cells. In high producer cultures more cells may be primed for activation. In this case, a mere inhibition of DNA synthesis by ara-c or low concentrations of IUDR may cause activation of a greater number of cells.

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