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EFFECT OF NEOCARZINOSTATIN ON THE CULTURED BURKITT LYMPHOMA CELLS, WITH PARTICULAR REFERENCE TO THE ENHANCEMENT OF PRODUCTION OF EPSTEIN-BARR VIRUS*1

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Synopsis

In order to elicit a relation between the synthesis of Epstein-Barr (EB) virus and the growth of host cell, effect of an antibiotic, Neocarzinostatin, on the Burkitt lymphoma cell line, P3HR–1, was examined. One μg/ml of Neocarzinostatin was sufficient to inhibit cell division but it induced a formation of giant cells. Percentage of EB virus-bearing cells in the Neocarzinostatin-containing culture was higher than that in the control culture, as revealed by immunofluorescence. The virus particle counting by electron microscopy showed that actual production of EB virus particles in the Neocarzinostatin-treated culture was remarkably higher than that in the control culture. No difference was observed by electron microscopy in the morphology of EB virus particles between the Neocarzinostatin-treated and control cells. Autoradiographic studies on the cell cultures in the presence of Neocarzinostatin indicated that the number of cells incorporating ^{3}H–thymidine was much higher in the fluorescent (EB virus-bearing) cells than in the non-fluorescent cells. These results strongly suggest that Neocarzinostatin reduces or prevents the synthesis of host cellular DNA and the cell division but not the synthesis of viral DNA or formation of viral particles in the cells.

INTRODUCTION

A herpes-type virus, Epstein-Barr (EB) virus was first found in the cultured cells isolated from a Burkitt tumor by Epstein *et al.*[2] in 1964. Since then, EB virus or EB virus-related virus, if not identical, has been observed in a number of established hematopoietic cell lines[10, 13, 14, 16, 18] which were derived from Burkitt tumor, peripheral leucocytes of patients with malignant or non-malignant diseases, and of healthy individuals, or lymph nodes of various patients. In these cells, it has been noted that EB virus is detected only in a small portion of the cells in culture. However, the previous studies[4, 5] by a cell-cloning technique which was made on the Burkitt lymphoma cell lines, P3 (Jijoye) and P3HR–1, suggested that all or most of the cells in the Burkitt lymphoma cell lines may have the viral genome to form EB virus particles or antigens. Supporting this suggestion, a marked increment of EB virus-bearing cells was observed in the P3HR–1 cell cultured under the particular condition which did not permit cell

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growth.[7,11] Then the question may arise whether the synthesis of cellular macromolecules or cell division affects the viral synthesis in this cell line. This leads to the attempt to examine the effect of Neocarzinostatin[9,12] on a Burkitt tumor cell line, P3HR–1, particularly the relation between the synthesis of EB virus and the cell growth. Neocarzinostatin is an acidic polypeptide antibiotic isolated from the culture filtrate of a streptomyces species. It has been reported that Neocarzinostatin exhibits antitumor activity *in vivo*[1,9] and a selective inhibition of the synthesis of DNA but not RNA and protein in *Sarcina lutea*[17] or HeLa cells[8] *in vitro*.

The present communication shows that Neocarzinostatin inhibits the synthesis of host cell DNA and cell division but not the synthesis of EB virus.

Materials and Methods

Burkitt Lymphoma Cells and Media The P3HR–1 cell line, which was one of the clonal cell lines derived from the P3 (Jijoye) line,[5] was used. This line produces a relatively large amount of EB virus and its properties were fully described previously.[7] The growth medium for the cells was Eagle's minimum essential medium supplemented with 20% bovine serum, 100 μg/ml of streptomycin, and 100 units/ml of penicillin. The Raji cell line,[19] which is non-producer of EB virus,[3] was used in some parts of the experiments. The medium used for the latter cell line was the same as that used for the P3HR–1 cell line except for the use of 20% fetal calf serum instead of 20% bovine serum. Total and viable cells were counted by a hemocytometer with Trypan Blue exclusion method. Throughout the present experiments, the incubation temperature was 37°.

Neocarzinostatin The antibiotic (Lot. 18 for clinical trial) was kindly supplied from the Kayaku Antibiotics Research Laboratory (Tokyo).

Immunofluorescence For the detection of EB virus antigen-bearing cells, the indirect method of immunofluorescence was employed. The techniques have been fully described.[7]

Procedures for Extraction and Enumeration of Virus Particles For counting of the virus particles, an electron microscopic technique was employed. The procedures were fully described in the previous paper.[6]

Autoradiography The procedures of autoradiography were principally the same as described by Zur Hausen *et al.*[22] After addition of Neocarzinostatin to the culture, tritiated thymidine (specific activity 5.0 Ci/mM) at a final concentration of 1 μCi/ml was given to the cell cultures and incubated for 2 hr. The cells were washed three times with a fresh medium, resuspended in the medium containing Neocarzinostatin, and then incubated. At various intervals, the cell samples were collected by a light centrifugation. After washing once with phosphate-buffered saline (PBS), the cells were resuspended in PBS, and smeared on microscope slides. After fixation with acetone, the cells were stained by immunofluorescence. Between 50 and 300 well-isolated fluorescent and non-fluorescent cells were selected per slide, and their locations were read from the scale of the mechanical microscope stage, and then the slides were coated with emulsion, type NR–M2 (Sakura, Japan). These autoradiograms were exposed for 2 weeks in a dark chamber at 4°. Development was made with Copinal (Fuji, Japan)

for 5 min and fixation with Fuji fixer. After Giemsa staining, the preselected cells were relocated under the microscope and examined for the presence of grains.

Results

Effect of Neocarzinostatin on Cell Growth and EB Virus Antigen-bearing Cell Fraction in P3HR-1 Cell Culture

After several preliminary experiments, the effect of 1 and 10 μg/ml of Neocarzinostatin on the growth of P3HR-1 cells in actively growing state was examined. In the same experiment, the fraction of EB virus antigen-bearing cells was also investigated by immunofluorescence. The results are summarized in Figs. 1 and 2.

In the presence of either concentration of Neocarzinostatin, the cell growth was completely inhibited and the viability diminished progressively. A characteristic feature of the Neocarzinostatin-treated cells was the development of giant cells, which was consistent with the previous finding in the Neocarzinostatin-treated HeLa cells.[15] Appearance of giant cells was first noted 24 hr after the addition of Neocarzinostatin and the size of cells became maximum on the 3rd to 4th day, with 2 to 6 times the diameter of the control cells.

The percentage of EB virus antigen-bearing cells in the Neocarzinostatin-treated cultures was about 2 times higher than that in the control culture (Fig. 2). However, based on total cell counts, the actual number of fluorescent cells in the Neocarzinostatin-treated cultures was smaller than that in the control cultures. Some of the giant cells in the Neocarzinostatin-treated cultures showed a brilliant fluorescence.

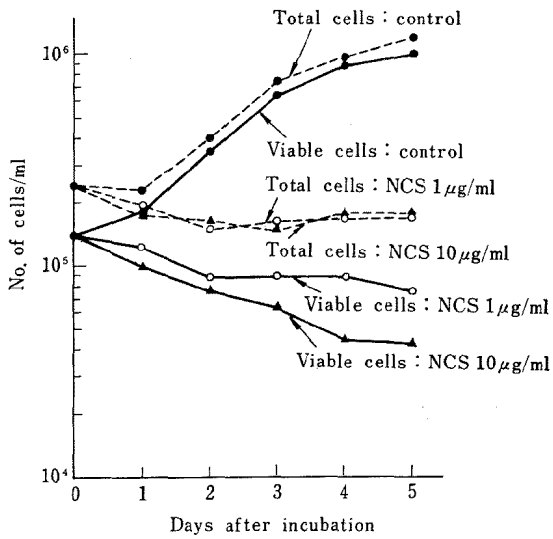

Fig. 1. Effect of Neocarzinostatin (NCS) on the growth of P3HR-1 cells

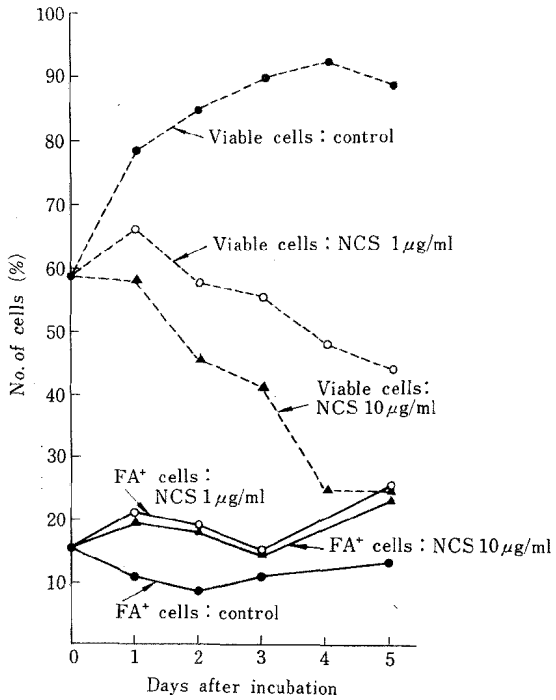

Fig. 2. Percentage of viable cells and immunofluorescence-positive (FA^+) cells in the experiment shown in Fig. 1

Effect of Neocarzinostatin on the Production of EB Virus in P3HR–1 Cell Culture, as Revealed by Electron Microscopic Counts

In order to examine quantitatively the production of EB virus in P3HR–1 cells in the presence of 1 μg/ml of Neocarzinostatin, electron microscopic counts of EB virus particles were carried out at appropriate intervals. The immunofluorescence examination of the cells was also performed in parallel. The summarized results are shown in Figs. 3, 4, and 5.

The total cell number in the control culture increased to 2.5×10^6/ml from 1.0×10^6/ml inoculum, but no cell growth was observed in the Neocarzinostatin-treated culture (Fig. 3). However, the increase in actual number of fluorescent cells in the Neocarzinostatin-treated culture was only slightly smaller than that in the control culture (Fig. 3). Accordingly, the percentage of fluorescent cells apparently increased in the Neocarzinostatin-treated culture but not in the control culture (Fig. 4). These results strongly suggested that the synthesis of EB virus was not inhibited in the Neocarzinostatin-treated culture in which cell division was prevented by Neocarzinostatin. As was expected, absolute number of EB virus particles per culture in the Neocarzinostatin-treated culture was apparently larger than that in the control culture, as shown in Fig. 5. The number of cell-associated virus particles was 8×10^7 per culture when the cultures were started and the number of EB virus particles progressively increased by prolonged incubation after Neocarzinostatin treatment. The number reached the

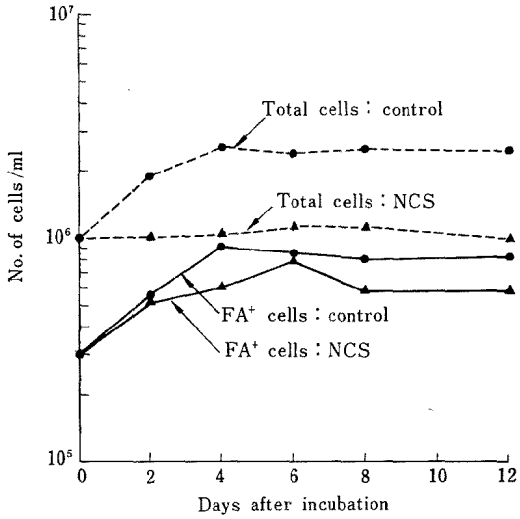

Fig. 3. Number of immunofluorescence-positive (FA^+) cells and total cells in the P3HR-1 cell culture in the presence of 1 μg/ml of Neocarzinostatin (NCS)

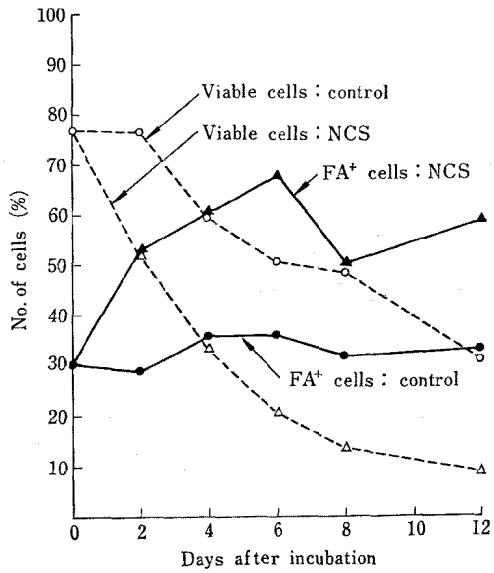

Fig. 4. Percentage of viable cells and immunofluorescence-positive (FA^+) cells in the experiment shown in Fig. 3

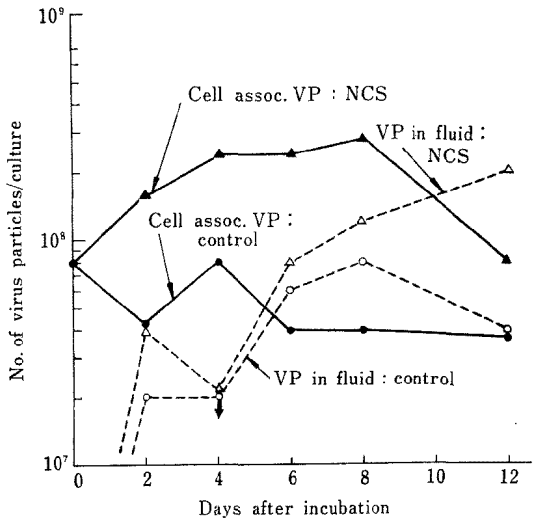

Fig. 5. Production of EB virus particles (VP) in the cell cultures shown in Fig. 3

maximum of 2.8×10^8 on the 8th day of incubation, and then gradually decreased. On the other hand, the number of the cell-associated virus particles in the control culture did not exceed 8×10^7 of the initial number. The rate of accumulation of the virus particles in the culture fluid was not so greatly affected by Neocarzinostatin treatment up to the 8th day, but the treatment with Neocarzinostatin yielded about 5 times the number of particles in the control culture 12 days after incubation. These results strongly suggested an enhancing effect of Neocarzinostatin on the production of EB virus in P3HR-1 cells.

Morphology of EB Virus Particles in Neocarzinostatin-treated Cells

The cells of P3HR-1 at 4 days after incubation in the presence of 1 μg/ml of Neocarzinostatin were thin-sectioned and examined by electron microscopy as described in the previous report.[21] The morphology of virus particles observed in the nucleus, in the cytoplasm, or in the extracellular space was not significantly different from that observed in the non-treated cell culture in the previous experiments.[21]

Autoradiographic Studies on Neocarzinostatin-treated Cells

Zur Hausen *et al.*[22] observed by a combination of autoradiography with immunofluorescence that when the Burkitt cells were irradiated with appropriate doses of X-ray, the synthesis of cellular DNA was markedly reduced whereas that of viral (EB virus) DNA was not affected. In order to confirm the preceding suggestion that Neocarzinostatin prevents the synthesis of cellular DNA but not viral DNA, autoradiographic studies were carried out. For this, P3HR-1 cells and Raji cells were used, the latter being known as a non-producer of EB virus. The P3HR-1 cells were incubated in the presence of 1 μg/ml of Neocarzinostatin, while the Raji cells were treated with 0.05 μg/ml of the antibiotic, since the latter cells were found to be about 20 times more sensitive than the former to Neocarzinostatin in a growth inhibition test.

Fig. 6 shows the results of autoradiographic analysis of the synthesis of DNA in the P3HR–1 and Raji cell cultures. The upper part of this graph again shows an increase in the percentage of fluorescent cells in P3HR–1 cell cultures and a decrease in viability as a result of Neocarzinostatin treatment. When a 2–hr isotope pulse-labeling was applied at 1 or 4 days after the addition of Neocarzinostatin, the percentage of tritium-labeled cells in the P3HR–1 cells was apparently higher than that in the Raji cells, as shown in the lower part of Fig. 6. The high percentage of labeled cells in the P3HR–1 cell cultures, however, cannot directly be attributed to the involvement of the synthesis of EB virus, because the origin and experimental conditions of the Raji cell line were different from those of the P3HR–1. Concerning the localization of the grains, however, there was a significant difference between the two cell lines. The autoradiographic grains in the Raji cells were clearly localized only over the nucleus but in the P3HR–1 cells, the grains were distributed not only in the nucleus but also in the cytoplasm. The Neocarzinostatin-treated P3HR–1 cell cultures were also examined by immunofluorescence in combination with autoradiography. Fig. 7 shows the results of autoradiographic analysis of the synthesis of DNA in the fluorescent and non-fluorescent cells. When the isotope pulse-labeling was applied one day after Neocarzinostatin treat-

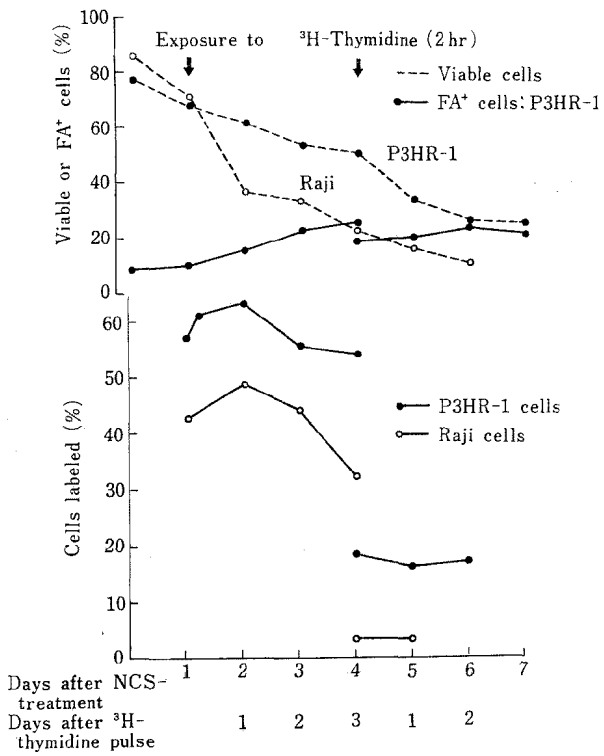

Fig. 6. Percentage of ^{3}H–thymidine-labeled viable or immunofluorescence-positive (FA^{+}) cells in P3HR–1 or Raji cell cultures in the presence of Neocarzinostatin (NCS)

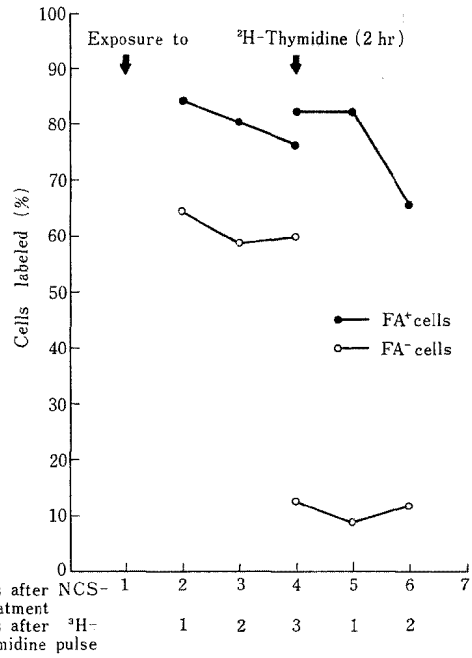

Fig. 7. Preferential labeling of fluorescent (FA^+) cells as compared to non-fluorescent (FA^-) cells of P3HR-1 cell culture in the experiment shown in Fig. 6

ment, the percentage of the tritium-labeled cells in both the fluorescent and non-fluorescent cells was found to be about 80 and 60%, respectively, during the first 3 days. On the other hand, when the pulse labeling was applied 4 days after the addition of Neocarzinostatin, the percentages of labeled cells in the non-fluorescent cells decreased to 10%, but those in the fluorescent cells were not significantly affected by prolonged incubation after the addition of Neocarzinostatin. This indicates that the synthesis of DNA is not prevented in a majority of the EB virus antigen-bearing cells in the presence of Neocarzinostatin.

These results denote the identity of immunofluorescent and virus-containing cells. Furthermore, it was evident that the EB virus as a DNA virus is insensitive to Neocarzinostatin, while the synthesis of host cellular DNA is reduced or prevented by this antibiotic.

Discussion

The present studies indicated that Neocarzinostatin inhibits the growth of P3HR-1 cells, in which EB virus is being carried over probably by intracellular transfer mechanism, as well as the growth of Raji cells, in which EB virus has not been detected, although the sensitivity of the latter cells to Neocarzinostatin was remarkably higher than that of the former cells. One of the possible mechanisms of inhibitory action of Neocarzinostatin on the cell growth due to an interaction of the antibiotic with cellular

DNA has been suggested.[8,17] Autoradiographic studies revealed a marked reduction of the incorporation of ^{3}H-thymidine into majority of non-fluorescent cells among the P3HR-1 cell culture. Furthermore, preliminary studies[20] indicated that Neocarzinostatin prevents the gross synthesis of DNA in the cells of P3HR-1 but not the synthesis of RNA or protein, as revealed by the incorporation of radioisotope-labeled precurser compounds into the respective macromolecules.

Under the condition in which Neocarzinostatin inhibited the synthesis of host cellular DNA and cell division, the production of EB virus was rather enhanced. This was indicated by the direct counting of virus particles in the culture fluid and of the cell-associated virus particles with an electron microscope. The percentage of EB virus-bearing cells as revealed by immunofluorescence in the Neocarzinostatin-containing culture was higher than in the non-treated culture, although the actual number of EB virus-bearing cells in the former culture was rather lower than that in the latter. Furthermore, it was calculated that the amount of virus particles per fluorescent cell in the Neocarzinostatin-containing culture was apparently higher than that in the control culture. As suggested by the experiment with autoradiography, the synthesis of viral DNA in the cells, in which the synthesis of cellular DNA was markedly inhibited, was not affected by Neocarzinostatin. Hence, it is probable that Neocarzinostatin does not inhibit the synthesis of viral DNA and sequential or concomitant formation of viral particles. In our preliminary experiments,[20] the DNA inhibitors other than Neocarzinostatin, i.e., Methotrexate, Mitomycin-C, or 5-fluoro-2'-deoxyuridine, had no activity to increase the percentage of EB virus-bearing cells in the P3HR-1 cultures under the conditions which inhibited cell multiplication. In this respect, Neocarzinostatin may be a unique agent which differentiates the synthesis of cellular DNA from that of viral DNA. Supporting this view, we preliminarily[20] found that the growth of herpes simplex virus was not inhibited at all by Neocarzinostatin. It is unknown yet, however, whether Neocarzinostatin does not affect the synthesis of DNA of all of the other DNA-type viruses.

Concomitant occurrence of the enhancement of EB virus production and the inhibition of cellular growth in the Neocarzinostatin-treated culture may suggest an existence of a regulatory mechanism for the synthesis of EB virus in an actively growing host cell, by which a virus carrier state is being maintained. This leads to the possibility that the active synthesis of cellular DNA and/or cell division may prevent viral growth. However, it seems unlikely that the cellular DNA synthesis alone prevents EB virus growth, since our studies[20] showed the enhancement of EB virus production by Colcemid, which prevented cell division but not cellular DNA synthesis. Therefore, it may be acceptable that the viral growth prevented by the host cell-devision was released by Neocarzinostatin.

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REFERENCES

1) Bradner, W. T., Hutchison, D. J., *Cancer Chemotherapy Rept.*, **50,** 79 (1966).
2) Epstein, M. A., Achong, B. G., Barr, Y. M., *Lancet*, **1,** 702 (1964).
3) Epstein, M. A., Achong, B. G., Barr, Y. M., Zajac, B., Henle, G., Henle, W., *J. Natl. Cancer Inst.*, **37,** 547 (1966).
4) Hinuma, Y., *GANN Monograph*, **7,** 65 (1969).
5) Hinuma, Y., Grace, J. T., Jr., *Cancer*, **22,** 1089 (1968).
6) Hinuma, Y., Konn, M., Yamaguchi, J., Grace, J. T., Jr., *J. Virol.*, **1,** 1203 (1967).
7) Hinuma, Y., Konn, M., Yamaguchi, J., Wudarski, D. J., Blakeslee, J. R., Jr., Grace, J. T., Jr., *ibid.*, **1,** 1045 (1967).
8) Homma, M., Koide, T., Saito, T., Kamo, I., Seto, M., Kumagai, K., Progress in Antimicrobial and Anticancer Chemotherapy. *Proc. 6th Intern. Congr. Chemotherapy*, **2,** 410 (1970).
9) Ishida, N., Miyazaki, K., Kumagai, K., Rikimaru, M., *J. Antibiotics, Ser. A.*, **18,** 68 (1965).
10) Iwakata, S., Grace, J. T., Jr., *N.Y. State J. Med.*, **64,** 2279 (1964).
11) Konn, M., Yamaguchi, J., Grace, J. T., Jr., Hinuma, Y., *Japan. J. Microbiol.*, **13,** 215 (1969).
12) Maeda, H., Kumagai, K., Ishida, N., *J. Antibiotics, Ser. A.*, **19,** 253 (1965).
13) Moore, G. E., Gerner, R., Franklin, H. A., *J. Am. Med. Assoc.*, **199,** 519 (1967).
14) Moore, G. E., Grace, J. T., Jr., Citron, P., Gerner, R., Burns, A., *N.Y. State J. Med.*, **66,** 2757 (1966).
15) Nishikawa, T., Kumagai, K., Kudo, A., Ishida, N., *J. Antibiotics, Ser. A.*, **18,** 223 (1965).
16) Oboshi, S., Seido, T., Hatano, R., Hinuma, Y., *GANN*, **60,** 427 (1969).
17) Ono, Y., Watanabe, Y., Ishida, N., *Biochim. Biophys. Acta*, **119,** 46 (1966).
18) Pope, J. H., Achong, B. G., Epstein, M. A., *Intern. J. Cancer*, **3,** 171 (1968).
19) Pulvertaft, R. J. V., *Lancet*, **1,** 238 (1964).
20) Sairenji, T., Hinuma, Y., unpublished data.
21) Yamaguchi, J., *GANN Monograph*, **7,** 77 (1969).
22) Zur Hausen, H., Henle, W., Hummeler, K., Diehl, V., Henle, G., *J. Virol.*, **1,** 830 (1967).