Effect of Various Inhibitors on the Expression of Polyoma
Virus-induced Surface Antigen in BHK21 Cells

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SUMMARY
BHK 21 cells infected with high multiplicities of polyoma virus show transiently
various characteristics of transformed cells, including the appearance of the
polyoma specific surface antigen (S antigen), as detected by immunofluorescence.
Various inhibitors were tested for their effect on S antigen. Drugs interfering with
DNA synthesis were found to block the appearance of this antigen, but did not
affect its disappearance. On the other hand, actinomycin D did not block S antigen
formation, but blocked its disappearance, and appeared to favour the maintenance
of the BHK21 cell in the abortively transformed state. Inhibitors of protein syn-
thesis prevented the appearance of S antigen. These results are discussed in relation
to other results on the effect of inhibitors on virus-induced antigens or on virus-
induced ‘unmasking’ of some lectin sites.

INTRODUCTION
BHK 21 cells infected with high multiplicities of polyoma virus undergo an abortive
cycle during which they exhibit some properties of transformed cells, a phenomenon called
‘abortive transformation’ (Stoker, 1968). The polyoma T antigen (Meyer, Lhérissón &
Bonneau, 1967) and the polyoma specific S antigen (Lhérissón, Meyer & Bonneau, 1967;
Malmgren, Takemoto & Carney, 1968) are present during this abortive cycle and may be
demonstrated by immunofluorescence in tests with the appropriate antisera.

S antigen was detected early after infection and the maximum number of positive cells
was observed 19 h after infection at a percentage which then decreased at about 30 h (Meyer,
antigen and the stimulation of DNA synthesis showed (Stoker et al. 1972) that in cultures
for which serum stimulation was much reduced, the maximum for S antigen was observed
at 24 h after infection. However, the stimulation of DNA synthesis was first detected by
autoradiography at about 28 h after infection (Taylor-Papadimitriou, Stoker & Riddle,
1971). We report studies in this abortive cycle, on the effect on S antigen of various inhibitors
of macromolecular synthesis.

METHODS
Cells. Baby hamster kidney cells of the BHK21 C13 line (Stoker & Macpherson, 1964)
were used. Cells from various polyoma-transformed cell lines served as controls. The cells
were usually grown in BHK medium (Eurobio) containing 10%, calf serum (Eurobio) and
10% bactotryptose phosphate (Difco) and were passaged by weekly trypsinization. For the
experiments, cells were sometimes seeded at low densities and grown for three days before infection in BHK medium containing 0.2% calf serum (Taylor-Papadimitriou et al. 1971).

BHK21 cells were infected at an input multiplicity of 100 p.f.u./cell. Three hours after infection, the unadsorbed virus was removed and replaced by medium.

**Virus.** The polyoma virus used was the small plaque Toronto strain produced in secondary mouse embryo cells (Crawford, 1962). Virus used for the experiments was either purified by centrifuging in caesium chloride gradients or was a crude virus suspension. Purified and unpurified preparations gave similar results.

**Antisera.** They were produced as described by Meyer & Birg (1970). The rabbit anti-hamster globulin serum, conjugated with fluorescein isothiocyanate, was obtained commercially (Institut Pasteur). Before use, it was adsorbed on mouse and hamster liver powder and on BHK21 cells, to eliminate reactivity to hamster cells.

**Inhibitors.** These were 5-fluorouracil (5 FU) (Roche), 5-bromodeoxyuridine (5 BUDR) (Nutritional Biochemical Corporation), mitomycin C and cytosine arabinoside dihydrochloride (Sigma), puromycin dihydrochloride (Nutritional Biochemical Corporation) and cycloheximide (Calbiochem). Concentrated stock solutions were filtered and kept frozen until use.

Media and solutions were phosphate buffered saline (PBS), 199 medium (Institut Pasteur) and buffered glycerin solution (80% glycerin in 0.1 M-phosphate buffer solution, pH 7.8).

**Autoradiography.** Cells grown in plastic Petri dishes (35 mm diam., Falcon Plastics) were labelled for 4 h with [3H]-thymidine (1 μCi/ml, [6-3H]-thymidine, CEA, France; specific activity: 26 Ci/m-mol.) At the end of the labelling period, the medium was sucked off and the cells washed with tris-HCl solution (mM, pH 7.4), 5% trichloroacetic acid and absolute alcohol. Cells were then dried and covered with a stripping film (AR-10, Kodak), and after a suitable exposure time, developed (D-19, Kodak), fixed (Ilford Hypam) and counted after Giemsa staining.

**Determination of RNA synthesis.** Cells were labelled for 2 h with [3H]-uridine (2 μCi/ml, [5-3H]-uridine, CEA, France; specific activity: 20 Ci/m-mol) and RNA extracted by a hot phenol procedure. RNA was precipitated with 20% trichloroacetic acid solution and the precipitates collected on glass fibre filters (GF/C, Whatman), washed with 10% TCA and alcohol and counted in a Packard scintillation counter in a toluene: POP:POPOP solution.

**Immunofluorescence** has been described (Meyer & Birg, 1970).

**Fluorescence microscopy.** Cells were viewed with a Leitz u.v. microscope with a HBO 200 high-pressure mercury lamp. Filters used were BG 12, BG 38 and a barrier filter K 530 (Leitz). The proportion of viable positive cells in 500 cells counted was estimated for each preparation. Each experimental point represents at least three determinations.

### RESULTS

**Inhibitors of DNA synthesis**

The various inhibitors tested were 5 BUDR, 5 FU, cytosine arabinoside and mitomycin C. All gave similar results in blocking the appearance of S antigen. Preliminary results with 5 BUDR have been reported (Meyer, 1970). When the cells were grown in the presence of 5 BUDR (30 μg/ml, 10⁻⁴ M) for 18 h before infection, the appearance of S antigen on the infected cells was blocked completely. However, the addition of the drug 18 h after infection had no effect on the disappearance of S antigen, which was similar to that observed in the absence of the drug. Control non-infected cells, treated or not with 5 BUDR, remained negative; 5 BUDR was slightly toxic.
Inhibitors and polyoma S antigen

Another type of inhibitor of DNA synthesis, 5 FU, was used at a concentration of 6·5 μg/ml (5 × 10⁻⁵ M) and added to the medium either 24 h before infection or with the virus (Fig. 1). In neither case did S antigen appear. Control non-infected BHK 21 cells, treated or not with the drug, remained negative. In this case, cells were cultured before infection for 3 days in medium containing 0·2 % calf serum, as described under Methods. We verified by autoradiography that incorporation of [³H]-thymidine was inhibited by 5 FU (40 to 50 % inhibition after a 24 h treatment with the drug).

When 5 FU was added to the cells 3 h after infection, and thymidine (10⁻⁶ M) added to the medium at 6 h, the block by 5 FU was reversed and S antigen appeared. The increase and decrease in positive cells was similar to that observed in non-treated cells (Fig. 2).

Mitomycin C (0·5 or 1 μg/ml) and cytosine arabinoside (0·2 μg/ml) also inhibited the appearance of S antigen. These results indicate a need for cellular or virus DNA synthesis, very early after infection.

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Fig. 1. BHK21 cells grown for three days in medium containing 0·2 % calf serum were infected with 100 p.f.u./cell of polyoma virus and subsequently grown in the same medium. Cells received 5 FU (5 × 10⁻⁵ M) either 24 h before infection at time zero (▲—▲) or with the virus (■—■). Infected cells without inhibitor (●—●). Non-infected cells, treated or not with inhibitor, remained negative for S antigen and are not shown on the Fig. 5 FU was left in the medium until the cells were harvested for immunofluorescent staining.
We tested the effect on the appearance of \( S \) antigen of actinomycin D, a drug known to block RNA synthesis. Cells were treated with drug (0.5 or 5 \( \mu \)g/ml) either before infection, with the virus or 3 h after infection. Actinomycin D added before infection remained in the medium until infection. When added with the virus or 3 h later, it remained in the medium until the cells were harvested. Actinomycin D, at 0.5 \( \mu \)g/ml, was effective since it inhibited RNA synthesis in BHK21 cells by more than 95\% when determined by \([\text{H}]\)-uridine incorporation after treatment for 12 or 24 h.

The results show (Fig. 3) that actinomycin D had the same effect irrespective of the time of addition. \( S \) antigen, which normally disappeared at about 30 h after infection, did not now disappear. On the contrary, the number of positive cells increased to 90 to 95\% at 30 h after infection, and remained unchanged as long as the cells were viable. Non-infected BHK21 cells treated with the same doses of actinomycin D remained negative for \( S \) antigen.
Inhibitors and polyoma S antigen

![Graph](image)

**Fig. 3.** BHK21 cells grown in medium containing 10% calf serum, infected at time zero with 100 p.f.u./cell of polyoma virus and subsequently grown in the same medium. Actinomycin D (0.5 μg/ml) was added to the medium either 24 h before infection (▲—▲) or 3 h after infection (■—■). Infected cells without actinomycin D (●—●).

Inhibitors of protein synthesis

Cycloheximide (10 μg/ml) added 3 h after infection, delayed the appearance of S antigen within 24 h. Higher doses of cycloheximide were cytotoxic. Puromycin (2.5 or 25 μg/ml) completely inhibited the appearance of S antigen when added to the medium 3 h after infection. Cycloheximide (10 μg/ml) and puromycin (2.5 μg/ml) inhibited [14C]-amino acid incorporation into TCA-precipitable material by 70 to 80% after a 24 h treatment. At the same time, incorporation of [3H]-thymidine was inhibited by 40 to 50% by those drugs.

Cycloheximide and puromycin therefore exhibit an effect very similar to that of inhibitors of DNA synthesis, perhaps by inhibition of DNA synthesis.

**DISCUSSION**

The expression of a surface modification of BHK21 cells by the appearance of S antigen occurs soon after infection by polyoma virus and is probably a complex phenomenon. It is inhibited by the addition to the cells of inhibitors of either DNA or protein synthesis but is insensitive to the addition of actinomycin D. The presence of S antigen at the surface mem-
brane of abortively transformed cells temporarily confers on them one of the characteristics of transformed cells.

The different inhibitors of DNA synthesis have different mechanisms of action. The addition of 5 BUDR to the cells before infection allowed its incorporation into cellular DNA but probably also inhibited other biosynthetic systems of the cell and especially some enzyme of the glycosylation system (Schubert & Jacob, 1970). Inhibition by 5 FU, mitomycin C and cytosine arabinoside were therefore used in attempts to distinguish between the two possible effects of 5 BUDR in the cell. Their effect on the appearance of S antigen was exactly the same. The addition of thymidine reversed the block by 5 FU, thus showing that DNA synthesis was blocked and that restoration of DNA synthesizing capacity also allowed appearance of S antigen. However, all virus functions were not sensitive to 5 FU. The addition of a similar drug (5-fluoro 2'-deoxyuridine) to mouse embryo cells infected with polyoma virus did not prevent the appearance of the polyoma T antigen as detected by immunofluorescent staining (Sheinin, 1964). In contrast to our findings with polyoma virus, Gergely, Klein & Ernberg (1971) demonstrated that the addition of different DNA antagonists to Epstein–Barr virus-infected Raji cells did not inhibit the appearance of the membrane antigen. Marin & Basilico (1967) showed that the addition of FUDR to polyoma-infected BHK 21 cells led to a decreased frequency of transformation, as tested by cloning efficiency in soft agar.

It should be emphasized that although stimulation of DNA synthesis (Taylor-Papadimitriou et al. 1971) as detected by autoradiography, occurs later in the abortive cycle than the appearance of S antigen (Stoker et al. 1972), inhibitors of DNA synthesis may block nevertheless the appearance of S antigen. A requirement for cellular DNA synthesis cannot be ruled out since autoradiography may not be sensitive enough. Stimulation of DNA synthesis is followed by mitosis (Taylor-Papadimitriou et al. 1971). Consequently, S antigen can be expressed independently of mitosis. In contrast, mitosis is indispensable to agglutination by concanavalin A during the abortive infection of 3T3 cells with simian virus 40 (Ben-Bassat, Inbar & Sachs, 1970). Sheppard, Levine & Burger (1971) and Eckhart, Dulbecco & Burger (1971) also showed that DNA synthesis was a pre-requisite in different virus-cell systems for the surface changes which led to agglutination by wheat germ agglutinin or concanavalin A, since addition of FUDR inhibited agglutination.

The conversion of the cell surface from a normal (S antigen -) to a transformed state (S antigen +) was apparently unaffected by actinomycin D. This may be due to a low sensitivity of the virus DNA to actinomycin D or to the virus bringing its own RNA polymerase. In mouse embryo cells infected with polyoma virus, actinomycin D did not prevent the appearance of the polyoma T antigen (Bowen, Hughes & Dmochowski, 1969).

On the other hand, in the presence of actinomycin D, the cells remained positive for S antigen and the conversion from the transformed (S antigen +) to the normal state (S antigen -) was blocked. Possibly cellular RNA synthesis has a higher sensitivity to this drug. Mallucci, Poste & Wells (1972) showed that the regeneration of the cell coat after trypsin treatment of both normal and polyoma tumour cells was more sensitive to actinomycin D in normal than in transformed cells. They favoured the hypothesis of an enhanced stability of the messenger RNAs involved in the synthesis of the coat material in transformed cells. A similar phenomenon could occur in polyoma-infected BHK21 cells.

The results obtained with cycloheximide and puromycin showed that a de novo protein synthesis was a pre-requisite for the appearance of S antigen. Alternatively, those drugs may inhibit indirectly DNA synthesis, thus blocking the appearance of S antigen.

These results agree with the findings that a prolonged treatment of polyoma-infected
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BHK21 cells by puromycin reduced the transformation rate (Marin & Basilico, 1967), whereas a short treatment enhanced it.

The appearance of S antigen is one of the first manifestations of abortive transformation and precedes the stimulation of host DNA synthesis. In view of the hypothesis of Pardee (1971) on the relationship between the cell surface and the cell cycle, those surface modifications could be an essential step in transformation.

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REFERENCES


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