Control of Polyomavirus T-Antigen and DNA Synthesis in Mouse Embryo Fibroblast Cells by Vitamin A

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ABSTRACT

Vitamin A (retinoic acid, 10^{-6} m) treatment of confluent mouse embryo cells for only 7 h resulted in optimal inhibition of Polyomavirus replication. Depending upon the input multiplicity of virus, one could wait until between 12 and 18 h postinfection to add vitamin A and still observe maximal inhibition of virus yields. Taken together, and assuming the same kinetics before and after virus infection, these results suggested that the inhibitory action of vitamin A occurred between 19 and 25 h into the Polyomavirus replication cycle. In this model system, such a time corresponded to the onset of T-antigen expression and virus-induced cellular DNA synthesis. Analysis of both viral and virus-induced cellular DNA synthesis by the method of Hirt (J. Mol. Biol., 26: 365–369, 1967) and by cesium chloride gradients suggested that vitamin A preferentially inhibited viral, more than virus-induced cellular, DNA synthesis in confluent cell monolayers. Vitamin A also concomitantly inhibited Polyomavirus T-antigen expression in such confluent cultures. In contrast, viral DNA synthesis and infectious virus yields were not significantly inhibited by vitamin A in subconfluent cell cultures. The antagonistic effect of vitamin A on Polyomavirus replication in confluent monolayers could be blocked with cycloheximide, a reversible protein synthesis inhibitor. This suggested that vitamin A inhibition of Polyomavirus replication was indirect and mediated by a newly synthesized protein. Taken together, these results suggest that vitamin A induced a protein in confluent, but not subconfluent, cells, which blocked the expression of Polyomavirus T-antigen expression. Decreased amounts of T-antigen most likely reduced Polyomavirus and cellular DNA synthesis and virus yield.

INTRODUCTION

Evidence that vitamin A might inhibit in vivo papovavirus infections (10, 11, 13–15) by acting at the level of the infected cell, rather than through some indirect host-mediated mechanism, has come from in vitro studies showing that vitamin A can inhibit Polyomavirus replication in confluent mouse embryo cells (18). We had hypothesized that vitamin A might inhibit Polyomavirus replication in confluent, nondividing cells by maintaining normal repression of cellular DNA synthesis-associated genes against antagonistic induction by this virus. This idea stemmed from previous findings that vitamin A can regulate the expression of a specific gene at the transcriptional level (2). In the initial experiments, the inhibitory effect of vitamin A on Polyomavirus replication was found to be cell density dependent (greater inhibition as cells approached confluency) and was not observed with other viral groups that lack a requirement for induction of host cell DNA synthesis-associated enzymes for replication in quiescent cells. These studies suggested that vitamin A inhibition of Polyomavirus was dependent upon both the prevailing level of cellular DNA synthesis and the ability of this virus to increase the DNA synthetic apparatus in quiescent cells. Subsequent experiments revealed both a reduced amount and rate of total DNA synthesis in vitamin A-treated Polyomavirus-infected confluent cells. However, these studies did not discriminate whether the effect was primarily on viral or virus-induced cellular DNA synthesis. This paper presents data which strongly suggest that vitamin A inhibition of Polyomavirus replication results from a selective inhibition of viral DNA synthesis by suppression of T-antigen expression. Evidence is provided for the indirect mediation of this effect by a newly synthesized vitamin A-induced protein.

MATERIALS AND METHODS

Cell Cultures and Polyomavirus. MEC® were prepared from 11- to 14-day-old Swiss Webster mouse embryos by trypsinization according to standard procedures. The cells were passaged in tissue culture flasks (3510; Costar, Cambridge, MA) and maintained at 37°C in a 4% CO2 atmosphere in EMEM (Grand Island Biological Co., Grand Island, NY) containing 10% FCS (Flow Laboratories, McLean, VA) and 100 units of penicillin per 100 μg of streptomycin per ml. Polyomavirus (strain LID-1, VR-252; American Type Culture Collection, Rockville, MD) was grown on confluent, secondary MEC in tissue culture flasks. Following ether extraction and partial purification, the protein content (3) of the original stock virus preparations was typically between 200 and 400 μg/ml.

Standard Experimental Procedure. MEC suspended in EMEM containing 10% FCS were seeded at 1.5 × 10⁵ cells/ml with 1 ml/well added to sterile, 24-well multiwell tissue culture plates (Falcon 3047 Becton Dickinson Labware, Oxnard, CA). Following incubation at 37°C in a 4% CO2 incubator for 48 h, the overlying medium was discarded. To lower the endogenous levels of DNA synthesis, the monolayers were incubated for an additional 72 h in EMEM containing 0.5% FCS. Each supernatant fluid was then supplemented with 0.1 ml of retinoic acid (trans-vitamin A acid, type XX, Sigma Chemical Co.) to the indicated concentration or the appropriate dilution of 95% ethanol or medium as controls. Except for kinetic experiments, retinoic acid was added 24 h prior to virus infection. In kinetic experiments, vitamin A was added at the indicated time relative to virus. Polyomavirus was added and allowed to adsorb for 1 h. One ml volumes of the original pooled media were then returned to the appropriate wells and replenished with retinoic acid on a daily basis. Spent medium was used in these experiments to maintain a low level of cellular DNA synthesis. Supernatant fluids from at least 2 replicate wells were harvested between 48 and 120 h postinfection. Samples were frozen at −70°C for subsequent determination of virus titer.

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The abbreviations used are: MEC, mouse embryo cells; EMEM, Eagle's minimal essential medium; FCS, fetal calf serum; HAU, hemagglutination units; TCID50, 50% tissue culture infectious dose.
Polyomavirus Assays. Polyomavirus titers were determined by hemagglutination assay in microtiter plates with U-bottom wells using a 0.3% suspension of guinea pig RBC. The microtiter plates were incubated at 37°C for 15 min prior to a 3- to 5-h refrigeration at 4°C and subsequent reading. The hemagglutination titer is the reciprocal of the last 2-fold dilution showing complete hemagglutination and is expressed as HAU/0.1 ml. A TCID₅₀ assay of Polyomavirus was performed as follows. MEC (1.5 × 10⁶ cells/well) in Multiwell II plates (Falcon Plastics) were grown to confluence. Two-fold dilutions of each virus sample were added in 0.1-ml volumes of EMEM containing 2% FCS (replicates of 8 or 12). The reciprocal of that dilution of virus which resulted in detectable cytopathic effects in 50% of the replicates after 8 to 9 days was used as the TCID₅₀ titer of a given sample. The method of Reed and Muench, as reported in Ref. 7, was used to determine any 50% end points lying between 2 consecutive virus dilutions. It was determined that, when using replicates of 8 and 12, the fold difference (or percentage of inhibition) between 2 titers to allow a statistical significance of P < 0.05 was 3.0 (67%) and 2.4 (58%), respectively. Polyomavirus titers were also determined by plaque assay on confluent MEC (3 × 10⁶ cells/ml) in 60- × 15-mm tissue culture dishes (25010; Corning Glass Works, Corning, NY). On Day 9 postinfection, monolayers were stained with filtered 0.01% neutral red (N-2880; Sigma) in medium containing 0.9% agar. Dishes were incubated in the dark, and the number of plaque-forming units was determined about 24 h later.

Discriminating between Viral and Cellular DNA on CsCl Gradients. At various times following vitamin A pretreatment and infection by Polyomavirus according to the standard experimental procedure described above, monolayers of MEC were pulsed for 6 h with 1 μCi of [methyl-³H]thymidine (New England Nuclear) per well. This radioactive medium was then removed, and the cells were washed twice and subsequently chased with 1-ml volumes of fresh medium (0.5% FCS) for an additional 20 h. The medium was then removed, and the DNA was extracted from the cells in a fashion similar to that of Crouch and Rapp (4). Cellular digests were placed on CsCl (Beckman Instruments) gradients with an initial density (ρ) of 1.6177 g/ml. CsCl solution (3.8 ml) was added to each cellulose nitrate centrifuge tube (Beckman), followed by 200 μl of the appropriate digested sample, and 25 μl of ethidium bromide (20 mg/ml; Sigma). The final CsCl density that resulted from these additions was 1.5916 g/ml. Each solution was mixed by inverting the covered tubes 3 times. Viral and cellular DNA were separated in CsCl gradients (17) using a 40.3 rotor (Beckman Instruments) at 30,000 rpm for 66 h in a Beckman L5-65 ultracentrifuge at 20°C. Fractions were collected from the bottom of the gradients onto Whatman No. 3MM paper filters (2.3 cm, qualitative; Whatman, England), and acid-insoluble radioactivity was measured in 10 ml of Scintiverse.

Determination of the Density of Form I Polyomavirus DNA. A Polyomavirus input multiplicity of 0.3 TCID₅₀ per cell was adsorbed to 3 multiwell plates (3047; Falcon) of confluent MEC for 1 h and then radiolabeled with 1 μCi of [³H]thymidine (New England Nuclear) per well at 18 h postinfection. The radiolabel was allowed to remain in the cultures until most cells were destroyed by virus. The [³H]-labeled Polyomavirus was then ethy:ether extracted (1:2, v/v), and aliquots (0.7 ml) of the concentrated virus were loaded onto a small column (1-cm diameter x 18.5 cm, Econo-column chromatography columns; Bio-Rad Laboratories) containing Sephacryl S-300 (Superfine; Pharmacia) beads. The 2 peak-excluded fractions were combined to yield about 52,000 counts in an 0.8-ml volume with a hemagglutination titer of 8 HAU/0.1 ml. The entire sample was then added to 2.7 ml of CsCl solution having an initial density of 1.4135 g/ml to yield a final density of 1.3201 g/ml. Since full polyoma virions are known to migrate in CsCl gradients to a density of 1.34 g/ml (18), 4 fractions forming a large peak at this density were pooled and protein-digested at 37°C for 18 h. Following such treatment, 200 μl of the digest were added to replicate cellulose nitrate tubes containing 3.8 ml of CsCl (1.6177 g/ml) and 25 μl of ethidium bromide (20 mg/ml). All subsequent steps were the same as for ultracentrifugation to discriminate viral and cellular DNA. The results indicated that the span of Form I DNA present in such gradients ranged between the extremes of 1.608 and 1.601 g/ml, although the usual density reached was about 1.604 g/ml. These results are consistent with those of other investigators (17) who used a slightly reduced concentration of ethidium bromide and a shorter centrifugation time.

RESULTS

Kinetics of Vitamin A Inhibition of Polyomavirus Replication. Kinetic studies were undertaken to establish that point along the Polyomavirus replication cycle where vitamin A acts. We first determined whether or not vitamin A needed to be present on MEC continuously, or only for a short duration, to exert maximal inhibitory effects. Chart 1 shows that vitamin A (retinoic acid, 10⁻⁶ M) did not need to be continuously present to inhibit Polyomavirus replication. Indeed, these data indicated that vitamin A could remain on cells only about 7 h and still demonstrate optimal inhibition of Polyomavirus replication.

[Graph showing the percentage inhibition of Polyomavirus yield vs. hours of treatment with retinoic acid.]

Chart 1. The minimum length of time of vitamin A treatment for maximum inhibition of Polyomavirus replication. MEC seeded (1.5 × 10⁶ cells/ml) according to the standard experimental procedure were infected with either 0.44 TCID₅₀ units/cell (O) or 0.001 TCID₅₀ unit/cell (C). Retinoic acid (10⁻⁶ M) was added immediately following virus adsorption and removed with washing (4 times) at the indicated times postinfection. The higher-input multiplicity samples were harvested 54 h postinfection; the lower-input multiplicity samples were harvested at 120 h postinfection.

Determination of the latest time postinfection one can add vitamin A to discriminate viral and cellular DNA. The results indicated that the span of Form I DNA present in such gradients ranged between the extremes of 1.608 and 1.601 g/ml, although the usual density reached was about 1.604 g/ml. These results are consistent with those of other investigators (17) who used a slightly reduced concentration of ethidium bromide and a shorter centrifugation time.

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[Chart showing the percentage inhibition of Polyomavirus yield vs. hour of retinoic acid addition.]

Chart 2. Determination of the latest time postinfection one can add vitamin A and still observe maximum inhibition of Polyomavirus replication. MEC were infected with either 1 (△), 0.3 (○), or 0.1 (□) TCID₅₀ unit/0.1 ml. The appropriate replicate wells were treated with retinoic acid to yield a final concentration of 10⁻⁶ M at the indicated times postinfection. Supernatant fluids were harvested at 120 h, and virus yields were determined by hemagglutination assay.
Experiments were then performed to establish how late after infection vitamin A could be added and still provide a maximal inhibitory effect on Polyomavirus replication. Chart 2 shows that, depending on the multiplicity of infection, vitamin A could be added between 12 and 18 h postinfection and still provide maximal inhibition of Polyomavirus yields. These results indicated that using a lower input multiplicity of virus allowed for later addition of vitamin A without losing a maximal inhibitory effect. More importantly, consideration of a 7-h minimum treatment time for maximum inhibition of virus yields taken together with the ability to wait 12 to 18 h postinfection before adding vitamin A suggested that vitamin A acted between about 19 and 25 h into the replication cycle of Polyomavirus. This, of course, assumes there was no change in the kinetics of vitamin A action following virus infection.

Effect of Vitamin A on Viral and Virus-induced Cellular DNA Synthesis in Confluent and Subconfluent Cells. To determine whether the putative time of vitamin A action (19 to 25 h) postinfection coincided with the onset of DNA synthesis, we determined the kinetics of viral and virus-induced cellular DNA synthesis by analysis on CsCl gradients in the presence of ethidium bromide (17). Chart 3 shows that control virus-induced cellular DNA synthesis was just beginning at 12 h postinfection and had almost peaked by 24 h. Polyomavirus DNA synthesis lagged slightly behind the cellular response. Thus, the onset of virus-induced cellular DNA synthesis corresponded to the time of vitamin A action, and the kinetics of the response in our system is similar to that observed by others (1, 8, 16, 20). These data are summarized in Chart 4. More importantly, vitamin A caused a 12-h delay in the onset of virus-stimulated cellular DNA synthesis and a 20% reduction in the total amount synthesized. The marginal suppression of virus-stimulated cellular DNA synthesis was contrasted by a marked inhibition of Polyomavirus DNA synthesis (63%) and virus yield (75% reduction). Further, Polyomavirus DNA synthesis in vitamin A-treated cultures lagged about 12 h behind the controls. Virtually identical results were obtained when viral and cellular DNA were separated based on differential solubility according to the method of Hirt (12) as described in Ref. 9 (data not shown).

Since our previous studies had shown that vitamin A inhibition of Polyomavirus replication was cell density dependent (greater inhibition at confluency), we determined the effect of vitamin A on Polyomavirus DNA synthesis in subconfluent cells. Table 1 shows that vitamin A did not inhibit either viral DNA synthesis or the infectious virus yield from subconfluent cells. However, significant inhibition of both aspects of virus replication was observed in confluent cultures. Taken together, these results strongly suggest that vitamin A inhibited Polyomavirus replication by a selective suppression of viral DNA synthesis. Since Polyomavirus T-antigen expression is required for viral DNA synthesis as well as stimulation of cellular DNA synthesis, we determined the effect of vitamin A on T-antigen expression. Table 2 shows that vitamin A reduced the number of T-antigen-positive cells. The suppression of T-antigen expression was coincident with the suppression of viral and cellular DNA synthesis. Thus, it is tempting to speculate that the preferential inhibition of viral DNA synthesis resulted from an inhibition of T-antigen synthesis.

Vitamin A Inhibition of Polyomavirus Replication Blocked by Cycloheximide. We next determined whether vitamin A directly suppressed Polyomavirus replication or acted indirectly through the induction of a new protein. To distinguish between these 2 possibilities, we attempted to block the effect of vitamin A with a reversible inhibitor of protein synthesis. Table 3 shows that, when cycloheximide was present during vitamin A pretreatment of cells and removed prior to infection, it blocked the inhibitory effect of vitamin A on Polyomavirus replication. This suggested that vitamin A action was indirectly mediated through a newly synthesized protein. This newly synthesized protein apparently was not interferon, since no interferon was measurable in vitamin A-treated Polyomavirus-infected cultures (data not shown), and vitamin A was shown previously to block interferon production (2).

DISCUSSION

A prerequisite to papovavirus replication in confluent, nondividing cells is the derepression of several cellular genes associated with DNA synthesis (19). Such an inductive event is apparently not required in subconfluent dividing cells, since the cellular enzymes required for virus replication are already present. Our previous findings that vitamin A inhibited Polyomavirus replication in confluent, but not subconfluent, MEC suggested that the expression of the DNA synthetic apparatus might be the site of vitamin A action. In this paper, kinetics studies showed that the point of vitamin A inhibition of Polyomavirus was 19 to 25 h postinfection. CsCl gradient analysis of the kinetics of DNA synthesis showed that this time also corresponded to the onset of virus induction of cellular DNA. In the presence of vitamin A, this event was delayed by 12 h and reduced by 20%. Viral DNA synthesis was similarly delayed but more markedly reduced. In contrast to these findings, vitamin A caused neither an inhibition of viral DNA synthesis nor Polyomavirus yield in subconfluent, dividing cells. These data strongly suggest that the inhibition of Polyomavirus replication in confluent cells results from a preferential inhibition of viral DNA synthesis. It seems probable that this inhibition may be caused by vitamin A suppression of Polyomavirus T-antigen, since the expression of this viral gene was blocked by vitamin A, and this occurred concomitantly with induction of DNA synthesis. The finding that cycloheximide blocked the vitamin A effect indicates that the suppression of T-antigen, viral DNA synthesis, and replication is not mediated directly by vitamin A but, rather, by a newly synthesized protein. In summary, the data seem to point to the following possible sequence of events. Vitamin A treatment of confluent cells results in the de novo synthesis of a protein. This protein, in turn, acts to suppress viral T-antigen expression. Since this gene product is required for the induction of virally required cellular enzymes as well as viral DNA synthesis, the end result is an inhibition of virus replication.

In the future, it will be important to identify the vitamin A-induced protein which is ultimately responsible for the suppression of virus replication. Further, since early events in replication and transformation by Polyomavirus are thought to be very similar, if not identical, vitamin A might be expected to inhibit Polyomavirus transformation of confluent cells through a similar mechanism. Indeed, there are several examples which are suggestive of such an effect (10, 11, 13–15), although these studies are in vivo and do not distinguish between the cellular and systemic effects of vitamin A. Such studies should eventually help to elucidate the mechanism of vitamin A inhibition of papo-
Chart 3. The effect of vitamin A on viral and virus-stimulated cellular DNA synthesis as determined by CsCl gradient analysis. After pretreatment with retinoid acid (10^{-6} M), cells were infected with Polyomavirus at an input multiplicity of 1.4 TCID_{50} units/cell. DNA synthesis was monitored at the indicated times postinfection by [\textsuperscript{3}H]thymidine incorporation. Cells were pulsed for 6 h and chased with nonlabeled medium for an additional 2 h prior to extraction by a method similar to that of Crouch and Rapp (4). Samples (200 μl) from such digests were combined with ethidium bromide in CsCl to generate gradients by ultracentrifugation. Fractions (6 drops) were collected on filters for subsequent determination of cold acid-insoluble counts. To define the density gradients formed, every tenth fraction was collected in a small vial for refractive index determination. Arrows indicate the density of Form I Polyomavirus DNA (17), which is about 1.604 g/ml in these gradients. Retinoic acid (10^{-6} M) inhibited Polyomavirus yields determined by TCID_{50} assay (2-fold dilutions, replicates of 12) by 75% (P < 0.01). A, B, C, D, E, and F correspond to 12, 24, 36, 42, 48, and 54 h postinfection, respectively. DNAs from infected and noninfected cells are designated by closed or open circles, respectively.
each noninfected peak from the total counts in the corresponding virus-infected chart illustrates that viral IA), more than virus-induced (B), cellular DNA synthesis by adding the cpm in each constituent fraction. Subtraction of the total counts in was inhibited by retinoic acid (10^{-6} M). The areas under each peak were determined control infected cells were compared.

were observed for positive immunofluorescence.6 Retinoic acid inhibited virus yield by 75% relative to the control at 54 h to Polyomavirus T-antigen (1:80), followed by a fluoresceinated goat antibody to were fixed with 95% ethanol. Coverslips were then stained with hamster antiserum indicated times, the coverslips were washed with phosphate-buffered saline and 24 h. Cells were then infected with 1.4 TCID of Polyomavirus per cell. At the antiserum to Polyomavirus target T-antigen for the immunofluorescence studies.

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