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Mechanism by Which Fiber Antigen Inhibits Multiplication of Type 5 Adenovirus[1]

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Purified fiber antigen of type 5 adenovirus inhibited the multiplication of type 5 adenovirus by 50% when 35 μg of fiber antigen protein was added to 10^6 KB cells in suspension culture. Although the fiber antigen reduced the number of virions adsorbed per cell when a multiplicity of infection of 50,000 plaque-forming units (PFU)/cell was employed, the number of cells infected was not diminished under these conditions. If a low multiplicity of infection (1.1 PFU/cell) was used, viral adsorption was not detectably decreased. The fiber antigen did not reduce the capability of virions to liberate their viral deoxyribonucleic acid (DNA). The biosyntheses of DNA, ribonucleic acid (RNA), and protein were blocked about 20 to 25 hr after the addition of fiber antigen to cultures of uninfected or type 5 adenovirus-infected KB cells. Most of the fiber antigen protein became cell-associated between 22 and 36 hr after it was added to cells. The hexon antigen neither inhibited viral multiplication nor blocked the biosynthesis of DNA, RNA, or protein. Moreover, the hexon did not attach to KB cells. The profound effects of the fiber antigen were not due to the induction of an interferon-like substance, for actinomycin D did not reduce the ability of the fiber to inhibit multiplication of type 1 poliovirus.

Infection of KB cells with type 5 adenovirus exerts a pronounced influence on the host cell's biosynthetic capabilities. Replication of KB cell deoxyribonucleic acid (DNA) stops 8 to 10 hr postinfection, at about the same time that viral DNA synthesis starts, and synthesis of host cell messenger ribonucleic acid (RNA) and protein begins to decline 16 to 20 hr after infection (12). When viral DNA synthesis is prevented, viral structural proteins are not synthesized (10) and synthesis of host cell proteins is not inhibited (Bello and Ginsberg, *submitted for publication*). These facts suggested that the viral structural proteins, of which there are at least three produced during adenovirus infection (25, 28, 29), may participate in the inhibition of host cell macromolecular synthesis. These viral proteins have been termed (14) hexon (L or A antigen), penton (toxin or B antigen), and fiber (E or C antigen).

Pereira (20) demonstrated that fiber antigen, but not the hexon, inhibited the multiplication of type 5 adenovirus, vaccinia virus, and type 1 poliovirus. In these studies a substantial reduction in viral multiplication was obtained when cells were incubated with antigen prior to challenge infection. Two facts suggested that the fiber protein might possess some unique biological properties: the antigen appeared to act on the host cell itself, and the fiber inhibited several viruses that differed greatly in their characteristics. These data, coupled with the indication that the viral structural antigens may play a part in the inhibition of host cell processes, led us to investigate the basis of the fiber antigen's ability to inhibit viral multiplication. It is the objective of this communication to present data confirming Pereira's (20) findings and to report that purified fiber protein combines irreversibly with KB cells and blocks DNA, RNA, and protein biosynthesis.

MATERIALS AND METHODS

Viruses. The prototype strain of type 5 adenovirus (18) was used throughout these studies. The Lederle Laboratory vaccine strain of type 1 poliovirus was employed in several experiments.

[1] Presented by A. J. Levine to the Faculty of the Graduate School of Arts and Sciences of the University of Pennsylvania in partial fulfillment of the requirements for the Ph.D. degree. A preliminary report of this investigation was presented at the annual meeting of the American Association of Immunologists, 9-14 April 1965.

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Tissue cultures. Spinner cultures of KB cells were employed. Cells were propagated in Eagle's minimal essential medium (MEM) supplemented with 10% calf or human serum by methods previously described (7, 8).

Preparation of viral pools. Cells in suspension culture were resuspended in MEM supplemented with 5% calf serum at a concentration of approximately 300,000 cells/ml. They were infected at an input multiplicity of about 100 plaque-forming units (PFU) per cell and were harvested after incubation at 36 C for 36 to 40 hr. The cells were concentrated 10-fold and virus was released by six cycles of freezing and thawing. The clarified viral suspensions were stored at −28 C in maintenance medium (13).

Infectivity assay. Infectious virus was quantitated in monolayer cultures of KB cells by use of a plaque assay. The details are as given by Gilead and Ginsberg (11) with one exception: a second overlay containing neutral red (1:15,000) was added on day 9 or 10 and the plaques were counted on day 13 or 14.

Preparation of antisera. Rabbits were immunized with 10-fold concentrated extracts of KB cells infected with type 5 adenovirus. The rabbits received three injections according to the following schedule: day 0, 3-ml intravenous inoculations; day 10 or 11 and day 20 or 24, 5-ml intraperitoneal inoculations. The rabbits were bled 10 days after the last inoculation. The whole blood was allowed to clot overnight at 4 C. The clarified and separated antisera were heated at 50 C for 30 min and stored at −28 C.

Complement-fixation test. The microtechnique was employed (22). Antigen titers are expressed as the highest dilution that completely fixed 1.5 to 2 exact units of guinea pig complement in the presence of 4 to 8 units of antibody.

Chemical determination of DNA, RNA, and protein. DNA was measured by the Burton (5) modification of the diphenylamine reaction; thymus DNA was used as a standard. RNA was assayed by the orcinol method, with D-ribose used as a standard (6). The method of Lowry et al. (19) was employed to determine protein concentrations, and crystalline bovine albumin was employed as the standard.

To determine the levels of cellular DNA, RNA, and protein, cold 5% trichloroacetic acid was added to the cell pellets and the precipitate was washed with cold 5% trichloroacetic acid. The precipitate was resuspended in 5% acid and heated to 90 C for 45 min. The hot acid supernatant fluids were assayed for DNA and RNA. The residue was dissolved in 0.1 N NaOH and tested for protein content.

Radioisotopes and techniques of measurement. ^{14}C-L-valine, ^{3}H-thymidine, and ^{14}C-algal hydrolysate (all New England Nuclear Corp., Boston, Mass.) were employed for several experiments. ^{32}P-orthophosphate (carrier-free; E. R. Squibb & Sons, New Brunswick, N.J.) was used to label viral DNA. ^{32}P was measured by plating samples on planchets and counting in a windowless gas-flow counter (Nuclear-Chicago Corp., Des Plaines, Ill.). ^{3}H and ^{14}C were measured in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Samples were usually plated on filter pads (Whatman, 2.3 cm), precipitated and washed once with 100 ml of cold 5% trichloroacetic acid and once in 100 ml of cold acetone, and dried in air. The filter pad was then placed in 5 ml of toluene - 1,4 - bis-2 - (5 - phenyloxazolyl) - benzene (POPOP) phosphor and counted. Toluene-POPOP phosphor contains 6 g of 2,5-diphenyloxazole and 0.5 g of dimethyl POPOP in 1 liter of toluene (reagent grade).

Determination of cell number. Cells were counted directly in a hemocytometer. Triplicate determinations were made.

Assay of viable cells. The number of viable cells were determined in plastic petri dishes (60 by 15 mm). Regular Eagle's medium (19) supplemented with 10% calf serum and two times the usual concentrations of glutamine and serine (100 mg/liter) was used in all experiments. The medium was kept at *p*H 7.2 and was warmed to 37 C before use. The cells were removed from a spinner culture, counted, and diluted in regular Eagle's medium (7) to a concentration of 100 cells/ml. A 1-ml sample of cells was added to 4 ml of medium in a plastic petri dish. These cells were incubated for 1 week at 37 C in an atmosphere of 5 to 7% CO_2. At that time the medium was gently removed and the colonies were fixed by allowing them to dry in air. The colonies were stained with Giemsa tissue stain for 1 hr, washed with distilled water, and counted. Each result represents the average of five plates per determination. The efficiency of the viable cell assay varied between 43 and 55%.

Determination of the percentage of infected cells. The materials and procedures were identical to those used for viable cell assays with one exception: a 1:20 dilution of type 5 adenovirus antiserum was included in the medium. The antiserum was sufficient to neutralize greater than 99.99% of the total virus present. A sample of infected cells was removed from the spinner culture and centrifuged from suspension. The cells were resuspended in warm medium containing antiserum, counted, and diluted to a concentration of 100 cells/ml. A 1-ml sample was added to a plastic petri dish containing 4 ml of medium with antiserum. The cultures were incubated for 1 week at 37 C in an atmosphere of 5 to 7% CO_2. The colonies were fixed, stained, and counted as described above.

Purification of viral antigens. Cells in suspension culture were adjusted to a concentration of about 300,000 cells/ml in MEM supplemented with 5% calf serum. The cells were infected with an input multiplicity of about 100 PFU/cell and harvested after incubation at 36 C for 36 to 40 hr. The infected cells were washed once with 0.15 M NaCl, buffered at *p*H 7.1 with 0.01 M sodium phosphate (PBS), and stored at −28 C as cell pellets. The thawed, infected cell pellets were resuspended in 0.01 M sodium phosphate buffer (*p*H 7.4) and disrupted by incubation with 0.25% sodium deoxycholate for 15 min at 37 C. To precipitate nucleic acids, the extract was chilled and streptomycin was added to a final concentration of 1%. This crude suspension was kept at 4 C for 30 min, after which it was centrifuged at 7,850 $\times$ *g* for 30 min. The supernatant fluid was mixed with an equal volume of Freon 113 (DuPont) and centrifuged at 1,200 $\times$ *g* for 15 min. The aqueous fraction was removed and,

to inactivate the toxinlike action of the penton, the solution was incubated with 33 μg (per ml) of trypsin (twice crystallized; National Biochemical Co., Chicago Ill.) at 37 C for 1 hr, after which an equal amount of soybean trypsin inhibitor (crystalline; Worthington Biochemical Corp., Freehold, N.J.) was added (9). Any precipitate which formed at this stage was removed by centrifugation at 7,850 $\times$ g for 30 min. The clarified cell extract was dialyzed for 16 to 20 hr at 4 C against 500 to 1,000 volumes of 0.01 M sodium phosphate buffer (pH 8.0). The dialysate was adjusted to 40% ammonium sulfate by the addition of a saturated solution at pH 8.0. The precipitate was centrifuged at 7,850 $\times$ g for 30 min and resuspended in a small volume (20 to 30 ml) of 0.001 M sodium phosphate (pH 7.5). The extract was dialyzed for 16 to 20 hr at 4 C against 500 to 1,000 volumes of the same buffer solution.

$CaHPO_4$ for column chromatography was prepared by the procedure of Taverne et al. (23) and was used within 24 hr from the time it was made. A column of $CaHPO_4$, 2.2 by 17 to 18 cm, was washed with 250 to 500 ml of 0.001 M sodium phosphate buffer (pH 7.5). The extract was then applied, after which a large excess of 0.001 M phosphate buffer was washed through the column until protein could not be detected in the wash fluid. The protein was eluted with a linear gradient of 0.001 M (250 ml) to 0.35 M (250 ml) sodium phosphate (pH 7.5). A flow rate of 0.3 to 0.5 ml per min was established, and 10-ml fractions were collected. Two large peaks of protein were eluted. The first peak, which eluted at about 0.05 to 0.06 M sodium phosphate, was the fiber antigen. The second protein peak, the hexon antigen, eluted at approximately 0.09 to 0.1 M phosphate buffer. Each of these proteins was dialyzed at 4 C for 16 to 20 hr against 500 to 1,000 volumes of 0.01 M sodium phosphate buffer (pH 7.2), applied to a diethylaminoethyl (DEAE)-cellulose column, and was eluted with stepwise increases in concentration of NaCl (26). The fiber antigen eluted at 0.08 M NaCl and the hexon at 0.24 M NaCl. This procedure yielded milligram quantities of the two viral structural proteins. The final yields for each antigen was about 10 to 15%.

Preparation of antigens for biological experiments. The purified viral antigens were dialyzed extensively against large volumes of Eagle's spinner medium (8). Amino acids, antibiotics, and vitamins were added to the protein solutions in the correct proportions to make complete spinner medium (8). The pH of the solution was adjusted with 5 N NaOH, and the medium was supplemented with 10% calf serum and serine (100 mg/liter). The complete medium containing antigen was passed through a membrane filter and stored at −28 C until used. To initiate the experiment, the antigen preparation was mixed with an equal volume of cells in complete Eagle's medium supplemented with 10% calf serum. Glutamine was added to all cultures (3.4 mM, final concentration) at the start of each experiment.

Analytical ultracentrifugation measurements. Analytical ultracentrifugation was carried out in a Spinco (model E) ultracentrifuge with a schlieren optical system. The protein concentration was adjusted to 1 to 3 mg/ml in a 0.01 M sodium phosphate (pH 7.8) buffer containing 0.2 M NaCl and 4 mM sodium decylsulfate. Centrifugation was carried out at 20 C at 52,640 rev/min. Measurements of the schlieren patterns were made with a microcomparator (Bausch & Lomb) and $S_{20,w}$ values were computed.

RESULTS

Purity of the viral antigens. To estimate the efficiency of purification, isotopically labeled proteins of uninfected KB cells were mixed with unlabeled infected cells, and the radioactivity which remained after purification of the viral proteins was measured. Assuming that the isotopic label accurately represents the host cell contamination, it should decrease as the unlabeled (viral) protein is purified. Thus, a decrease in the specific activity (counts per minute per milligram of protein) with increasing purity of the unlabled viral proteins will result.

A 250-ml suspension culture of uninfected KB cells (150,000 cells/ml) was incubated at 36 C for 46 hr with 200 μc of ^{14}C-valine (160 μc/mg). The washed uninfected KB cells, containing approximately 1.2 $\times$ 10^7 counts/min, were added to unlabeled infected cells. This mixture was then fractionated according to the purification scheme presented in Materials and Methods. At each step in the procedure, the protein, radioactivity, complement-fixing activity, and number of PFU were measured. These data, presented as the average of three separate purification experiments, are given in Table 1. Less than 0.5% of the host cell protein was present in the purified preparations of the viral antigens. Similar results were obtained when uninfected cells were labeled with ^{14}C-algal hydrolysate instead of ^{14}C-valine, indicating the general validity of these results. Less than 500 PFU/ml of infectious type 5 adenovirus remained in the viral antigen preparations at the end of the purification procedure.

Direct chemical analysis of the purified antigen preparations indicated that less than 1% DNA and 0.1% RNA was found with either antigen. Both fiber and hexon antigens gave an ultraviolet spectrum of a typical protein. The analytical ultracentrifuge pattern [in 4 mM sodium decyl sulfate, 0.2 M NaCl, and 0.01 M sodium phosphate (pH 7.8)] showed single symmetrical schlieren peaks of 6.1 and 12.1 $S_{20,w}$ for the fiber and hexon antigens, respectively. The immunological and morphological properties of the antigens were identical to those previously reported (1, 25, 26, 29).

Dose-response experiment. To determine the effect of various concentrations of purified fiber antigen on viral multiplication, 2 ml of KB cells in suspension cultures (185,000 cells/ml) was

TABLE 1. *Purification of the viral structural proteins*

Fraction	Specific activity[a]	Contamination	CF[b] (total)	PFU[b] (total)
		%		
Starting material[c]	17,024	100	143,360	1.3×10^{12}
Deoxycholate + streptomycin	15,603	91	145,360	3.5×10^{11}
Freon	14,110	82	135,168	1.4×10^{11}
Trypsin	11,880	69	143,360	
$(NH_4)_2SO_4$ precipitate	5,908	34	81,920	8.5×10^{8}
$(NH_4)_2SO_4$ supernatant fluid	16,442	96	480	5.6×10^{9}
$CaHPO_4$, peak I	2,057	12	38,840	$<5 \times 10^{3}$
$CaHPO_4$, peak II	628	3.6	30,720	$<5 \times 10^{3}$
DEAE, fiber	64	0.3	20,460	$<5 \times 10^{2}$
DEAE, hexon	16	0.1	15,360	$<5 \times 10^{2}$

[a] Expressed as counts per minute per milligram of protein.

[b] Data (complement-fixing units and plaque-forming units) taken from a single experiment. The other data represent the arithmetic means of the results from three different purification experiments.

[c] Mixture of uninfected ^{14}C-valine-labeled cells (1.2×10^7 counts/min) and unlabeled infected cells.

incubated with antigen in screw-cap tubes for 6 hr at 36 C in a 5 to 7% carbon dioxide atmosphere. The cells were then infected with type 5 adenovirus at a multiplicity of 100 PFU/cell. The infected cultures were incubated an additional 36 hr (in the presence of antigen), after which the cells were harvested, washed, and resuspended in PBS. The washed cells were disrupted by six cycles of freezing and thawing and the debris was removed by centrifugation. Viral multiplication was assayed by testing the clarified supernatant fluids for complement-fixing activity and number of PFU. Duplicate cultures of each antigen concentration were employed. The cells from the duplicate cultures were pooled at the termination of the experimental period, and determinations of complement-fixing activity and infectivity were made. The results of a representative experiment are presented in Fig. 1. After an initial plateau at low antigen concentrations, the inhibition of viral multiplication, whether measured by complement fixation or infectivity, decreased with increasing concentrations of antigen. Approximately 30 complement-fixing units or 35 μg of antigen protein per million cells reduced the infectivity by 50%. The hexon antigen had little or no effect on viral multiplication. Pereira (20), using less purified preparations of fiber (C) antigen, showed a similar dose-dependence inhibition of production of cytopathic effects and complement-fixing antigens.

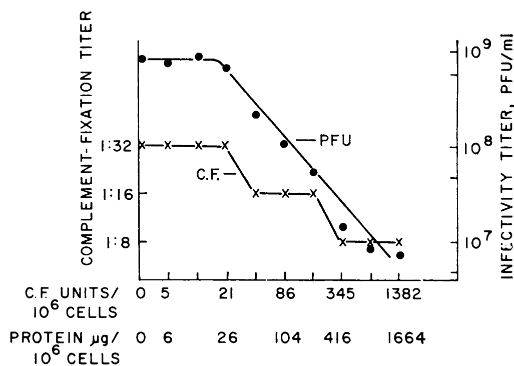

FIG. 1. *Effect of various concentrations of purified antigen on the multiplication of type 5 adenovirus. Each twofold dilution of purified antigen was incubated with two suspension cultures of KB cells (185,000 cells/ml; 2 ml) for 6 hr at 36 C. The cells were then infected with type 5 adenovirus (100 PFU/cell). After an additional 36 hr, the cells were harvested, washed, and disrupted by freezing and thawing. Viral multiplication was assayed by testing the clarified supernatant fluids for complement-fixing activity and plaque-forming units.*

Effect of fiber antigen on viral adsorption. The fiber antigen is a structural component of the type 5 virion as well as a soluble antigen (26, 28, 29). Fiber antigen attaches to and agglutinates red blood cells (21), and it has been suggested that this antigen may be responsible for the attachment of adenovirus to the cell (25). These characteristics suggested that the data summarized in Fig. 1 might result from an excess of soluble fiber antigen in the medium, blocking adsorption of the virus to cell receptors and thus protecting cells from infection. To test this possibility, four suspension cultures of KB cells (400,000 cells in 2 ml), two in the presence and two in the absence of fiber antigen, were incubated for 22 hr at 36 C in a 5 to 7% CO_2 atmosphere. The final concentration of fiber antigen was 210 μg/ml of culture medium. After this preincubation period, an equal number of cells from each

TABLE 2. *Effect of fiber antigen (210 μg/ml) on adsorption of type 5 adenovirus to KB cells*[a]

Preincubation (22 hr at 36 C) with	Infected (1.1 PFU/cell) medium containing	Viral adsorption[b]
		%
Fiber antigen	Fiber antigen	47.7
Fiber antigen	Untreated	48.3
Untreated	Fiber antigen	43.3
Untreated	Untreated	42.3

[a] Suspension cultures containing 200,000 cells/ml.

[b] Adsorption period, 3.5 hr at 36 C; cells were then centrifuged from suspension, and the concentration of viruses in the supernatant fluid was determined.

group were suspended in media without or with the same concentration of antigen protein. Type 5 adenovirus was added to all tubes at an input multiplicity of 1.1 PFU/cell, and viral adsorption was allowed to proceed for 3.5 hr. The cells were then centrifuged and the supernatant fluids were titrated for free virus. (Table 2). The antigen preparation employed reduced viral multiplication by 98.2%, but no difference in adsorption of virus to cells was detectable under these conditions. A similar result was obtained by Pereira for poliovirus and to a less certain degree for adenovirus (20).

Effect of fiber antigen on viral uncoating. The possibility that fiber antigen affected the uncoating of virus was next explored. Cells were incubated in spinner cultures in the presence or absence of antigen (190 μg/ml) for 20 hr. The cells were then sedimented by centrifugation, resuspended in 2 ml of medium, and infected at 0 C with a high multiplicity (50,000 PFU/cell) of purified ^{32}P-labeled type 5 adenovirus. After a 20-min adsorption period, the cells were washed several times with chilled PBS and returned to prewarmed medium at 36 C. Samples were taken at various times, the cells were disrupted with deoxycholate, and the homogenate was assayed for the percentage of labeled DNA that was sensitive to deoxyribonuclease (Lawrence and Ginsberg, *in preparation*). The kinetics and extent of uncoating as measured by the susceptibility of viral DNA to deoxyribonuclease were not affected by the fiber antigen (Fig. 2).

Effect of the fiber antigen on adsorption with a high multiplicity of infection. The previous experiment demonstrated that the percentage of cell-associated viral DNA which became susceptible to deoxyribonuclease was the same in cultures treated with fiber antigen and in untreated cultures. An unexpected result, however, was that

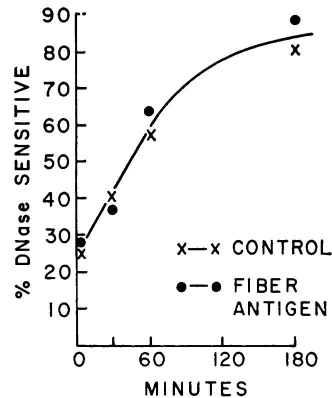

FIG. 2. *Effect of fiber antigen on viral uncoating. KB cells were incubated with or without fiber antigen (190 μg/ml) for 20 hr, and then infected with purified ^{32}P-labeled type 5 adenovirus (50,000 PFU/cell); the kinetics of "uncoating" were measured by the increased susceptibility of viral DNA to deoxyribonuclease after infection.*

TABLE 3. *Effect of fiber antigen on viral adsorption with a high multiplicity of infection*

Incubation with[a]	Viral adsorption[b]
Nothing	1,740
Fiber antigen (190 μg/ml)	840

[a] Suspension cultures of KB cells, 200,000 cells/ml, incubated for 20 hr at 36 C.

[b] ^{32}P-labeled purified virus (50,000 PFU/cell) was added to cultures for 20 min at 36 C. Cells were then washed three times with PBS at 0 C, and the ^{32}P still associated with the cells was counted (counts per minute per milliliter of cells).

the total number of ^{32}P counts (representing isotopically labeled virus) that were associated with antigen-treated and untreated cells was different. The results (Table 3) imply that the cells incubated with fiber antigen had adsorbed only half as much ^{32}P-labeled virus as the untreated cells.

These data appeared to be at variance with the previous conclusion, namely, that fiber antigen had no effect on viral adsorption. A striking difference between the two experiments existed, however. In the later experiment (Table 3), a large viral inoculum (a multiplicity of about 50,000 PFU/cell) was used; in the former experiments (Table 2), the input multiplicity of infection was only 1.1 PFU/cell. If the fiber antigen associates with only a proportion of the cell receptors, an inhibition of adsorption could be detected only when a high multiplicity of infection

TABLE 4. *Effect of fiber antigen on susceptibility of cells to type 5 adenovirus infection*

Incubation with[a]	Virus adsorbed[b]	Colonies formed[c]	
		Uninfected	Infected
Nothing	1,850	46.0×10^{-3}	1.0×10^{-3}
Fiber antigen (220 μg/ml)	450	43.6×10^{-3}	2.1×10^{-3}

[a] Suspension cultures of KB cells, 150,000 cells/ml, incubated for 25 hr at 36 C.

[b] ^{32}P-labeled purified virus (50,000 PFU/cell) was added to cultures for 3 hr at 36 C. Cells were assayed for their ability to adsorb virus as in Table 3.

[c] Cells were washed with prewarmed medium containing antisera, sufficient to neutralize greater than 99.99% of the virus present, counted, and plated for their ability to produce colonies. The plated cells were incubated for 7 days in Eagle's MEM, 10% calf serum, serine (100 mg/liter), and antiserum to type 5 adenovirus.

was employed. If this hypothesis is correct, then every cell treated with fiber antigen, and exposed to a high multiplicity of virus, should have been infected even though less virus was adsorbed to each of the treated cells.

To test this possibility, KB cells were incubated in suspension cultures (150,000 cells/ml, 25 ml) at 36 C for 25 hr with or without fiber antigen. The concentration of fiber antigen used was 220 μg/ml of culture. At the end of the preincubation period, both sets of cultures (treated and untreated) were infected with a high viral multiplicity (50,000 PFU/cell) of purified ^{32}P-labeled virus for 3 hr. The infected cells were then washed (with prewarmed medium containing specific antiserum) by centrifugation and counted, and the number of infected cells was determined by plating in the presence of antiserum (i.e., infected cells will not divide and produce colonies). Samples taken just prior to infection were also plated for viable colonies, and a sample of washed infected cells was assayed for cell-associated virus (Table 4). Even though treatment of these cells with fiber antigen reduced viral adsorption by 76%, the fiber-treated cells remained as susceptible to infection (96% infected) as did the untreated cell cultures (98% infected).

Comparison of the ability of fiber and hexon antigens to block viral adsorption. The results of the experiments described above indicated that it was possible to compare the effects of fiber and hexon antigens on viral adsorption. Four KB cell suspension cultures, one treated with fiber antigen (250 μg/ml), one mixed with hexon

TABLE 5. *Comparison of the effect of fiber and hexon antigens on adsorption of type 5 adenovirus to KB cells*

Incubation with[a]	Virus adsorbed[b]	Inhibition of adsorption
		%
Untreated	15,000	
Untreated	13,000	
Fiber antigen (250 μg/ml)	1,060	92
Hexon antigen (250 μg/ml)	11,100	21

[a] Suspension cultures of KB cells, 150,000 cells/ml, incubated for 24 hr at 36 C.

[b] ^{3}H-labeled purified virus (40,000 PFU/cell) was added to cultures for 2 hr at 0 to 4 C. Cells were then washed four times with PBS at 0 C, and the ^{3}H still associated with the cells was counted (counts per minute per milliliter of cells).

(250 μg/ml), and two untreated cell cultures, were incubated for 24 hr at 36 C. An equal number of cells from each spinner culture was sedimented by centrifugation, and a high multiplicity (40,000 PFU/cell) of purified tritium-labeled virus was added to the cultures. The cell-virus suspension was mixed for 2 hr at 0 to 4 C, after which the cells were washed four times (until labeled virus was no longer detectable in the washes) and the cell-associated radioactive virus was measured. The fiber antigen significantly reduced the adsorption of virus to susceptible cells, whereas the hexon had little or no effect on virus-cell association (Table 5). A second untreated culture was included in the experiment to indicate the variation one might expect in this assay. It should be noted that these experiments were carried out at 0 to 4 C so that viral adsorption, but not viral penetration or eclipse, could occur (Lawrence and Ginsberg, *in preparation*).

Effect of fiber antigen on the synthesis of macromolecules. To consider the possibility that the fiber antigen inhibits viral multiplication by acting on the biosynthesis of viral components, the effect of fiber antigen on the synthesis of macromolecules in infected and uninfected cells was investigated. Purified fiber antigen was added to two spinner cultures at a final concentration of 250 μg/ml. After 10 hr, an antigen-treated and an untreated culture were infected with 100 PFU/cell. At 10-hr intervals, 8-ml samples were removed and centrifuged. The sedimented cells were washed with PBS, and the concentrations of DNA, RNA, and protein were determined.

The results of a representative experiment showing the effect of fiber antigen on DNA synthesis are presented in Fig. 3. In the absence of antigen, there was a linear synthesis of DNA in

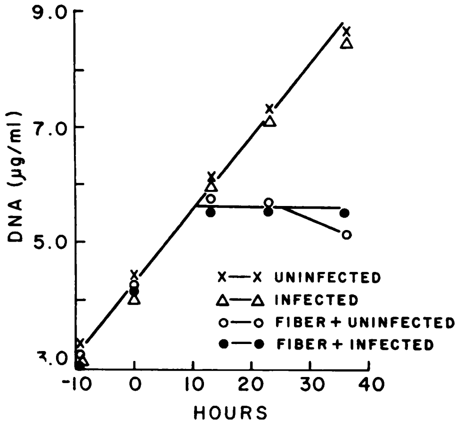

FIG. 3. *Effect of fiber antigen on DNA synthesis in infected and uninfected cells. To one-half of a series of four spinner cultures fiber antigen was added at a final concentration of 250 µg/ml. After 10 hr, a treated and untreated culture were infected with type 5 adenovirus (100 PFU/cell).*

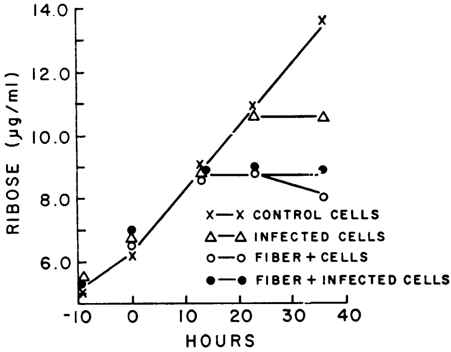

FIG. 4. *Effect of fiber antigen on RNA synthesis in infected and uninfected cells. The procedure was identical to that described in the legend for Fig. 3.*

both the infected and the uninfected cells. In contrast, DNA synthesis stopped 20 to 30 hr after antigen was added to either infected or uninfected cells.

In the uninfected, untreated culture, RNA synthesis was linear during the 46 hr in which the cultures were studied (Fig. 4). However, in the infected, untreated culture, synthesis was linear until about 23 hr after infection, after which time RNA synthesis ceased. On the other hand, by approximately 23 hr after addition of antigen (i.e., 13 hr after infection), RNA synthesis stopped in both infected and uninfected cells.

Protein was also synthesized linearly in both infected and uninfected untreated cells for the entire experimental period (Fig. 5). Like DNA and RNA, protein biosynthesis was also in-

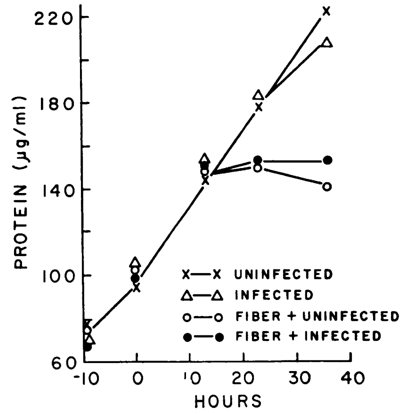

FIG. 5. *Effect of fiber antigen on protein synthesis in infected and uninfected cells. For details of the procedure, see legend of Fig. 3.*

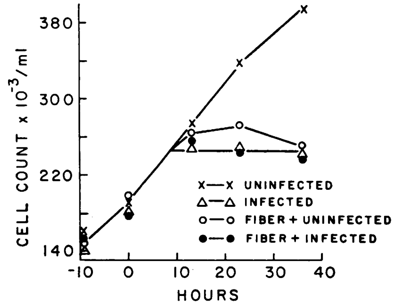

FIG. 6. *Effect of fiber antigen on cell division in infected and uninfected cells. Cell counts were performed in triplicate with a hemocytometer. For details of the procedure, see Fig. 3.*

hibited by 23 hr after antigen was added to the infected and uninfected cultures.

The effect of fiber antigen on cell division is shown in Fig. 6. Cell division was linear in uninfected, untreated cells for the 46-hr period. Cell division ceased by 8 to 10 hr after infection, whether or not the cells received antigen. Uninfected cells treated with fiber antigen stopped dividing by 23 hr after exposure to the antigen, at a time when syntheses of DNA, RNA, and protein were blocked. In the experiment from which the data described were obtained (Fig. 3-6), viral multiplication was inhibited by 98.9%. In contrast, purified hexon antigen did not affect cell division or the biosynthesis of DNA, RNA, or protein.

Kinetics of association between fiber antigen and KB cells. The experiments described above demonstrate that fiber antigen blocked the biosynthesis of macromolecules in KB cells. It was

puzzling, however, that more than 20 hr was required for fiber antigen to act. To investigate the basis for the long delay in the action of fiber antigen, the kinetics of association between antigen and KB cells was studied. For these experiments, ^{14}C-labeled fiber antigen was prepared by adding ^{14}C-algal hydrolysate (0.5 μc/mg) to suspension cultures of KB cells at 10 hr postinfection and subsequently purifying the antigens. The radioactive purified antigen (240 μg/ml), which had a specific activity of 171 counts per min per μg of protein was added to 50-ml suspension cultures of KB cells (150,000 cells/ml) at 36 C and incubated. At intervals up to 47 hr, 8-ml samples were removed; the cells were washed twice in PBS and stored at −28 C until used. The cell pellets were fractionated by procedures described previously, and the hot trichloroacetic acid insoluble residue was dissolved in 0.1 N NaOH; a portion of this was added to a filter pad (Whatman, 2.3 cm), dried, and counted in toluene-POPOP phosphor in a liquid scintillation spectrometer. The counts per minute in each sample represented the quantity of cell-associated antigen.

The kinetics of association between fiber antigen and KB cells are summarized in Fig. 7. The two points per time interval represent results from two different experiments. Included for reference purposes is the effect of the antigen on DNA synthesis. The fiber antigen became cell-associated in an unusual manner. The kinetics of association demonstrate that one-half of the total antigen that became cell-associated (during the 47 hr of incubation) did so between 22 and 36 hr after the fiber antigen was added to the culture. This was the same time interval (22 to 36 hr) during which host cell biosynthetic functions were inhibited. Of the total antigen added to the culture medium, only 0.3 to 0.4% became cell-associated during the 47-hr experimental period. In addition, all of the radioactive label in the culture medium remained acid-precipitable, and none of the cell-associated labeled protein became acid-soluble during the duration of these experiments. Similar experiments done with ^{14}C-labeled hexon antigen demonstrated that this protein did not combine with KB cells.

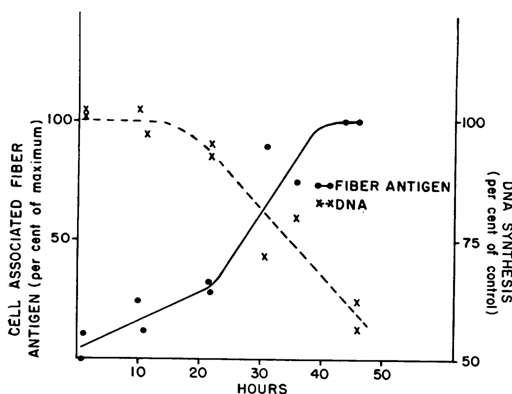

FIG. 7. *Kinetics of association of fiber antigen with KB cells. Purified labeled fiber antigen (240 μg/ml; 171 counts per min per μg) was incubated with KB cells in suspension cultures at 36 C for 47 hr. At prescribed intervals, samples were removed from the culture, washed, fractionated, and assayed for DNA and cell-associated antigen.*

Effect of actinomycin D on the ability of the fiber antigen to inhibit poliovirus multiplication. The evidence presented demonstrates that the fiber antigen effectively inhibited the propagation of type 5 adenovirus and concomitantly blocked synthesis of DNA, RNA, and protein. It was possible that the fiber antigen reduced viral replication indirectly by inducing the synthesis of a second substance (e.g., interferon) which produced the actual inhibitory effect. The induction of such a hypothetical inhibitory substance would probably require synthesis of messenger RNA and protein, as has been demonstrated for interferon production (16, 24). This possibility was tested by utilizing actinomycin D to inhibit synthesis of messenger RNA and hence synthesis of protein. Poliovirus, whose multiplication is inhibited by the fiber antigen (20) but not by actinomycin D (30), was employed to determine whether the fiber antigen could block viral propagation in the presence of actinomycin D. The antigen preparations used for these experiments consisted of the partially purified ammonium sulfate fraction. Adenovirus was removed from these preparations by two consecutive cycles of ultracentrifugation (105,000 × *g* for 1.5 hr). At the end of each cycle, only the top one-half of the extract was used (27).

Four 10-ml suspension cultures were employed as follows: actinomycin D (0.05 μg/ml) was added to one culture; partially purified fiber protein (2 mg/ml) was added to a second; actinomycin D (0.05 μg/ml) and fiber antigen (2 mg/ml) were added to a third; and no additions were made to a fourth culture. After incubation for 23 hr, all four cultures were infected with type 1 poliovirus at a multiplicity of 40 PFU/cell. At 8 hr postinfection, the cells were centrifuged, washed, and resuspended in Eagle's medium containing 10% calf serum. The cells were disrupted by six cycles of freezing and thawing, and the debris was removed by centrifugation. The content of poliovirus in each cell

TABLE 6. *Effect of actinomycin D (0.05 μg/ml) on the inhibition of type 1 poliovirus multiplication by fiber antigen (2.00 mg/ml)*

Treatment[a]	Poliovirus titer (PFU/ml)	Inhibition
		%
None	7.6×10^7	
Actinomycin D	9.0×10^7	
Fiber antigen	3.0×10^7	59
Fiber antigen and actinomycin D	4.25×10^7	53

[a] Suspension cultures of KB cells, 150,000 cells/ml, were incubated for 23 hr at 36 C and then infected with type 1 poliovirus (40 PFU/cell). Each value represents the mean of duplicate plaque assays made 8 hr after infection.

extract was titrated by plaque assay. Actinomycin D did not reduce the ability of the antigen to inhibit viral multiplication (Table 6).

In the above experiment, the cell cultures were treated with actinomycin D for 31 hr. The prolonged incubation time required, and the consequent cell toxicity, made it necessary to use less than the optimal concentrations of actinomycin D required for rapid inhibition of RNA synthesis. Hence, the concentration of actinomycin D employed, 0.05 μg/ml, reduced RNA synthesis by only 80% at 2 to 3 hr after the addition of antigen, although synthesis of RNA was inhibited 96% by 13 hr after exposure of the cells to fiber antigen.

DISCUSSION

The evidence presented confirms the studies of Pereira (20) that the fiber antigen but not the hexon inhibits multiplication of viruses. Although the fiber antigen can diminish the adsorption of adenoviruses, this phenomenon cannot account for the blockade of viral propagation. Thus, at concentrations of antigen that reduced the production of infectious virus by greater than 90%, more than 95% of the cells were infected and could not divide.

The decrease in viral adsorption was directly proportional to the amount of antigen incubated with the cells and could be observed only when high multiplicities of infection were employed. These data suggest that the fiber antigen associates with viral receptors on the cell membrane and prevents virus from adsorbing to these sites. The evidence further implies that the fiber antigen, and not the hexon which cannot combine with the host cells, is the structural protein responsible for viral adsorption. Valentine and Pereira (25) reached a similar conclusion based on the role of the fiber antigen in hemagglutination and on its structural location in the virion.

Just as the inhibition of viral multiplication by fiber antigen cannot be explained by the decrease in viral adsorption, it also cannot be accounted for by an effect of the fiber antigen on the penetration or the uncoating of the virus; i.e., cell penetration by virus and viral uncoating are not prevented in cells mixed with large amounts of fiber antigen.

It may be deduced from this evidence that the fiber antigen deranges some intracellular processes and, by this effect, blocks viral replication. Indeed, the data obtained testify that the fiber protein antigen added to KB cells interrupts biosynthesis of macromolecules. Production of DNA, RNA, and protein stops about 20 hr after fiber antigen is added to uninfected or adenovirus-infected cells. This profound effect on synthesis of nucleic acids and protein should prevent replication of all viruses whether they contain DNA or RNA, just as do synthetic chemicals and antibiotics which produce similar effects on biosynthetic reactions. In fact, the fiber antigen prevents multiplication of viruses as different as type 5 adenovirus, polioviruses, and vaccinia virus (20).

The possibility was considered that the fiber antigen interrupts biosynthetic processes and inhibits viral multiplication by inducing the synthesis of an interferon-like protein. However, fiber antigen inhibited the multiplication of type 1 poliovirus equally in the presence and absence of actinomycin D, indicating that the inhibition was not dependent upon the synthesis of RNA transcribed from a DNA molecule or upon the subsequent synthesis of a protein.

Initially, the long delay between addition of fiber antigen to a culture and the initial depressive effects on the biosynthesis of macromolecules was puzzling. It was then discovered that the combination of protein with cell was very slow, and 50% of the fiber antigen that became cell-associated did so between 22 and 36 hr after antigen was added to the culture. Thus, the period during which most of the antigen combined with cells (22 to 36 hr) was the same as that during which host cell biosynthetic functions were inhibited. Whether this large increase in cell-associated antigen is a major cause of the inhibition or whether it occurs as a result of the inhibitory process is not clear. It should be noted that removal of fiber antigen from the culture medium (after 20 to 25 hr of incubation) allows KB cells to recover their ability to form viable colonies (Table 4).

Only 0.3 to 0.4% of the antigen added to the culture medium becomes cell-associated. Therefore, each KB cell, which contains 400 to 500 $\mu\mu$g of protein, complexes with 2 to 3 $\mu\mu$g of antigen protein. The antigen in or on an inhibited cell, therefore, represents about 0.4 to 0.8% of the total cell protein. The molecular weight of the fiber antigen has been estimated to be about 200,000 (25); each cell would then contain about 10^6 to 4×10^6 molecules of the fiber protein. Because the molecular weight of the fiber protein has not been determined precisely, these numbers can only represent approximations. Furthermore, it seems likely in this artificial system that all of the cell-bound antigen is not in a position to inhibit host cell functions.

The penton also produces profound effects upon cells; 4 to 6 hr after addition to monolayer cultures (considerably earlier than the biosynthetic blockade produced by free fiber protein), marked cytopathic effects are noted (9). The mechanism by which the penton effects cell injury has not been adequately studied because of the difficulties encountered in obtaining pure penton in the absence of free fiber antigen (26). However, because the penton is composed of the fiber protein and a base (12, 25), it is possible that the intact viral structural unit can produce cellular biosynthetic changes similar to its component fiber. These biochemical alterations are probably unrelated to the early cytopathic changes induced by the penton, for such early cytopathic alterations are not induced by purified fiber protein.

That the fiber protein reduces viral propagation by blocking the syntheses of DNA, RNA, and protein seems likely. How the fiber protein effects this action is not known. In vitro, the fiber and hexon proteins can combine with DNA and inhibit the action of DNA and DNA-dependent RNA polymerases (15; Levine and Ginsberg, *in preparation*). Whether these viral proteins inhibit the syntheses of macromolecules intracellularly by this action, as do histones (1, 3, 4, 16, 18), or by some more novel effect, such as an action on cell membranes, requires clarification. These biochemical effects of the fiber and hexon antigens further suggest that they could be responsible for the block in syntheses of DNA and RNA in adenovirus-infected cells (15).

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