Soluble Antigens of Vaccinia-infected Mammalian Cells

II. Time Course of Synthesis of Soluble Antigens and Virus Structural Proteins

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ABSTRACT

Virus-induced soluble antigens produced in mammalian cells after infection with vaccinia virus can be divided into two classes on the basis of molecular weight. Synthesis of the low molecular weight antigens begins early in the course of infection (1 to 2 hr), and is switched-off rather abruptly 4 to 5 hr after infection in a manner similar to that reported for the early enzymes characteristic of this same system. It was demonstrated, however, that these antigens do not include virus-induced thymidine kinase, a major virus-induced enzyme, nor is it likely that the low molecular weight antigens described here share identity with any of the virus-induced enzymes. A portion of the low molecular weight antigens appear to be incorporated into the structure of newly synthesized virus, probably as internal proteins. In contrast, synthesis of the high molecular weight antigen class is initiated later in the course of infection (4 to 5 hr), just prior to the appearance of newly synthesized virus. Antiserum directed specifically against virus structural proteins forms precipitin bands with all of the high molecular weight antigens recognizable by immunoelectrophoresis. This evidence, coupled with the observation that the high molecular weight antigen fraction elicits production of specific virus-neutralizing antibody, strongly suggests that this antigen class represents virus structural subunits produced in excess.

The virus-induced soluble antigens characteristic of the vaccinia virus-KB cell system can be divided into classes on the basis of molecular weight (2). The antigens comprising these classes differ also in certain biological characteristics; i.e., the high molecular weight (HMW) species elicit production of neutralizing antibody, whereas the low molecular weight (LMW) antigens show little or no capacity in this regard (2). It is well-established that infection of mammalian cells with members of the poxvirus group elicits production of virus-induced proteins that differ in their time-course of synthesis in infected cells. These are referred to as "early" and "late" proteins. The late proteins, which appear 4 to 6 hr after infection, represent, in part at least, virus structural proteins (6, 10, 15). The early proteins appearing 1 to 2 hr after infection include a number of virus-induced enzymes (4, 7). It is the

purpose of this communication to demonstrate the following. (i) In terms of temporal relationships, the LMW antigens represent "early" proteins and the HMW antigens represent "late" proteins. (ii) Both "early" (LMW) and "late" (HMW) antigens are incorporated into newly synthesized virus as structural components. (iii) Although the LMW antigens appear early in the course of infection (at the same time, in fact, as the virus-induced enzymes), they do not share identity with these enzymes but appear to represent separate entities.

MATERIALS AND METHODS

Cell culture. Suspension cultures of KB cells were employed. Cells were propagated in Eagle's minimal essential medium supplemented with 10% calf serum.

Virus. A substrain of the vaccinia IHD strain, which does not elicit production of hemagglutinin, was used throughout these studies. This strain is designated as IHD⁻.

Titration of virus. A standard plaque titration with primary chick embryo monolayers was employed (13).

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Virus neutralization tests. A simple plaque reduction test was used as described elsewhere (2). The greatest dilution of serum causing a 70% or greater reduction of plaque count, as compared with virus preimmune serum mixtures, was arbitrarily selected as the neutralization titer.

Degradation of purified virus. Virus obtained from homogenates of infected KB cells was purified in the manner described by Joklik (5). After purification by this procedure, virus was banded in 25 to 40% sucrose gradients two additional times to free virus completely from contaminating soluble antigens. The titer of such suspensions ranged from 2×10^7 to 1×10^{11} plaque-forming units (PFU) per ml. Virus thus purified was dialyzed against carbonate-bicarbonate buffer (0.2 M, pH 10.5) for 48 hr. After dialysis, sodium dodecyl sulfate (SDS) was added to the virus suspension to a final concentration of 0.2%. This mixture was incubated at 37 C for 1 hr with occasional shaking and then was sonically treated for 2 min. As a result of this procedure, the original opalescent virus suspension became almost clear. The resultant material was dialyzed against several changes of 0.01 M phosphate buffer (pH 7.5) for 4 days at 4 C with constant agitation to remove SDS. The final product is referred to as degraded virus.

Preparation of soluble antigens. HMW and LMW antigen classes were prepared by fractionation of a concentrated soluble antigen mixture by means of gel filtration (Sephadex) as previously described (2).

Preparation of antisera. Antisera directed against degraded virus and high and low molecular weight antigen classes were prepared by immunizing rabbits with a series of three weekly intramuscular injections of the appropriate antigens suspended in Freund's complete adjuvant. Precautions taken to insure that such antigen preparations were free from infectious virus are described elsewhere (2). Antisera were selected for the following experiments on the basis of their specificity. For this reason, antisera against the LMW antigens (anti-LMW) did not react with HMW antigens, as evidenced by complement-fixation, double-diffusion in agar, or immunoelectrophoresis. Anti-HMW sera were equally specific by these criteria. Antiserum against virus and, presumably, the entire spectrum of virus-induced proteins was produced by infecting rabbits with purified virus via the intradermal route. All antisera were adsorbed with extracts of normal KB cells to remove antibodies directed against normal cellular and calf serum components.

Complement-fixation. A microtechnique described by Sever (14) was employed. The test system utilized 4 units of antibody. Titers are expressed as the highest dilution of antigen yielding complete fixation of complement.

"Pulse"-labeling experiments. Suspension cultures of logarithmically growing KB cells were adjusted to a concentration of 4.5×10^5 cells per ml in Eagle's medium containing one-fifth the normal concentration of valine and were infected with vaccinia virus at an input multiplicity of 80 to 100 PFU per cell. After 60 min of adsorption at 37 C, prewarmed medium was added to adjust the cell concentration to 1.5×10^5 cells per ml. At appropriate time intervals after

infection, 100 ml of culture was removed to individual spinner flasks, and 20 μ C of 14 C-valine (specific activity 200 mc/mmol) was added. Incorporation of label was stopped at the end of a 30-min period by pouring the cells over iced phosphate-buffered saline (PBS). The cells were then washed four times with cold PBS, concentrated, and resuspended in cold PBS containing 1% bovine serum albumin (BSA) to a volume of 3 ml; the suspensions were frozen for subsequent assay.

Indirect immune precipitation technique. The method employed was essentially the same as that described by Shatkin (15). Pulse-labeled cell preparations were treated for 1 min with a 60-w MSE ultrasonic disintegrator. Sonically treated samples were centrifuged at $800 \times g$ for 30 min to remove cell debris. To 0.1 ml of the resultant supernatant fluid was added 0.1 ml of absorbed immune sera, treated as previously described (2); control preparations which received an equivalent amount of preimmune rabbit serum. After 2 hr of incubation at 37 C in an atmosphere of 5% CO₂, goat anti-rabbit γ -globulin was added in an amount calculated to achieve optimal proportions in order to precipitate completely γ -globulin. An additional 2 hr of incubation followed this procedure. The resulting precipitates were collected by centrifugation at $800 \times g$ for 30 min, and were washed three times with 4 ml of chilled saline. Washed precipitates were dissolved in 0.25 ml of 0.1 N NaOH, plated on stainless-steel planchets, and counted in a gas-flow counter. Results are expressed as the difference between the number of counts precipitated by immune serum and those precipitated from a duplicate sample by preimmune serum.

Thymidine kinase assay. The procedure employed for the assay of thymidine kinase was that of M. J. Bessman of Johns Hopkins University. The assay mixture contained, in a final volume of 0.25 ml: MgCl₂, 1.5 moles; adenosine triphosphate, 1.5 μ moles; tris(hydroxymethyl)aminomethane buffer (pH 7.8), 12.5 μ moles; thymidine-2- 14 C, 7.0 m μ moles (1,500 counts per min per μ mole); and 100 μ g of cell protein. After 60 min of incubation at 37 C, the reaction was stopped by the addition of 0.1 ml of 25% trichloroacetic acid and 1 ml of water, the mixture was centrifuged, and 1.0 ml of clear supernatant fluid was carefully removed. To this was added 0.1 ml of 5 N KOH, and then 0.1 ml of 1 M BaBr₂ and 1.0 ml of ethyl alcohol. After stirring, this mixture was held at 4 C for 10 min. The resultant precipitate was collected by centrifugation and washed three times with a mixture consisting of one part 5 N KOH and nine parts ethyl alcohol. These procedures were carried out at 4 C. The washed precipitate was suspended in 0.5 ml of 0.5 N HCl, plated on a stainless-steel planchet, and counted in a gas-flow counter. Specific activity of thymidine kinase is expressed as millimicromoles of thymidine converted to the phosphorylated form per hour per microgram of protein.

RESULTS

Degradation of purified virus. To explore the relationship of the soluble antigens to virus

structural proteins and to investigate directly the time course of synthesis of this latter class of proteins, it became necessary to obtain antiserum directed solely against virus structural proteins. In this regard, the use of degraded, highly purified virus for the preparation of such antiserum seemed most suitable for two reasons. First, it obviates the necessity of finding an animal suitable for antiserum production in which vaccinia virus will not multiply. Virus multiplication with the concomitant synthesis of virus-induced proteins would, of course, result in the production of antibodies directed against all of these moieties as well as the virus structural proteins. Second, since vaccinia virus is complex in structure, it is possible that degradation of the particle reveals "internal" antigens which, because of their location, might not elicit synthesis of specific antibody in animals in which the killed, undegraded virus particle does not become totally disrupted. This may be the explanation for the failure of certain viruses to produce antibody against internal antigens under various conditions (8).

The procedure employed for degrading virus is described in Materials and Methods. Infectivity and ability to fix complement with appropriate antiserum at various stages of the degradation procedure is shown in Table 1. The final product, referred to as degraded virus, failed to produce plaques in tissue culture either before or after several blind passages through KB cell monolayers. In addition, intradermal injection of undiluted, degraded virus into rabbits did not produce a demonstrable infection. It was concluded, therefore, that the procedure employed for degradation resulted in a total loss of infec-

tivity. The neutralizing potency of antiserum prepared with degraded virus was compared with antisera prepared with HMW and LMW soluble antigens (Table 2). These data indicate that the antigens obtained from degraded virus maintained their capacity to elicit production of potent neutralizing antibody. This result, coupled with the observations that degraded virus exhibited no loss in complement-fixing capacity (Table 1) and that antiserum directed against the complete soluble antigen mixture (anti-SAM) revealed the characteristic seven lines (2) after immunoelectrophoresis of degraded virus, led to the conclusion that the virus structural proteins maintained their antigenic integrity after the degradation procedures. Other properties of antiserum made in response to degraded virus will be discussed later in this communication.

Experiments were undertaken to characterize some of the physical changes in the virus particle accompanying degradation. Purified virus labeled in the protein moiety with ^{14}C -valine was prepared. The specific activity of such preparations ranged from 5×10^{-5} to 5×10^{-6} counts per min per PFU. Part of the preparation was degraded; the remainder served as a control. Labeled virus, degraded and undegraded, was centrifuged at $23,000 \times g$ in 25 to 40% sucrose density gradients for 45 min. Drops were collected from the bottom of the centrifuge tubes, and 15-drop fractions were tested for infectivity, radioactivity, and ability to fix complement with appropriate antiserum. As shown in Fig. 1, essentially all of the labeled virus structural proteins were found at, or very near, the top of the sucrose gradient after degradation. As might be expected, essentially all of the complement-fixing activity also shifted to this area after degradation. The total loss of infectivity and failure to observe antigenic activity in the virus area of the gradient indicate a major disruption of the virus particle. In contrast, although treatment with SDS alone destroyed infectivity, it did not cause a major dissociation of protein antigens from the virus particle.

Time course of synthesis of high and low molec-

TABLE 1. *Effect of various steps in the degradation procedure^a upon infectivity and complement-fixing capacity of vaccinia virus*

Virus prepn	Infectivity (PFU/ml)	CF iter per 0.025 ml ^c
Purified virus untreated	1.4×10^{11}	1:16
Purified virus (pH 10.5 for 48 hr)	6.1×10^5	1:16
Purified virus (0.2% SDS ^b for 1 hr)	No detectable infectivity	1:8
Purified-degraded virus	No detectable infectivity	1:16

^a See Materials and Methods for degradation procedure.

^b Sodium dodecyl sulfate.

^c Antiserum directed against virus-specific proteins was employed for these tests.

TABLE 2. *Virus-neutralizing capacity of antisera specific for various preparations*

Antiserum versus	Neutralizing titer ^a
Low molecular weight antigens.....	<1:8
High molecular weight antigens.....	1:4,096
Degraded, purified virus (IHD ⁻)....	1:1,024
Purified, undegraded virus (IHD ⁻)...	1:2,048

^a Greatest serum dilution still capable of causing a 70% or greater reduction in plaque titer.

FIG. 1. Sucrose gradient centrifugation of degraded and undegraded vaccinia virus. Purified, vaccinia virus labeled with ^{14}C -valine was employed. Linear gradients ranging from 25 to 40% sucrose were utilized. Centrifugation was carried out at $23,000 \times g$ for 45 min in a Beckman Spinco SW-25 head. Antiserum directed specifically against virus proteins was employed for complement-fixation tests.

ular weight virus-induced antigens. Suspension cultures of KB cells (2×10^5 cells per ml) were infected with virus at an input multiplicity of 80 to 100 PFU per cell. At intervals thereafter, 100-ml samples were removed. Infected cells collected in this manner were washed three times with cold PBS, concentrated into a final volume of 5 ml, and frozen. At the termination of the experiment, concentrated cell suspensions were sonically disrupted and were centrifuged at $800 \times g$ to remove cell debris. Supernatant fluids thus obtained were tested for complement-fixing activity by use of antisera directed specifically against the high and low molecular weight soluble antigens (2). The results of one such experiment (Fig. 2) indicated quite clearly that synthesis of the LMW soluble antigens began early in the course of infection. Samples taken at the time of infection (0 hr) and 1 hr after infection, as well as extracts of normal control cells, showed no background of complement-fixing activity when tested with antisera against either HMW or LMW antigens.

It is apparent from these data, therefore, that specific virus-induced antigens first appear between 1 and 2 hr after infection. It appeared, furthermore, that production of these antigens was either greatly reduced or "switched-off" 4 to 5 hr after infection. In contrast, production of virus-induced HMW antigens was not apparent until later in the course of infection (4 to 5 hr postinfection), at roughly the same time that synthesis of the "early" antigens was reduced or stopped and just prior to the appearance of newly synthesized virus. The possibility that a relationship exists between the onset of synthesis of late antigens and the "switch-off" of early antigens remains to be investigated, although data to be reported elsewhere suggest that such a relationship may not exist.

To substantiate the preceding data, with particular-reference to the apparent "switch-off" of LMW antigen synthesis, experiments were designed to study the rate of synthesis of the HMW and LMW antigens at various times after infection. In brief, spinner cultures of KB cells were "pulsed" for 30-min intervals with ^{14}C -valine at various times after infection; cells were harvested immediately after this treatment. By means of the indirect immune precipitation technique (15) with the use of antigen-specific antisera, the HMW and LMW antigens present

FIG. 2. Time course of synthesis of high and low molecular weight soluble antigen classes as shown by complement-fixation (CF). Symbols: $\circ$, CF titers of extracts with antiserum directed against low molecular weight antigens; $\bullet$, CF titers of the same samples with antiserum directed against the high molecular weight soluble antigens; Δ , plaque titers of newly synthesized virus.

in these samples were precipitated; the procedures used are described in Materials and Methods. Rate of antigen synthesis was expressed as the amount of ^{14}C -labeled antigen found in the specific precipitate at various times after infection. Results of one such experiment are shown in Fig. 3. It was apparent from these data that the synthesis of the LMW antigens attained a maximal rate between 2 and 4 hr after infection, and then rapidly declined to approach background levels. This result is entirely consistent with the complement-fixation data previously described. In contrast, synthesis of HMW antigens did not reach maximal rates until 8 hr after infection. It was notable once again that the increase of HMW antigen synthesis to a maximal rate coincided with a rapid decline in the rate of LMW antigen synthesis.

Two things seem clear from these data. First, the HMW and LMW antigens differed not only in physical-chemical characteristics and the ability to induce production of neutralizing antibody, but also in the time at which they were synthesized in infected cells. Second, the LMW antigens are "early" proteins, comparable in this respect (time of synthesis) to the "early" enzymes observed in vaccinia-infected cells by McAuslan and others (3, 7, 11, 12). In view of these results, two questions were asked: first, are early (LMW) as well as late (HMW) antigens incorporated into virus particles as structural components, and,

second, are the early (LMW) antigens identical with the early enzymes reported by others in vaccinia-infected cells. These points were considered in the sequence listed.

Relationship of HMW and LMW antigen classes to proteins obtained from degraded virus. To determine whether antigens of both classes were incorporated into mature virus particles, several experiments were undertaken. Antiserum specific for degraded, purified virus was employed to demonstrate the presence of virus structural proteins. The ability of this antiserum to fix complement, in conjunction with purified HMW and LMW antigen preparations obtained by Sephadex filtration (2), was tested. As shown in Table 3, antiserum specific for virus protein (antidegraded virus) combined with antigens of both molecular weight classes to fix complement. This would appear to indicate that either all or some of the antigens of both the HMW and LMW preparations are virus structural proteins produced in excess. No valid relationship between complement-fixation titers and protein concentrations of the HMW and LMW antigen preparations can be drawn from these data, because, unlike HMW antigen preparations, the LMW material is rather heavily contaminated with host-cell protein. On the other hand, one may calculate from the data in Table 3 that, in terms of specific activity (complement-fixing units per milligram of protein), the HMW antigens appear to be four to five times more potent than the degraded virus preparation. This apparent paradox is due largely to the observation that the antigenic subunits in degraded virus preparations are aggregated to a considerable extent. Fresh soluble antigen preparations do not exhibit this property.

Immunoelectrophoresis and double diffusion

FIG. 3. Rate of synthesis of virus-specific high and low molecular weight soluble antigens determined by pulse-labeling. Points indicate the times at which samples were taken. ^{14}C -valine was added to the culture 30 min before sampling (30-min pulse). Amount of ^{14}C -labeled protein precipitated by nonimmune serum was subtracted from that precipitated by antigen-specific antiserum. Symbols: ●, radioactivity precipitated by antiserum versus low molecular weight antigens; ○, radioactivity precipitated by antiserum versus high molecular weight antigens; △, number of PFU per sample harvested at the indicated times.

TABLE 3. Reaction of antiserum directed against the proteins from degraded, purified virus with soluble antigen proteins of the high and low molecular weight classes

Antigen prepn	Concn (mg/ml)	CF titer per 0.025 ml
High molecular weight antigens ^a	0.17	1:256
Low molecular weight antigens ^a	0.17 ^b	1:32
Normal KB cell extract.....	0.17	<1:2
Degraded purified virus.....	0.05	1:16

^a Prepared by gel filtration on Sephadex.

^b This value does not accurately reflect the concentration of virus-induced antigens in this preparation, since it is contaminated to a considerable extent with normal host-cell proteins.

in agar reveal four LMW (early) antigens and three HMW (late) antigens (2), a total of 7 readily identifiable virus-induced antigens. The data presented in Table 3 do not indicate whether all or only some of the antigens comprising these groups are immunologically identical to virus structural proteins. Two lines of evidence bear upon this question. First, immunoelectrophoresis of unfractionated soluble antigen preparations by use of antiserum against virus structural proteins (anti-degraded virus) revealed the full complement of seven arcs in the positions characteristic for the HMW and LMW antigens (2). Second, the reciprocal test, immunoelectrophoresis of concentrated-degraded virus by use of antiserum against all of the soluble antigens (anti-SAM), revealed six of the seven arcs. The relative density, but not the position, of these arcs differed from those seen with soluble antigen preparations. It is probable that the missing arc does not indicate the absence of a particular antigen in this case, but rather indicates that it is present in small amount in virus particles. It would appear, therefore, that all of the soluble antigens described in this communication represent virus structural proteins produced in excess. In addition to this, a study was undertaken to follow specifically the time course of synthesis of virus structural proteins. Soluble antigen samples were obtained from infected cells at hourly intervals after infection. Each sample was tested for complement-fixing activity by use of antiserum specific for virus structural proteins (anti-degraded virus), as well as for infectivity. The results of this experiment (Fig. 4) indicated that virus structural proteins appeared very early (around 2 hr) in the infectious cycle, at a time characteristic for the appearance of the LMW (early) proteins (Fig. 2). The biphasic shape of the curve suggested that synthesis of a second class of virus structural protein was initiated later in the course of infection. Indeed, if one extrapolates the second steep rise in antigen production to the base line, a pattern is obtained virtually identical to that seen when these same samples are tested with antiserum specific for HMW (late) virus-induced antigens (Fig. 2). These data also indicate that early-appearing (LMW) as well as late-appearing (HMW) proteins contribute to the structure of newly synthesized virus.

Relationship of the "early" (LMW) soluble antigens to the "early" virus-induced enzymes. In view of the obvious temporal similarities existing between the kinetics of synthesis of LMW antigens described in this communication and the "early" virus-induced enzymes appearing in vaccinia-infected cells (7), the possibility was entertained that the LMW antigens were, in fact,

FIG. 4. Time course of synthesis of virus structural proteins. Extracts from infected cells, harvested at the times indicated, were tested for the presence of virus structural proteins by use of antiserum directed against virus proteins (anti-degraded virus). For purposes of comparison, each sample was also tested for complement-fixing (CF) activity with antisera directed against the high and low molecular weight antigen classes. Symbols: ○, CF titers with antiserum vs. degraded virus; ●, CF titers with antiserum vs. high molecular weight soluble antigens; ×, CF titers with antiserum vs. low molecular weight soluble antigens.

virus-induced enzymes. Kinetic studies were undertaken, therefore, to measure the effect of vaccinia infection of KB cells upon the synthesis of thymidine kinase, perhaps the most widely studied of the virus-induced early enzymes. Methods employed for the preparation of samples and the assay of thymidine kinase are described in the appropriate section. Inasmuch as the results of these experiments serve merely to illustrate that the induction of thymidine kinase activity in infected KB cells is similar, if not identical to that often reported for similar systems (6, 7, 11), the following brief summary should be adequate. (i) Within 30 to 60 min after infection, increased levels of thymidine kinase were detectable. (ii) Within 4 to 6 hr after infection, thymidine kinase activity attained levels 7 to 10 times greater than those seen in uninfected control cells. (iii) At 4 to 6 hr after infection, further increase in thymidine kinase ceased or was "switched-off", and enzyme levels remained more or less constant. (iv) The increase of thymidine kinase activity in infected cells treated with 5-fluorodeoxyuridine (3×10^{-6} to 6×10^{-6} M) was not "switched-off"

4 to 6 hr after infection, but continued for as long as 10 to 12 hr after infection.

With this information at hand, experiments were designed to determine whether the early-appearing virus-induced proteins designated as LMW antigens were virus-induced enzymes. Advantage was taken of procedures which have been developed for partial purification of virus-induced thymidine kinase (9), the object being to determine whether or not the purified enzyme would react with antisera directed against LMW antigens, as well as antisera against purified degraded virus, HMW antigens, and the entire soluble antigen spectrum. In this regard, both the ability of such sera to fix complement with purified enzyme and to interfere with enzyme activity was tested. Calculations based on the data presented in Table 4 revealed that the purification procedure accomplished an 11-fold increase in the specific activity of virus-induced thymidine kinase. When crude enzyme extracts (infected cell sonic extracts) and purified enzyme preparations were tested in parallel for complement-fixing activity with the use of various antisera, the results shown in Table 5 were obtained. These data also show the capacity of various antisera to inhibit thymidine kinase activity. These results indicate that the enzyme purification procedure, as employed, resulted in a total removal of soluble antigen material. It was apparent also that antisera specific for the various soluble antigen fractions and for virus structural proteins obtained from degraded, purified virus do not react with virus-induced thymidine kinase to fix complement. For this reason, one may conclude that the "early" LMW antigens referred to in this communication did not represent thymidine kinase. This conclusion

is further substantiated by the observation, also shown in Table 5, that antiserum directed against either HMW or LMW antigens, or against virus structural proteins obtained from degraded virus, did not inhibit thymidine kinase activity. In contrast, antiserum made in response to concentrated, unfractionated infected cell lysates (anti-SAM) exhibited considerable inhibitory activity. Despite the fact that these data indicate that the LMW antigens found in purified-degraded virus were not enzymes, it is interesting to note that traces of thymidine kinase can be detected (0.1% of the amount of enzyme found in the crude virus preparation) in purified virus that has undergone only a single banding in the

TABLE 4. *Partial purification of virus-induced thymidine kinase*

Prepn	Total protein (mg)	Total enzyme units ^a	Specific activity	
			Enzyme ^b	Antigens ^c
Infected-cell sonic extract (100,000 × g)	106.0	1,123.60	0.0106	327.8
Purified enzyme preparation	4.2	488.46	0.1163	0

^a One unit of enzyme is expressed as that amount of enzyme which will produce 1 μ mole of product in 60 min.

^b Enzyme units per microgram of protein.

^c Complement-fixing units per microgram of protein with antiserum directed against the entire spectrum of soluble antigens (anti-SAM).

TABLE 5. *Reaction of various antisera with crude and purified thymidine kinase*

Antiserum vs	Prepn ^a	CF titer	Inhibition ^c of enzyme activity (%)
Concentrated soluble antigen mixture	Infected cell sonic extract	16,384	—
	Purified enzyme	0	46
Three times sucrose banded degraded virus	Infected cell sonic extract	4,096	—
	Purified enzyme	0	0
Low molecular weight "early" antigens	Infected cell sonic extract	32	—
	Purified enzyme	0	0
High molecular weight "late" antigens	Infected cell sonic extract	512	—
	Purified enzyme	0	0

^a The crude enzyme preparation (infected cell sonic extract) was adjusted to the same protein concentration (500 μ g/ml) as the purified enzyme preparation for comparative complement-fixation titers.

^b CF titers are expressed as the reciprocal of the greatest antigen dilution yielding complete fixation of complement.

^c Enzyme inhibition tests employed equal volumes of purified enzyme and undiluted serum. The final enzyme concentration was 50 μ g per test. Normal rabbit serum was used for controls.

25 to 50% sucrose gradient. No enzyme was detected in virus banded two or three times. It is noteworthy, however, that three-times banded virus, which had been subsequently degraded, reacted with antiserum to the LMW antigens to fix complement. One may reasonably assume, therefore, that the "early" (LMW) antigens are incorporated into newly synthesized virus as structural components.

DISCUSSION

It was previously shown that the virus-induced soluble antigens of vaccinia-infected mammalian cells can be divided into two classes on the basis of molecular weight (2). These same antigen classes differ also in their capacity to elicit synthesis of specific neutralizing antibody (2). The data presented in this communication demonstrate still another striking difference between the antigens comprising these classes with respect to their time of synthesis in infected cells. In this regard, it seems appropriate to class the LMW antigens as "early" proteins and the HMW antigens as "late" proteins.

It appears reasonably certain that the HMW antigens represent virus structural proteins synthesized in excess. There are several lines of evidence in favor of this interpretation. (i) The high molecular weight antigens elicit synthesis of high-titer neutralizing antibody, the implication being that one or more of these antigens represent "coat" protein. (ii) Antiserum against purified virus structural proteins (anti-degraded virus) fixes complement and forms precipitin lines when reacted with the HMW antigens. (iii) Antiserum against the HMW antigens fixes complement and forms precipitin lines when reacted against virus structural proteins obtained from degraded, purified virus. The data presented in this communication indicate that the LMW antigens also represent virus structural proteins, despite the fact that these antigens do not elicit synthesis of neutralizing antibody. This evidence may be summarized as follows. Antiserum directed against the LMW antigens fixes complement when reacted with virus structural proteins obtained from degraded, purified virus. Antiserum versus virus structural proteins forms the four precipitin lines characteristic of the LMW antigens when reacted against soluble antigen preparations. This same antiserum reveals the synthesis of complement-fixing antigens early in the course of infection at a time characteristic of the appearance of the LMW antigens. In view of the striking similarity between the LMW antigen and the early virus-induced enzymes as regards their time course of synthesis, the possibility that the LMW antigens do, indeed, represent the

virus-induced enzymes was entertained. This possibility proved unlikely, however, for the following reasons. (i) Antiserum specific for the LMW antigens did not fix complement with or form precipitin lines with ("see") partially purified, virus-induced thymidine kinase. (ii) Antiserum directed against the virus structural proteins similarly did not react with the partially purified enzyme. (iii) Antiserum against either the LMW antigens or virus structural proteins did not inhibit the activity of thymidine kinase. It appeared, therefore, that the LMW antigens share no homology with the early, virus-induced enzymes, but that they do, indeed, represent virus structural proteins. Since these antigens do not elicit formation of neutralizing antibody, it is possible that they represent internal proteins, perhaps closely associated with virus deoxyribonucleic acid. This possibility is in keeping with the observation that these proteins arise very early in the course of infection, close to the onset of viral deoxyribonucleic acid synthesis.

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