Soluble Antigens of Vaccinia-infected Mammalian Cells

III. Relation of "Early" and "Late" Proteins to Virus Structure

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The structural proteins of vaccinia virus can be divided into two classes on the basis of their times of synthesis in the infected cell. The production of one of these classes of proteins begins prior to the onset of viral deoxyribonucleic acid (DNA) replication. These are referred to as "early" proteins. Synthesis of the second class of structural proteins follows the onset of viral DNA replication; hence, the term "late" proteins for this class. We are able, by immunological procedures, to identify three "early" virus-structural proteins. These materials, when incorporated into virions, appear to be associated with the "core" of the virion and do not elicit production of virus-neutralizing antibody. It would seem, therefore, that those virus-structural proteins synthesized early in the course of infection act as internal components of the virion. The "late" proteins may be subdivided into two groups on the basis of certain physical properties and molecular weight differences. The first of these groups, comprised of at least two proteins, corresponds to the classical LS antigens and elicits production of neutralizing antibodies. These proteins, when incorporated into virions, are found only in the outer ("coat") fraction of the virion. The second group of "late" antigens, also comprised of two proteins, termed the G antigens, do not elicit synthesis of neutralizing antibody. One of these proteins is associated with the virus "core"; the other is found in the "coat" fraction of the virion and appears to occupy an intermediary, subsurface position. Procedures suitable for the isolation of the G antigens are described, in addition to the partial characterization of these antigens.

Virus-induced soluble antigens synthesized in KB cells after infection with vaccinia virus have been divided into two classes on the basis of their separation by gel filtration (1). These classes were termed, respectively, the "high molecular weight" (HMW) and the "low molecular weight" (LMW) antigens. The onset of synthesis of the LMW antigens occurs within the first 2 hr after infection, at a time preceding replication of viral deoxyribonucleic acid (DNA). Synthesis of the HMW proteins, on the other hand, is a late function, beginning after the onset of viral DNA replication. These antigens, therefore, will be referred to as "late" proteins.

In addition to certain virus-induced enzymes (4, 6-8), four "early" proteins have been identified

by immunoelectrophoresis. Evidence is presented which would indicate that at least three of these antigens are virus-structural components, produced in excess (19). A number of late proteins, also virus-structural components, have been similarly identified. The present communication describes the further separation and characterization of the antigens comprising the "late" protein fraction, and presents evidence pertaining to the position of the "early" and "late" proteins within the structure of the mature virion.

MATERIALS AND METHODS

Procedures employed for the cultivation of KB cells, production of vaccinia virus (IHD⁻ strain), and preparation of the "early" (LMW) and "late" (HMW) soluble antigen preparations have been described in detail elsewhere (1). Virus obtained from homogenates of infected KB cells was purified in the manner described by Joklik (4) and then was banded in 25 to 40% sucrose density gradients two additional times to remove contaminating soluble antigens. Immuno-

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logical procedures (complement fixation, virus neutralization, immunoelectrophoresis, and immunodiffusion) utilized for these studies have also been described in previous communications (1, 19), as have the methods employed for the production of antigen-specific antisera.

Preparation of virus-specific proteins and viral subunits. Virus structural proteins were obtained by two methods. The first of these entailed degradation of highly purified virus by sodium dodecyl sulfate at a pH of 10.5. This procedure, which resulted in the total dissolution of the virus particles without destroying the antigenicity of the component proteins, has been described in detail in a previous communication (19). The product is referred to as degraded virus. The second procedure employed to obtain virus structural proteins involves a partial degradation of the virions to yield "coat" proteins and virus "cores." Highly purified virus was treated with a nonionic detergent (Nonidet P-40, Shell Chemical Co., Ltd. London, England) and mercaptoethanol in the manner described by Easterbrook (3). After extensive dialysis to remove detergent and mercaptoethanol, the treated virus suspension was layered onto 36% sucrose in 0.001 M tris(hydroxymethyl)aminomethane (Tris), pH 8.0, and was centrifuged for 80 min at $26,000 \times g$. The pellet, consisting of "cores," was washed twice by centrifugation ($26,000 \times g$, in 0.001 M Tris buffer, pH 8.0). Prior to use, these preparations were sonically treated (60-w MSE ultrasonic disintegrator) for 5 min to disrupt the "cores" (3). After this treatment, the small amount of intact virus remaining was removed by centrifugation, and the disrupted "core" material was employed to immunize rabbits. The infectivity of core preparations before sonic treatment was variable, ranging from 0.0001% of the original titer to no detectable infectivity. The 36% sucrose supernatant fluid containing the outer components of the treated virus was dialyzed against 0.001 M Tris buffer (pH 8.0) to remove sucrose and then was concentrated by vacuum dialysis to a protein concentration of about 500 $\mu\text{g}/\text{ml}$. This material, referred to as "coat" protein, exhibited no infectivity.

LS complex. In 1936, Cragie and Wishart identified a vaccinia-induced soluble antigen and described a simple method of isoelectric precipitation for concentrating and purifying this material (2). This material, believed at that time to represent the only soluble antigen, was termed the LS antigen. Subsequent work by Shedlovsky and Smadel led to the conclusion that the LS antigen was a single protein capable of eliciting two distinct antibodies (17). More sophisticated techniques (immunodiffusion, immunoelectrophoresis) that have become available in the intervening years reveal that the soluble antigen fraction described by Cragie and Wishart (2), by convention the LS antigen, is actually a complex of antigens numbering at least two and perhaps more (1, 11, 18). It is doubtful for this reason whether it is appropriate any longer to refer to this material in the singular as the LS antigen. To preserve the original terminology, which has gained wide acceptance and has historical significance, we have chosen to refer to these antigens collectively as the LS complex, regardless of the means by which they are obtained (1).

RESULTS

Fractionation of the "late," virus-induced antigens by gel-filtration. Preparations of "late" antigens were obtained by gel-filtration with the use of Sephadex G-200 as previously described (1). Examination of these preparations by immunoelectrophoresis revealed three precipitin arcs (Fig. 1). Two of these migrate towards the anode and are immunologically identical to the LS complex. The third, less prominent arc, which we have designated as the G antigen, appears to be basic in nature, inasmuch as it migrates towards the cathode under the conditions employed (pH 8.5). This latter antigen bears no immunological relationship to the LS complex, the "early" antigens, or normal host-cell antigens. To characterize the G antigen further, efforts were undertaken to separate this material from the other "late" virus-induced proteins. Concentrated "late" antigen preparations (5 to 7 mg/ml) were passed through Biogel 300 columns (Bio-Rad, Richmond, Calif.) Figure 2 illustrates the elution profile of the "late" antigens traced by testing successive eluates for antigen-specific, complement-fixing (CF) activity with two different antisera specific for "late" antigens. Two peaks of CF activity are evident. The first of these, designated as peak 1, eluted with the void volume and fixed complement when tested with antiserum directed against the "late" antigen fraction but did not react with antiserum directed against the LS complex, the components of which have previously been demonstrated to be "late" proteins (19). In contrast, eluates comprising the second peak of antigenic activity fixed complement with both antiserum preparations. Immunological analysis of the peak 2 fraction employing antiserum directed specifically against this material, as well as known anti LS serum, revealed that the peak 2 material is identical to the LS complex prepared by the usual procedures (1, 2). These data indicate that the "late" proteins may be separated into two groups by gel filtration on an appropriate medium: a fraction with a molecular weight of 300,000 or greater, which is immunologically unrelated to the complex, and the LS complex proper, the components of which exhibit molecular weights close to 200,000. To characterize the peak 1 antigens further and to establish the relationship of this material to the G antigen component identifiable by immunoelectrophoresis, antiserum directed specifically against the peak 1 fraction was made. Eluates under peak 1 (Fig. 2) were pooled, concentrated, and subjected to three cycles of centrifugation ($26,000 \times g$ for 30 min) to sediment virus. After each cycle of centrifugation, the top two-thirds of the super-

FIG. 1. Immunoelectrophoretic comparison of the crude virus-induced soluble antigens and the "late" antigen fraction derived from this preparation. In the drawings shown above, the cathode is on the left-hand side of the figure. (A) Crude soluble antigen (900 $\mu\text{g/ml}$); (B) "late" soluble antigen fraction (750 $\mu\text{g/ml}$). The antisera employed are coded as follows: 1 = antiserum against the crude soluble antigen mixture; 2 = antiserum directed specifically against the "late" antigen fraction; 3 = antiserum directed against the LS antigens.

nant fluids were carefully collected and re-centrifuged. Three cycles of centrifugation resulted in a product exhibiting approximately 50% of the original CF activity, and no infectivity

as ascertained by plaque assay before or after three blind passages of the material through KB cell monolayers. This material was employed to make antiserum designated, for the time being, as anti-peak 1.

Relation of peak 1 fraction to the G antigen. Concentrated "late" antigen preparations, LS complexes prepared by various procedures (1), and pooled, concentrated (700 $\mu\text{g/ml}$) peak 1 eluates were compared by immunoelectrophoresis and immunodiffusion. Antisera specific for each of the antigen preparations listed above were employed for these tests. Figure 3 illustrates the results obtained with these techniques. It is apparent from these data that the peak 1 eluates do indeed represent the isolated G antigen component of the "late" protein fraction. It is notable also that the use of antiserum directed specifically against the G (peak 1) material reveals that this fraction is comprised of two antigenic moieties. As expected, neither of the G antigens exhibited any immunological relationship to the LS complex, the "early" virus-induced proteins, or normal host-cell components. It would appear, therefore, that gel filtration of the "late" antigens on Biogel 300 permits the separation of this antigen class into the G and LS fractions, each comprised of at least two antigenic

FIG. 2. Gel filtration of the "late" antigen fraction through Biogel 300. Elution profile of complement-fixing activity employing antiserum directed against (●) the "late" antigens and (○) the LS complex. The column used was 2.5×87 cm; flow rate, 8 ml/hr, fraction volume, 3.0 ml. The buffer system was 0.1 M NaCl in 0.05 M phosphate buffer (pH 7.2).

moieties. In view of these data, antiserum directed against the peak 1 material will be referred to as anti-G for the remainder of this communication.

Further characterization of the "late" antigen fractions. One of the biological characteristics of the virus-induced "late" proteins is the ability of this class of antigens to stimulate production of specific virus-neutralizing antibody (1, 19). Separation of the "late" antigens into the G and LS complexes may permit further identification of the antigens responsible for this ability, which, in turn, provides a basis for speculation regarding the position of these antigens in the virus structure. One might expect, for example, that only viral surface proteins would elicit production of neutralizing antibody. The data presented in Table 1 demonstrate that antiserum directed against "early" proteins does not neutralize vaccinia virus. One may suggest, on the basis of these data, that the early antigens represent internal components of the virion. In contrast, antiserum directed against the unfractionated "late" antigens does exhibit neutralizing activity. It is apparent from Table 1 that this activity can be attributed to the ability of the LS components of this antigen class to elicit synthesis of neutralizing antibody, since antibody directed against the remainder of the "late" proteins (the G antigens) exhibits no neutralizing capacity whatsoever. This was a surprising result in view of our previous observation, shared with others, that the LS complex does not elicit production of neutralizing antibodies (1, 20). Our success in this instance is probably related to the fact that 11 to 15 times as much antigen, in terms of CF units, were employed over the course of immunization, thus magnifying the probability of obtaining the active site in an undenatured state. The alternative possibility, i.e., that the rabbits were infected by small amounts (undetected) of residual virus in the antigen preparation, is ruled out by the failure of the resultant antiserum to react with any of the other soluble antigens; infected rabbits produce sera which react with the entire spectrum of soluble antigens. It is further notable that Marquardt and co-workers have recently isolated a soluble antigen fraction that elicits synthesis of neutralizing antibody (12). These antigens, designated e and f by these workers, are similar to the LS complex, both immunologically and chemically. Assuming that only the surface proteins of the virion are involved in the neutralization reaction, it would appear that one or both of the LS antigens represent surface components of the virion, a conclusion which is consistent with the observations of others (2, 11). On this same basis, the

FIG. 3. Relation of the peak 1 (Biogel) fraction to the G antigen. (a) Comparison of the peak 1 antigens with "late" and LS complex by immunoelectrophoresis. The cathode is represented on the left-hand side of the above drawings. Antisera in troughs are coded as follows: 1 = antiserum versus "late" antigen fraction; 2 = antiserum versus the peak 1 (Biogel) fraction; 3 = antiserum versus the LS complex. (b) Comparison of the peak 1 (Biogel) fraction with the "late" antigens and the LS complex employing immunodiffusion.

TABLE 1. Virus neutralizing capacity of various antisera specific for soluble-antigen fractions

Antiserum versus	Relative neutralizing titer ^a
"Late" antigen class.....	1:4,096
(a) G antigen fraction (peak 1, Fig. 2).....	<1:16
(b) LS complex	
1. Prepn 1 ^b	1:2,048
2. Prepn 2 ^c	1:2,048
"Early" antigen class.....	<1:16

^a Arbitrarily chosen as the greatest dilution of serum resulting in a 70% or greater reduction in plaque titer (200 plaque-forming units used per test).

^b LS complex prepared by classical acid-precipitation technique.

^c LS complex prepared by gel-filtration (Biogel 300) of "late" antigen class (peak 2, Fig. 2.)

data presented in Table 1 suggest either that the G antigens are internal virus proteins or, alternatively, that, although virus-induced, the G antigens are not virus structural components. This possibility was further investigated.

Relation of the "early" and "late" soluble antigens to virus structure. The relationship of soluble antigens to virus structural components was investigated in two ways, both dependent upon the use of virus structural proteins, obtained from the complete degradation of highly purified virus, and virus subunits ("cores"), obtained from the partial degradation of highly-purified virions (see Materials and Methods). In the first instance, antisera prepared against the various soluble antigen preparations were tested for their ability to react with virus structural proteins and subunits ("cores"). Alternatively, antisera prepared against virus structural components and virus "cores" were tested against the various soluble antigen fractions. It is possible in this fashion to determine which of the soluble antigens represent virus structural components and, to a limited extent at least, to learn something about the position of these components within the structure of the virion. It is well established that a number of virus-induced enzymes are produced early in the course of infection. Available evidence suggests that two of these early enzymes, thymi-

dine kinase and DNA polymerase, are coded for by the virus genome (7). We have previously demonstrated that none of the four early antigens that we have identified by immunodiffusion and immunoelectrophoresis is thymidine kinase; nor is it likely that DNA polymerase is included among these antigens (19). Comparison of virus structural proteins, obtained by degradation of purified virus, with the "early" soluble antigen fraction indicates quite clearly that three of the "early" antigens thus far identified are immunologically identical to virus structural components (Fig. 4a). The data presented in Fig. 4b indicate that these same "early" proteins are recognizable in virus "cores" but do not occur, in detectable quantities at any rate, in the "coat" protein fraction obtained from the controlled degradation process employed to make "cores." A comparison of the "late" antigens with virus structural proteins reveals that all of the "late" antigens are immunologically identical to virus structural components (Fig. 4c). With one exception, these antigens appear to be limited to the outer ("coat") components of the virus (Fig. 4d). A single "late" protein was consistently found in both the "core" and the "coat" fractions of partially degraded virus (Fig. 4c and d). It can be seen from Fig. 4d that this is one of the two G antigens. Although washing "core" preparations with

FIG. 4. Relation of the "early" and "late" soluble antigens to virus structure as determined by immunodiffusion. Wells containing antisera are shaded. "Early" refers to "early" virus-induced antigen fraction; "late" refers to "late" virus-induced antigens; G refers to the G antigen components of the "late" antigens obtained by gel filtration; d-virus refers to virus structural proteins, both "coat" and "internal," obtained by the complete degradation of purified virus particles (see Materials and Methods); "core" indicates virus "cores" obtained by the procedure described in Materials and Methods; and "coat" refers to the more external or "coat" proteins obtained as a by-product of the preparation of "cores" from purified virus preparations.

0.001 M Tris buffer (pH 8.0) failed to remove the "core-associated" G antigen, prolongation of the degradation process used to obtain "cores" did accomplish this objective. It is suggested, therefore, that the presence of this G antigen in the "coat" preparation is merely a reflection of heterogeneity in the virions with respect to resistance to degradation.

One may conclude from these data that the "early" virus structural proteins represent internal virus proteins. This is in agreement with the observation that these antigens do not elicit production of neutralizing antibody. The antigens of the LS complex of the "late" protein fraction are confined to the outer ("coat") structures of the virion. This is consistent with the data which demonstrate that antiserum directed against this antigen fraction is able to neutralize virus. The G antigens appear to occupy intermediary positions, one more closely associated with the "coat" the other with the "core." In view of the complexity of the vaccinia agent, this would not be surprising and would explain the inability of the latter antigens to elicit synthesis of neutralizing antibody.

DISCUSSION

Although the vaccinia genome is very large, with a molecular weight of the order of 160 million, containing roughly 500 cistrons, only a relatively few virus-induced proteins have been identified in infected cells. These virus-induced proteins are of two types, enzymes whose synthesis begins during the early period of infection (6, 9), and virus structural proteins which vary in their time of synthesis (19, 14). The virus structural proteins can, in turn, be divided into two classes based upon their times of synthesis in the infected cells. These classes have been termed the "early" and the "late" structural proteins. The onset of synthesis of the "early" structural proteins begins prior to replication of viral DNA and continues even in the absence of this event. We are able to identify three such proteins with some certainty, the role of a fourth protein of this class remains unclear. These "early" proteins are of relatively low molecular weight (5×10^4 to 10×10^4), a situation which may be related to the observation that the messenger ribonucleic acid (mRNA) molecules coding for the "early" proteins have been reported to be relatively small (13). The "early" proteins are associated with the internal structure ("cores") of the virion, an observation which is consistent with the inability of these antigens to elicit production of neutralizing antibody, and related perhaps to the fact that synthesis of these molecules closely precedes replication of viral

DNA. Production of the "late" virus structural antigens begins after the onset of viral DNA replication and is dependent upon this event to the extent that inhibition of DNA synthesis by treatment with 5-fluorodeoxyuridine or hydroxyurea prevents the production of the "late" antigens (*unpublished data*). It has been previously noted in this regard that replication of viral DNA was necessary for the formation of a complete spectrum of virus-specific proteins (8, 16). The recent work of Oda and Joklik (13), which indicates that parental or "input" viral DNA transcribes only for "early" mRNA, whereas replicating viral DNA transcribes for "early" as well as "late" mRNA, would appear to bear upon these observations. We have been able, in any event, to identify clearly four "late" proteins. It is probable that there are more, existing, most likely, as minor components. The "late" virus structural proteins may be divided into two groups on the basis of their electrophoretic properties, their apparent molecular weight, and their ability to elicit production of neutralizing antibody. The LS complex, containing a minimum of two components, migrate toward the cathode at pH 8.5, have molecular weights close to 200,000, and elicit production of potent neutralizing antibody. These proteins are found exclusively in the outer ("coat") fraction of the virion and probably, for the reasons listed, represent the outermost layer of the virion; this observation is in agreement with the findings of other investigators (2, 11). The two remaining "late" proteins thus far identified are referred to as the G antigens. The criteria for their identification are as follows: migration towards the cathode under conditions of electrophoresis in agar (pH 8.5); molecular weights of 300,000 or greater; no immunological relationship to the other "late" antigens; and inability to elicit production of neutralizing antibody. One of the G antigens is clearly an internal protein, inasmuch as it is closely associated with the virus "core." The second of the G antigens is found in the proteins "stripped-off" the viral particles by the procedure employed to obtain "cores." Inasmuch as the G antigens do not elicit production of neutralizing antibody, it appears likely that this antigen represents a subsurface component. The procedure employed to obtain viral "cores" leaves the structures known as the "lens bodies" intact (3). The relationship of these structures to the G antigens is being investigated.

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