Herpesvirus Type 2-related Antigens and Their Relevance to Humoral and Cell-mediated Immunity in Patients with Cervical Cancer

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Summary

The central theme of this communication is the interaction of herpes simplex virus type 2 with its host. In addition to the productive infection, we are confronted by latency and, as suggested by recent studies, by cancer. The possible mechanisms of latency and the role it may play as a precursor of carcinogenesis are discussed. If virus is to coexist with its host, a defined level of molecular interaction between host and viral gene products must exist. The association of AG-4 with active tumor growth and its identification as a minor virion protein, also exposed on the surface of the infected cell, open new vistas in the understanding of the role virus-host cell interactions may play in tumor growth. The modulation of the host immune response by the results of this interaction may play a significant role in cancer control. In these terms, the observation that antibody to AG-4 is a macroglobulin and that, therefore, immunity to AG-4 may be T-cell independent, should be given further consideration.

Problems

The biological and morphological characteristics identifying the herpesviruses, large DNA viruses infecting man and all animal species investigated to date, have been reviewed extensively (26, 37). The problems dealt with in this presentation arise from 3 considerations. First, many species including man, can be infected by more than 1 of the herpesviruses. However, in the body, the cells infected by the various herpesviruses are different. In man, HSV-1 and HSV-2 are capable of causing disseminated disease; commonly, however, each virus is preferentially found in a specific organ. HSV-1 is associated with lesions of the eye and of mucocutaneous junctions of the face, and HSV-2, with lesions of the urogenital tract. The possibility that this organ predilection is not only a consequence of the mode of virus transmission but also results from properties (antigenic and/or biochemical) characteristic of each virus type has major implications on their individual relationship to disease and their respective modulation of immune responses. Second, whereas, in vitro, herpesviruses destroy the cells in which they multiply, they are notorious for their ability to persist in the infected host in a latent state, i.e., without causing clinically obvious disease. Recently, they have also been associated with cancer (21) and thus the ability to confer upon infected cells uncontrolled replication and immortality. The products of virus expression during latency, their regulation, and their respective role in modulating host immune responses must be given serious consideration. Finally, high levels of circulating antibody to the various antigens of HSV-1 and HSV-2 are generally observed in most populations and persist for long time intervals. These represent various immunoglobulins and possibly even allotypes, differing in properties and under different control (34). Their presence in the infected host, together with various populations of memory and effector cells possibly regulated independently of the humoral responses, must be interpreted in terms of the multitude of Herpesvirus antigens (type common or specific); their expression during clinical disease, productive, latent or neoplastic; their role in the modulation of immune responses; and the probable in vitro interaction of all these factors. The relevance of these phenomena to the control and therapy of herpesvirus infections and ultimately cancer constitutes the focus of many present investigations. The purpose of this paper is to discuss these problems in light of the information available to date, and to point to avenues of research which might furnish answers to at least some of the questions raised.

Herpesvirus Type and Its Affinity for Various Organs

The significant nucleic acid homology existing between HSV-1 and HSV-2 (29), 2 herpesviruses infecting man, suggests that the 2 viruses may have arisen from the same parent virus, possibly as the result of selective pressure imposed by the type of infected cells, genital or buccal. Such pressures might result from different physiologically and/or genetically determined regulatory mechanisms operating in the mucosal cells of these organs. Indeed, infected cells can select mutants that are best suited to replicate in them. Thus, dog kidney cells cannot support the growth of a HSV-1 strain; however, when passaged serially, they ultimately...
ultimately die due to the replication of mutants capable of multiplying in them (45). These mutants differ from the parent strain in antigenic specificity, thermal stability, and other phenotypic properties indicating that mutation has resulted in protein changes. Protein structure differences between various HSV-1 isolates have been reported (19). If accepted, this hypothesis predicts the formation of: (a) an increased number of defective particles and (b) mutant viruses capable of expressing hitherto repressed genetic information possibly responsible for controlling the rate of cellular DNA synthesis. The cell selective-pressure hypothesis would therefore suggest that the cell itself plays an active, albeit, indirect role in its own transformation, and provides a fascinating explanation for a paradox of the hypothesis that HSV-2 causes cervical cancer, namely, the relatively large populations of HSV-2 seropositive women who do not develop neoplasia (2, 37).

The pertinent facts concerning latency may be summarized as follows. (a) The primary exposure to HSV-1 occurs generally early in life, in approximately 85% of people (26); primary infection with HSV-2 follows puberty (37) and, at least in some populations (28), is relatively rare. (b) Following recovery from the primary infection, a fraction of the population suffers from recurrent lesions appearing at or near the site of the primary infection. (c) Recurrent lesions can be predicted by the individual and are induced by a variety of stress provocations such as sun, strong wind, fever, hormone therapy, menstruation, etc. The predictable character of recurrences and their occurrence always at the same site on the body suggest that they are not due to reinfection but, rather, are derived from an endogenous virus residing in the body in the interim between recrudescences. These conclusions are corroborated by the observation that people suffering from recurrences have high levels of circulating neutralizing antibody. The key problems are the nature of virus expression during latency and its modulation of host immune responses. Specifically bearing on this point are questions on: (a) the mechanisms by which the virus remains in the body, (b) the viral antigens that are expressed during this apparently quiescent interim between recurrences, and (c) the immune response humoral and/or cellular that is generated.

**Mechanisms of Virus Persistence in the Body.** Several lines of evidence suggest that HSV-1 is maintained in sensory neurons. Thus, in rabbits and mice latently infected with HSV-1, viral infection could not be demonstrated in any organ by virological or histological means; virus could be induced to multiply by explanting the surgically removed ganglia and maintaining them for some time in vitro, or by implanting them to virgin animals (52). The high frequency of isolation of HSV-1 from trigeminal ganglia removed from randomly picked cadavers (7) suggests that HSV-2 may persist in the cells it has originally infected.

Two hypotheses have been advanced by Roizman (43) to explain the mechanisms of virus persistence in the host in the absence of obvious disease. The one, designated “dynamic state,” suggests that persistence results from continuous multiplication of the virus in confined pockets of chronically infected tissue. Induction of recurrence would result from an increased susceptibility of these same tissues following stress. It predicts successful isolation of the virus from the tissues in the interim between recrudescences. According to the alternative hypothesis, designated “static state,” the viral genome is maintained in a nonproductive state in the cells it has infected and resumes a productive infection when the cells are induced as a consequence of stress provocations. Although no rigorous differentiation between these 2 hypotheses is possible at present, there is merit in discussing their independent requirements and predictions.

**Dynamic State Hypothesis.** The dynamic state hypothesis is unsatisfactory from 2 points of view. First a characteristic of the productive infection is the appearance of viral antigens on the plasma membrane (20). In cell culture, these surface-modified cells are lysed by convalescent human serum and complement (50). If virus persists in neurons, it is difficult to imagine that chronically infected nerve cells reacting with serum and complement would persist without provoking some clinical symptoms. It cannot be assumed that these patients suffer from an immune deficiency, because they are seropositive (2, 26, 28, 37). The dynamic state hypothesis is also unsatisfactory because it fails to explain how tissues in which virus is continuously replicating become more susceptible after stress provocation.

Despite such apparent paradoxes, experimental evidence
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in support of the dynamic state hypothesis is available. Thus, in rabbits and man with recurrent type HSV-1 disease, virus is shed in the tears far more frequently than indicated by clinical corneal disease. The shedding is associated with a chronic infection of the lacrimal glands and the conjunctiva (27), suggesting virus persists in these cells (rather than in neurons) and according to the requirements and predictions of the dynamic state hypothesis. Similarly, in man, HSV-1 was cultured from oral and nasal secretions (31, 46) of seropositive individuals in the absence of clinical lesions, and HSV-2 was isolated at a relatively high frequency (9) from male genitourinary samples also in the absence of clinical lesions.

Herpesvirus infections occur in mucocutaneous junctions in which the prominent antibody is secretory IgA. It may be postulated that antibody to Herpesvirus in this IgA attaches to the chronically infected cells and restricts virus spread. As IgA cannot fix complement, the infected cells are not lysed but, rather, are protected from the cytotoxic activity of serum IgG. Decrease in secretion (and therefore IgA) levels would result in an immediate burst of virus replication and spread (increased infectivity), followed by cell lysis due to the cytotoxic activity of serum IgG (restriction of infectivity). Significantly, the stress conditions inducing herpetic recurrences share in common the ability to decrease mucus levels. The advantage of such an interpretation of the paradoxes raised by the dynamic state hypothesis is that it is amenable to laboratory investigation.

Static State Hypothesis. The static state hypothesis requires that the viral genome present in the cells express only as much information as is compatible with the survival of the cells. Cell death, defined as the inhibition of host macromolecular functions, occurs early in the virus reproductive cycle (44). If it is virus specified, the only 2 events possibly preceding it are transcription of viral DNA and its translation. Deproteinated HSV-1 DNA is infectious (48), and the viral genome is almost totally transcribed in the absence of protein synthesis (49), suggesting that early in infection the virus may be utilizing a host polymerase. According to these interpretations, the static state hypothesis predicts that, in cells harboring the virus, early transcription of viral DNA does not occur. This could be due to the fact that: (a) the cell lacks the specific transcriptase capable of reproducing the virus, (b) the transcriptase is inactive, possibly because the cell is nonreplicating; significantly, neurons are such cells, and (c) a cellular repressor blocks its initiation. Following exposure to stress, either transcriptase activity is regained or the repressor breaks down.

Evidence in support of the static state hypothesis comes from the work of Stevens and Cook (52) with HSV-1, indicating the absence of virus and virus antigens in infected ganglia before stress induction, and that of Aurelian et al. (6) with HSV-2, indicating the absence of virus and virus antigens in cervical tumor cells grown in culture prior to exposure to conditions of stress. Ultimately, however, the best evidence will depend on the in vitro establishment of a latent culture in which virus replication could be induced and repressed at will, and in which infectivity could not be demonstrated during the repressed stage.

The possibility that the cell of virus persistence is individually determined and that the mechanism of latency is a function of the type of cell (neuron or epithelial) in which virus persists cannot be excluded at present.

Immune System in Latency. The specific virus polypeptides that are immunogenic, modulating humoral and/or cellular-type responses, are still unknown. However, recent studies indicate that the immune system plays a role in the maintenance of latency. Stevens and Cook (53) have shown that IgG in latently infected mice prevents total viral reactivation in transplanted syngeneic latently infected ganglia. However, Lodmell et al. (32) suggested that both the humoral and the cellular immune systems must operate together in a synergistic fashion to suppress herpesvirus infections. Indeed, the data of Wilton et al. (57) indicate that predisposition to recurrent lesions is a function of the absence of macrophage-migration inhibition factor and lymphocyte cytotoxin. Immunosuppressive therapy and syndromes associated with alteration of the humoral and cell-mediated immune systems (18) increase the number and/or severity of lesions due to herpesvirus recurrences; and, finally, lymphocytes, independently or in concert with macrophages, may produce interferon, resulting in reduced or limited viral replication in neighboring uninfected cells (42).

HSV-2 and Squamous Cancer of the Human Cervix

The difficulty in establishing the etiological role of HSV-2 in human cancer arises from 3 considerations. First, the virus is ubiquitous, infecting between 30 and 70% of the people studied (28), whereas the cancer is relatively rare. Second, unlike the situation in acute infections, a long time interval lapses between time of infection and development of cervical neoplasia (38), rendering prospective epidemiological studies difficult to perform. Finally, in view of the nature of the host, experimentation that might lead to more acceptable evidence is rendered impossible. Assuming that HSV-2 causes cervix cancer, 3 questions arise. First, does the virus cause the transformation of the cell from normal to neoplastic or does it act upon the transformed cell as a selective agent leading to the establishment of a neoplastic alone? Second, if the virus transforms the cell, is it latent in it before transforming it? Finally, since the stress conditions capable of inducing virus multiplication still occur even after transformation, why is it they no longer induce virus replication and cell death? The question of the role played by HSV-2 in the original transformation of the cell has infected cannot be unequivocally answered. In animal models, in vitro transformation by inactivated HSV-2 (and HSV-1) has been reported. However, with 1 exception (40), transformed cells contain the C-type virus genome (17), raising doubts as to the real involvement of HSV. This is further questioned by the failure of inactivated virus to induce cellular DNA synthesis (35). Also there still is some controversy as to the criteria acceptable as indicative of transformation. With the exception of 1 oncogenic clone (41), HSV-2-induced transformation has been defined as the presence, in the cells, of virus antigens and virions in the absence of infection (12).

Precancerous lesions of cervical carcinoma are easily identifiable, and it is conceivable that atypia is actually an in...
vivo counterpart of in vitro transformation. Evidence that HSV-2 causes atypia is indirect. It consists of studies indicating that (a) infection with the virus precedes even its earliest detectable form (2), (b) women with cytological evidence of HSV-2 infection develop cervical atypia at a higher frequency (38), and (c) the presence of HSV-2 antigens in the dysplastic cells from 80% of cervical atypia cases (4), a criterion similar to that used for most in vitro transformants.

Considering the ubiquitous nature of the recurrence-inducing stress conditions, why is it they do not act upon the transformed cells? Possibly, only cells maintaining the appropriate defective genome become transformed. This interpretation predicts that (a) functional and defective viral genomes can be maintained in a latent state by cells other than neurons, (b) defective virions are produced during productive infection, and (c) defective genomes can transform cells. Bronson et al. (8) have shown that defective DNA is produced and incorporated into virions in cells infected at a high multiplicity of infection. The nature of defectiveness remains to be established (11). Also, only a fragment of the HSV-2 DNA was found in 1 cervical tumor biopsy (16). However, the presence of complete HSV particles in 2 of 14 cervical tumors (22), argues against this interpretation. Absence of viral DNA in cells transformed in vitro (11) is more difficult to interpret, as they contain small amounts of mRNA (11) and virus particles (40). Another possibility, not necessarily discounted by the susceptibility of cervical tumor cells to reinfection with HSV-2 (6), is that transformed cells contain host-to-virus factors ("repressors") that block virus multiplication.

Precancerous lesions (atypia and carcinoma in situ) can regress or progress to invasive cervical cancer (25). The isolation of infectious HSV-2 from cultured cells obtained from a precancerous lesion (6) and the observation that it had persisted according to the predictions of the static state hypothesis, argue in favor of the interpretation that HSV-2 is latent in the cells it transforms. In order to prove unequivocally that latency is a precursor step to transformation, it remains to establish bona fide latent cultures in vitro, define the operative mechanisms that will transform these cells, determine whether transformation requires the presence and maintenance of the same sequences of viral DNA as those present in latency, and show that all cancers associated with this virus contain at least some of the same set of DNA sequences.

**Virus Expression in Cervical Tumor Cells.** In light of the considerations discussed above, studies were designed to enquire into the nature of the products of translation of the viral genome in cervical tumor cells. Originally, we reported that HSV-2 causes transformation in an artificial mixture with HSV-MP (2) by the multiplicity analysis previously described (2), and (c) stain HSV-2 cells infected with HSV-2 for 4 and 24 hr by the indirect immunofluorescent procedure, using Evans blue as a counterstain. The results of these studies indicated that AG-4 antigen is IgM, whereas antibody to HSV-2 resides in the IgG fraction if assayed by complement fixation and immunofluorescence, and in both the 7 S and 19 S immunoglobulins, if assayed by neutralization (3).

The effect of physicochemical criteria, such as antigenic structure and size, on the predilection of some antigens to elicit only 19 S antibodies, even after many years, is not known (33). IgM is the preferential response to a sarcoma-specific antigen (14), and agglutinins for sheep erythrocytes in sera of patients with infectious mononucleosis are also macroglobulins (30). In view of the tumor specificity of AG-4, it may be of particular significance that antibody to AG-4 is IgM, as it suggests that immunity to AG-4 may be T-cell independent (36). The exact significance of this observation in terms of tumor growth and/or control must await further investigation.

**Cellular Location of AG-4**

Various dilutions of IgM fractions were mixed with a HSV-negative human serum (serving as a source of complement) and used in ACIF staining of HEP-2 cells infected with HSV-2 for 4 and 24 hr. These periods are optimal for the synthesis of AG-4 and virus, respectively. IgM from all AG-4-positive and 2 of 4 AG-4-negative sera gave rise to cytoplasmic and surface fluorescence (Fig. 1) on both 4- and 24-hr-infected cells. The surface nature of this fluorescence was confirmed by staining unfixed cells. For most sera, antibody titers calculated as the highest staining IgM dilution, were similar for the 4- and 24-hr-infected cells.

Virus-coded glycoproteins common to infected cell membranes and virion envelopes have been described (20); however, AG-4 must be distinct, since it does not block serum-neutralizing potential (5). To explore this interpretation, IgM fractions were adsorbed prior to ACIF assays with: (a) 24-hr-infected HEP-2 cells, (b) virus pelleted by centrifugation for 1.5 hr in a Spinco SW 27 rotor at 25,000 rpm, or (c) 4-hr-infected HEP-2 cells. The results (Table 1) indicate that, following adsorption with virions or 24-hr-infected cells, the IgM from AG-4-positive sera become negative for 24-hr-

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*1 A laboratory variant of herpesvirus type 1.*
infected cells but maintain their ability to stain a small number (2 to 5%) of HEp-2 cells infected with HSV-2 for 4 hr. IgM from AG-4-negative sera no longer stain 24- or 4-hr-infected cells. Following adsorption with cells infected for 4 hr IgM from both AG-4-positive and -negative sera does not stain 4- or 24-hr-infected cells. It may be concluded that the ability of IgM to fix complement with AG-4 correlates with its ACIF staining potential for 2 to 5% of the 4-hr-infected cells and that AG-4 is located on the cell surface. However, unequivocal evidence to this effect must await the preparation of monospecific sera.

AG-4: A Structural Viral Protein

The experiments described in the subsequent sections were designed to enquire into the nature (viral or cellular) of AG-4.

In order to determine the number, electrophoretic mobility, and hence (47, 56) the molecular weights of the virus-specific proteins in the purified virions, virus was prepared in HEp-2 cells labeled with [35S]methionine between 6 and 24 hr postinfection and purified according to the method of Spear and Roizman (51). Briefly, packed cells were disrupted by Dounce homogenization, and sucrose was added to yield a final concentration of 0.25 M. The cytoplasm was separated from the nuclei by centrifugation at 15,000 rpm for 10 min in a PR-2 International refrigerated centrifuge, and the cytoplasmic extract was centrifuged on 2 consecutive Dextran-10 gradients (1.04 to 1.09 g/ml) at 20,000 rpm for 1 hr in a Spinco SW 27 rotor. This procedure was shown to separate virions from soluble proteins and most cellular membrane vesicles which remain on top of the gradient, and from aggregates of virions, cytoplasmic organelles, and large debris that pellet. The virions found in a diffuse light-scattering band just above the middle of the tube were collected, made 0.5 M with respect to urea, and treated sonically for 5 sec to dissociate aggregates of virus particles and host membrane vesicles. Following dilution with 0.01 M Tris buffer (pH 7.4), they were pelleted by centrifugation at 25,000 rpm for 1.5 hr in a Spinco SW 27 rotor. Immediately prior to electrophoresis, the proteins in the virus pellets were denatured and solubilized by the addition of small volumes of 0.05 M Tris-hydrochloride (pH 7.0), 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, and 0.005% bromothymol blue, followed by boiling for 2 min. The solubilized proteins (100 to 150 µg) were subjected to electrophoresis on acrylamide gels, utilizing the discontinuous buffer system modified by the inclusion of sodium dodecyl sulfate (13). The 8.5% concentration of acrylamide was chosen as it gives the best overall resolution of virus proteins (51). Autoradiography of gel cylinders cut in half longitudinally was done according to the method of Fairbanks et al. (15).

The yield and purity of the virion preparations were determined by negative-stain electron microscopy and by estimates of the recovery of infectivity and virus-specific label. The virus band consisted almost exclusively of intact enveloped nucleocapsids with very rare, if any, membrane contaminants. By monitoring the purification procedure for
Table 1
ACIF staining of HSV-2-infected HEp-2 cells with immunoglobulins adsorbed with HSV-2

<table>
<thead>
<tr>
<th>Immunoglobulin</th>
<th>ACIF of HSV-2-infected HEp-2 cells</th>
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<tbody>
<tr>
<td></td>
<td>4 hr</td>
</tr>
<tr>
<td>25 IgM</td>
<td></td>
</tr>
<tr>
<td>Unabsorbed (1/40)</td>
<td>+ (9)</td>
</tr>
<tr>
<td>Adsorbed with HEp-2 cells</td>
<td>+ (9)</td>
</tr>
<tr>
<td>HEp-2-HSV-2 for 24 hr</td>
<td>+ (5)</td>
</tr>
<tr>
<td>HEp-2-HSV-2 for 4 hr</td>
<td>-</td>
</tr>
<tr>
<td>8 IgM, 10 IgM</td>
<td></td>
</tr>
<tr>
<td>Unabsorbed (1/2)</td>
<td>+ (6)</td>
</tr>
<tr>
<td>Adsorbed with HEp-2 cells</td>
<td>+ (4)</td>
</tr>
<tr>
<td>HEp-2-HSV-2 for 24 hr</td>
<td>-</td>
</tr>
<tr>
<td>17 IgM</td>
<td></td>
</tr>
<tr>
<td>Unabsorbed (1/10)</td>
<td>+ (16)</td>
</tr>
<tr>
<td>Adsorbed with HEp-2 cells</td>
<td>+ (12)</td>
</tr>
<tr>
<td>Pelleted HSV-2</td>
<td>+ (2)</td>
</tr>
<tr>
<td>HEp-2-HSV-2 for 24 hr</td>
<td>-</td>
</tr>
<tr>
<td>160 IgM</td>
<td></td>
</tr>
<tr>
<td>Unabsorbed (1/4)</td>
<td>+ (4.1)</td>
</tr>
<tr>
<td>Adsorbed with HEp-2 cells</td>
<td>+ (4)</td>
</tr>
<tr>
<td>Pelleted HSV-2</td>
<td>+ (1.7)</td>
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<tr>
<td>HEp-2-HSV-2 for 4 hr</td>
<td>-</td>
</tr>
<tr>
<td>63 IgM</td>
<td></td>
</tr>
<tr>
<td>Unabsorbed (1/10)</td>
<td>+ (10)</td>
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<tr>
<td>Adsorbed with HEp-2 cells</td>
<td>+ (10)</td>
</tr>
<tr>
<td>Pelleted HSV-2</td>
<td>+ (3)</td>
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<tr>
<td>HEp-2-HSV-2 for 4 hr</td>
<td>+ (1)</td>
</tr>
<tr>
<td>63 IgG</td>
<td></td>
</tr>
<tr>
<td>Unabsorbed (1/30)</td>
<td>+ (12)</td>
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<td>Adsorbed with HEp-2 cells</td>
<td>+ (15)</td>
</tr>
<tr>
<td>Pelleted HSV-2</td>
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<tr>
<td>HEp-2-HSV-2 for 4 hr</td>
<td>-</td>
</tr>
<tr>
<td>12 IgG</td>
<td></td>
</tr>
<tr>
<td>Unabsorbed (1/60)</td>
<td>+ (10)</td>
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<td>Adsorbed with HEp-2 cells</td>
<td>+ (7.8)</td>
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<tr>
<td>Pelleted HSV-2</td>
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<td>HEp-2-HSV-2 for 4 hr</td>
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<tr>
<td>HEp-2-HSV-2 for 24 hr</td>
<td>-</td>
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<tr>
<td>25 IgG</td>
<td></td>
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<tr>
<td>Unabsorbed (1/40)</td>
<td>+ (5)</td>
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<td>Adsorbed with HEp-2 cells</td>
<td>+ (5)</td>
</tr>
<tr>
<td>Pelleted HSV-2</td>
<td>-</td>
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</table>

* Numbers preceding Ig, patient number.

b Dilution of immunoglobulin.

* Numbers in parentheses, percentage of staining cells in each preparation.

Humoral and Cell-mediated Immunity

Analyses of the virion proteins by autoradiography following solubilization and electrophoresis are highly reproducible. They indicate there are 24 virus-specific proteins in the virions designated VP 1 through VP 24 (Fig. 2). Molecular weights of the viral proteins were estimated by determining their migration rates relative to proteins the molecular weights of which are well characterized (47). In 8.5% acrylamide, the standard proteins larger than 100,000

![Fig. 2. Autoradiograms of electrophoretically separated [35S]methionine-labeled virion polypeptides (purified from cells and labeled 6 to 24 hr postinfection). Solubilized labeled virions were subjected to electrophoresis in 8.5% gels and processed for autoradiography. A, reproduction of photograph of the original autoradiogram; B, absorbance profiles of the same autoradiogram. Virus-specific protein bands have been assigned numbers from 1 to 24 (VP 1 to VP 24).](image-url)
daltons migrate faster than would be expected if they had 
obeyed the inverse logarithmic function between molecular 
weight and relative migration rate. However, a smooth 
curved line was drawn to fit the points, and molecular 
weights were estimated from it (51). The average molecular 
weights of the viral proteins range from 16,000 to 250,000 
daltons. These results agree with those obtained by Spear 
and Roizman (51) for HSV-1, both with respect to the num-
ber of virion proteins and the range of molecular weights. 
On the other hand, relatively little similarity is observed in 
the molecular weights and abundance of the individual 
polypeptides as reported by Honess and Roizman (23).

Analyses of polypeptides made in HEP-2 cells infected 
with HSV-2 for 4 hr and used in AG-4 assays revealed the 
presence of 53 ICP's of which, 24 corresponded in electro-
phoretic mobilities to the virus proteins, subjected to elect-
rophoresis simultaneously. Accordingly, to correlate AG-4 
activity with a specific polypeptide, it seemed reasonable to 
calculate the polypeptide profiles of cell extracts with and 
without AG-4 activity. This approach should cause the de-
pletion of specific polypeptides that can be reproducibly 
associated with the loss of AG-4 complement-fixing activi-
ty.

Effect of Inhibition of Infected Cell Protein Synthesis on 
Subsequent Synthesis of AG-4. The experiments described 
in this section deal specifically with the synthesis of virus 
polypeptides immediately after removal of cycloheximide 
present from the time of infection. HEP-2 cells were infected 
with 10 plaque-forming units of HSV-2 per cell and incu-
bated at 37° for 8 hr in the presence of 50 μg of cyclohexi-
mide per ml. At this time, they were overlayed with minimal 
esential medium containing [14C]methionine (10 μCi/ml) 
and 10 μg of actinomycin D and reincubated for 4, 6, and 8 
hr. The rationale for this approach, based on the studies of 
Hones and Roizman (24) with HSV-1, is 2-fold. First, the 
HSV-1 polypeptides made immediately upon removal of cy-
cloheximide represent a subset of the ICP made in un-
treated cells at this time. They correspond to proteins nor-

mally made early in infection and do not require prior in-
fected cell protein synthesis in order to be made. Second, 
actinomycin D prevents or reduces the decline in the rates 
of synthesis of this subset of proteins, at the same time 
precluding the synthesis of other sets of viral polypeptides. 
Complement-fixing activity with AG-4-positive but not -nega-
tive sera is borderline (10% fixation) in HEP-2 cells infected 
with HSV-2 and exposed to actinomycin D for 8 hr after 
cytoheximide removal and quite high (20 to 30% fixation) in 
similarly infected cells exposed to actinomycin D for 4 or 6 
hr.

Autoradiograms of the electrophoretically separated poly-
peptides present in these cells are shown in Fig. 3, together 
with polypeptides labeled in uninfected cells treated and 
untreated in a similar fashion. Comparison of these samples 
showed that most ICP's made during the pulse interval after 
removal of cycloheximide cannot be differentiated from the 
polypeptides of the similarly treated mock-infected cells. 
The exception is 7 ICP's unique to the infected cells and 
respectively designated ICP 7, VP 4, VP 5, ICP 25, ICP 26, VP 
12, and VP 14, according to their correspondence in elec-
trophoretic mobility to polypeptides from purified virions (VP)

or from untreated infected cells labeled at the same time 
intervals in the virus growth cycle (B. C. Strnad and L. 
Aurelian, in preparation).

Of major significance in terms of the interpretation of 
these results is the observation that, whereas the relative 
abundance of 6 of the 7 ICP's unique to the infected cells is 
similar in preparations labeled for 4, 6, and 8 hr after re-
moval of cycloheximide, VP 4 is made in largest amounts in 
cells exposed to actinomycin D for 4 and 6 hr. In view of the 
borderline (10%) complement-fixing AG-4 activity of the 
extracts from the cells exposed to actinomycin D for 8 hr 
after removal of cycloheximide, the data suggest that com-
plement-fixing ability with AG-4-positive cervix cancer sera 
may be associated with the virion polypeptide designated 
VP 4. The observation that treatment of uninfected cells 
with cycloheximide does not have a marked selective effect 
on the polypeptides made after drug removal is not unex-
pected (24). However, the presence of host polypeptides in 
the infected cells treated with cycloheximide was not ob-
served with HSV-1 (24).

Effect of Virus Passage History on Synthesis of VP 4 in 
Presence of Cycloheximide. The next series of experiments 
was designed to consider the possibility that the passage 
history of the cells in which AG-4 is prepared, or of the virus
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strain used to make it, may play a significant role in its synthesis. In view of the possible association between VP 4 and AG-4 complement-fixing activity, experiments essentially similar to those described in the preceding section were performed. Two lines of HEp-2 cells were used: (a) “HEp-2” originally obtained from Microbiological Associates (Bethesda, Md.) and maintained in our laboratory in Medium 199 with 10% calf serum. They were passaged with trypsin and appeared epithelial-like in morphology, and (b) “HEp-R,” morphologically, more fibroblastic-like than the HEp-2 cells, were originally obtained from Dr. B. Roizman (University of Chicago; Chicago, Ill.), grown in minimal essential medium, supplemented with 10% fetal calf serum and transferred with EDTA. The HSV-2 stocks used were: “G,” a virus generally used in AG-4 preparation, originally obtained from Dr. B. Roizman, and passed 60 times in our laboratory in HEp-2 cells at a high multiplicity of infection, and “GW,” a low-passage seed of G-virus, further passed only 3 times in our laboratory in HEp-R cells at a low multiplicity of infection.

Cells were infected with the virus stocks in presence of 50 μg of cycloheximide per ml. After 8 hr, the drug was removed and the infected cells were exposed to [35S]methionine in the presence of actinomycin D for 6 hr. Extracts from these cells were assayed for their ability to fix complement with AG-4-positive sera. Good fixation (38 and 45%) was observed in HEp-2 and HEp-R cells infected with G-virus, whereas extracts from GW-infected HEp-R cells did not fix complement with AG-4-positive sera. The following points were made evident by the analysis of the labeled polypeptides separated by polyacrylamide gel electrophoresis (Fig. 4).

1. The morphological differences between HEp-2 and HEp-R cells are reflected in differences in the species and abundance of their polypeptides.

2. The polypeptides made in HEp-R cells infected with G-virus after removal of cycloheximide are host polypeptides similar to those made in uninfected cells and the same 7 ICP that are also present in HEp-2 cells infected with G-virus. The relative abundance of these ICP is also similar to that observed in HEp-2 cells infected with G-virus.

3. One ICP (VP 4) present in both HEp-2 and HEp-R cells infected with G-virus is not made in HEp-R cells infected with GW and labeled with [35S]methionine for 6 hr in the presence of actinomycin D after removal of cycloheximide. Another ICP (VP 5) is made, but in much lower amounts than in G-virus-infected cells. These observations confirm the association between VP 4 and the ability of infected cell extracts to fix complement with AG-4-positive sera. They further indicate that this is a virus-specified function, apparently affected by virus history passage.

**Immunoprecipitation of 35S-labeled AG-4.** The experiments described in this section are a modification of radioimmunooassays using [35S]-labeled antigen, and AG-4-positive and -negative sera. In an effort to limit the number of ICP's available for precipitation, the antigen is an extract of

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Fig. 5. Autoradiograms of 8.5% polyacrylamide gels containing electrophoretically separated: (a) immune precipitates formed by the addition of AG-4-positive Sera 12 and 9 or AG-4-negative Serum 15 to supernatant fluid from high-speed centrifugation of infected cells lysate labeled with [35S]methionine for 6 hr, starting immediately after removal of cycloheximide; (b) immune precipitates formed by the addition of antiserum specific for SAM proteins (A) or HSV-1 (C), to supernatant fluid (B) from untreated cells labeled with [35S]methionine for the same time interval. Numbers on the sides refer to virus-specific polypeptides precipitated by the various antisera; h represents host polypeptides. Cacx, cervix cancer; Con, control.

it is not precipitated by Sera 9 and 12. Of major significance is the observation that VP 4 is the major polypeptide precipitated by both AG-4-positive sera (Sera 9 and 12), but not by AG-4-negative Serum 15.

Purification of 35S-labeled AG-4. Chromatography on Sephadex G-200 followed by calcium phosphate (Brushite), prepared according to the method of Taverne et al. (55), were used. AG-4 was made in HEp-2 cells infected with G-virus, as previously described (1). Eluates were assayed for complement-fixing activity with AG-4-positive and -negative sera. The AG-4-positive fractions from the Sephadex G-200 column were pooled, dialyzed overnight against 0.005 M phosphate buffer (pH 7.0), and chromatographed on calcium phosphate. Eluates obtained in a step-wise fashion with increasing concentrations of phosphate buffer were dialyzed against Veronal buffer, assayed for complement-fixing activity with AG-4-positive and -negative sera, solubilized, and subjected to electrophoresis on polyacrylamide gels. Two fractions, one eluted with 0.04 M and the other with 0.1 M phosphate buffer, fix complement with AG-4-positive but not with AG-4-negative sera. AG-4 reactivity is not observed in any other fraction. The major complement-fixing activity (55% fixation) resides in the fraction eluted with 0.04 M buffer, whereas only 15% fixation is obtained with the fraction eluted with 0.1 M buffer. Autoradiograms of the polyacrylamide gels of these fractions (Fig. 6) indicate that the 0.04 M eluate contains 13 major polypeptides including VP 4 and VP 5, whereas only 6 major polypeptides are observed in the 0.1 M calcium phosphate fraction. The only polypeptide unique to both these fractions and absent from all others is VP 4. Furthermore, its relative abundance in the 2 fractions corresponds to their complement-fixing ability, which is much higher in the 0.04 M than in the 0.1 M eluate.

The studies described in these sections associate AG-4 complement-fixing activity with VP 4, a virion protein with a molecular weight of 161,000 daltons. The presence of AG-4 on the surface of infected cells does not argue against this interpretation since (a) all virus-specific proteins binding to membranes are also structural proteins of the virus, (b) not all surface HSV-1 antigens are glycosylated, and (c) in the virion, the virus-specific glycoproteins are structural components of the envelope usually derived from the inner lamella of the nuclear membrane (20). The location of VP 4 in the virion is unknown. However, in view of these considerations, and since it does not block the neutralizing potential of anti-HSV-2 sera (5), it can be concluded that VP 4 is a subsurface virion component. Absolute proof that VP 4 is indeed responsible for fixing complement with sera from patients with squamous cervix cancer, but not controls, will depend on further purification of crude AG-4, preparation of monospecific sera, and demonstration of the presence of VP 4 in cervical biopsies. Such studies are now in progress in our laboratory.

The observation that AG-4 is a virion protein encourages the following predictions in terms of the immune response...
in patients with cervix cancer and controls. First, during the primary infection, when lesion is extensive, but not necessarily in recurrent infections in which lesions are minimal, immune responses are expected. Indeed, control HSV-2 seropositive patients are AG-4 negative (1). Second, since neoplastic transformation is associated with unregulated expression of VP 4, AG-4-positive humoral and cellular immune responses are expected in cancer patients. Association of cancer with a positive AG-4 humoral response has been established (1, 5).

Three hypotheses could explain the possible involvement of the cellular response to VP 4 in cervical cancer.

1. The development of cervical neoplasia may be the result of an alteration of the immunological surveillance system (cell-mediated immunity) manifested specifically by an impaired ability to recognize and/or eliminate tumor cells displaying VP 4. It would then follow that women who are seropositive for HSV-2, and who develop cervical atypia, do not exhibit a similar immunological impairment.

2. The development of cervical cancer is accompanied by the appearance, on tumor cells, of VP 4 which, in turn, stimulates clones of antigen-reactive lymphocytes. Implicit in this hypothesis is that lymphocytes from HSV-2-seropositive individuals without evidence of cervical neoplasia would react either minimally or not at all to these antigens.

3. There is neither an impaired nor an enhanced immunological reactivity at the effector lymphocyte level. However, tumor induction is accompanied by the development of specific antibody populations that can act as blocking antibody, either by binding to antigen recognition sites or to VP 4 on the tumor cells, rendering them inaccessible to effector lymphocytes.

The possible implications of these interpretations for cancer control must await further investigation.

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References
Herpesvirus Type 2-related Antigens and Their Relevance to Humoral and Cell-mediated Immunity in Patients with Cervical Cancer

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