

Marek's Disease Herpesviruses

I. Production and Preliminary Characterization of Marek's Disease Herpesvirus A Antigen¹

PHILIP A. LONG,² PARVENEH KAVEH-YAMINI, AND LELAND F. VELICER*

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan, 48824

Received for publication 29 October 1974

A method was developed for the large-scale production of Marek's disease herpesvirus A antigen in duck embryo fibroblast roller bottle cultures in quantities sufficient to permit its purification and characterization. Maximum yield was obtained in serum-free culture medium harvested daily. The Marek's disease herpesvirus A antigen was stable at pH 2.0 and was a glycoprotein based on its sensitivity to trypsin, specific immune co-precipitation of radioactive amino acids and glucosamine, and detection of radioactive glucosamine by immunodiffusion and autoradiography. The antigen aggregated and lost titer upon storage but dissociated readily and regained titer in 1 or 2 M urea and 0.05% Brij 35. Fresh unaggregated antigen or antigen dissociated with urea and Brij 35 sedimented at 3.7S on sucrose gradients. The apparent molecular weight of the glycoprotein antigen was estimated to be 44,800 by gel filtration on Sephadex G-200 in the presence of 2 M urea and 0.05% Brij 35.

Marek's disease herpesvirus A antigen (MDHV-A) is detected by immunodiffusion analysis of both cell extracts and concentrated culture fluid from infected cells using sera from naturally infected chickens (5, 6). In addition to being the antigen most consistently detected by antibody in the sera of naturally infected birds (6), MDHV-A antigen is of interest for several other reasons. Churchill et al. (6) concluded that the simultaneous loss of pathogenicity and MDHV-A antigen production by infected cells after 33 passages in culture are related phenomena, whereas others have reported that they are unrelated (1, 21). Preparations containing unpurified MDHV-A antigen elicit a delayed hypersensitivity response in MDHV-infected chickens (2, 9). The relationship between MDHV-A antigen and its common counterpart (HVT-A antigen), from cells infected with the herpesvirus of turkeys vaccine virus (19, 22, 30), and its role, if any, in protection against Marek's disease remain to be elucidated. Also the potential relationship of MDHV-A antigen to cell surface membrane antigens (4, 18) and virion structural proteins (3) is still unknown.

Adequate assessment of the role of MDHV-A antigen in the above situations will require that it be purified and characterized thoroughly.

¹ Article no. 6964 from the Michigan Agriculture Experiment Station.

² Present address: 3 M Company, Bioscience Research Center, St. Paul, Minn. 55101.

Although some early work was recently reported (20, 23, 24), the antigen is not yet purified and characterized adequately. In this paper we report the production of MDHV-A antigen in quantities sufficient for biochemical and biological analysis and describe some characteristics of the unpurified antigen. A subsequent paper (16) will deal with the purification of the antigen and some additional physical and chemical properties.

(This paper was presented in part at the 72nd and 73rd Annual Meetings of the American Society for Microbiology, 23-28 April 1972, Philadelphia, Pa., and 6-11 May 1973, Miami Beach, Fla., respectively.)

MATERIAL AND METHODS

Preparation and propagation of primary duck embryo fibroblast (DEF) cells. Large-scale preparation of primary DEF cells was by published methods (13, 25, 26), modified to include four or five 20-min trypsinization periods with a trypsin-EDTA solution containing 0.05% trypsin (1:250, Difco) and 0.02% EDTA in a balanced salt solution. Cells not used on the first day were stored on ice for 10 days with 50% or greater viability for use as needed (13). Cells were seeded in 150-mm diameter plastic tissue culture dishes (4×10^7 freshly prepared cells) or large roller bottles (4×10^8 freshly prepared cells) as described (13, 25) with the standard Medium 199 and nutrient mixture F-10 combination (13, 25, 26) containing 2% calf serum.

Virus stock. MDHV strain GA-infected (21) DEF

cells were obtained from K. Nazerian and co-workers (USDA Regional Poultry Research Laboratory) in the 17th passage of virus. After several passages onto normal DEF cells, quantities of stock virus were frozen as infected cells in the 21st passage. The cells from each 150-mm petri dish were stored in a 1-ml ampule. Infected cells were never used later than the 26th passage of virus.

Infection of cell cultures for MDHV-A antigen production. The method for large-scale production of virus-infected cells in large roller bottles (13, 14) was modified only slightly to produce large quantities of MDHV-A antigen. Infection of roller bottle cultures with the cell-associated virus was by virus-infected cells (13, 14) in amounts optimal for maximum cytopathic effect and antigen production within 4 to 5 days. In this study the amount of infecting cells was based on the surface area of confluent monolayers rather than cell counts or titration of virus-infected cells (13). One ampule of the frozen infected cells was used to infect six or seven confluent DEF monolayers (150-mm plate) so maximum cytopathic effect occurred in 3 to 5 (usually 4) days. The infected cells were then trypsinized and reseeded at a sixfold dilution on 36 to 42 confluent DEF monolayers which in turn were used to infect 20 nearly confluent DEF roller bottle monolayers (approximately two plates per roller bottle) after another 4 days.

Harvesting, concentration, and storage of MDHV-A antigen. To harvest MDHV-A in the absence of serum, each roller bottle was washed with three 50-ml volumes of Hanks balanced salt solution at 72 h after infection, and then reincubated with 25 ml of serum-free medium. The serum-free medium was replaced daily for up to 6 to 8 days. The harvested fluid was pooled, centrifuged at 10,000 rpm for 30 min at 4 C in a Sorvall GSA rotor, and then stored at -20 C after adding 0.1 ml of 10% NaN₃ per 100 ml. Samples (25 ml) of each daily harvest were concentrated 50-fold at room temperature by negative pressure dialysis against TES buffer (0.01 M Tris, pH 7.4, 0.05 M NaCl, 0.01 M EDTA) for assay of MDHV-A yield. Stored culture medium containing adequate antigen was filtered through an XM 300 membrane and concentrated 100-fold on a PM 10 membrane by diafiltration.

Preparation of antisera. Chickens infected naturally with MDHV by contact transmission were bled by cardiac puncture to obtain serum with antibody primarily against MDHV-A. By avoiding serum from chickens infected by inoculation of infected DEF cells, antibody to calf serum components and possibly duck cells antigens was avoided. As a further precaution serum from each chicken was tested by immunodiffusion to select those sera that formed visible precipitin lines only with MDHV-A. Although these selected sera appeared monospecific for MDHV-A by visual observation of immunodiffusion lines and were adequate for routine assay, they could not be considered truly monospecific.

Rabbit anti-chicken immunoglobulin (Ig) G serum was prepared with chromatographically purified chicken IgG (Nutritional Biochemical Corp., Cleveland, Ohio). The first injections were in each footpad

with equal portions of an emulsion of 1.25 ml of IgG (2.5 mg of protein) in 1.25 ml of complete Freund adjuvant. Subsequent injections were in the footpads and one other subcutaneous site with an emulsion containing 1.25 mg of protein and incomplete Freund adjuvant. Bleeding was by cardiac puncture at 48-h intervals on 3 different days starting 4 days after each reimmunization.

Immunodiffusion analysis of MDHV-A. Immunodiffusion was performed as described (5, 6, 25) with the following modifications: (i) Noble agar (Difco) was clarified by several washes with distilled water until no further color appeared, one wash with 70% ethanol and one wash with acetone followed by air drying; (ii) agar gels contained 1.2% agar, 8% NaCl, and 0.01 M barbital buffer (pH 7.2), or 0.05 M Tris-barbital buffer (pH 8.8); and (iii) 0.001% NaN₃ was used as a preservative. MDHV-A antigen titers were determined as the reciprocal of the highest two-fold serial dilution that produced a visible precipitin line. For quantitation the titer of a concentrated antigen preparation was assumed to be the number of arbitrary units of MDHV-A per milliliter of concentrate. Relative antigen yield and recovery was determined by multiplying the titer by the volume, in milliliters of each sample.

Assay of MDHV for pathogenicity and virulence. One-day-old chicks (RPL line 15 × 7) were inoculated intraperitoneally with 1 or 2 × 10⁷ MDHV-infected DEF cells and were held in modified Horsfall isolators with approximately equal numbers of uninoculated chicks that served as direct contact controls. All birds were monitored daily for clinical disease and deaths were examined for typical gross and microscopic Marek's disease lesions by established procedures (29, 30), either at the time of death or when the experiment was terminated after 70 days.

Sucrose gradient analysis of MDHV-A. Fresh or stored unpurified MDHV-A concentrated 50-fold by negative pressure dialysis was clarified at 25,000 rpm for 1 h in the SW27 rotor (Beckman) at 4 C. Then 0.4-ml samples were analyzed on 4.8-ml linear gradients of 5 to 20% (wt/vol) sucrose in TES buffer at pH 7.4. When samples were treated with 1 or 2 M urea and 0.05% Brij 35, the respective gradient were prepared with urea and Brij 35 at the same concentration. Centrifugation was for 11 to 12 h at 50,000 rpm in the SW50.1 rotor (Beckman) at 20 C. Separate but identical gradients with bovine serum albumin were centrifuged in parallel for sedimentation coefficient determinations. Approximately 35 fractions (0.14 ml) were collected through the bottom of the tube and assayed for MDHV-A activity by immunodiffusion. At the 1:8 dilution each fraction of MDHV-A gradients was analyzed for optical density at 280 nm. Gradients with bovine serum albumin were diluted eightfold directly and analyzed for optical density. Approximate sedimentation coefficients were calculated by the method of Martin and Ames (17), assuming a $s_{20,w}$ of 4.41S for bovine serum albumin (15).

Molecular weight estimation. Gel filtration was carried out at room temperature in a column (1.2 by 90 cm) of Sephadex G-200 equilibrated with TES

alone or TES, 0.05% Brij 35, and 2 M urea. Samples (0.5 ml) of antigen or marker protein were made to 5% sucrose, layered on the column under the eluting buffer, and then eluted at a flow rate of 10 to 12 ml/h. Two-milliliter fractions were collected and assayed for absorbance at 280 nm and for antigen activity by immunodiffusion. The apparent molecular weight of MDHV-A antigen was determined by extrapolating from a standard curve (11).

Radioactive labeling of MDHV-A. Infected and uninfected DEF roller bottle cultures were labeled with 0.2 μ Ci of a 14 C-labeled amino acid mixture per ml (New England Nuclear, Boston, Mass.) or 2.0 μ Ci of [3 H]leucine (30 to 50 Ci/mmol; New England Nuclear) in 50 ml of serum-free, amino acid-deficient, or leucine-deficient minimal essential medium after preincubation for 8 h to deplete the respective amino acid pools. When the 14 C-labeled amino acid mixture was added, 1/20 volume of normal media was included to provide amino acids not present in the labeling mixture. Infected cells were labeled 120 h postinfection and uninfected cells were labeled 96 h after seeding. The culture medium was harvested 48 h after labeling and processed as described above.

Infected and uninfected roller bottle cultures were also labeled with [14 C]glucosamine (45 to 55 mCi/mmol; New England Nuclear) or [3 H]glucosamine (5 to 15 Ci/mmol; New England Nuclear). Each infected bottle received 0.2 μ Ci of 14 C per ml or 1.0 μ Ci of 3 H per ml in 25 ml of serum-free media just as the cytopathic effect became extensive, usually 96 h postinfection, and labeling continued for 48 h. Uninfected cultures were labeled in parallel. The culture medium was harvested and processed as described above.

Immune co-precipitation analysis of MDHV-A antigen. Radioactively labeled MDHV-A preparations and uninfected cell culture media were incubated with five- and 20-fold greater quantities of MDHV-A-positive chicken serum to demonstrate that antibody excess and maximum precipitation was achieved (28). Equal amounts of MDHV-A-negative sera from specific pathogen-free chickens were used in parallel control reactions. After 2 h at 37 C, rabbit anti-chicken IgG serum (Microbiological Associates, or prepared as described previously) was added at an optimal ratio of six parts to one part chicken serum. After further incubation (12 h at room temperature or 2 h at 37 C), the precipitates were pelleted, washed, and solubilized as described (28). Then 0.1-ml samples of the precipitates and supernatant fluid were spotted on separate filter disks and counted as described below to determine the percentage of radioactivity precipitated (28).

Radioactivity assays. Samples (0.01 to 0.2 ml) from immune co-precipitation analysis or from various labeling or preparative procedures were prepared and assayed by standard procedures (11).

pH treatment. Small samples of a single unpurified antigen preparation in TES buffer were adjusted to the desired pH and same final volume by adding HCl, NaOH, and TES buffer as needed. The samples were held at 4 C for 7 days and analyzed for antigen titer by immunodiffusion after 1, 5, and 24 h, and after 7 days. After day 7, the pH was again deter-

mined and in every sample it had remained within 0.1 to 0.2 pH units of the starting pH.

autoradiography. [14 C]glucosamine-labeled culture medium was concentrated 50-fold by negative pressure dialysis and clarified at 30,000 rpm for 1 h in a SW50.1 rotor to avoid excess nonspecific background radioactivity due to aggregates trapped in the agar surrounding the antigen wells. Immunodiffusion was done with sufficient labeled antigen to insure the formation of a sharp precipitin line within 18 to 36 h which contained at least 200 counts/min. After the precipitin line was formed but still sharp, the agar was washed in 8% NaCl for 3 to 4 weeks to remove nonspecific background radioactivity. When background levels were constant for 3 to 4 days, the agar was fixed in 10% trichloroacetic acids for 1 to 3 h and washed with glass distilled water 4 to 5 h. The precipitin line was photographed during either procedure and the washed agar was air dried on a glass slide overnight, after covering it with moist filter paper to prevent distortion. Dried agar slides were then used to expose X-ray film (Kodak No-Screen) and the film was developed.

RESULTS

Pathogenicity of GA-MDHV. The reported simultaneous loss of pathogenicity of MDHV and loss of MDHV-A production by the infected cells after passage 33 (6) suggested that certain precautions were needed to ensure good antigen yield. Infected cells with virus in passage 28 in culture were assayed for pathogenicity and ability to produce MDHV-A. Clinical disease and gross lesions (Table 1), as well as microscopic lesions (P. Long, data not shown), were found in both inoculated and uninoculated direct contact chickens, indicating that the infected cells used to inoculate birds and the virus released from those birds were pathogenic. For MDHV production passage 26 in cell culture was never exceeded and good antigen yield was obtained.

Optimal conditions for production of MDHV-A antigen. When a careful comparison was made between MDHV-A recovered in the culture medium and cells, the antigen titer was 32- to 128-fold greater in the former (Table 2). Furthermore, elimination of the calf serum from the medium after infection had no significant effect on overall MDHV-A production. Since the cell-associated nature of MDHV results in cell-to-cell spread of infection through a monolayer over several days, antigen production may continue through the infection cycle. Consequently, a study was undertaken to determine the parameters for maintenance of infected monolayers and for optimal production and recovery of MDHV-A. Daily harvests of antigen were optimal and produced nearly eightfold

TABLE 1. Assay of tissue culture passage 28 GA-MDHV for pathogenicity and virulence

| Expt | No. of birds | Treatment | Gross lesions ^a | Deaths ^b | Average days to death ^c |
|------|--------------|-------------------------|----------------------------|---------------------|------------------------------------|
| 1 | 8 | Inoculated ^d | 6/8 | 4/8 | 41 |
| | 9 | Direct contact | 4/9 | 3/9 | 41 |
| 2 | 10 | Inoculated ^e | 10/10 | 10/10 | 47 |
| | 10 | Direct contact | 1/10 | 1/10 | 57 |

^a Number of positive birds/number of experimental birds.

^b Number of specific deaths/number of experimental birds.

^c Days to death for each bird divided by number of deaths.

^d Each chick was inoculated intraperitoneally with 2×10^7 MDHV-infected DEF cells containing virus in tissue culture 28 passage.

^e Each chick was inoculated as above with 10^7 cells.

TABLE 2. Comparison of MDHV-A antigen yield from culture medium and cells in the presence and absence of sera

| Expt | Serum (%) | Antigen titer ^a | | Day of harvest post-infection |
|------|-----------|-----------------------------|--------------------|-------------------------------|
| | | Culture medium ^b | Cells ^c | |
| 1 | 2 | 128 | 4 | 6 |
| | 0 | 64 | 4 | 6 |
| 2 | 2 | 32 | 4 | 6 |
| | 0 | 64 | 2 | 6 |

^a Antigen titers were obtained from culture medium and cells are expressed as indicated in Materials and Methods.

^b The medium from five plates (20 ml/plate) was concentrated 50-fold by negative pressure dialysis to 2 ml and was clarified along with the cells as described above.

^c Cells were scraped from five plates and washed with Hanks balanced salt solution twice, and the final pellet was suspended in 5 ml of Hanks balanced salt solution. Cells were disrupted by dounce (tight pestle) homogenization and centrifuged at 30,000 rpm in a SW50.1 rotor. All clarified cell extracts were then concentrated to 0.5 ml, which represents a fourfold increased concentration in relation to that of the culture medium.

more MDHV-A (relative total antigen; Table 3) over a 6-day interval, when compared to the yield from roller bottles held without a media change for the entire period. Daily harvests

resulted in a 1.2-fold greater yield compared to harvests at 2-day intervals. In preliminary experiments, 25 and 50 ml of serum-free media were required for 24- and 48-h harvest intervals, respectively, for optimal cell maintenance and prolonged MDHV-A production. Although much of the difference in antigen yield may have been due to variation in the condition of the monolayers, it appears that MDHV-A made early in the cycle, when the cells were in good condition, was not accumulated or maintained in the culture fluid over long periods (Table 3).

The 252 U accumulated over six daily harvests (Table 3) were per ml of concentrated medium (see Materials and Methods) and represented two roller bottles. Consequently, the maximum weekly yield was as high as 126 U per bottle. However, daily antigen titers were usually 16 to 32 and the weekly yield was 38 to 62 U per bottle.

TABLE 3. MDHV-A antigen recovery from roller bottle cultures maintained in the absence of serum

| Harvest time days after infection | MDHV-A antigen titer at various harvest intervals ^a | | | |
|-------------------------------------|--|---------------------|---------------------|---|
| | 1 day ^b | 2 days ^c | 3 days ^d | Single harvest on last day ^e |
| 4 | 4 | | | |
| 5 | 8 | 16 | | |
| 6 | 32 | | 64 | |
| 7 | 128 | 128 | | |
| 8 | 64 | | | |
| 9 | 16 | 64 | 8 | 32 |
| Relative total antigen ^f | 252 | 208 | 72 | 32 |

^a Antigen titers were obtained from concentrated serum-free medium pooled from three roller bottles at each harvest time and are expressed as indicated in Materials and Methods. Serum-free medium was added 3 days postinfection and the serum-free media harvest times and intervals were as indicated.

^b 25 ml of serum-free medium per bottle concentrated to 0.5 ml/bottle.

^c 50 ml of serum-free medium per bottle concentrated to 0.5 ml/bottle.

^d 100 ml of serum-free medium per bottle concentrated to 0.5 ml/bottle.

^e 100 ml of serum-free medium per bottle concentrated to 0.5 ml/bottle. In this case the medium was held with only pH adjustment for the entire 6-day period of the experiment.

^f Arbitrary units of antigen per milliliter of concentrated medium was calculated as described in Materials and Methods and the total amount for all harvests was calculated.

Effect of trypsin and dissociating agents on MDHV-A antigen titer. The immunological activity of MDHV-A, in both a partially purified preparation (Table 4) and a crude preparation (L. Velicer, unpublished results), was reduced 94% in 2 h and was completely destroyed within 8 h at 37 C in the presence of trypsin. The control sample did not decrease in titer and the addition of soybean trypsin inhibitor had no effect on the titer.

Concentrated, clarified, and stored antigen, especially if frozen and thawed repeatedly, consistently had reduced titers. One likely explanation was that the antigen aggregated, either with itself or other proteins. Severe aggregation was also a problem in preliminary attempts to purify MDHV-A by isoelectric focusing (16). The reports that Brij 35 alone (10) or in combination with urea (12) could reduce aggregation and precipitation suggested a means for analyzing and purifying MDHV-A more effectively. An aggregated MDHV-A preparation that had undergone a fourfold drop in titer (64 to 16) was dissociated with varying concentrations of urea in the presence of Brij 35 detergent to assess their effect on antigenic activity and to determine if aggregation reduced the antigen titer. The MDHV-A titer increased fourfold after 5 h at 37 C in the presence of 1 or 2 M urea and Brij 35 (Table 5).

Sucrose gradient sedimentation analysis of MDHV-A antigen. To obtain information about the size of MDHV-A and to confirm that the loss in titer described above was probably due to aggregation, concentrated crude antigen preparations were analyzed by sucrose gradient sedimentations. Fresh high titered preparations of antigen sedimented as a single peak with a sedimentation coefficient of 3.5 to 3.8S and with

TABLE 4. *Effect of trypsin on MDHV-A antigen titer*

| Treatment ^a | Antigen titer at various times of treatment (hours) | | | |
|------------------------|---|----|-------|----|
| | 0 | 2 | 4 | 8 |
| Trypsin | 64 | 4 | Trace | 0 |
| Untreated | 64 | 64 | 64 | 64 |

^a 2 mg of trypsin (Worthington, Freehold, N.J.) was added to 1.0 ml of concentrated antigen partially purified by DEAE-Sephadex column chromatography (16) and was incubated at 37 C. A portion was removed at the specified times and the trypsin was inactivated with an equal amount of soybean trypsin inhibitor (Worthington). The untreated sample was handled in an identical manner except that the trypsin solution was replaced by an equal volume of TES buffer. Immunodiffusion analysis was then carried out as described in Materials and Methods.

TABLE 5. *Effect of dissociating agents on MDHV-A antigen titer*

| Treatment | Antigen titer at various times of treatment (hours) | | | | | | |
|------------|---|----|----|----|----|-----------------|----|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| Untreated | 16 | 16 | 16 | 16 | 16 | 16 | 16 |
| 2.0 M urea | 16 | 32 | 32 | 32 | 64 | 64 | 64 |
| 1.0 M urea | 16 | 32 | 32 | 32 | 32 | 64 ^b | 64 |
| 0.5 M urea | 16 | 32 | 32 | 32 | 32 | 32 | 32 |

^a All treated samples contained 0.01 M Tris (pH 7.4) and 0.05% Brij 35, and the urea concentration varied as indicated. All samples were held at 37 C for the number of hours indicated.

^b Immunodiffusion—trace reaction.

a noticeable shoulder on the leading edge, but no antigen was detected in the lower part of the gradient (P. Long and L. Velicer, data not shown). When the stored preparation with the reduced titer (Table 5) was analyzed in the absence of dissociating agents, the characteristic peak was still present (Fig. 1A). However, the leading shoulder was more pronounced, the antigen was in the lower fractions of the gradient, and a significant amount of antigen activity was in the pellet. When the same preparation was treated with 1 or 2 M urea and Brij 35 detergent for 4 to 6 h (Table 5), the possible aggregates were dissociated and all of the MDHV-A was in a single sharp peak at about 3.7S (Fig. 1B). The average sedimentation coefficient was 3.7S (average of six determinations).

Gel filtration analysis of MDHV-A antigen. Unpurified, high titered antigen (up to 450 U/0.5 ml loading volume) had an apparent molecular weight of 44,800 ($\pm 1,100$, average of three determinations) in the presence of 2 M urea and 0.05% Brij 35 (Fig. 2B) based on the elution volume of the sharp symmetrical antigen peak. In the absence of urea and Brij 35, the antigen eluted in a lower and broader peak skewed toward the high-molecular-weight region (Fig. 2A). Apparent molecular weight estimates based on the center of these broader, asymmetrical peaks were less precise but a value of approximately 55,800 ($\pm 3,100$, average of three determinations) was obtained.

Immune co-precipitation analysis of MDHV-A antigen. Immune co-precipitation of amino acid- and glucosamine-labeled antigen preparations was used to show that MDHV-A contains carbohydrate as well as protein and to attempt quantitation of these molecules. The specificity and sensitivity (28) of the method permitted analysis of unpurified or only par-

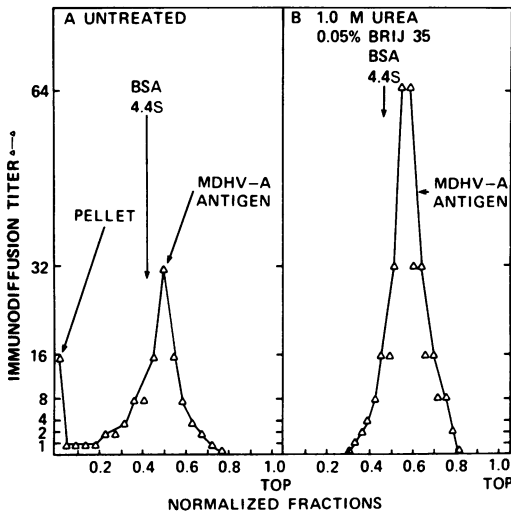


FIG. 1. Sucrose gradient sedimentation analysis of MDHV-A antigen. A 2.0-ml sample of the 50-fold concentrated culture medium which contained MDHV-A that had aggregated during 5 months storage at 20 C (same preparation as in Table 5) was concentrated further to 1.0 ml and dialyzed against 0.01 M Tris, pH 7.4. Then, two 0.4-ml samples were processed simultaneously by the following two methods along with two bovine serum albumin (BSA) samples in the same buffer. (A) One antigen sample and one BSA sample were untreated and incubated for 5 h at 37 C and then analyzed on parallel 5 to 20% (wt/vol in TES buffer) sucrose gradients by centrifugation for 12 h at 50,000 rpm in a SW50.1 rotor at 15 C. (B) The other antigen and BSA samples were made up to final concentrations of 1.0 M urea and 0.05% Brij 35, incubated 5 h at 37 C, and centrifuged in parallel with those in (A) except that the gradients were also made up to 1.0 M urea and 0.05% Brij 35. The antigen and BSA gradients were then analyzed for antigen titer and absorbance at 280 nm as described.

tially purified antigen. First, a mixture of ^{14}C -labeled, amino acid-labeled infected cell culture medium and ^3H leucine-labeled uninfected cell culture medium, which had been carried through the DEAE-Sephadex step in purification (16), was analyzed to demonstrate that antibody against MDHV-A would specifically precipitate protein and that the nonspecific trapping control with SPF serum (serum from uninfected chickens in a specific pathogen-free flock) was correct (Table 6, expt. 1). Although only 2.7% of the ^{14}C -labeled amino acid label from infected cells was formed in the precipitate, this was considerably greater than the 0.2% of ^3H leucine from uninfected cells. Similar analysis of two different ^{14}C glucosamine-labeled and unpurified preparations (Table 6, expt. 2, 3A, and 4), with titers of 4 and 8, revealed that 1.57 and 2.2 to 2.4%, respectively, of the radioactivity could be specifically precip-

itated. In contrast, only 0.33% of uninfected cell glucosamine label precipitated (Table 6, expt. 3B).

Autoradiographic analysis of MDHV-A antigen. To provide additional and more conclusive evidence that MDHV-A is a glycoprotein, autoradiographic analysis was performed with unpurified antigen preparations. A clear line of ^{14}C glucosamine radioactivity coincides exactly (left half of Fig. 3) with the distinct single MDHV-A line when infected cell culture medium was reacted with the standard chicken antisera, whereas there was no line in either case with uninfected cell material. To rule out nonspecific trapping in the precipitin line, the labeled control material from uninfected cells was mixed with unlabeled, partially purified antigen and then reacted with the standard sera. The unlabeled antigen formed the characteristic sharp line but no uninfected cell radioactivity was associated with it (right half of Fig. 3).

Effect of pH on MDHV-A antigen titer. Since antigen made in chick kidney cells was reported to be resistant to low pH (24), the pH stability of MDHV-A antigen made in DEF cells was tested. The antigen (Table 7) was extremely labile at pH 12.0, very stable from pH 11.5 down to pH 2.0 for up to 7 days, and only slightly labile when held at pH 1.5 for 7 days.

DISCUSSION

MDHV-A antigen could be produced in quantities sufficient for purification and analysis, but high yield depended on those parameters described (Table 1 to 3), precise conditions for optimal infection, and the condition of the cells at the time of infection (P. Long and L. Velicer, unpublished results). Production was facilitated by having primary cells available on ice for up to 10 days for seeding as needed. Furthermore, harvesting antigen in serum-free culture medium facilitated both concentration and purification by reducing contamination with cell and serum proteins. Production methods and high yield are emphasized because adequate antigen was essential to determine physical and chemical properties precisely by detecting peaks of immunological activity (Fig. 1 and 2) (16). In contrast, most reported data are based on antigen-positive regions rather than sharp peaks (20, 23) and could be subject to error.

The reduced titer after storage appeared to result from aggregation rather than degradation since freezing would minimize proteolytic activity and antigen titer was regained by dissociation (Table 5). MDHV-A antigen had an aver-

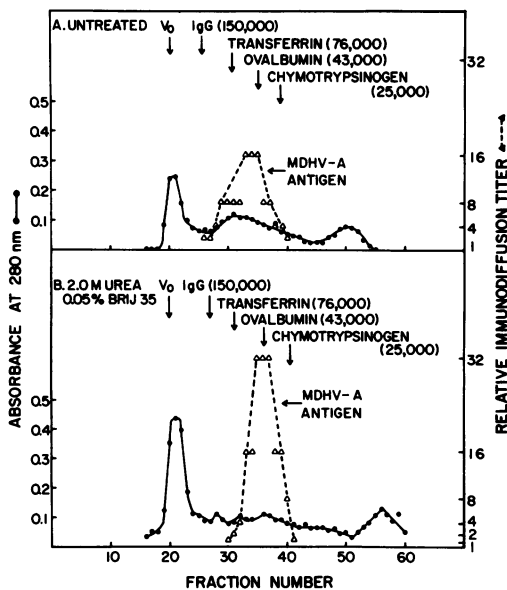


FIG. 2. Gel filtration analysis of MDHV-A antigen. An unpurified antigen preparation (250 ml) was concentrated 200-fold (to 1.25 ml) by negative pressure dialysis against TES buffer and had a final immunodiffusion titer of 1,024. (A) A 0.5-ml sample was diluted to 0.6 ml with TES to form an untreated control sample and was incubated along with the sample in (B). (B) Another 0.5-ml sample received the amount of dry urea and 5% Brij 35 necessary to achieve a final concentration of 2.0 M urea and 0.05% Brij 35 when brought to a final volume of 0.6 ml with TES buffer. The sample was then incubated at 37 C for 4 h with the control in (A). After the incubation period, 0.15 ml of both samples (A) and (B) were analyzed on sucrose gradient with or without urea and Brij, as in Fig. 1, to determine the degree of aggregation (data not shown). The remaining 0.45 ml of each sample was then analyzed by gel filtration on Sephadex G-200 in the presence of TES alone (A) or TES with 2.0 M urea and 0.05% Brij 35 (B) as described. To obtain a better estimate of the antigen peak in spite of the extensive dilution during chromatography, all fractions were spotted initially to fill immunodiffusion wells four times, twice, and once. All fractions that were positive after a single spotting of undiluted material were then titered by twofold serial dilution. To represent this data all fractions positive only when spotted four times were assigned an arbitrary relative immunodiffusion titer of one and the relative titers of the other positive fractions were calculated accordingly.

age sedimentation coefficient of 3.7S and sedimentation analysis provided evidence for aggregation during storage (Fig. 1). The ease of aggregation indicated that precautions would be necessary during antigen purification. However, the effective dissociation by urea and Brij 35 without destroying immunological activity (Table 5, Fig. 1) helped overcome aggregation

during the isoelectric focusing step (10, 12, 16).

Molecular weight estimates range from 80,000 \pm 10,000 for MDHV-A antigen from DEF cells (23) to 46,000 \pm 4,000 from quail cells and 33,000 \pm 5,000 from feather tips (20). These estimates appear to be based on the center of a range of positive fractions, rather than on a sharp peak of antigen activity (20, 23). In this study, the use of high-titered antigen resulted in sharp antigen peaks, and urea and Brij 35 minimized aggregation and improved the accuracy of our estimates (Fig. 2B). The apparent molecular weight of 44,800 \pm 1,100 in the presence of urea and Brij agrees best with the estimate of 46,000 by Onuma et al. (20). In the absence of urea and Brij, the antigen had an apparent molecular weight of 55,800, but this estimate was high because the peaks were shorter, broader, and skewed to the high molecular weight due to aggregation (Fig. 2A). All molecular weight estimates must be considered apparent molecular weights because the antigen is a glycoprotein and branching of the carbohydrate may prevent it from behaving the same as the marker proteins (27). Variations in the amount of carbohydrate added in different cell systems may explain some of the variable molecular weight estimates, such as the 33,000 for feather tip antigen (20). However, this would not explain the high estimate of 80,000 daltons (23) for antigen also produced in DEF cells. Assuming the estimate obtained in urea and Brij 35 is correct, higher estimates may be high due to interaction with other molecules in partially purified preparations. The skewed peak in the absence of urea and Brij 35 (Fig. 2A) suggests this type of interaction rather than aggregation of the antigen with itself to form dimers or trimers. Furthermore, the estimate of 44,800 daltons correlates reasonably well with an estimate of 53,160 daltons calculated from an average sedimentation coefficient of 3.7S (Fig. 1) using the formula of Martin and Ames (17).

The available evidence strongly supports the conclusion that MDHV-A antigen is a glycoprotein. Its sensitivity to trypsin (Table 4) clearly indicates that the antigenic determinant is at least part protein. However, difficulty in detecting protein associated with antigen during purification (16) suggested that the amount is limited. Furthermore, periodic acid-Schiff staining of 200-fold purified antigen (16) indicated that A antigen contained carbohydrate and confirmed similar observations by Ross and Biggs (23). However, small amounts of a contaminating glycoprotein in 200- (16) or 20-fold (23) purified antigen could have caused false-positive results.

TABLE 6. Immune co-precipitation analysis of MDHV-A antigen

| Expt | Source of labeled supernatant fluid | Antigen titer | % Radioactivity precipitated ^a | | |
|------|--|---------------|---|--------------------------------|-------------------------------------|
| | | | RAC and anti-MDHV-A ^b | RAC and SPF serum ^c | Specific precipitation ^d |
| 1 | A. ¹⁴ C-labeled, amino acid-labeled infected cells ^e | 8 | 5.70 | 3.00 | 2.70 |
| | B. [³ H]leucine-labeled uninfected cells ^e | | 3.40 | 3.20 | 0.20 |
| 2 | [¹⁴ C]glucosamine-labeled infected cells ^f | 4 | 1.80 | 0.23 | 1.57 |
| 3 | A. [¹⁴ C]glucosamine-labeled infected cells ^g | 8 | 3.21 | 1.01 | 2.20 |
| | B. [¹⁴ C]glucosamine-labeled uninfected cells ^g | | 1.31 | 0.98 | 0.33 |
| 4 | [¹⁴ C]glucosamine-labeled infected cells ^h | 8 | 3.62 | 1.2 | 2.42 |

^a Percent radioactivity precipitated: counts per minute in precipitate/(counts per minute in precipitate plus counts per minute in supernatant) × 100 (28). All data are the average of duplicate samples or more as indicated below.

^b Anti-MDHV-A serum is the standard chicken reference serum used throughout this study. RAC (rabbit anti-chicken IgG) in experiments 1 and 2 was from a commercial source and in experiments 3 and 4 was prepared as described in Materials and Methods.

^c SPF serum was from uninfected chickens in a specific pathogen-free flock (USDA Regional Poultry Laboratory, East Lansing, Mich.) and was free of antibody to MDHV-A as determined by immunodiffusion.

^d The percentage of counts per minute precipitated by the SPF serum-RAC precipitate was subtracted from the percentage precipitated by the antiserum-RAC precipitate to correct for nonspecific trapping (28).

^e The mixture of partially purified material contained 6,800 counts/min of ¹⁴C and 2,000 counts/min of ³H in the 20 μl added to each reaction mixture.

^f Unpurified supernatant fluid containing 5,700 counts/min in 2 μl was added to each reaction mixture.

^g In this experiment, the unpurified infected and uninfected cell supernatant fluids were assayed separately with 3,600 and 3,000 counts/min, respectively, in 1 μl added to each reaction mixture.

^h These data represents three experiments (six samples) using the same materials as in Expt 3A.

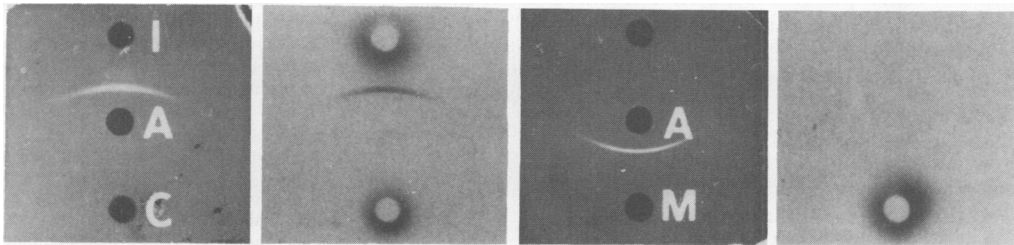


FIG. 3. Autoradiographic analysis of [¹⁴C]glucosamine-labeled MDHV-A antigen. The standard reference antisera from naturally infected chickens (A) was reacted with [¹⁴C]glucosamine-labeled culture medium from infected cells (I), [¹⁴C]glucosamine-labeled culture medium from uninfected cells as a control (C), or a mixture of C and unlabeled antigen partially purified through isoelectric focusing (M). When the immunodiffusion lines were well developed and still sharp, the slides were washed extensively to remove background radioactivity, fixed, photographed, dried, and used to expose X-ray film as described. In each half of the photograph, the left panel with the white line and letters represents the immunodiffusion photograph and the right panel with the black line or no line represents the developed autoradiograph. The absence of a center well in each autoradiograph is expected since the center well always contained antisera which had no radioactivity.

The co-precipitation data (Table 6) also support the conclusion that MDHV-A antigen is a glycoprotein. However, these data are not rigorous proof alone, since the antiserum was not

proven monospecific and small amounts of antibody to another glycoprotein could have given false-positive results. However, the serum was selected to give only one immunodiffusion

TABLE 7. *Effect of pH on MDHV-A antigen titer*

| Time of treatment | Antigen titer ^a after treatment at the following pH | | | | | | | | |
|-------------------|--|-------------------|-----|-----|-----|-----|------|-------------------|----------------------------|
| | 1.5 | 2.0 | 2.5 | 5.0 | 7.4 | 9.5 | 11.0 | 11.5 | 12.0 |
| 1 h | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | Tr, undiluted ^b |
| 5 h | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | Negative |
| 24 h | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | Negative |
| 7 days | 4 | Tr 8 ^b | 8 | 8 | 8 | 8 | 8 | Tr 8 ^b | Negative |

^a Reciprocal of last dilution giving a visible precipitin line.

^b Tr before a number denotes a trace reaction at that dilution.

line and the problem was probably minimal. In light of other evidence that MDHV-A antigen is a glycoprotein (Fig. 3), the co-precipitation data indicate that MDHV-A contained only 1.6 to 2.4% of the total radioactive glucosamine in preparations with titers of 4 and 8, respectively. Although quite low, these percentages were three- to eightfold higher than the nonspecific trapping background and were considered significant. The co-precipitation of a small amount of amino acid label was consistent with the conclusion that at least part of the antigen is protein.

The autoradiographic data (Fig. 3) support the similar original observation by Ross and Biggs (23) and provide the most convincing proof that MDHV-A antigen is a glycoprotein. Our data included a control experiment not previously reported, which ruled out nonspecific trapping of another glycoprotein in an unlabeled MDHV-A antigen precipitin band (Fig. 3), rather than just in a heterologous precipitate (23). The only possible concern remaining is that radioactive glucosamine was reutilized and incorporated into protein (8). Since reutilization does not occur in chicken cells (8) it might not occur in duck cells. This must be proven however, and possible reutilization will be ruled out when sufficient purified labeled antigen is available to show, by chemical analysis, that the radioisotope is still in carbohydrate and not in protein (8). Autoradiographic analysis was extended to show that MDHV-A antigen also contained fucose and galactose, and that the common antigen produced in herpesvirus of turkey-infected cells also contained all three carbohydrates (L. Velicer and P. Kaveh-Yamini, manuscript in preparation).

The pH sensitivity data (Table 7) confirm and extend the original observations by Settnes (24). The antigen preparation was stable up to pH 11.5 and as low as pH 2 for up to 7 days, which represents greater stability than previously reported (20, 24). Settnes suggested MDHV-A antigen was an interferon-like substance based on virus plaque inhibition and

similar properties such as stability at pH 2.0, but he recognized an important difference in that it is antigenic in chickens. It is unlikely that MDHV-A antigen is interferon or an interferon-like substance for the above and following reasons. The antigen preparation used to demonstrate interferon-like activity was not purified at all (24) and could have contained chicken interferon. Finally, the fact that interferon (7) and MDHV-A antigen (Fig. 3) (23) are both glycoproteins suggests that they might have similar properties, such as stability at pH 2.0, because they have similar chemical compositions rather than the same biological activities. Final resolution of this question will require assay of higher purified antigen for interferon activity. The following paper (16) will describe the purification and further characterization of MDHV-A antigen.

ACKNOWLEDGMENTS

We thank Constance L. Wenke, Linda C. Cieresewski, Catherine S. Sekerke, and James L. Clark for excellent technical assistance. We also wish to thank the staff at the USDA Regional Poultry Research Laboratory for their generous support and cooperation in making available virus, sera, embryos, animals, use of their facilities for animal work, and frequent consultation and collaboration without which this work would not have been possible.

This research was supported by Cooperative Agreement no. 12-14-100-10, 652(44) from the U.S. Department of Agriculture, Agricultural Research Service through the Regional Poultry Research Laboratory in East Lansing, Mich., and by a grant from the Elsa U. Pardee Foundation. Additional support, especially for purchase of equipment in early stages of this investigation, was provided by grants from the Jane Coffin Childs Memorial Fund for Medical Research and the Anna Fuller Fund. P. A. Long was supported by Public Health Service Research Fellowship 4F02 AI 45555 from the National Institute of Allergy and Infectious Diseases. L. F. Velicer is a recipient of Public Health Service Research Career Development Award CA70808 from the National Cancer Institute.

Most of this work is from a thesis submitted by P. A. Long in partial fulfillment of the requirement for the Ph.D degree.

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