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Marek's Disease Herpesviruses

II. Purification and Further Characterization of Marek's Disease Herpesvirus A Antigen[1]

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Marek's disease herpesvirus A antigen was purified greater than 200-fold with a 24% recovery by ion exchange column chromatography, isoelectric focusing, and preparative polyacrylamide gel electrophoresis. The antigen had an isoelectric point of 6.68 ± 0.03 in the presence of 1 M urea and 0.05% Brij 35, a nonionic detergent, and approximately 6.5 in the absence of dissociating agents. When analyzed by electrophoresis on analytical polyacrylamide gels, the purified antigen migrated as a single broad band which stained for both protein and carbohydrate, suggesting that it was a highly purified heterogeneous glycoprotein. However, the antigen was not purified to homogeneity as determined by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate and by immunodiffusion analysis. Antibody to Marek's disease herpesvirus A antigen was prepared in a rabbit, and antibody to two contaminating antigens was removed by adsorption to yield monospecific antisera.

A method for large scale production of Marek's disease herpesvirus A antigen (MDHV-A antigen) yields sufficient antigen to facilitate its purification and physical and chemical characterization (8). Initial characterization of unpurified or partially purified antigen in this laboratory (8) and by others (11, 13, 14) revealed that MDHV-A antigen is a glycoprotein stable at pH 2. The antigen sediments at about 3.7*S* (8) to 4.2*S* (11), its apparent molecular weight is estimated to range from 33,000 (11) to 44,850 (8) and 80,000 (13), and its isoelectric point ranges from 4.5 to 6.35 (11, 13). Because of these variations (8, 11, 13), it became clear that the antigen would have to be purified more extensively to obtain precise estimates of physical parameters, as well as to determine its biological properties. Initial purification ranges from 12.2-fold, not 305-fold (11) (see below), to 20-fold (13) with recoveries of 20 to 45%. Their purified antigen preparations were not analyzed rigorously for homogeneity and additional purification seemed necessary. In this paper, a method is described for purifying MDHV-A antigen more than 200-fold with 24% recovery, the preparation of monospecific rabbit antiserum against the highly purified antigen, and several of the antigen's physical and chemical properties.

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MATERIALS AND METHODS

MDHV-A antigen production. The procedures for MDHV-A antigen production and concentration have been described (8).

Radioactive labeling of MDHV-A antigen. The methods for labeling the antigen with radioactive amino acids and glucosamine have been described (8).

DEAE-Sephadex ion exchange chromatography. DEAE-Sephadex A25 or A50 (Pharmacia) was prepared according to manufacturers instructions and equilibrated with initial buffer (0.01 M Tris, pH 7.4). The column size was based on a maximum ratio of 1 mg of protein per 1 cm^3 of gel bed, to ensure complete adsorption of MDHV-A. The column was washed with 10 volumes of initial buffer before the sample was applied in small aliquots at 30-min intervals. Adsorption was continued for 2 h prior to stepwise elution with initial buffer and 0.2 and 2.0 M NaCl in 0.01 M Tris, pH 7.4. Each step was initiated only after the absorbance at 280 nm of the previous eluate remained at base line for one column volumn. Fractions were assayed for MDHV-A by immunodiffusion (8) and positive fractions were pooled, dialyzed with initial buffer, concentrated, and stored at −20 C.

Isoelectric focusing. Antigen from DEAE-

Sephadex columns was dialyzed and isoelectric focused in a 110-ml capacity glass column (model 1801, LKB Instruments Inc.) according to manufacturers directions for the anode at the bottom with the following modifications: the light and dense solutions were only 50 ml each and contained 1 M urea and 0.05% Brij 35 (4, 7, 8); and the light solution contained 5 g of sucrose. Constant voltage was applied at 300 to 400 V and increased to 700 or 800 V at 100 to 200-V increments as the amperage allowed, to avoid exceeding 1.5 to 2.0 W. Maximum voltage was maintained until the amperage was constant for 6 h. At the end of the focusing period (40 to 48 h) 1.5- to 2.0-ml fractions (60 to 80) were collected and assayed for pH, MDHV-A by immunodiffusion (8), absorbance at 280 nm, and radioactivity as described later. Antigen-positive fractions were pooled, dialyzed extensively with 0.01 M Tris (pH 7.4), concentrated, and stored at −20 C for refocusing or other use.

Analytical polyacrylamide disc gel electrophoresis. Polyacrylamide disc gel electrophoresis (PAGE) under nondenaturing conditions was on 7.5% polyacrylamide gels by the acid-neutral protein method of Davis (2), with the following modifications: a 1.0-cm spacer gel of 3% polyacrylamide was formed from acryalmide and bisacrylamide in a ratio of 20:1.2; polymerization was with (*N,N,N',N'*-tetramethylethylenediamine and ammonium persulfate; and samples were made to 5% sucrose. Electrophoresis was at 1 mA per gel initially and a constant 1.5 mA per gel for 3 to 4 h after the phenol red tracking dye entered the resolving gel. The gels were analyzed by: amido-black 10 B staining (2) and scanning at 580 nm (0.5-mm slit, Gilford gel scanner); periodic acid-Schiff (PAS) staining (3) and scanning at 560 nm as above; and slicing into 1-mm cross sections with a razor blade device (Diversified Scientific Instruments) for immunodiffusion as described later.

PAGE in the presence of sodium dodecyl sulfate (SDS) was on gels as described above except that: (i) the 9-cm resolution gel was 10% polyacrylamide, (ii) the spacer gel buffer was 0.13 M Tris, pH 6.8; (iii) the resolution gel buffer was 0.24 M Tris, pH 8.8; (iv) both gels contained 0.1% SDS; and (v) the chamber buffer was 0.025 M Tris-0.190 M glycine (pH 8.0) and 0.1% SDS. Samples were made 1% SDS, 0.01% 2-mercaptoethanol, and 5% sucrose, heated to 90 C for 30 min, and clarified at 30,000 rpm and 18 C for 30 min in a SW50.1 rotor (Beckman) prior to loading. Electrophoresis was at 50 V for 1 h and 100 V for 3 to 4 h. The gels were fixed in 7% acetic acid for 24 h to remove SDS, stained with amido black 10 B (2), and scanned as above.

Preparative PAGE. Analytical PAGE was adapted to preparative scale by forming a 3-cm spacer gel of 3% polyacrylamide on a 1.5-cm resolution gel of 10% polyacrylamide in a medium size column (Canalco). Elution and electrode buffers were 0.005 M Tris-0.038 M glycine (pH 8.0) and 0.025 M Tris-0.190 M glycine (pH 8.0), respectively. A 1.5-ml sample with 25 mg of protein, 5% sucrose, and phenol red tracking dye in spacer gel buffer was layered on the spacer gel. Electrophoresis was with constant current held at 2 mA for 1 h, raised to 5 mA until the dye entered the resolution gel, and raised to 8 mA for the remainder of the run. The column was cooled to 0 C throughout and eluted at 1 ml per min. Fractions (1.5 ml) were collected and assayed for MDHV-A activity by immunodiffusion (8) and for radioactivity as described below. Antigen-positive fractions were pooled, concentrated, titered, and stored at −20 C.

Preparation of antisera. Preparation of the standard chicken sera reactive against MDHV-A was described (8). Rabbits were inoculated with the 200-fold purified antigen by the footpad method (5, 8) except that only 55 μg of protein were used each time, the second inoculation was 30 days later, and all booster injections were in the footpad and one other subcutaneous site with emulsions made with incomplete Freund's adjuvant. Bleeding was also by cardiac puncture at 48-h intervals starting 4 days after each subsequent inoculation (8) to match the time of optimal antibody response to MDHV-A.

Adsorption of antisera. Sera from rabbits immunized with 200-fold purified MDHV-A antigen were adsorbed with sonically treated extracts of uninfected cells, either alone or in combination with concentrated culture medium from uninfected cells and/or calf serum. The optimal amounts of adsorbing material for each serum sample were determined in preliminary experiments to achieve complete adsorption of antibody against two contaminating antigens without excess adsorbent. Mixtures of rabbit serum and adsorbing material were incubated 3 to 4 h at 37 C and overnight at 4 C before the serum was centrifuged for 30 min at 1,800 × *g* to remove the precipitate.

Immunodiffusion analysis of MDHV-A. The immunodiffusion method with standard chicken serum (8) was used to detect antigen-positive fractions from analytical and preparative procedures. One-millimeter cross-sections of polyacrylamide gels were placed in large wells, overlayed with agar, and analyzed with chicken serum. Immunodiffusion with rabbit serum was in 1.5% agar in phosphate buffer saline (0.01 phosphate, pH 7.2, 0.85% NaCl) with merthiolate (1 part in 10,000) as a preservative (5).

Determination of protein content. Protein concentrations were determined by the method of Lowry et al. (9) using crystalline bovine serum albumin as the standard.

Radioactivity assays. Samples (0.01 to 0.2 ml) of fraction from various procedures were prepared and assayed by standard methods (5, 8).

RESULTS

DEAE-Sephadex ion exchange chromatography. Preliminary experiments led to a simple four-step elution (Fig. 1) which became the first procedure in the purification scheme. In this particular experiment, 40 ml of a stored, partially aggregated (8), unlabeled antigen preparation were mixed with 20 ml of fresh unaggregated [^{3}H]leucine-labeled antigen preparation to determine the effect of aggregation on elution. All the antigen adsorbed to the column in

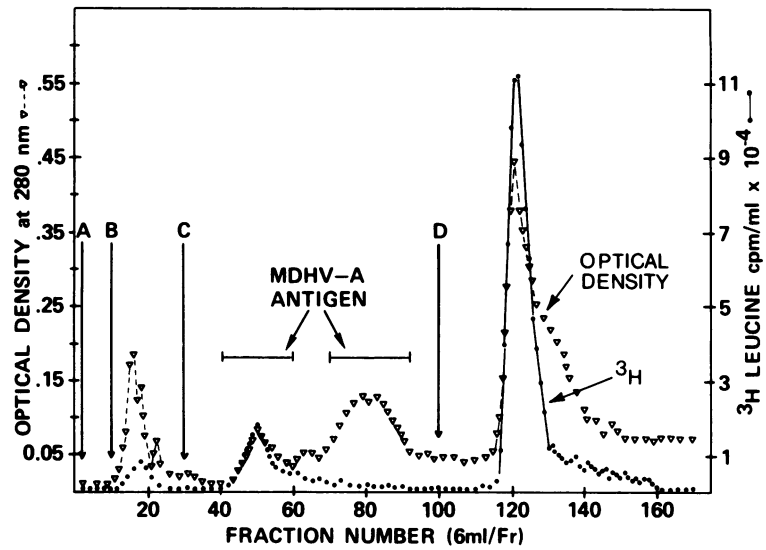

FIG. 1. *Ion exchange chromatographic analysis of MDHV-A antigen. A 60-ml sample consisting of 20 ml of concentrated fresh [^{3}H]leucine-labeled antigen and 40 ml of concentrated, stored, unlabeled antigen was dialyzed extensively against 0.01 M Tris (pH 7.4) and then applied to a column (1 by 90 cm) of DEAE-Sephadex A25. The application and elution involved the following four steps which started at the points indicated by the letters and arrows: (A) application, (B) wash with 0.01 M Tris (pH 7.4), (C) elution with 0.2 M NaCl in 0.01 M Tris (pH 7.4), (D) elution with 2.0 M NaCl in 0.01 M Tris (pH 7.4). Six-milliliter fractions were collected and assayed for [^{3}H]leucine. Optical density at 280 nm and antigen were determined as described. All antigen eluting with 0.2 M NaCl, even if in two separate peaks due to aggregation, was pooled and applied to isoelectric focusing as in Fig. 2.*

step 1 (A, Fig. 1), and none eluted with initial buffer during the second step (B, Fig. 1). The third step, elution with 0.2 M NaCl (C, Fig. 1), removed the antigen but only 15 (P. Long, unpublished data) to 32% (Table 1) of the total protein as determined by optical density and radioactivity. The antigen was in two regions corresponding to two protein peaks as determined by optical density. In contrast, the fresh [^{3}H]leucine-labeled material was in only one peak. The two protein peaks and two antigen regions apparently result from partial aggregation of stored antigen, since fresh preparations consistently yielded one antigen region and one protein peak (P. Long, unpublished data). The remaining protein was eluted with 2.0 M NaCl in the final step (D, Fig. 1) but no antigen was in this eluate.

Isoelectric focusing. Antigen purified through the DEAE step isoelectric focused in a peak at about pH 6.5 in preliminary experiments on a pH 3 to 10 gradient, but a precipitate containing significant antigen settled to the lower part of the gradient. After the results of others (4, 7) and the success in dissociating MDHV-A (8), 1 M urea and 0.05% Brij 35 were included in the sucrose gradient during subsequent electrofocusing on pH 5 to 8 gradients to obtain sharper peaks. In a typical purification experiment (Fig. 2) the bulk of antigen was in a sharp peak between pH 6.16 to 7.06 with the peak fraction at pH 6.69. Isoelectric focusing with urea and Brij was reproducible and the average pI of MDHV-A under these conditions was 6.68 ± 0.03 pH units (average of five determinations) with 25 to 75 mg of total protein put in the column (P. Long, unpublished data). Most protein either focused or precipitated in low pH portions of the column and only approximately 10% of the total protein applied, as measured by [^{3}H]leucine, was in the main area of the peak (titer ≥ 4) which contained 80% of the antigen (Fig. 2).

Preparative PAGE. Successful separation of

FIG. 2. *Isoelectric focusing analysis of MDHV-A antigen. Antigen-containing fractions from the DEAE-Sephadex column (Fig. 1) were pooled, concentrated to 5 ml, dialyzed extensively against 0.001 M Tris (pH 7.4), and focused in a linear sucrose gradient with 1 M urea, 0.05% Brij 35, and 1.2% ampholine (pH range 5 to 8). The sample, which contained 25 mg of protein, was added to the light solution with the dissociating agents and incubated at 37 C for 4 h. Then the focusing and the assay of fractions for pH, radioactivity, and MDHV-A antigen were carried out as described.*

TABLE 1. *Analysis of MDHV-A antigen purification by differential double labeling*

Method	Recovery during purification (%)					Purification factor of MDHV-A antigen[b]
	Infected		Uninfected [^{3}H]leucine	Total protein[a]		
	Antigen	^{14}C-labeled amino acids		L	C	
Initial concentrated preparation[c]	100	100	100	100	100	
Clarification	100[d]	82	74	86	80	
DEAE-Sephadex	60	34	19	32	29	2-fold
Isoelectric focusing[e] ..	30	0.92	0.60		0.81	37-fold
Preparative PAGE ...	24	0.15	0.056		0.11	218-fold

[a] L, Lowry procedure (addition of ampholines at the isoelectric focusing step interfered with accurate analysis); C, counts per minute per milligram of original materials.

[b] The purification factor was determined by multiplying the fold reduction in total protein content (100% at start/percent remaining after each step) as determined by total radioactivity times the percent recovery of antigen.

[c] The initial preparation contained 1,288 mg of total protein, 840 mg from infected cells and 448 mg from control cells, with 2.49×10^7 counts/min of ^{14}C and 9.74×10^7 counts/min of ^{3}H, respectively.

[d] Fresh antigen preparations exhibiting no aggregation.

[e] Isoelectric focusing gradient fractions containing MDHV-A antigen were pooled, concentrated, and refocused before preparative PAGE.

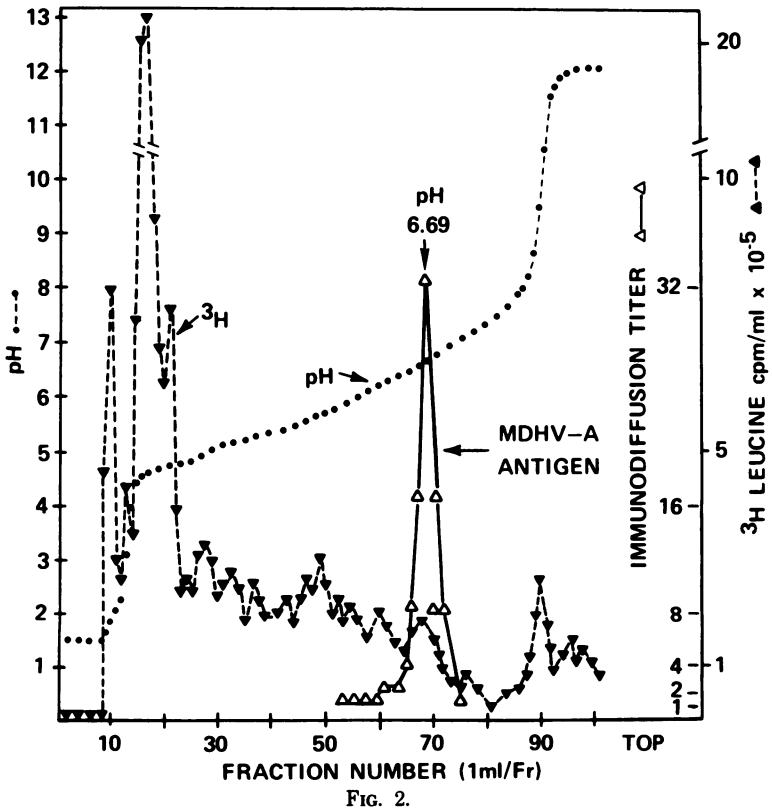

FIG. 2.

the antigen from other proteins in preparations purified through isoelectric focusing by preliminary analytical PAGE (see Fig. 4B) led to the development of the preparative PAGE method. The data in Fig. 3 are results of preparative PAGE analysis of a mixture of infected and uninfected material in a differential, double label experiment designed to measure the removal of host cell proteins. The two preparations were actually mixed at the beginning and purified by ion exchange chromatography (Fig. 1) and isoelectric focusing (Fig. 2) first, as will be summarized later (Table 1). Most of the remaining uninfected and infected cell proteins eluted in the first 20 fractions, and most of the antigen (the highest titered regions detected by filling immunodiffusion wells only once) eluted in fraction 25 to 32. However, the peak of ^{14}C-labeled amino acid label from infected cells in the antigen region was not distinct, and the ratio (Fig. 3) of the ^{14}C to ^{3}H was calculated to better demonstrate the little radioactivity associated with MDHV-A. The ratio began to increase around fraction 16, reached a peak in fractions 30 to 32 at the right side of the antigen region, and returned to base line levels at about fraction number 40. The increased ratio corresponded closely to the entire range of antigen detected only by multiple filling (up to four times) of immunodiffusion wells (Long and Velicer, unpublished data). Only the peak fractions indicated in Fig. 3 were pooled for further analysis. The slight enrichment for ^{14}C is also evident since 16% of the infected cell radioactivity and only 9% of the uninfected cell radioactivity was in the region containing 80% of the antigen.

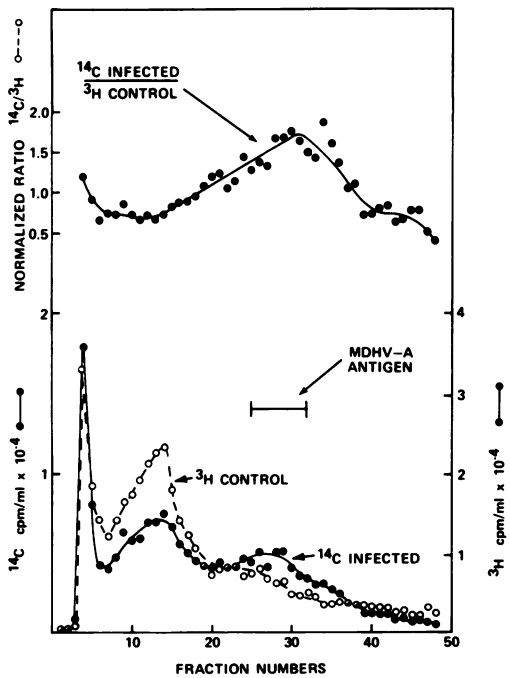

FIG. 3. *Preparative PAGE analysis of MDHV-A antigen. A sample of the mixture of ^{14}C-labeled amino acid-labeled infected and [^{3}H]leucine-labeled uninfected cell supernatant fluids that had been purified through DEAE-Sephadex and isoelectric focusing (Table 1) and contained 120 U of antigen was further purified by electrophoresis on a 3% acrylamide spacer gel and a 10% acrylamide resolution gel. Analysis of fractions for radioactivity and antigen were as described. The data from this figure and the entire double label experiment are summarized in Table 1.*

Analytical PAGE. Antigen preparations, purified through DEAE-Sephadex, isoelectric focusing, and preparative PAGE were analyzed by analytical PAGE under nondenaturing conditions to assess the extent of purification and the migration properties of the antigen. The respective stained gels had 16, 10, and 1 protein bands (Fig. 4), but the single band was quite broad (Fig. 4C). However, purified antigen was also in the same wide region of the gel (Fig. 4C), suggesting it was heterogeneous in charge.

PAS staining of purified MDHV-A antigen. Although trypsin sensitivity indicates that the antigenic determinant of MDHV-A includes some protein (8), the limited recovery of amino acid label or stainable protein during purification (Fig. 2, 3, 4), and the negative results of attempts to in vitro label protein (P. Long and L. Velicer, unpublished data), suggests that the amount of protein may be quite limited. To provide additional evidence that the antigen is a glycoprotein, and to assess the effectiveness of the purification procedure in respect to other glycoproteins with limited protein content, highly purified material from preparative PAGE was analyzed by analytical PAGE and stained for carbohydrate with PAS. The antigen did stain with PAS, forming a broad flat peak, or possibly two poorly separated peaks that coincided with the antigen region (Fig. 4E) and with the amido black stain (Fig. 4C). The extent of purification from other carbohydrate-containing materials was also apparent when purified antigen (Fig. 4E) was compared with a partially purified preparation from DEAE-Sephadex which was PAS stained in at least five of its 16 protein bands (Fig. 4A, D).

Evaluation of MDHV-A antigen purification by differential double labeling. To quantitate the degree of purification and especially the removal of host cell contaminants, recovery of antigen and protein was determined through-

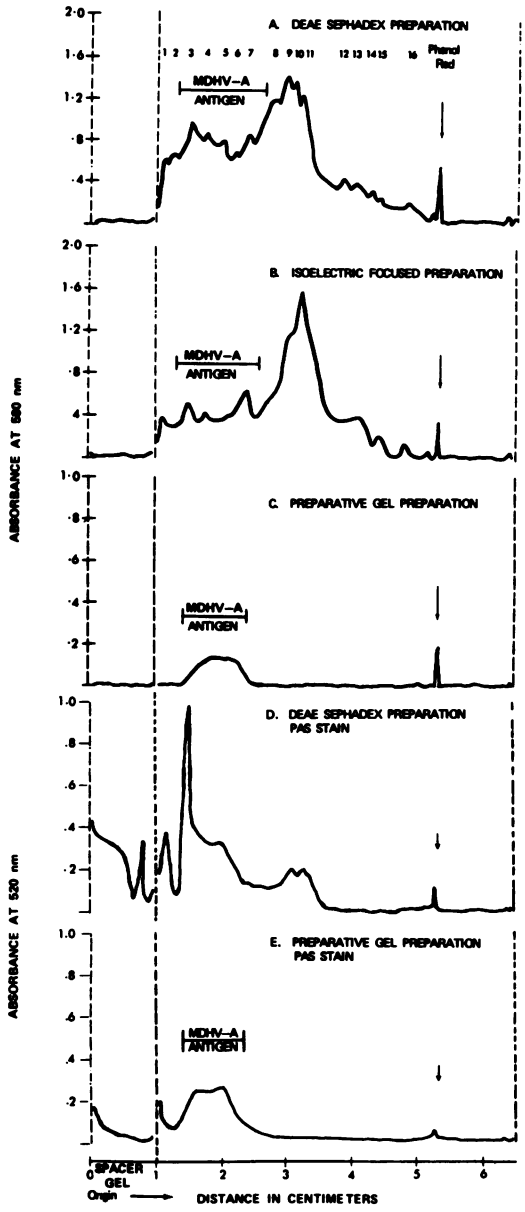

FIG. 4. *Analytical PAGE analysis of purified MDHV-A antigen. Samples for electrophoresis were obtained as follows: (A and D) DEAE-Sephadex (Fig. 1), 300 μg of protein/gel; (B) isoelectric focusing (Fig. 2), 300 μg of protein/gel; (C and E) preparative PAGE (Fig. 3), 100 μg of protein/gel. Preparation of the sample, electrophoresis on a 3% acrylamide spacer gel and 7.5% acrylamide resolving gel, staining the gels with amido black (A, B, and C) and PAS (D and E), and scanning of stained gels were as described. Detection of MDHV-A antigen (brackets) was done by first cross-sectioning duplicate gels and then performing immunodiffusion analysis, as described in the text, to allow comparison of antigen position with stained bands. The numbers at the top of (A) represent the minimum number of amido black-stained proteins discernible in the gel scan.*

out the purification of MDHV-A from a mixture of culture medium from ^{14}C-labeled amino acid-labeled infected cells and [^{3}H]leucine-labeled uninfected cells (Table 1). The recovery of 0.11% total protein was a 910-fold overall reduction, whereas the 24% antigen recovery was a 4.15-fold overall reduction. Based on these values, the antigen was purified 218-fold. Due to the interference of ampholines with the Lowry procedure, estimates of total protein after isoelectric focusing were based on starting specific activities of the ^{14}C and ^{3}H. The validity of this method is evident from a comparison of estimates obtained by both methods after the clarification and DEAE-Sephadex steps. Although estimates based on radioactivity were slightly lower, the difference was 10% or less. Therefore the estimate that the antigen was purified 200-fold or more was assumed valid unless the little protein remaining had a specific activity much lower than the starting material. Removal of ^{3}H from uninfected cell material was approximately threefold greater than removal of ^{14}C from infected cell material, reflecting the slight enrichment for ^{14}C already seen in Fig. 3.

SDS-PAGE analysis of purified MDHV-A antigen. Although estimates of purification (Table 1) and analytical PAGE analysis were useful, a more rigorous criterion of purity was the number of polypeptides remaining as determined by SDS-PAGE analysis. The extensive purification described earlier (Fig. 4, Table 1) was confirmed when the profile of material from preparative PAGE (Fig. 5C) was compared to the profile of the DEAE-Sephadex preparation (Fig. 5B). However there were still four polypeptides with approximate molecular weights of 82,000, 57,000, 52,000, and 21,000 (Fig. 5C) in relation to standard marker proteins (Fig. 5A). The presence of multiple polypeptides strongly suggested that the highly purified MDHV-A was not homogeneous unless all the polypeptides were subunits of the antigen.

Immunodiffusion analysis of highly purified MDHV-A antigen. When the highly purified antigen from the preparative PAGE step was used to inoculate rabbits, antibody reactive with more than one antigen was detected upon subsequent immunodiffusion analysis. When the rabbit serum was reacted with the inoculating antigen, two precipitin lines were produced (Fig. 6, upper left panel). When the serum was reacted with a partially purified antigen from isoelectric focusing, three distinct precipitin

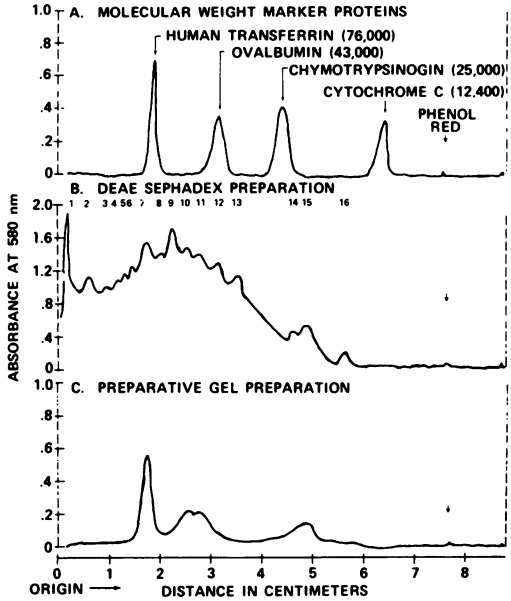

FIG. 5. *SDS-PAGE analysis of purified MDHV-A antigen. Samples for electrophoresis were obtained as follows: (A) mixture of marker proteins; (B) DEAE-Sephadex (Fig. 1), 300 μg of protein/gel; (C) preparative PAGE (Fig. 3), 100 μg of protein/gel. The samples were dissociated in 1% SDS and 0.01% 2-mercaptoethanol and electrophoresed for 1 h at 50 V and 3 to 4 h at 100 V on a 1-cm spacer gel of 3% acrylamide and a 9-cm resolution gel of 10% acrylamide. Further details of electrophoresis and staining of gels with amido black are described in the text.*

lines were present (Fig. 6, upper right panel). These results indicate that the 200-fold purified MDHV-A was still contaminated with at least two other antigens assuming that one precipitin band represented rabbit antibody to MDHV-A. To prove that antibody to MDHV-A was actually made, a line of identity between the standard chicken reference serum and the rabbit serum was necessary. However, since precipitin lines form optimally with chicken serum in high salt (8% NaCl) and with rabbit serum in low salt (0.85% NaCl), it was necessary to do the same experiment under both conditions. The rabbit serum again produced three precipitin lines in low salt agar, and the single line with chicken serum formed a line of identity with the outer line from rabbit serum (Fig. 6, center left panel). These photographs were taken after only 24 h of incubation when the lines were visually the sharpest. After longer incubation the middle line from rabbit serum had crossed the one common line. Therefore only the outer rabbit serum line is identical to the chicken serum line. The line of identify between the two sera was confirmed in high salt agar (Fig. 6, center right). Although rabbit antibody did not form sharp precipitin lines in 8% NaCl, and the two contaminant lines were joined, the MDHV-A line was distinct and continous with the single line formed by chicken serum.

Preparation of monospecific antisera. Adsorption of the rabbit serum with sonically treated extracts of uninfected cells in amounts

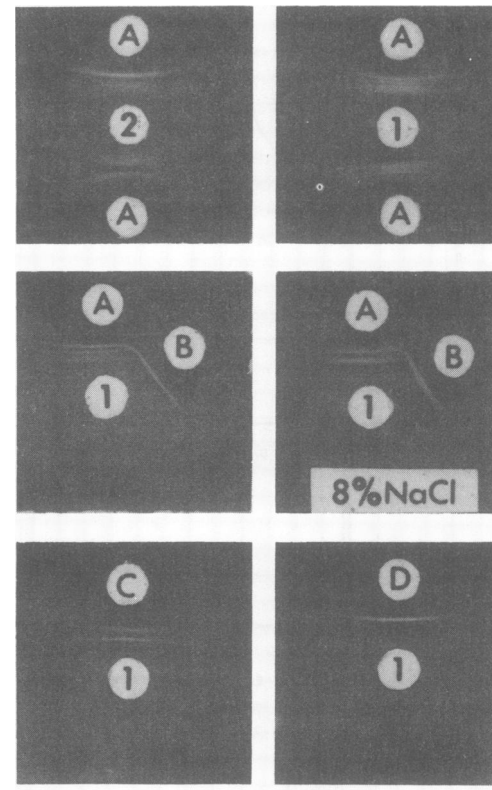

FIG. 6. *Immunodiffusion analysis of highly purified MDHV-A antigen. Antisera used include (A) sera from a rabbit immunized with MDHV-A antigen purified through preparative PAGE (bottom wells were filled twice in two upper panels, other wells were filled four times), (B) standard reference sera from chickens naturally infected with MDHV (each well was filled once, (C) sera as in (A) adsorbed with a soluble extract of sonically treated uninfected duck embryo fibroblast cells (each well was filled four times), (D) sera adsorbed as in (C) and also with concentrated serum-free culture medium from uninfected duck embryo fibroblast roller bottle cultures and/or calf serum (each well was filled four times). Antigens used included (1) partially purified MDHV-A antigen obtained from isoelectric focusing (Fig. 2), and (2) highly purified MDHV-A antigen obtained from preparative PAGE (Fig. 3) which was also used for inoculation of the rabbit. Immunodiffusion was in 1.5% agar in PBS as described, except for the right center panel which was in 8% NaCl (8).*

necessary for optimal precipitation removed antibody to only one contaminant (Fig. 6, lower left). Further adsorption with concentrated culture medium from uninfected cells and/or calf serum in optimal amounts removed antibody to the remaining contaminant and yielded monospecific antisera as determined by immunodiffusion analysis (Fig. 6, lower right). In control experiments the single remaining line was identical with a line formed by chicken serum just as in Fig. 6, left center.

DISCUSSION

Purification of MDHV-A antigen was complicated by two problems, logistics of production and the tendency of the antigen to aggregate readily during storage (8). Furthermore, the necessity of daily harvest and storage until sufficient material was available for large-scale purification precluded work with large amounts of fresh unaggregated on a routine basis. Elution of stored aggregated antigen and fresh unaggregated antigen at the same salt concentration (Fig. 1) made it possible to achieve a two-fold purification and an essential 60% reduction in total protein prior to isoelectric focusing regardless of the extent of aggregation. The slower elution of stored antigen at the same salt concentration (Fig. 1) is not understood. Clearly it was not due to antigen degradation either in respect to antigenic activity or size (8). The apparent heterogeneity of the antigen (Fig. 3 and 4) precluded elution with linear gradients and the slight variation in elution at the 0.2 M NaCl step presented no problem since the antigen was neither lost in the initial wash nor retained until the 2.0 M NaCl elution step (Fig. 1).

Isoelectric focusing was an effective purification procedure, particularly when urea and Brij 35 were used to maintain solubility and reduce aggregation (Fig. 2) and the antigen was refocused (Table 1). It is realized that the pI of 6.68 (Fig. 2) is defined only under these conditions and may actually be different than the true pI of the antigen in the absence of any dissociating agents. However, the values of 6.5 for unaggregated antigen (P. Long, unpublished data) and 6.35 for feather-tip antigen (11) in the absence of these reagents suggest that the pI was not altered significantly. This conclusion is also supported by the report that Brij 35 alone does not seem to alter the pI of at least two other proteins (4). However, Onuma et al. (11) reported a low pI of 4.5 in the absence of dissociating agents for a cell fluid antigen and Ross and Biggs (13) reported the antigen had a heterogeneous charge with a pI of 4.5 to 5.5 in 2 M urea. In each case no data were presented to indicate whether the antigen focused as a sharp peak or as a broad band. In this study highly aggregated antigen was also in a broad range at low pI in the absence of urea and Brij 35 (P. Long and L. Velicer, unpublished data). Therefore, unless these other A antigen preparations are very different, it is likely that their low pI values are due to aggregation and/or precipitation. However, this conclusion would imply that 2 M urea alone (13) is of no value in preventing these problems. We have not determined whether urea or Brij 35 works well alone in our system or whether a combination of both reagents is necessary for optimal results. A report by Friesen et al. (4) suggests that Brij 35 alone may be sufficient.

The low amounts of in vivo incorporated radioactive amino acids, optical density at 280 nm, and amido black stain in fractions of preparative and analytical gels containing highly purified antigen (Fig. 2, 3, 4) suggest that protein is only a small part of the antigen. This was further substantiated by the fact that the antigen could not be in vitro labeled in its protein (Long and Velicer, unpublished data) either with ^{125}iodine (6) or [^{3}H]formaldyde (12). The presence of at least some protein is clearly indicated by the protease sensitivity (8, 11, 14) and immune coprecipitation data (8). The PAS staining (8, 13), immune coprecipitation (8), autoradiography (8, 13), and sodium periodate sensitivity (11) clearly indicate that MDHV-A contains carbohydrate. The proportion of protein and carbohydrate in this glycoprotein will be determined when sufficent antigen is purified to homogeneity to permit appropriate chemical analysis.

Analysis of highly purified antigen by analytical PAGE (Fig. 4) and differential double labeling (Table 1) clearly demonstrated extensive purification along with 24% recovery of antigen. Greater than 200-fold purification in relation to total protein recovered represents approximately 400-fold purification in relation to uninfected cell protein based on recovery of radioactive labeled protein. Ross and Biggs (13) purified A antigen only 20-fold with 20 or 45% recovery, depending on the methods used, and recognized the possibility of trace contaminants with the same electrophoretic mobility. Onuma et al. (11) reported a purification value of 305 for feather-tip antigen by gel filtration and electrofocusing. However, after recalculation of their data (Table 1 in reference 11), we conclude their purification was only 12.2-fold and the higher number resulted from erroneous use of a correction factor. Their titer (15 U with-

out allowing for a total volume of 100 ml) divided by the total protein (300 mg), which is really a form of specific activity (0.05 U/mg) assuming a 1-ml volume, was arbitrarily multiplied by a factor of 20 to obtain a value of one for the starting material. Since starting material prior to purification is always assigned a purification value of one, application of a correction factor to specific activity to achieve the numerical value of one was incorrect and resulted in an erroneously large estimate. When the data of Onuma et al. (11) are calculated as by Ross and Biggs (13) and as in this study (Table 1), the starting total protein (300 mg) divided by the final total protein (7.2 mg) represents a 41.7-fold reduction in total protein. Correction for 29.3% recovery of antigen indicates 12.2-fold purification. Recalculation of their data (11) on total volume, antigen titer, and total protein indicates an increase in actual specific activity from 5 to 61 U/mg, which also represents a 12.2-fold purification.

The lower purification by others (11, 13) is emphasized to caution against possible false conclusions about physical, chemical, and biological properties of partially purified antigen which may actually be due to contaminants as recognized by Ross and Biggs (13). For example, the preliminary report that A antigen stimulates virus neutralizing antibody (11), although possibly correct, may result from a contaminating viral antigen in their 12.2-fold purified material. Even 200-fold purified antigen (Table 1) was still not homogeneous when assayed by more rigorous criteria such as SDS-PAGE (Fig. 5) and immunodiffusion analysis with antisera prepared against the purified material (Fig. 6). Finding four polypeptides on SDS-PAGE indicated one or more contaminants were present unless the antigen consists of four different sized subunits ranging in size from 20,000 to 82,000 daltons (Fig. 5). This is unlikely since its sedimentation coefficent is between 3.5 and 4.0*S* (8, 11) and its molecular weight was estimated to be 80,000 (13) or less (8, 11).

The assay for homogeneity by immunodiffusion utilized the sensitivity of the immune response to magnify the presence of two trace contaminants as demonstrated in Fig. 6. Apparently one trace contaminant was present in 200-fold purified antigen in a concentration sufficient to stimulate the immune response but insufficient to form a precipitin line, whereas it was present in amounts adequate for immunodiffusion in the 37-fold purified isoelectric focusing preparation. In addition, the immunodiffusion data are evidence that rabbit antibody can be prepared against the 200-fold purified MDHV-A antigen. One contaminant was from calf serum which apparently remained associated with cells despite the use of serum free medium. The other contaminant was from normal duck embryo fibroblast cells, and antibody to each was removed by appropriate adsorption techniques (Fig. 6). The resulting monospecific rabbit antiserum is now being used to determine if the antibody against MDHV-A antigen can neutralize cell-free infectious MDHV and/or detect the cell membrane antigen seen in MDHV-infected cells (1, 10). The same rabbit serum should also prove valuable in developing an affinity chromatography method of purification and a complement fixation assay for MDHV-A antigen which will be more sensitive than immunodiffusion. In each case the availability of rabbit antibody should make it possible to avoid problems related to the use of chicken serum which have previously prevented these methods from being developed.

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