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MOLECULAR AND IMMUNOLOGIC CHARACTERIZATION OF MAREK'S DISEASE HERPESVIRUS ANTIGENS

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MOLECULAR AND IMMUNOLOGIC CHARACTERIZATION OF MAREK'S DISEASE HERPESVIRUS ANTIGENS

By

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ABSTRACT

MOLECULAR AND IMMUNOLOGIC CHARACTERIZATION
OF MAREK'S DISEASE HERPESVIRUS ANTIGENS

By

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The identification of Marek's disease herpesvirus (MDHV) A and B antigens was attempted by S.aureus immunoprecipitation followed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). Using MDHV-A antigen reactive sera and a highly purified MDHV-A antigen preparation (49), MDHV-A antigen was conclusively identified as a glycoprotein with an approximate molecular weight of 61-65,000. An antigen of apparently identical molecular weight was also demonstrated in the culture medium of herpesvirus of turkeys (HVT)-infected cells. Identification of MDHV-B antigen from infected cell lysates by immunoprecipation with MDHV-B antigen reactive sera (106) was inconclusive. ^{35}S-methionine labeling resulted in the immunoprecipitation of virus-specific polypeptides of 83,000 and 150,000 daltons while ^{14}C-glucosamine labeling resulted in the immunoprecipitation of only a 230,000 dalton molecule.

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INTRODUCTION

Marek's disease (MD) is a common neoplastic disease of chickens. Until the early 1960's, MD was classified with other neoplastic diseases of chickens as part of the avian leukosis complex. Careful clinical studies by Campbell (3) and Biggs (4) separated the complex into two diseases: Marek's disease which was later shown to be caused by Marek's disease herpesvirus (MDHV) (17,18,62); and lymphoid leukosis which has an RNA tumor virus as the etiologic agent (61). Until it was brought under control by vaccination in the early 1970's, MD was a major cause of economic loss to the poultry industry. In the United States alone, annual losses due to MD by the late 1960's were estimated at about 200 million dollars (75).

The antigens of the MD system have been defined by various immunological methods. The immunofluorescent technique has identified the membrane antigens of the productive infection (2,15,31,65,78,105), and the tumor associated antigen of the transformed state (54,56,59, 66,92,111). Immunodiffusion analysis has identified the predominantly extracellular A antigen, and the

predominantly cell-associated B antigen, both characteristic of the productive infection. Although the nature of the host immune response to MD has been extensively studied, the relatedness of the antigens defined by different immunological methods remains unknown. Also, the relationship of these antigens to the mature virion, their location in the infected cell, and their role in the disease process still remains to be elucidated.

The objective of this thesis was to further the immunologic and molecular characterization of the A and B antigens on polyacryamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) following S.aureus immunoprecipitation. The physical and chemical properties of these antigens have been extensively studied (49,50,106) and the ability to further study these antigens was dependent upon the availability of previously prepared, highly purified antigens and monospecific antisera (49,106).

The identification of the A and B antigens on SDS-PAGE will aid in the understanding of the relationship of these antigens to the MD syndrome, and will additionally serve as a foundation for further molecular studies of the carbohydrate and polypeptide portions of these antigens and future analysis of the MDHV genome.

LITERATURE REVIEW

Pathogenesis of MD

MD is characterized by the appearance of lymphoid tumors in viscera, muscle, and skin, as well as infiltration of lymphoid cells in peripheral nerves (63). Marek (52) first characterized the classical form of MD in 1907, which presents itself as a paralysis of the wings and legs with gross enlargements of the peripheral nerves. The acute form of MD, described by Biggs, et al. (5), has a high incidence of visceral lymphoid tumors and a high incidence of mortality. Lymphomas are composed of a heterogeneous combination of cells including small and medium lymphocytes, mature blast-type cells with abundant basophilic cytoplasm and large nuclei, and plasma cells (72). The target cell for transformation is the T-lymphocyte, since most cells present in lymphomas are T-cells (28,89,99) and cell lines developed from MD lymphomas are T-cell lines (66,68,75).

Properties of MDHV

The causative agent of MD was first isolated in cell cultures from tumor cells and identified as a group B herpesvirus (17,18,60). MDHV displays a typical

herpesvirus morphology. The nucleocapsid is about 100 nm and has an icosahedral symmetry with 162 hollow centered capsomeres. A herpesvirus isolated from normal turkeys, named herpesvirus of turkeys (HVT), was found to be morphologically and antigenically related to MDHV but apathogenic for both chickens and turkeys (34,87,112). Vaccination of chickens with HVT was highly protective against the development of MDHV-induced lymphomas (68) and HVT and MDHV appeared to share immunologically related antigens (21,87,106,109). The DNA of both MDHV and HVT is a double stranded molecule of 1×10^8 daltons. It has a bouyant density in CsCl of 1.706 g/cm^3 which corresponds to a guanine/cytosine content of 46% (44). Recent DNA homology studies indicate that there is apparently only a 2-5% homology between the two viral DNAs (26,35,47). A 2% DNA homology could allow for the production of only 2-3 HVT and MDHV common antigens with an average molecular weight of 5×10^4 daltons (26).

Analysis by Chen, et al. (14) of partially purified MDHV virions by SDS-PAGE revealed at least 8 polypeptides ranging in molecular weight from 110×10^3 to 155×10^3. Three polypeptides comprised about 50% of the total protein and two polypeptides could be labeled with glucosamine and were apparently associated with the viral envelope since pre-treatment of virions with

Nonidet P-40 eliminated these proteins. The relationship of these proteins to the MDHV antigens defined by various serological tests was not established.

Two types of virus-cell interactions can occur after infection with MDHV. Productive interaction results in the production of virus nucleic acid, virus proteins, sometimes virus particles, and cell death (59). Infection of feather follicles can result in fully enveloped and infectious virus (10), while infection of other cells of the chicken as well as tissue culture cells may result in the production of only virus specific antigens and noninfectious particles (1,21,60).

Nonproductive interaction is encountered in lymphoid cells and can lead to cell proliferation and tumor formation. Virus DNA may be present in multiple copies in these cells, but the expression of viral DNA is rarely observed (62,64). Evidence exists for the integration of viral DNA into host DNA (36). MDHV - transformed lymphoblastoid cell lines have been established (3,12,67,75) and all lymphoblastoid cell lines are of the T-cell type and carry a membrane associated tumor specific antigen (MATSA) (54,56,59,66, 92,111).

MDHV-Induced Antigens

Several antigens have been detected in MDHV-infected cells using various serological tests. The agar-gel immunodiffusion test has revealed the presence of a number of precipitating antigens. Chubb, et al. (16) initially detected only one precipitin line, although in later work (19) three major antigens were detected. The major antigen present in culture fluid of infected cells was designated A antigen and the two antigens detected in infected cell extracts were designated B and C antigens. It was also noted that the capacity of the virus to produce A antigen was diminished during attenuation by passage in tissue culture and this suggested a correlation between the presence of A antigen and pathogenicity. This conclusion was later proven false since it appeared that loss of A antigen can occur without loss of pathogencity (84), and a serologically identical A antigen was still detected in HVT as well as other apathogenic strains of MDHV-infected cell fluids (80,87,108).

A antigen is the antigen most commonly detected by sera from naturally infected birds (50,88). It can be produced in reasonably large amounts in tissue culture and is easily monitored by immunodiffusion (16,50). For these reasons, A antigen has been

extensively studied by many labs. An early attempt in the purification of A antigen was made by Ross, et al. (88) who reported a 20-fold purification with either a 45% recovery by electrophoresis in 5% acrylamide or a 20% recovery by chromatography on either DEAE-Sephadex A50 or Sephadex G-200. The antigen was produced in duck cells and was detected by convalescent chicken sera. It was also identified as a glycoprotein by autoradiography and was found to have a molecular weight range of 70,000 to 90,000 daltons by gel filtration and a heterogeneous charge (pI 4.5 to 5.5). In a study by Settnes (93), crude A antigen was found to be trypsin sensitive and resistant to pH 1.7.

Onuma, et al. (69) described a common antigen, presumed to be A antigen, that was associated with both MDHV and HVT infections. It appeared as a single band in immunodiffusion tests from extracts obtained from feather-tips of MDHV infected chickens and from tissue culture fluids of cultures infected with MDHV and HVT. The molecular weight of the feather-tip antigen, estimated by gel filtration, was 33,000 daltons and this antigen also had a pI of 6.35. The antigens from culture fluids of MDHV and HVT infected cells had molecular weights of 46,000 and 43,000 respectively, and both had a pI of 4.5. The common antigen was

unaffected by acetone or ether and was concluded to be a glycoprotein due to its sensitivity to pronase and sodium periodate. The antigen was soluble and separable from MDHV and HVT virions but it reacted in common with a virion antigen in immunodiffusion, leading to the conclusion that the antigen may be a virion envelope glycoprotein or an altered cell membrane glycoprotein. The authors indicated that inoculation with common antigen stimulated virus neutralizing antibody and that it might be part of the virus envelope. In later work, antisera against a partially purified common antigen, presumed to be A antigen, neutralized cell-free MDHV and HVT and also appeared to react with a late appearing membrane antigen defined by immunofluorescence (55,70, 71).

Ross, et al. (87) analyzed the B and C antigens present in extracts of chicken embryo cells infected with an attenuated strain of MDHV after partial purification of the antigens by gel filtration. The B antigen was relatively stable and of lower molecular weight on polyacrylamide gel electrophoresis than C antigen. B and C antigens were also found in the culture medium of infected cells at low levels but were distinguishable from A antigen. The results of immunodiffusion studies suggested that B antigen was common to MDHV and HVT and that C antigen was MDHV-specific.

A rigorous purification scheme for A antigen was developed by Long, et al. (49,50), and for B antigen by Velicer, et al. (106), so that A and B antigens could be purified apart from each other and produced in quantities sufficient to achieve sharp antigen peaks for analysis. Long, et al. (49) purfied A antigen 200-fold with a 25% recovery by a combination of ion-exchange column chromatography, isoelectric focusing, and preparative polyacrylamide gel electrophoresis. The antigen had a pI of 6.68 when 1 M urea and Brij 35 was used to maintain solubility, and a molecular weight of 44,800 by gel filtration on Sephadex G-200 and 53,160 from calculation from sedimentation coefficients. The antigen was apparently not purified to homogeneity since four polypeptides were visible on SDS-polyacrylamide gel electrophoresis analysis of the antigen. Antibody to A antigen was prepared in a rabbit, and antibody to two contaminating antigens was removed by adsorption to yield monospecific antisera.

Extensive characterization and purification of B antigen free of A antigen was achieved by Velicer, et al. (106) with Conconavalin A affinity chromatography, sucrose gradient sedimentation, isoelectric focusing, and gel filtration on Sephadex G-200. In the presence of 1-2 M urea and 0.05% Brij 35, purified B antigen had

a pI of 4.54 and a molecular weight of 58,250 by gel filtration. A greater than 200-fold purification of B antigen was achieved. Rabbits were immunized to prepare antisera that appeared monospecific for B antigen by immunodiffusion (106). A comparison of the published properties of highly purified A and B antigens is summarized in Table 1 (106).

In the previously cited purification work of Ross, et al. (87) and Onuma (69,70,71) antigens were purified only by gel filtration and data were based on antigen positive regions rather than sharp peaks of antigen activity. In light of this, Velicer, et al. (106) points out that the discrepancies in the molecular weight values for A antigen and common antigen that have been reported could have been a result of the overlap on gel filtration of the A and B antigen peaks; A and B antigens both being common between MDHV and HVT infected cells; A and B antigen precipitin lines appearing as one under certain immunodiffusion conditions; and untreated A antigen tending to aggregate. The more extensive purfication of A and B antigens, free of each other and in quantities large enough to produce distinct antigen peaks, provides a basis for an accurate physio-chemical analysis of the antigens.

TABLE 1.--Published Comparison of MDHV-A and MDHV-B Antigens.

	MDHV-A	MDHV-B
Detected by sera from naturally infected birds	Yes	Yes
Found in greatest concentration in	Medium	Cells
Common between MDHV and HVT	Yes	Yes
Glycoprotein	Yes	Yes
Resistant to pH 2.0	Yes	YES
Elute from Concanavalin A by α-Methyl-D-Manoside	Yes	Yes
Sensitive to trypsin	Yes	No
Resistant to 1 M urea, 0.05% Brij 35	Yes	Yes
Isoelectric point	6.68	4.50
Sedimentation coefficient	3.7S	4.4S
Apparent molecular weight determined by		
Gel filtration on Sephadex G-200	44,800	58,250
Calculation from sedimentation coefficient	53,160	69,000

In a preliminary study by van Zaane and Gielkins (113), polypeptides from cells infected with different strains of MDHV and HVT were analyzed by immunoprecipitation with homologous and heterologous sera from infected birds followed by SDS-polyacrylamide gel electrophoresis. The pattern of virus-specific polypeptides observed in immunoprecipitations with homologous sera from cells infected with pathogenic MDHV, apathogenic MDHV, and HVT were similar. Four major virus specific protein bands were immunoprecipitated and were apparently late gene products because their amounts increased late in infection. Immunoprecipitation with heterologous sera showed that the four virus specific proteins from these viral strains were antigenically related. Two-dimensional gel electrophoresis of these polypeptides revealed differences in the polypeptide patterns between HVT and the MDHV strains. In light of the DNA homology studies between HVT and MDHV, it is possible that these four polypeptides might be coded for by the homologous regions of the HVT and MDHV genomes. Immunoprecipitations of culture media from cells infected with the above strains of virus detected a protein, presumed to be A antigen, with a molecular weight of 60 - 65,000 that also appeared to be a glycoprotein. Immunoprecipitations with heterologous sera showed antigenic

similarities between the presumptive A antigen from all strains.

Four different antigens have been observed in infected cells by immunofluorescence: a diffuse nuclear antigen, a diffuse cytoplasmic antigen, a granular cytoplasmic antigen and a membrane antigen (MA) on the surface of infected cells (2,15,31,65,78,105). Cytoplasmic and nuclear antigens are present only in cells containing virus particles (23,65). The relationship between these antigens and viral structural proteins are still undefined. Nazerian and Chen (61) found that some cells positive for MA were not producing virus and suggested that MA could be similar to MA detected on cells infected by other herpesviruses such as Epstein-Barr virus. It was also reported that rabbits immunized with purified virus developed antibody to MA. Although it was recognized that the virus might not have been completely purified, it appeared that MA might be related to structural proteins of the virus. Nazerian (58) also reported the loss of MA in cells infected with attenuated MDHV that had also lost A antigen and concluded that A antigen and MA might be related, although it was recognized that these observations could also be coincidental.

Another MD related antigen, designated the MATSA antigen, was detected by immunofluorescence on the surface of MD tumor cells and on almost all cells in MD lymphoblastoid cell lines (54,56,67,75,111). MATSA was not related to viral structural proteins because it was not present on cells productively infected with MDHV (56,111). It appeared that MATSA was distinct from histocompatibility antigen and embryonic antigen. MATSA from different tumor cell lines appeared to be immunologically related but not identical (111). The exact role of MATSA in the host immune response to MD is not known, but it appears that chickens infected with MDHV and HVT do elicit a transient cell mediated response to MATSA (96,100).

Resistance to MD

Resistance to MD can be natural or acquired. Natural resistance to the disease is genetically inherited and falls under two classifications: early resistance, experienced at hatching, and late resistance, which is expressed with increasing age (95). Both early and late forms of natural resistance are expressed through lesion regression (95). After infection with MDHV, resistant chickens develop lesions of MD that eventually disappear while simultaneously infected, susceptible chickens will develop lesions that are

accompanied by high levels of mortality. Natural resistance does not appear to be antibody dependent (94,100) but cell-mediated immunity appears to be important (102). A recent study by Lam and Linna (42) indicated that late natural resistance could be transferred to newly hatched, susceptible chickens with spleen cells from resistant chickens. These cells were apparently neither T-cell, B-cell, nor macrophage in origin, but possibly part of the "third lymphocyte" population which shares many characteristics with natural killer cells in ability to kill a wide range of tumor targets.

Acquired resistance is by vaccination, either by natural exposure to apathogenic strains of virus, or by artificial vaccination with attenuated or apathogenic strains of MDHV or with antigenically related HVT (68,83,95). The Marek's disease system is the only example of a malignant disease that can be completely protected against by vaccination. The first important vaccine was made by Churchill (19) who found that chickens could be protected by inoculation with a virus that was attenuated by repeated passage in tissue culture. Since then, a number of live vaccines have been developed but HVT is the strain most widely used in vaccination (63,68).

Host Immune Response to MDHV Infection

Infection with MDHV results in the initial suppression of both the humoral and cell-mediated responses of the host (72). This initial impairment might be a factor leading to lymphoma formation, or might be a consequence of the degenerative and proliferative changes that occur in the lymphoid tissues of infected birds.

There is a distinct humoral response to infection with MDHV, but the significance of the humoral response in the overall protection of the host is not well understood. Anti-viral antibody has been detected by immunodiffusion (16), immunofluorescence (78,79), virus neutralization (9,24), indirect hemaglutination tests (22) and complement fixation test (53). Antibodies appear 3-5 weeks post-infection and are present for a long time, possibly due to the persistent infection caused by MDHV (63). Passively acquired maternal antibody has been shown to have a significant protective effect against morbidity and mortality caused by MDHV (7). In chickens with maternal or passively administered antibody, the initial productive infection and acute inflammation was greatly reduced (72). The number of tissues with viral antigen and the amount of antigen in positive tissues was lower and it appeared that the

antibody exerted its effect by reducing the extent of the initial virus infection. Chickens with a high titer of antibody during infection appeared to survive the disease or live longer than those with a low antibody titer (59). However, since MDHV infection will cause a severe degeneration of the bursa and thus affect antibody titer, it is not clear whether mortality in low antibody producing chickens is a cause or effect of antibody production. Sharma,et al. (100) showed that bursectomy of age-resistant birds and the resulting agammaglobulinemia did not affect the susceptibility of these birds to MD, implying that the mechanism of tumor regression that is characteristic of age-resistant birds is not dependent on humoral functions. In addition, bursectomized chickens immunized with an apathogenic strain of MDHV were still protected against a transplantable MD-induced tumor (90), however the protective effect of HVT vaccination was impaired though not abolished after bursectomy and x-irradiation (82). Comparative studies on active antibody synthesis in genetically resistant and susceptible chickens did not reveal any quantitative or qualitative differences (25). It appears that although humoral immunity has an amelioratory effect on the severity of Marek's disease, it is not a crucial part of the host's immune response to the disease.

A considerable amount of evidence exists demonstrating the importance of the cell-mediated immune response in the host's defense against MD. Fauser, et al. (24) first provided evidence for cell-mediated immunity to MD based upon delayed hypersensitivity and MIF tests using a semi-purified A antigen preparation. Byerly and Dawe (8) reported a stronger delayed hypersensitivity reaction with a crude MDHV-associated antigen preparation derived from infected cells, than with antigens from tissue culture medium or from feather follicles. The T-cell system, in addition to being the target cell for transformation by MDHV (28,89,99), has been implicated in immune surveillance against the development of lymphomas. Sharma, et al. (102) showed that genetically resistant chickens made deficient in T-cells by neonatal thymectomy and gamma-irradiation became highly susceptible to MD while their untreated hatchmates remained resistant to tumor formation. The availability of MD lymphoblastoid cell lines (3,12,67,75) has allowed for the in vitro study of immune responses to the antigens present on these cells. Sharma, et al. (96) used a ^{51}Cr-release microcytotoxicity test to demonstrate a specific cell-mediated response in spleen cells from MDHV infected chickens directed against ^{51}Cr-labeled lymphoblastoid target cells. The response was expressed

in the absence of antibody and complement and occurred briefly after virus infection and early in the disease process, before gross lymphomas appeared. The cytotoxic response, presumably directed against MATSA, indicated that cell-mediated immunity was also important in mediating the host response against MDHV-inducted tumors.

Lee, et al. (43,45,46) has reported on the existence of suppressor macrophages in the spleens of MDHV-infected chickens, that suppressed the PHA proliferation response of T-cells and also inhibited the DNA synthesis of lymphoblastoid cells. However, Sharma (97) demonstrated that spleens of normal chickens contain suppressor macrophages that are inhibitory to cells of a rapidly dividing MD tumor cell line and also inhibited mitogen-induced blastogenesis of lymphocytes. It is not known if the mechanism of inhibition of MD infected chicken derived macrophages is the same as that of macrophages from normal chickens but it appears that suppressor macrophages may play a nonspecific role in anti-tumor immunity in the chicken.

Vaccinal Immunity

Vaccination with nononcogenic virus results in the persistent viremia of the vaccinating virus. These nononcogenic viruses have been shown to be lymphotrophic with isolation patterns very similar to oncogenic

viruses (11,77,110). However, unlike oncogenic viruses, virus replication and antigen expression is minimal and tissue necrosis is absent (77).

Purchase and Sharma (81) first demonstrated the involvement of the immune system in HVT vaccination. They found that HVT did not protect chickens that had previously been treated with high doses of cyclophosphamide, a drug that causes a permanent impairment of the humoral response and a temporary impairment of the cellular immune response. Although they could not discern which of these two host responses was important in vaccinal immunity, subsequent work done by Else (23) and Rennie, et al. (79) showed that bursectomy and the resulting agammaglobulinemia did not substantially impair vaccine protection.

Ross (85), using a plaque inhibition test as a measure of cell-mediated immunity, showed that plaques formed by MDHV-infected cells could be inhibited by T-lymphocytes present in the peripheral blood of chickens vaccinated with attenuated MDHV. When the same procedure was attempted using sensitized lymphocytes from HVT vaccinated birds, only a low level of plaque inhibition was observed (86). However, when antisera from both HVT or attenuated MDHV vaccinated birds was supplemented with normal spleen cells, equally

good plaque reduction was observed. The author suggests that two mechanisms might exist for inhibiting growth and spread of MDHV in tissue culture; one mediated by sensitized lymphocytes and the other by an antibody-dependent cell-mediated cytotoxicity.

The nature of the important antigens involved in vaccinal immunity to MD was first examined by Kaaden, et al. (33). The protective effects of purified HVT virions and HVT-infected cellular membranes was studied. Purification of HVT from infected cells yielded viral preparations of low infectivity that contained many unenveloped nucleocapsids. This viral preparation provided an effective immunity to vaccinated chickens, reducing specific mortality by 74%. When cellular membrane fractions from HVT infected cells, purified on sucrose gradients, were used to vaccinate chickens, specific mortality was reduced by 94%. After solubilization, the membranes from HVT-infected cells formed two specific precipitin lines in immunodiffusion tests with MDHV-specific chicken sera. In subsequent work, (32) plasma membranes obtained from MDHV and HVT infected cells were highly purified by isopynic centrifugation in dextran and determined to be free of virus particles by electron microscopic examination. Two specific precipitin bands were formed in the

immunodiffusion assays with solubilized plasma membranes from infected cells, and polyacrylamide gel electrophoresis analysis of this material also showed two virus induced protein bands. Antisera prepared against plasma membranes from MDHV or HVT infected cells neutralized extracellular infectious HVT. When chickens were inoculated twice with plasma membranes from HVT infected cells, MD mortality was reduced by 94%. This work indicated that virus infection with MDHV or HVT induced the formation of unique proteins on the infected cell surface that were also immunogenic. However, this work still leaves open the question of the relationship of these membrane components to viral structural proteins as well as to the soluble antigens defined by immunodiffusion.

Work concerning the antigenic nature of vaccinal immunity was done by Lesnik and Ross (48). Chickens were immunized with noninfectious materials extracted with nonionic detergents from cells infected with attenuated MDHV. Protection was obtained with both soluble and insoluble antigens of Nonidet P-40 extracts, but only with the insoluble fractions of deoxycholate extracts. The growth and spread of MDHV was reduced in immunized chickens based on the observed proportion of circulating blood leukocytes that contained virus

and the proportion of birds having A antigen in their feather follicles. In addition, the authors noted that when soluble and insoluble fractions were examined for the presence of precipitating antigens, it appeared that the amount of antigen in the preparation did not correlate with the capacity to protect, suggesting that immunity could be conferred by antigens that did not take part in the precipitation tests.

Solubilized glycoproteins from membrane-rich fractions of HVT-infected cells were isolated by Wyn-Jones and Kaaden (112) with Concanavalin A affinity chromatography. These glycoproteins elicited heavy precipitin lines in immunodiffusion test with chicken sera from MDHV-infected birds, although their relationship to the already defined A and B antigens were not determined. Analysis of this glycoprotein material by PAGE showed three polypeptide bands in the molecular weight range of 100×10^3 - 120×10^3 that were not present in uninfected cell glycoprotein extracts. This material, when purified by preparative PAGE and then inoculated into chickens, resulted in the production of low titer neutralizing antibody and partial protection against challenge with virulent MDHV. The work demonstrated that HVT can induce production of unique glycoproteins in infected chicken cells and also

demonstrated the possible involvement of these glycoproteins in eliciting neutralizing activity, although the resulting neutralizing activity induced in vaccinated chickens was of low titer and only a small number of chickens were actually tested.

A number of studies have been concerned with the anti-tumor nature of vaccinal immunity. Powell (74) immunized chickens against MD with repeated injections of gluteraldehyde-fixed cells of an MD-lymphoblastoid cell line. The resulting protection was attributed to immunity against MATSA antigen. Using a ^{51}Cr-release assay, Sharma, et al. (101) demonstrated a T-cell-mediated immune response to MD tumor cells in HVT or attenuated MDHV vaccinated chickens. In addition, other studies have shown that HVT inoculated birds developed transient lymphoproliferative lesions (110), MATSA positive cells have been detected in the tissues of HVT and attenuated MDHV vaccinated chickens (91) and a chicken thymus-derived lymphoblastoid cell line has been established from HVT inoculated chickens in which 95% of the cells from this line demonstrated MATSA (39). HVT could be rescued from this cell line by co-cultivation with chicken embryo fibroblasts.

Although it has been assumed that the MATSA antigen was the most likely target for an anti-tumor

immune response, recent work by Schat and Murthy (92) indicated that the exact role of MATSA in the host immune response is still questionable. MATSA positive cells of a MD-lymphoblastoid cell line were made MATSA negative by treatment with papain. These papain treated cells had become negative for membrane fluorescence, and they did not lyse in the presence of anti-MATSA antibody and complement. These results were not seen if cells were treated with trypsin or mixed glycosidases. When these MATSA negative cells were used as target cells for MD-sensitized spleen cells in a chromium release cytotoxicity assay, no significant decrease in the specific release of chromium was observed as compared to MATSA positive cells. The authors concluded that MATSA was not the target antigen in chromium release assay tests, and is probably not an important antigen in cell-mediated immunity to MD. Other suggested antigenic alternatives for MATSA included the possibility of a tumor associated antigen that could not be detected by serological methods such as has been described on Epstein-Barr virus transformed B-lymphocytes, and the unlikely possibility of the chicken fetal antigen that is present on all transformed cell lines.

Although the exact nature of the antigens involved in vaccinal immunity is still unknown, the

fact that both anti-viral and anti-tumor responses are important lends support to the theory presented by Payne (73) concerning the mechanism of vaccinal immunity to MD. Payne proposed a two step mechanism of resistance in vaccinated birds. The first step involved both humoral and cell-mediated responses that are stimulated by the vaccinating virus against the replication and spread of infecting MDHV. Thus, by reducing the virus load on the host, there is a reduction in both the immunosuppressive effects of MDHV infection and the probability of neoplastic transformation of lymphoid cells. This permits the host to mount a more effective response to infection. The second step consists of a cell-mediated response directed against tumor cells. This anti-tumor response could be a result of transient lymphoproliferation induced by the vaccinating virus or a result of cell transformation due to the challenging MDHV.

This theory is further supported by work done by Powell, et al. (76). Chickens were immunized with either gluteraldehyde-inactivated, MDHV-infected chicken kidney cells containing virus specific antigens, or with gluteraldehyde-inactivated MDHV-transformed lymphoblastoid cells containing MATSA. They found that both types of immunizations protected against MD but the mechanisms

of protection were different. Immunization with viral antigens was associated with suppression of virus replication after challenge with virulent virus but cytotoxicity to MD tumor cells was not observed. Immunization with tumor antigens did not affect viral multiplication but some evidence of cell-mediated anti-tumor activity was found.

The above results were also confirmed by Murthy, et al. (57) who studied early pathological events following immunizations with viral antigens or tumor antigens and subsequent challenge with MDHV. They found that viral antigen vaccine inhibited both replication of challenge virus and tumor formation while tumor antigen vaccine only had an effect on subsequent tumor development. These results probably accounted for the observation that birds given viral antigen vaccines had a higher level of protection against tumor development than birds given tumor antigen vaccine.

MATERIALS AND METHODS

Cells and Virus

Primary duck embryo fibroblast cells (DEF) were prepared and propagated according to the procedures established by Long, et al. (50). Cells were seeded in 100 mm diameter plastic tissue culture dishes (1.0×10^7 cells/plate) with 10 ml per plate growth medium consisting of standard Medium 199 and nutrient mixture F-10 combination containing 2% calf serum. Growth medium was changed 16-24 hrs after seeding and the pH was adjusted with isotonic $NaHCO_3$ as needed.

MDHV strain GA-infected cells in 25^{th} passage and HVT strain FC-126 infected DEF cells in 16^{th} passage were used to generate stock supplies of MDHV and HVT infected DEF cells in 26^{th} and 17^{th} passages, respectively. Virus infected cells from 100 mm tissue culture dishes were frozen in 1 ml ampules for storage in liquid nitrogen. They were then titered and used in all subsequent experiments as a source of infective virus. DEF monolayers that were approximately 85% confluent, were infected with a 1:8 dilution of a 1 ml ampule of stock infected DEF cells. At this dilution

of infected cells, cytopathic effect (CPE) was barely noticeable at 24 hrs post infection (PI) and reached a maximum at approximately 48-72 hrs PI. Infected cell monolayers were maintained in 10 ml of growth medium without calf serum. The medium was changed 24 hrs PI and the pH adjusted with $NaHCO_3$ as needed.

Radioactive Labeling

Infected cells were labeled at 48-72 hrs PI, when more than 50% of the cells demonstrated CPE. Uninfected cells were handled in the same manner as infected cells. Labeling of protein with ^{35}S-methionine was accomplished by washing cell monolayers three times with warm Hanks Buffered Saline Solution (HBSS, Gibco) followed by a 24 hr incubation period in 5 ml of Dulbecco's Minimal Essential Medium (DMEM, Gibco) containing 1/20 the normal concentration of unlabeled methionine and ^{35}S-methionine (New England Nuclear) at 50 μCi/ml. Glycoproteins were labeled by incubating cell monolayers for 24 hrs in 5 ml growth medium containing 1% calf serum and ^{14}C-glucosamine (New England Nuclear) at 1 μCi/ml.

Preparation of Labeled Culture Media and Cell Lysates for Immunoprecipitation

Culture media from infected and uninfected cells were collected and clarified at 5,000 x g for 10 minutes followed by ultracentrifugation at 147,000 x g for 1 hr. Labeled cells were lysed according to the method of Witte, et al. (108). Cell monolayers were washed three times with phosphate buffered saline (PBS) at 4° followed by extraction into a detergent lysis buffer consisting of 0.01 M NaH_2PO_4-$NaHPO_4$ (pH 7.5)-0.1 M NaCl containing 1% Triton X-100, 0.5% sodium deoxycholate (NaDOC), and 0.1% sodium dodecyl sulfate (SDS). Cell lysates were clarified as described above for culture media.

Radioactivity Assays

For the determination of radioactive isotope incorporation in culture media or cell lysates, 100 µl of ^{14}C-glucosamine labeled material or 10 µl of ^{35}S-methionine labeled material was spotted on 2.3 cm diameter Whatman 3 mm filter discs. The discs were oven-dried at 60° and treated on ice with cold 5% Trichloracetic acid (TCA) for 20 minutes followed by a 1 minute wash in acetone. The discs were then dried as above and added to vials containing 5 ml of phosphor scintillation fluid (2,5, Diphenyloxazole [PPO] 22.7 gm;

1,4,bis-2-[4-methyl-5-phenyloxazolyl]-benzene (POPOP) 1.9 gm; toluene 8 pints) and counted in a Packard liquid scintillation spectrometer (Packard Inst. Co.).

Protein Determinations

Protein determinations were done according to the method established by Lowry, et al. (51) with crystalline bovine serum albumin (BSA) as the standard.

Antisera for Immunoprecipitations

All antisera were already available in the laboratory. Immune chicken serum (ICS), which demonstrates a stronger reactivity to A antigen then to B antigen by immunodiffusion analysis, was obtained from birds naturally infected with MDHV. Rabbit anti-A serum (RαA), negative for B antigen by immunodiffusion, and rabbit anti-B antigen sera (RαB), negative for A antigen by immunodiffusion, were prepared by inoculating rabbits with highly purified A and B antigens, respectively (49,105). Rabbit anitsera prepared against infected cell plasma membranes purified by Dextran T-40 gradients (RαPM), and found to be reactive with B antigen by immunodiffusion analysis, was also used. In certain instances, the above immune sera were absorbed with unlabeled, uninfected cell lysates or culture media in order to reduce the appearance of

background polypeptides on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The amount of material used for absorption was five times the volume of labeled material used for immunoprecipitation. After a 24 hr absorption period, on ice at 4°, the sera was centrifuged at 30,000 rpm for 1 hr in a type 50.1 rotor (Beckman) prior to use. In order to block the appearance of either A or B antigens, 40 µl of immune serum was incubated with either 1 unit of A antigen or 1 unit of B antigen (49,106) followed by a centrifugation at 30,000 rpm for 1 hr in a type 50.1 rotor (Beckman) at 4°. Both A and B antigen preparations were donated by Dr. Velicer and were partially purified through isoelectric focusing (106). As a control, normal rabbit serum (NRS) and serum from specific pathogen free birds (NCS) were also used in immunoprecipitation analysis.

Immunoprecipitation

Infected cell culture medium and lysate material were used as a source of A and B antigens, respectively. Immunoprecipitations were performed according to the methods of Witte, et al. (108) with the use of S.aureus Cowan strain I to collect antigen-antibody complexes. This procedure exploits the high specificity and adsorption capacity of the protein A molecule located

on the cell wall of the Cowan I strain of the S.aureus bacterium for the Fc region of many IgG subclasses (38). S.aureus Cowan I strain, prepared for use as an immunoadsorbent according to the method of Kessler (38) was provided by Dr. Velicer. Prior to use, the prepared S.aureus was pelleted at 5,000 x g for 10 minutes and resuspended to the original volume with detergent lysis buffer containing 1 mg/ml BSA. This S.aureus immunoadsorbant was then used at ten times the volume of antiserum used for immunoprecipitation.

Aliquots of culture medium containing 10^3 - 10^4 counts per minute (cpm)/µg of ^{35}S-methionine labeled protein and 10^3 - 10^5 cpm/µg ^{14}C-glucosamine labeled protein, and cell lysates containing 10^5 - 10^6 cpm/µg ^{35}S-methionine labeled protein and 10^5 - 10^7 cpm/µg ^{14}C-glucosamine labeled protein were used for immunoprecipitations. Prior to incubation with immune serum, the antigen containing material was precleared in order to remove material that nonspecifically adsorbs to either IgG or the S.aureus immunoadsorbant. After overnight incubation of the material at 0-4° with 40 µl of normal serum, 400 µl of the S.aureus immunoadsorbant was then added followed by an incubation period of one hour at 0-4° and centrifuged at 12,000 x g for 3 minutes in an Eppendorf centrifuge. The precleared

supernatant was then added to 40 µl of immune serum followed by an overnight incubation at 0-4°, an addition of 400 µl of S.aureus immunoadsorbant, incubation for 1 hr at 0-4°, and a centrifugation at 12,000 x g for 3 minutes in Eppendorf centrifuge. The supernatant was carefully removed by vacuum and discarded with a pasteur pipet attached to a vacuum flask so as not to disturb the bacterial pellet containing the antigen-antibody complex. The pellet was then washed three times with detergent lysis buffer at 0-4° and the wash supernatant carefully removed by vacuum and discarded as described above. In order to extract all the liquid from the pellet, after the last wash was removed, the pellet was centrifuged once more at 12,000 x g for 1 minute in an Eppendorf centrifuge and any remaining liquid was discarded. The pellet was then prepared for SDS-PAGE as described below.

SDS-PAGE and Fluorography

Discontinuous stack SDS-Polyacrylamide slab gels were prepared and run according to the methods of Laemmli (41) in a vertical gel apparatus (Hoeffer, model SE-520). Polyacrylamide gels containing a 5% stacking gel and a 6.5%, 7.5%, or a 10% separating gel were prepared from a stock solution of a 30:0.8 ratio of acrylamide to bis-acrylamide mixture. Final

concentration of the stacking gel was 0.0625 M Tris-HCL, pH 6.8, and 0.1% SDS. The final concentration of the separating gel was 0.184 M Tris-HCL, pH 8.8 and 0.1% SDS. The electrode buffer was 0.05 M Tris, 0.38 M glycine, and 0.1% SDS at pH 8.8.

The washed S.aureus pellet containing immunoprecipitates were handled for electrophoresis as described by Witte, et al. (108). Samples were denatured by placing them in 40 µl of Sample Buffer (SB) containing 0.05 M Tris-HCL, pH 6.8, with 1.0% mercapthoethanol, 10% glycerol, 1.0% SDS and 0.001% bromophenol blue, and heating at 68° for 15-20 minutes. After centrifugation in an Eppendorf centrifuge for 1 minute, one half of the SB supernatant was loaded onto the gel.

Acetone precipitates of ^{14}C-glucosamine labeled culture media and cell lysates were prepared by adding one volume of culture media or lysate to nine volumes of acetone, and kept overnight at -20°. The precipitate was pelleted at 12,000 x g for 30 minutes and the pellet air-dried before it was dissolved in 40 µl of SB and heat treated as described above, prior to SDS-PAGE analysis. Direct SDS-PAGE analysis of ^{35}S-methionine labeled proteins of culture media and lysates was achieved with a 1:2 dilution of labeled material in

double strength SB, followed by heat treatment as described above, prior to gel loading.

Standard molecular weight markers included ^{14}C-glucosamine labeled myosin (200,000 daltons), phosphorylase B (92,500 daltons), BSA (69,000 daltons), ovalbumin (46,000 daltons), and carbonic anhydrase (22,000 daltons) (New England Nuclear). Molecular weight estimations were calculated by interpolation between standard proteins, similar to Weber and Osborn (107).

Electrophoresis was carried out at a constant voltage of 150 volts (ISCO Electrophorsis Power Supply Model 1493) until the marker dye ran off the gel, usually after 1.5-2 hrs. Gels were fixed overnight in a solution containing 10% acetic acid and 10% methanol. Detection of labeled proteins and glycoproteins by fluorography was performed by impregnating the gel with PPO as described by Bonnar and Laskey (6). Gels were then dried under vacuum (Hoeffer Slab Gel Dryer Model SE-540), placed in contact with RP Royal X-OMAT Film RP/R2 (Kodak) and kept at -70° for varying lengths of time.

RESULTS

Analysis of ^{35}S-methionine and ^{14}C-glucosamine Labeled Polypeptides Found in Culture Media

Since A antigen has previously been characterized as a glycoprotein that is shed into the culture medium of MDHV-infected cells (19,49,50,88), an analysis of culture media from ^{35}S-methionine and ^{14}C-glucosamine labeled cells was performed on SDS-PAGE (Figure 1). Profiles of culture media from ^{35}S-methionine and ^{14}C-glucosamine labeled infected cells revealed a virus-induced polypeptide with an approximate molecular weight range of 61-65,000 (Figure 1A). Although culture medium from ^{35}S-methionine labeled cells showed apparently virus-specific polypeptides at 40,000 daltons and 67,000 daltons, the 61-65,000 dalton polypeptide appeared to be the major virus-induced polypeptide shed from the cell.

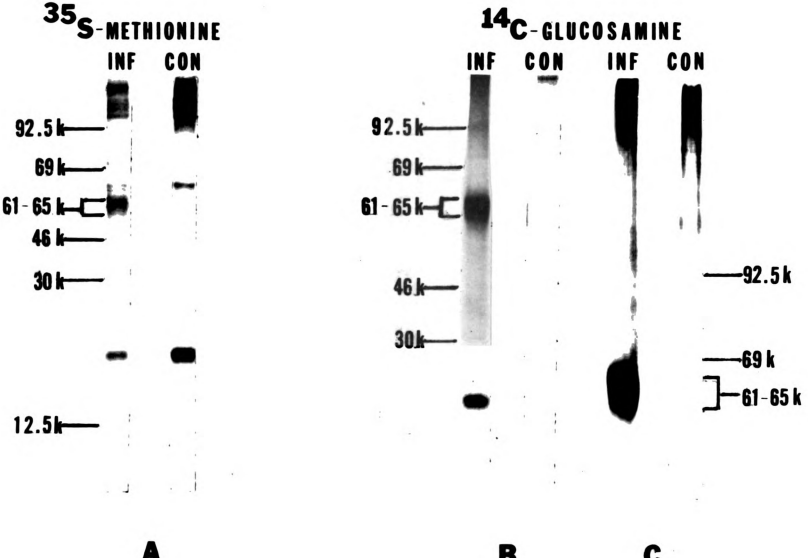
Analysis of acetone precipitates of culture medium from ^{14}C-glucosamine labeled cells also demonstrated a virus-specific, 61-65,000 dalton, glycosylated molecule (Figures 1B and 1C). Acetone precipitates from equal volumes of infected and

Figure 1.--Analysis of culture media from ^{35}S-methionine and ^{14}C-glucosamine labeled, MDHV-infected (INF) and uninfected (CON) cells.

A. Direct analysis, performed as described in materials and methods, of culture medium from ^{35}S-methionine labeled INF and CON cells, containing 4.5×10^5 and 6.0×10^5 cpm, respectively, on 10% SDS-PAGE, 24 hr fluorographic exposure.

B. Acetone precipates of culture medium from ^{14}C-glucosamine labeled INF and CON cells, containing 4.0×10^4 and 1.7×10^4 cpm, respectively, on 10% SDS-PAGE, 16 day fluorographic exposure.

C. Acetone precipitates of culture medium from ^{14}C-glucosamine labeled INF and CON cells, containing 6.0×10^4 cpm each, on 7.5% SDS-PAGE, 10 day fluorographic exposure.


35S-METHIONINE
INF
CON
92.5k
69k
61-65k
46k
30k
12.5k
14C-GLUCOSAMINE
INF
CON
INF
CON
92.5k
69k
61-65k
46k
30k
92.5k
69k
61-65k
A
B
C

uninfected cell culture medium revealed a ^{14}C-glucosamine labeled, 61-65,000 molecular weight molecule in infected cell culture medium, while uninfected culture medium did not reveal any ^{14}C-glucosamine labeled molecules (Figure 1B). Since incorporation of ^{14}C-glucosamine in uninfected cells was 2-4 times less than in infected cells, acetone precipitate samples, containing equal counts per ml of ^{14}C-glucosamine, were prepared for SDS-PAGE analysis in order to better ascertain the virus-specific nature of the labeled molecules observed (Figure 1C). This analysis confirmed the virus-specific nature of the 61-65,000 dalton molecule.

The observation that the 61-65,000 dalton molecule was the major ^{14}C-glucosamine labeled species shed into the culture medium of infected cells, suggested that it may be A antigen. However, in order to confirm rigorously the identity of this molecule as A antigen, highly specific immunological analysis was required.

Immunoprecipitation Analysis of Culture Medium of ^{35}S-methionine Labeled Cells Using RαA

A ^{35}S-methionine labeled, virus-specific polypeptide with a molecular weight range of 61-65,000, was immunoprecipitated from culture medium using RαA (Figure 2A). This polypeptide was not detected on

Figure 2.--Immunoprecipitation analysis of culture media from ^{35}S-methionine labeled, MDHV-infected (INF) and uninfected (CON) cells with rabbit anti-A serum (RαA).

A. 100 µl of culture media from INF and CON cells, containing 4.8 x 10^5 and 6.6 x 10^5 cpm respectively, were reacted with RαA. Immunoprecipitates were analyzed on 8.5% SDS-PAGE, 48 hr fluorographic exposure.

B. 100 µl of culture medium from INF and CON cells, containing 3.0 x 10^5 and 4.7 x 10^5 cpm, respectively, were reacted with RαA and normal rabbit serum (NRS) and immunoprecipitates analyzed on 7.5% SDS-PAGE, 7 day fluorographic exposure.

C. 100 µl of culture medium from INF cells were reacted with RαA and RαA blocked with A antigen and the immunoprecipitates analyzed on 7.5% SDS-PAGE, 48 hr fluorographic exposure.

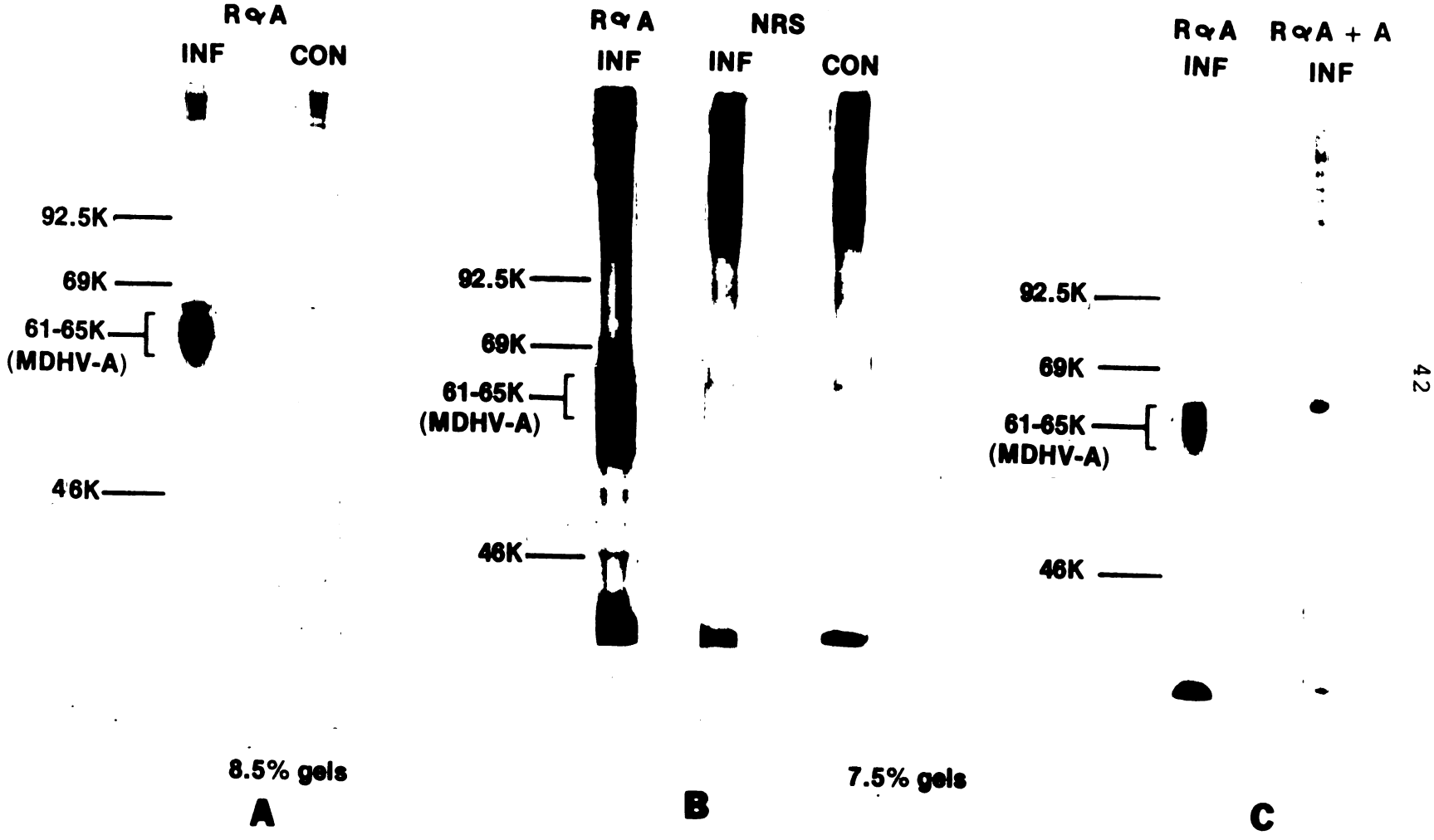
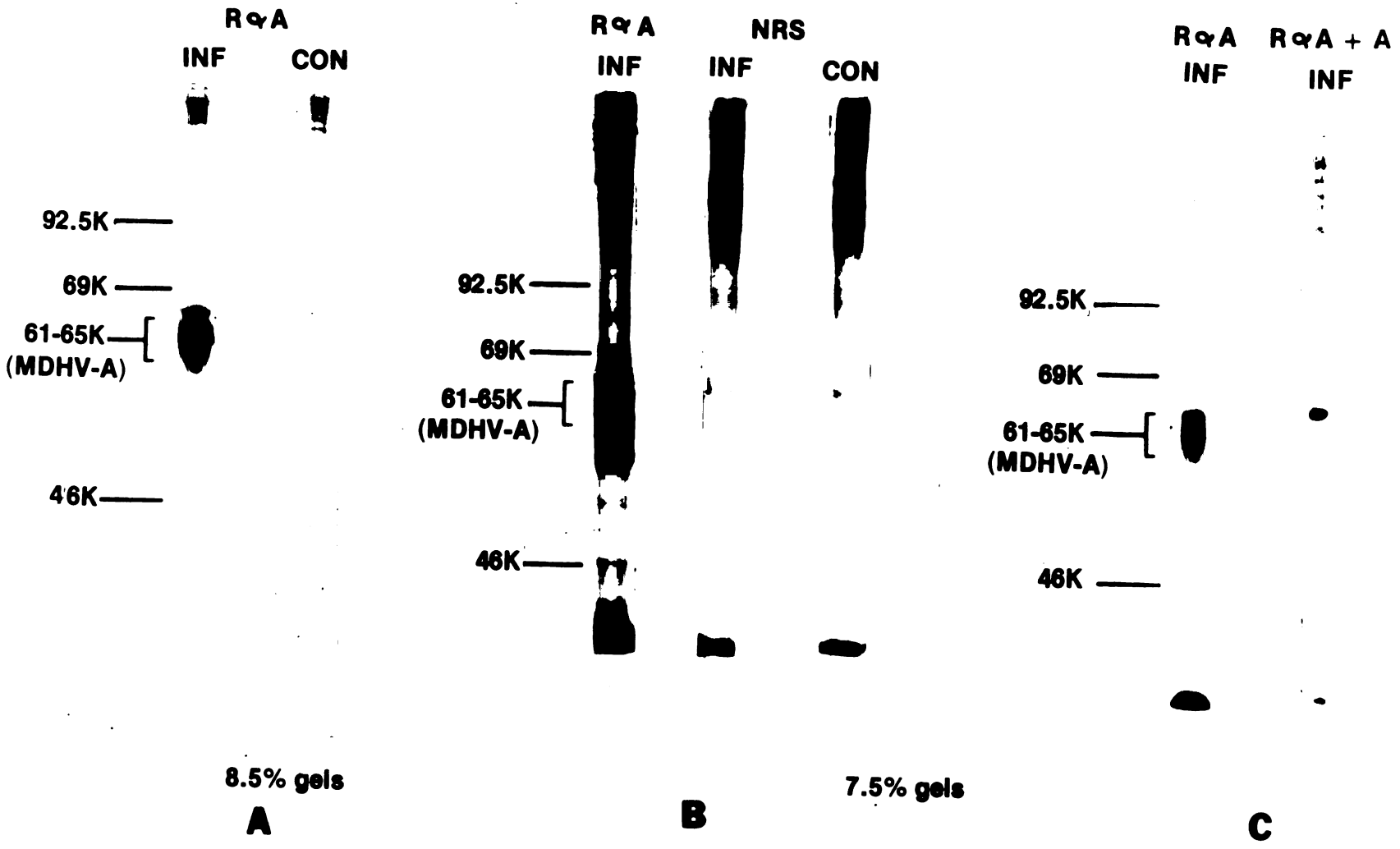

R∝A
INF
CON
92.5K
69K
61-65K
(MDHV-A)
46K
8.5% gels
A
R∝A
INF
NRS
INF
CON
92.5K
69K
61-65K
(MDHV-A)
46K
B
R∝A
INF
R∝A + A
INF
92.5K
69K
61-65K
(MDHV-A)
46K
7.5% gels
C

Figure 2.--Immunoprecipitation analysis of culture media from ^{35}S-methionine labeled, MDHV-infected (INF) and uninfected (CON) cells with rabbit anti-A serum (RαA).

A. 100 µl of culture media from INF and CON cells, containing 4.8 x 10^5 and 6.6 x 10^5 cpm respectively, were reacted with RαA. Immunoprecipitates were analyzed on 8.5% SDS-PAGE, 48 hr fluorographic exposure.

B. 100 µl of culture medium from INF and CON cells, containing 3.0 x 10^5 and 4.7 x 10^5 cpm, respectively, were reacted with RαA and normal rabbit serum (NRS) and immunoprecipitates analyzed on 7.5% SDS-PAGE, 7 day fluorographic exposure.

C. 100 µl of culture medium from INF cells were reacted with RαA and RαA blocked with A antigen and the immunoprecipitates analyzed on 7.5% SDS-PAGE, 48 hr fluorographic exposure.

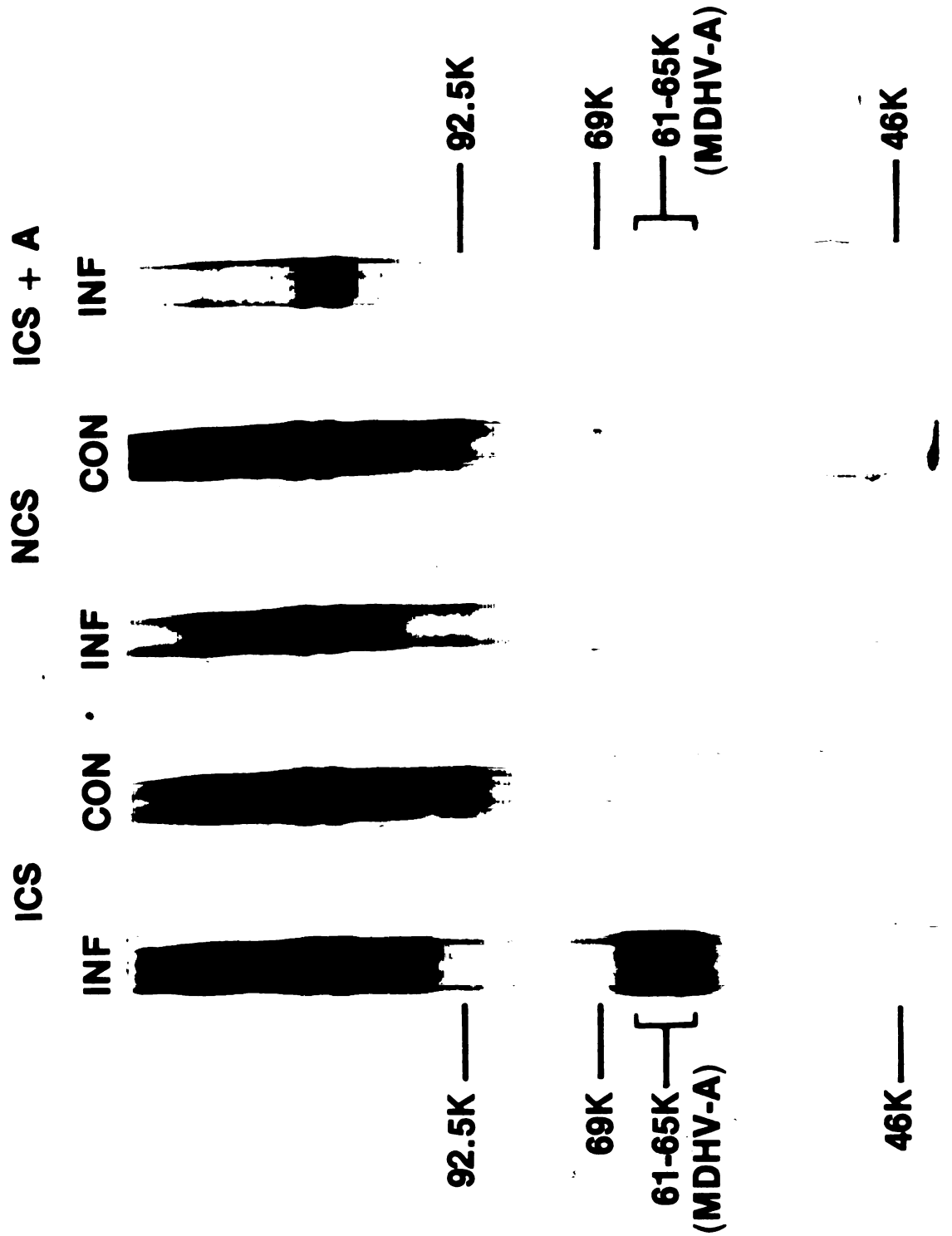

RαA
INF
CON
92.5K
69K
61-65K
(MDHV-A)
46K
8.5% gels
A
RαA
NRS
INF
INF
CON
92.5K
69K
61-65K
(MDHV-A)
46K
B
RαA
INF
RαA + A
INF
92.5K
69K
61-65K
(MDHV-A)
46K
7.5% gels
C

SDS-PAGE analysis of immunoprecipitates of infected or uninfected cell culture media with NRS, even with an overexposure of the gel (Figure 2B). In order to confirm the identity of the 61-65,000 dalton polypeptide as A antigen, blocking experiments were performed by pre-incubating RαA with A antigen that was purified through isoelectric focusing (49). Figure 2C shows that blocking immune serum with the A antigen preparation selectively eliminated the 61-65,000 dalton polypeptide.

Immunoprecipitation Analysis of Culture Medium of ^{35}S-methionine Labeled Cells Using ICS

The 61-75,000 dalton polypeptide was immunoprecipitated only from infected cell culture medium using ICS, and was not precipitated by NCS (Figure 3). Confirmation of the identity of this 61-65,000 dalton polypeptide as A antigen was also achieved by selectivity blocking the appearance of the polypeptide on SDS-PAGE with A antigen, purified through isoelectric focusing (49). The gel in Figure 3 was intentionally overexposed to demonstrate the virus-specific nature of the 61-65,000 dalton polypeptide and the thoroughness with which purified A antigen can block the precipitation of this polypeptide.

Figure 3.--Immunoprecipitation analysis of culture media from ^{35}S-methionine labeled, MDHV-infected (INF) and uninfected (CON) cells with immune serum from naturally infected chickens (ICS). 100 µl of culture medium from INF and CON cells, containing 5.0 x 10^5 and 6.7 x 10^5 cpm, respectively, were reacted with ICS, normal chicken serum (NCS) and ICS blocked with A antigen (ICS + A). Immunoprecipitates were analyzed on 7.5% SDS-PAGE, 5 day fluorographic exposure.


ICS
INF
CON
NCS
INF
CON
ICS + A
INF
92.5K
69K
61-65K
(MDHV-A)
46K
92.5K
69K
61-65K
(MDHV-A)
46K

Immunoprecipitation Analysis of Culture Medium of ^{14}C-glucosamine Labeled Cells

A antigen has been previously characterized as a glycoprotein and labeled with ^{14}C-glucosamine (49,88). In order to confirm further the identity of the 61-65,000 dalton polypeptide as A antigen, culture medium from ^{14}C-glucosamine labeled cells was examined by immunoprecipitation analysis. A virus-induced molecule, of molecular weight 61-65,000, was observed in immunoprecipitates obtained with RαA (Figure 4) and ICS (Figure 5), but was not visible in control precipitates obtained with either NRS or NCS. In addition, the appearance of this molecule could be blocked by purified A antigen, as shown in Figure 4 with RαA and in Figure 5 with ICS.

Immunoprecipitation Analysis of Culture Medium of HVT-Infected Cells

Immunodiffusion studies have demonstrated that HVT and MDHV infection results in the appearance of an antigenically related A antigen (49,108). When culture medium from ^{35}S-methionine labeled cells infected with HVT was examined by immunoprecipitation analysis with either RαA or ICS, a virus-induced polypeptide was observed with a molecular weight similar to that of the previously identified, MDHV-associated A antigen

Figure 4.--Immunoprecipitation analysis of culture media from ^{14}C-glucosamine labeled, MDHV-infected (INF) and uninfected (CON) cells with rabbit anti-A serum (RαA).

A. 600 µl of culture medium from INF and CON cells, containing 2.4 x 10^5 and 1.0 x 10^5 cpm, respectively, were reacted with RαA and normal rabbit serum (NRS). Immunoprecipitates were analyzed on 8.5% SDS-PAGE, 16 day fluorographic exposure.

B. Same as in A, with RαA blocked with A antigen (RαA + A) on 8.5% SDS-PAGE, 8 day fluorographic exposure.

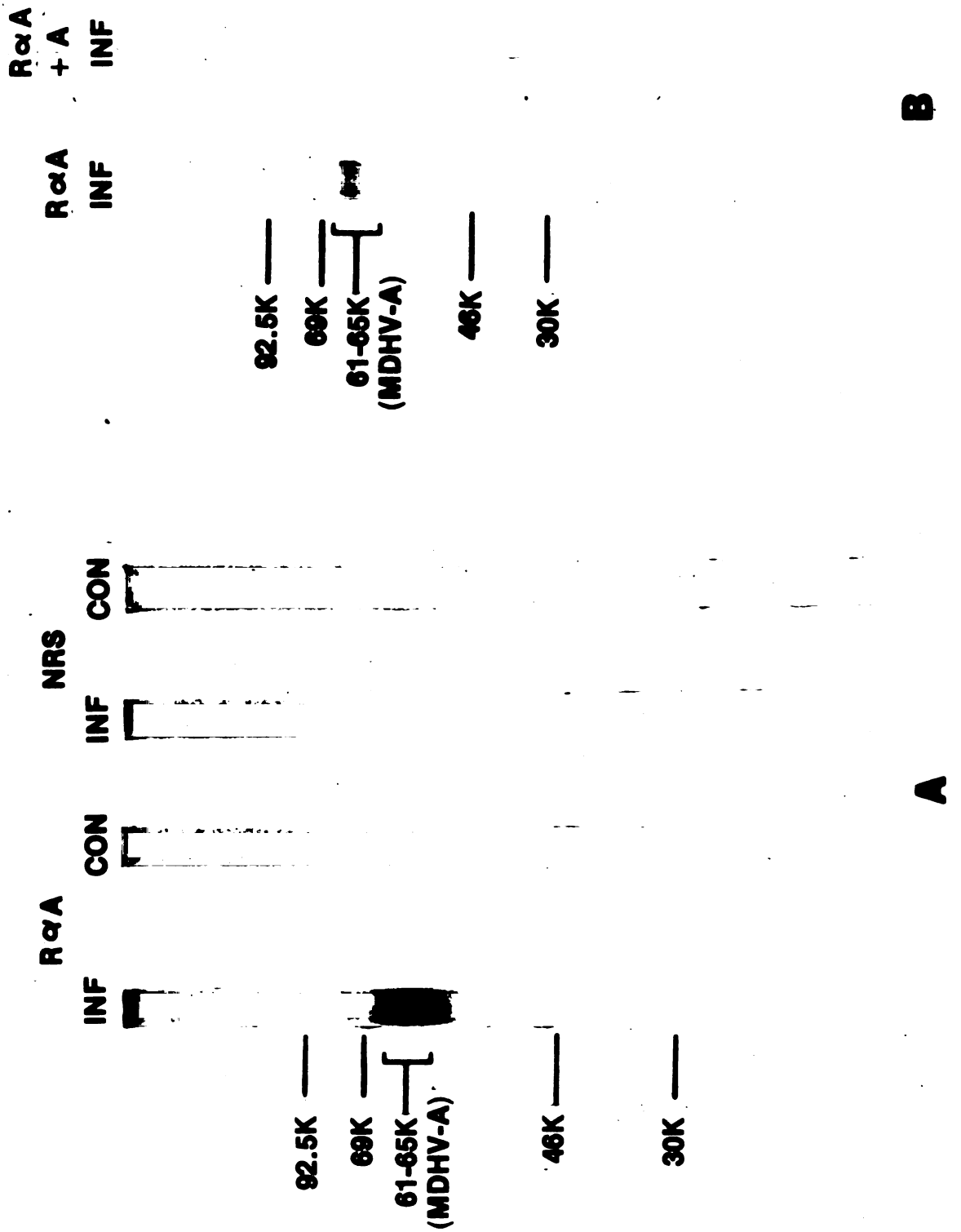

A
RαA
INF
CON
NRS
INF
CON
INF
92.5K
69K
61-65K
(MDHV-A)
46K
30K
B
RαA
INF
RαA
+A
INF
92.5K
69K
61-65K
(MDHV-A)
46K
30K

Figure 5.--Immunoprecipitation analysis of culture media from ^{14}C-glucosamine labeled, MDHV-infected (INF) and uninfected (CON) cells with immune serum from naturally infected chickens (ICS). 600 µl of culture medium from INF and CON cells, containing 4.0×10^5 and 8.0×10^4, respectively, were reacted with ICS, normal chicken serum (NCS) and ICS blocked with A antigen (ICS + A). Immunoprecipitates were analyzed on 7.5% SDS-PAGE, 8 day fluorographic exposure.

ICS+A
INF

NCS
INF CON

ICS
INF CON

61—65K [redacted]
(MDHV—A)

(Figure 6). This polypeptide was not visible in immunoprecipitations performed with either NRS or NCS.

Analysis of ^{35}S-methionine and ^{14}C-glucosamine Labeled Polypeptides of Cell Lysates

B antigen has been characterized as a soluble, cell-associated glycoprotein (105). In an attempt to identify B antigen by SDS-PAGE analysis, infected cells were disrupted and proteins solubilized by a lysis buffer containing the detergents SDS, Triton-X 100, and sodium deoxycholate. These cellular detergent lysates contained solubilized membrane-associated proteins (108).

Direct analysis of ^{35}S-methionine labeled cell lysates revealed a large number of polypeptides common to both infected and uninfected cells (Figure 7A). The two most obvious virus-induced polypeptides had approximate molecular weights of 83,000 and 150,000.

Acetone precipitates of ^{14}C-glucosamine labeled cell lysates resulted in indistinct bands on SDS-PAGE analysis. This is characteristic of the general appearance of glycoproteins on SDS-PAGE due to heterogeneity of glycosylation (102). Much of the label appeared to be associated with molecular weights of 69,000 and higher (Figure 7B and 7C). Infected cell lysates also appeared to contain a ^{14}C-glucosamine

Figure 6.--Immunoprecipitation analysis of culture medium from ^{35}S-methionine labeled, HVT-infected cells. 100 µl of culture medium from MDHV-infected cells containing 4.0×10^5 cpm, and 200 µl of HVT-infected cell culture medium containing 8.0×10^5 cpm, were reacted with rabbit anti-A serum that had been absorbed with unlabeled, normal cell culture medium (RαA), normal rabbit serum (NRS), immune serum from naturally infected chickens (ICS) and normal chicken serum (NCS). Immunoprecipitates were analyzed on 7.5% SDS-PAGE, 4 day fluorographic exposure.

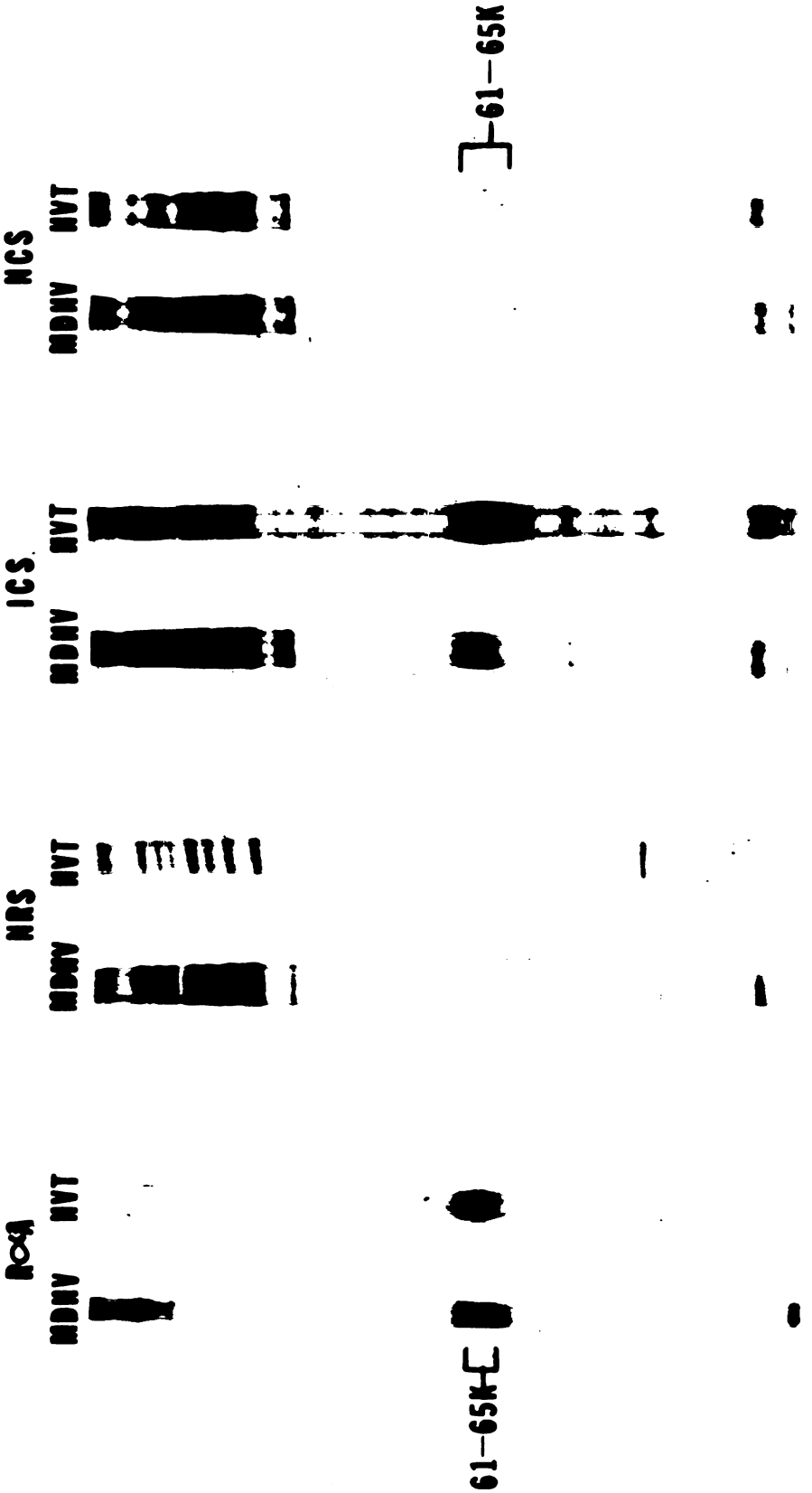

61-65K
NCS
NDNV
NVT
ICS
NDNV
NVT
NRS
NDNV
NVT
Rc4
NDNV
NVT
61-65K

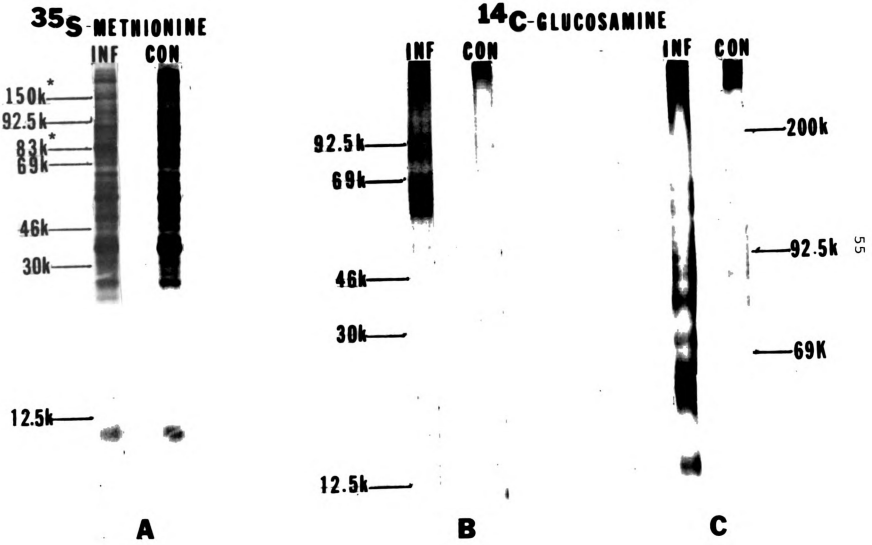
Figure 7.--Analysis of detergent lysates from ^{35}S-methionine and ^{14}C-glucosamine labeled, MDHV-infected (INF) and uninfected (CON) cells.

A. Direct analysis, performed as described in materials and methods, of lysates from ^{35}S-methionine labeled INF and CON cells, containing 1.8 x 10^5 and 1.6 x 10^5 cpm, respectively, on 10% SDS-PAGE, 24 hr fluorographic exposure.

B. Acetone precipitates of lysates from ^{14}C-glucosamine labeled INF and CON cells, containing 1.0 x 10^4 cpm and 8.0 x 10^3 cpm, respectively, on 10% SDS-PAGE, 1 month fluorographic exposure.

C. Acetone precipitates of lysates from ^{14}C-glucosamine labeled INF and CON cells, each containing 2.0 x 10^4 cpm, on 6.5% SDS-PAGE, 10 day fluorographic exposure.

*Indicates virus-specific polypeptides.


35S-METHIONINE
INF
CON
150k*
92.5k
83k*
69k
46k
30k
12.5k
A
14C-GLUCOSAMINE
INF
CON
92.5k
69k
46k
30k
12.5k
B
INF
CON
200k
92.5k
69K
C

labeled molecule with the approximate molecular weight of 60-65,000, which is similar to that of A antigen.

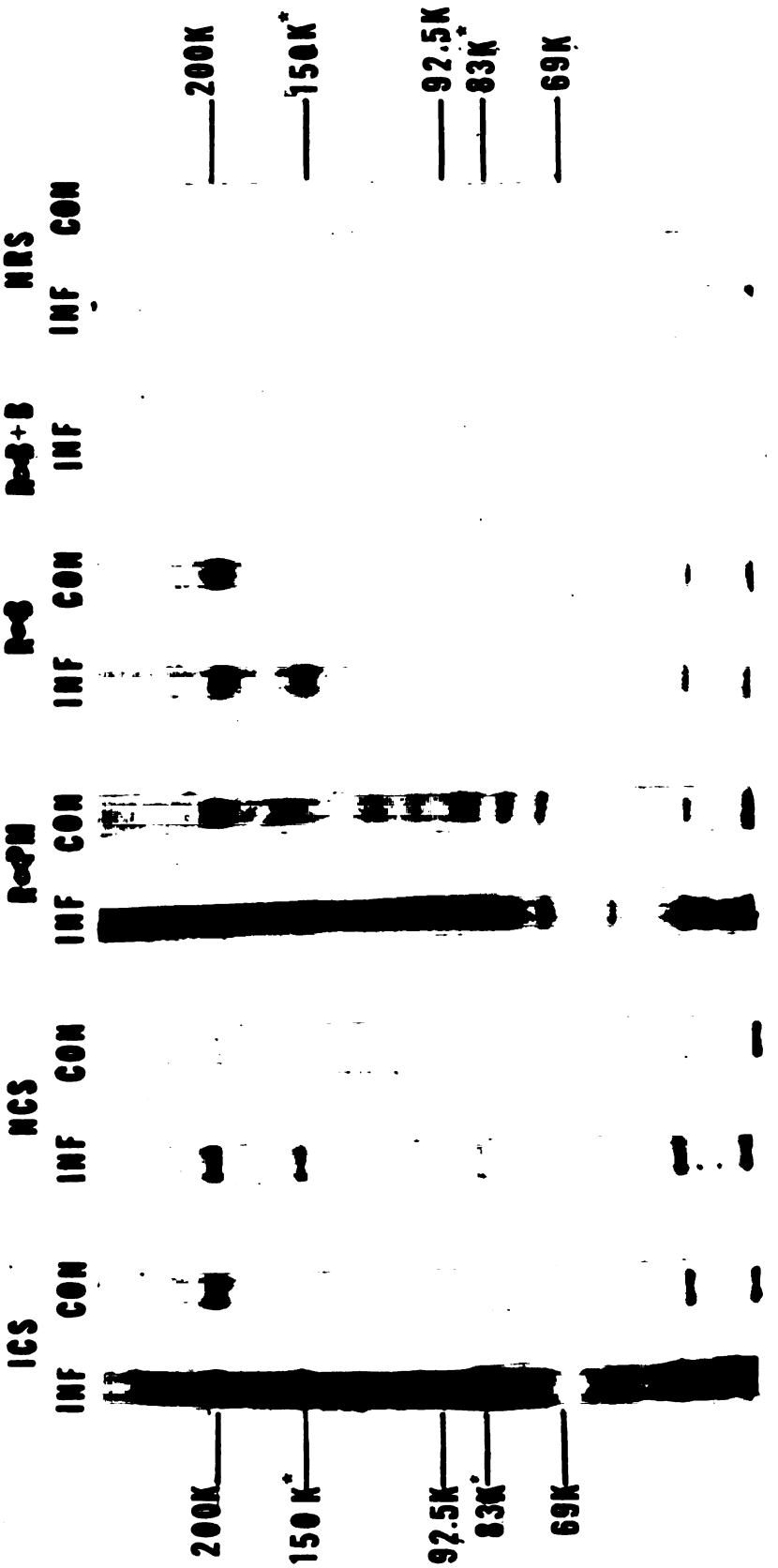
Because of the 2 to 4 fold less incorporation of ^{14}C-glucosamine in uninfected cells, few labeled molecules were detected in lysates from uninfected cells when equivalent volumes of infected and uninfected lysates were analyzed on SDS-PAGE (Figure 7B). SDS-PAGE analysis of acetone precipitates from infected and uninfected cell lysates, containing nearly equivalent counts per ml of ^{14}C-glucosamine, did not reveal any major differences, except for the virus-specific, 60-65,000 molecular weight molecule which probably represents A antigen (Figure 7C).

Immunoprecipitation Analysis of ^{35}S-methionine Labeled Cell Lysates with Sera Reactive With B Antigen

Detergent lysates from ^{35}S-methionine labeled infected and uninfected cells were subject to immunoprecipitation analysis with various sera that demonstrated a significant reactivity to B antigen by immunodiffusion analysis. ICS precipitated two major virus-induced polypeptides with approximate molecular weights of 83,000 and 150,000 (Figure 8). All other polypeptides appearing in infected lysate immunoprecipitates were also visible in control lysate precipitates

Figure 8.--Immunoprecipitation analysis of detergent lysates from ^{35}S-methionine labeled, MDHV-infected (INF) and uninfected (CON) cells with sera reactive with B antigen. 300 µl of INF and CON cell lysates, containing 6.3×10^4 and 6.2×10^4 cpm, were reacted with immune chicken serum (ICS), normal chicken serum (NCS), rabbit anti-plasma membrane serum (RαPM), rabbit anti-B serum (RαB), rabbit anti-B serum blocked with B antigen (RαB + B) and normal rabbit serum (NRS). Immunoprecipitates were analyzed on 6.5% SDS-PAGE, 6 day fluorographic exposure.

*Designates B antigen candidate polypeptides.


200K
150K*
92.5K
83K*
69K
NRS
INF CON
INF
INF CON
INF CON
NCS
INF CON
ICS
INF CON
200K
150 K*
92.5K
83K*
69K

after longer gel exposure time. Reduced amounts of the two virus-induced polypeptides were also present in infected cell lysate precipitates with NCS. However their appearance, at greatly reduced levels compared to the specific immunoprecipitates, were probably due to the nonspecific trapping of these polypeptides. Both RαB and RαPM also precipitated the virus-induced 83,000 and 150,000 dalton polypeptides. The appearance of these polypeptides with NRS, at greatly reduced levels, was again probably due to the nonspecific trapping of these polypeptides.

In order to identify conclusively B antigen on SDS-PAGE, and to determine the relationship of the virus-specific 83,000 and 150,000 dalton polypeptides to B antigen, blocking experiments were attempted with B antigen purified through isoelectric focusing (105). However, attempts at blocking B antigen reactive sera with purified B antigen were unsuccessful because blocking appeared to nonspecifically remove background polypeptides as well as virus-specific polypeptides. This is exemplified in the SDS-PAGE profile of an immunoprecipitation with B antigen blocked RαB (Figure 8). It was therefore impossible to conclusively identify B antigen on SDS-PAGE.

Immunoprecipitation Analysis of ^{14}C-glucosamine Labeled Cell Lysates with Sera Reactive with B Antigen

B antigen was previously identified as a glycoprotein (105). It was therefore of interest to examine the SDS-PAGE profiles of immunoprecipitates from ^{14}C-glucosamine labeled detergent lysates of MDHV-infected cells. The only virus-induced molecule immunoprecipitated had an approximate molecular weight of 230,000 (Figure 9). This carbohydrate containing molecule was present in immunoprecipitates of infected cell lysates with RαPM and RαB, but not with NRS.

Immunoprecipitation Analysis of Cell Lysates of MDHV and HVT-infected Cells with Sera Reactive with B Antigen

B antigen has been characterized by immunodiffusion analysis as a common antigen between HVT and MDHV infected cells (106,109). Immunoprecipitation analysis on SDS-PAGE, of ^{35}S-methionine labeled, HVT-infected cell lysates with B antigen reactive sera revealed the presence of both the 83,000 and 150,000 polypeptides (Figure 10). ICS precipitated comparatively more of the 150,000 dalton polypeptide than the 83,000 dalton polypeptide. RαPM immunoprecipitated approximately equal amounts of the 83,000 and 150,000 dalton polypeptides from HVT infected cell lysates.

Figure 9.--Immunoprecipitation analysis of detergent lysates from ^{14}C-glucosamine labeled, MDHV-infected (INF) and uninfected (CON) cells with sera reactive with B antigen. 1.0 ml of INF cell lysate and 1.5 ml of CON cell lysate containing 2.7×10^5 and 1.2×10^5 cpm, respectively, were reacted with rabbit anti-plasma membrane serum (RαPM), rabbit anti-B serum (RαB) and normal rabbit serum (NRS). Immunoprecipitates were analyzed on 6.5% SDS-PAGE, 21 day fluorographic exposure.

*Designates B antigen candidate polypeptides.

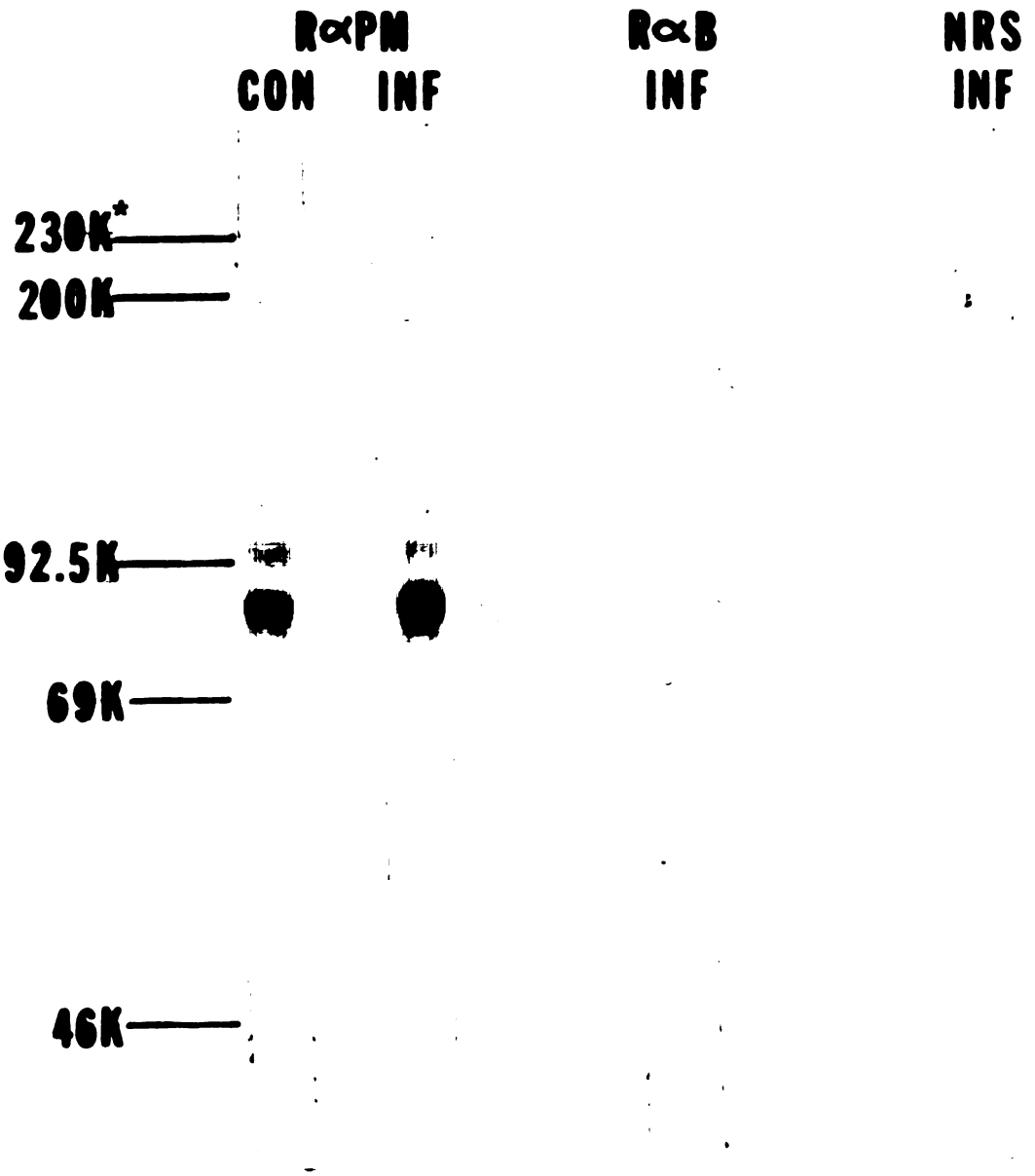
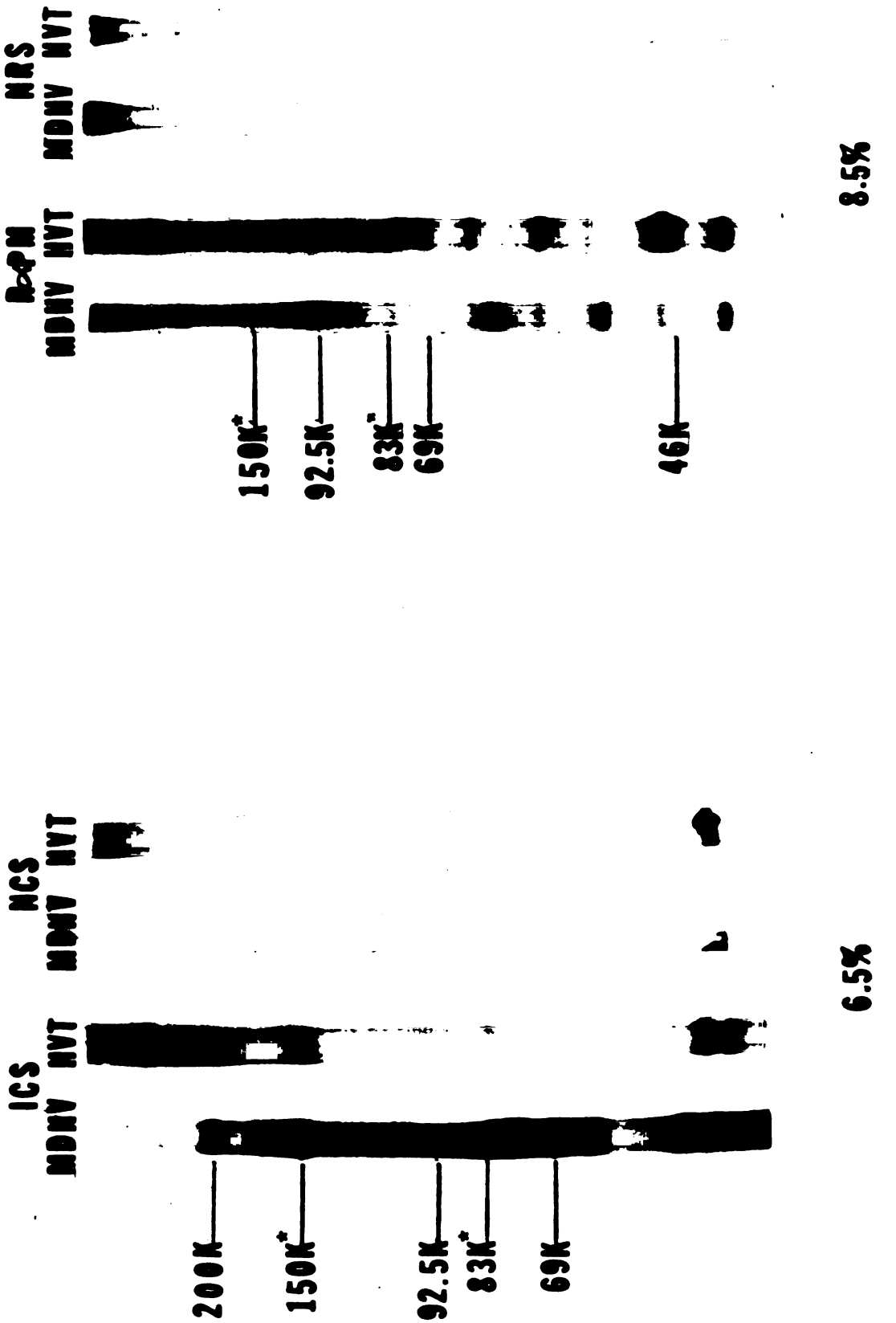

RαPM
CON
INF
RαB
INF
NRS
INF
230K*
200K
92.5K
69K
46K

Figure 10.--Immunoprecipitation analysis of ^{35}S-methionine labeled lysates from HVT-infected cells with sera reactive with B antigen. 100 µl of MDHV and HVT-infected cell lysates containing 1.4 x 10^6 and 2.3 x 10^6 cpm, respectively, were reacted with immune chicken serum (ICS) and normal chicken serum (NCS). Immunoprecipitates were analyzed on 6.5% SDS-PAGE, 4 day fluorographic exposure. The same amounts of infected cell lysates were reacted with rabbit anti-plasma membrane serum (RαPM) and normal rabbit serum (NRS) and immunoprecipitates analyzed on 7.5% SDS-PAGE, 4 day fluorographic exposure.

*Designates B antigen candidate polypeptides.


NRS
MDMV NVT
8.5%
MDMV NVT
150K*
92.5K
83K*
69K
46K
NCS
MDMV NVT
6.5%
ICS
MDMV NVT
200K
150K*
92.5K
83K*
69K

A small degree of non-specific trapping of these polypeptides could be seen with NCS and NRS.

DISCUSSION

The objective of this work is to further the molecular characterization of the A and B antigens of MDHV-infected cells by SDS-PAGE analysis following S.aureus immunoprecipitation. These antigens have previously been defined only by immunodiffusion (19,49, 50,88), and all previous attempts at biochemical and biophysical characterization (49,50,87,88,106) were based on this criteria. Molecular characterization and identification of these antigens on SDS-PAGE can subsequently serve as a foundation for further analytic studies of entire glycoproteins and their polypeptide portions.

A antigen proved to be the easiest antigen to characterize, probably because it is shed into the culture medium in large quantities. SDS-PAGE profiles, representing the overall ^{35}S-methionine labeled polypeptide content of infected and uninfected cell culture media (Figure 1A), show a major virus-specific polypeptide with the approximate molecular weight of 61-65,000. Direct analysis on SDS-PAGE of glycoproteins found in the culture medium of ^{14}C-glucosamine labeled

infected cells also show that a major glycoprotein with an approximate molecular weight of 61-65,000 is shed into the culture medium (Figures 1B and 1C).

Results of immunoprecipitations with A antigen reactive sera and blocking studies with purified A antigen show unequivocally that A antigen is a glycoprotein with an apparent molecular weight of 61-65,000 on SDS-PAGE. This is demonstrated by the immunoprecipitation of this polypeptide from the culture medium of infected cells using RαA (Figure 2) and ICS (Figure 3). Furthermore, the immunoprecipitation of this polypeptide with both sera could be selectively blocked by incubating the antisera with purified A antigen prior to immunoprecipitation. This is observed with both ^{35}S-methionine and ^{14}C-glucosamine labeled material and with all immune sera used (Figures 2,3 and 4).

Although early studies initially indicated that avian IgG does not have a high specificity for protein A (40), more recent examinations indicate that immunoglobulin affinity for protein A increases substantially when it is bound in an immune complex (38). This is probably the reason immunoprecipitation of the 61-65,000 dalton polypeptide could be achieved with ICS. This ability of the S.aureus protein A to bind to ICS sera should aide in future immunological studies of MDHV antigens.

While this work was in progress, van Zaane and Gielkens (113) reported the detection of a virus-specific glycoprotein of molecular weight 60-65,000 that is secreted into the culture medium of MDHV-infected DEF cells. This glycoprotein was immunoprecipitated with complex sera obtained from MDHV immunized birds and observed on SDS-PAGE, but no further attempt was made to rigorously confirm its identity as A antigen. While this work provided preliminary data suggesting that this glycoprotein might be A antigen because of its appearance in the culture medium of infected cells, the study done for this thesis proves conclusively that A antigen appears on SDS-PAGE as a glycoprotein of 61-65,000 molecular weight. The rigorous proof of the identity of A antigen presented here was made possible due to the availability of A antigen-specific antisera and purified A antigen for use in immunoprecipitation analysis and blocking studies, respectively.

The work reported here is an extension of the initial purification and characterization of A antigen by Long, et al. (49,50). A antigen was purified greater than 200 fold with a 24% recovery on ion exchange column chromatography, isoelectric focusing, and preparative PAGE. However, the analysis of the purified antigen by immunodiffusion and by SDS-PAGE

showed that it was not purified to homogeneity. Three precipitin lines were observed in immunodiffusion tests when serum from rabbits inoculated with the highly purified antigen was used. However, absorption of the rabbit serum with sonicated extracts of uninfected cells, cell culture medium, and calf serum removed antibody to the contaminants, resulting in antiserum monospecific for A antigen in immunodiffusion analysis. When purified A antigen was analyzed on SDS-PAGE, four polypeptides with approximate molecular weights of 21,000; 52,000; 57,000; and 82,000 were observed (49). A re-examination of the data strongly suggests that the two polypeptides with molecular weights of 52,000 and 57,000 are actually two poorly resolved peaks of a broad band that may represent the 61-65,000 molecular weight A antigen observed in this study. This is significant in light of the observation that under certain gel conditions A antigen sometimes appears as a doublet. The difference in molecular weight values observed in this study and the study by Long, et al. (49) could be accounted for by the different SDS-PAGE buffer systems used.

Long found purified A antigen had an apparent molecular weight of 44,800 by gel filtration in Sephadex G-200 and an apparent molecular weight of

53,160 by calibration from sedimentation coefficient. The differences in the molecular weight values observed by Long using gel filtration and those found in this study by SDS-PAGE following immunoprecipitation are probably a consequence of the technical differences involved in these different analytical procedures. It is also difficult to achieve an accurate assessment of the molecular weight of glycosylated proteins by SDS-PAGE since glycosylated polypeptides may bind SDS anomalously, resulting in migration rates not strictly inversely proportional to the logarithm of the molecular weight (103). Thus, as indicated above, the size estimates resulting from this molecular characterization study must be considered apparent molecular weights.

It has been previously demonstrated by immunodiffusion that HVT and MDHV infected cells share an immunologically similar A antigen (49,109). SDS-PAGE immunoprecipitation analysis of culture medium from HVT infected cells with sera specific for MDHV-A antigen also demonstrates a 61-65,000 polypeptide similar to that of MDHV (Figure 6). This result also agrees with the preliminary immunoprecipitation studies of van Zaane and Geilkens (112). The finding that both HVT and MDHV infected cells share an immunologically similar A antigen is significant in light of

hybridization studies which indicate that HVT and MDHV share only a 2-5% DNA homology. This would allow MDHV and HVT infected cells to share coding potential to only three or four proteins of approximately 5 x 10^4 daltons (47). Presumably, MDHV-A antigen, or at least a portion of the polypeptide responsible for its antigenic identity, would be encoded by this region of homology. Since vaccination with HVT confers a highly effective immunity against Marek's disease, these common proteins maybe of major importance when considering anti-viral immunity to Marek's disease.

Because A antigen is a common antigen, it would be of major interest to determine the functional role of A antigen in the host's response to Marek's disease. Attempts by many laboratories have been hampered by poor purification of the antigen, but preliminary work cited by Velicer, et al. (106) suggested that the monospecific rabbit anti-A serum did not neutralize cell-free MDHV. It also appeared that both A and B antigens were associated with the plasma membrane of infected cells.

A similar procedure and general approach used for the identification of A antigen on SDS-PAGE, was applied in an attempt to detect B antigen. Due to the cell-associated nature of B antigen, detergent lysates

of infected cells were examined. Direct analysis of polypeptides found in ^{35}S-methionine labeled infected and uninfected cell lysates on SDS-PAGE show a very complex pattern. The most noticeable virus induced polypeptides have molecular weights of 83,000 and 150,000 (Figure 7A). SDS-PAGE analysis of acetone precipitates from ^{14}C-glucosamine labeled cell lysates did not show the presence of any major virus-specific glycoproteins, except for the 60-65,000 dalton molecule which could be A antigen (Figures 7B and 7C). The presence of A antigen in cell lysates probably represents cell-associated A antigen (106). It could also be a result of contamination of cell lysates with culture medium, despite the fact that cell monolayers were washed several times before lysis.

Immunoprecipitation analysis of ^{35}S-methionine labled lysates most consistently reveal virus-specific polypeptides of molecular weight 83,000 and 150,000 on SDS-PAGE (Figure 8). These bands are evident in infected cell lysates immunoprecipitated with RαPM, RαB, and ICS. The 83,000 and 150,000 molecular weight polypeptides are also visible after reaction of infected cell lysates with NCS and NRS, but to a much lesser degree than with immune sera. The appearance of these polypeptides at low levels with nonimmune sera is

probably due to nonspecific binding of these polypeptides to the IgG molecule or to the S.aureus immunoadsorbant. Since the 83,000 and 150,000 molecular weight polypeptides are much more intensely visible after immunoprecipitation with all three sera known to have specificity for B antigen, these polypeptides appear to be immunoprecipitated and can be considered major candidates for B antigen.

SDS-PAGE analysis of immunoprecipitates prepared from lysate material with B antigen specific sera could not conclusively identify B antigen. The fact that infected cell lysates contain a large number of normal cell components, as well as the tendency of immune sera to react nonspecifically with normal cell components, are factors that hindered the identification of B antigen by contributing to the complexity and variability of the SDS-PAGE profiles of infected cell lysates. Due to the cell-associated nature of MDHV, it is impossible to achieve a synchronized infection, and consequently infected cell lysates always contain an array of infected and uninfected cell polypeptides. The overall appearance of the immunoprecipitation profile of infected cell lysates on SDS-PAGE is therefore influenced by the extent in which the monolayer is infected. If the monolayer is

lysed when the bulk of the cells are well advanced in the infectious cycle, then most of the labeled proteins will be virus-induced. If cells are lysed when only a fraction of the monolayer is infected, virus-induced precursor polypeptides, including proteins in different stages of glycosylation, will be more apparent.

Results of blocking experiments with purified B antigen are not conclusive enough to determine the relationship of either of the candidate B antigen polypeptides to B antigen. The results shown in Figure 8 demonstrate the prior incubation of rabbit anti-B sera with B antigen purified through isoelectric focusing not only eliminated the appearance of both the 83,000 and 150,000 molecular weight polypeptides from the immunoprecipitation profile on SDS-PAGE, but eliminated many of the background polypeptides as well. It was, therefore, difficult to assess the ability of the B antigen preparation to specifically block B antigen related polypeptide(s). This could be due to contaminating proteins in the partially purified B antigen preparation which might be reacting with components in the immune sera. As a result, both background polypeptides and candidate B antigen polypeptides may be blocked. Alternatively, due to a high carbohydrate content, B antigen might be a glycoprotein

which adheres to other polypeptides or to immune sera, thus entirely blocking the immunoprecipitating capabilities of the sera.

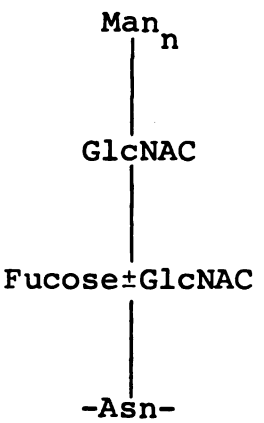
B antigen has been characterized as a common antigen between HVT and MDHV infected cells (106,109). For this reason, HVT infected cell lysates were also subject to immunoprecipitation analysis with B antigen reactive sera (Figure 10). The fact that both the 83,000 and the 150,000 molecular weight polypeptides were visible on SDS-PAGE is significant in light of the minimal amount of DNA homology between the two viruses (26,35,47). This observation also supports the possibility that the two polypeptides are related to an antigen(s) common to HVT and MDHV infected cells, which may include B antigen.

Analysis of ^{14}C-glucosamine labeled lysates by immunoprecipitation with B antigen reactive sera followed by SDS-PAGE did not provide any evidence for the glycosylation of either the 83,000 or the 150,000 candidate B antigen polypeptides. Figure 9 shows that the only major virus-induced, ^{14}C-glucosamine labeled molecule that is immunoprecipitated by both RαPM and RαB has a molecular weight of approximately 230,000. However, a virus-induced polypeptide of 230,000 molecular weight is not visible on SDS-PAGE

immunoprecipitation profiles of ^{35}S-methionine labeled material, although it is possible that its presence is obscured by background polypeptides, especially if methionine constitutes only a small fraction of the glycopeptide. Alternatively, the SDS-PAGE conditions used may not have maximized resolution in that region of the gel, and further analysis on a 5% gel, rather than a 7½% gel, may be necessary.

The preliminary finding that the ^{35}S-methionine and ^{14}C-glucosamine labeled lysates yield very different immunoprecipitation profiles on SDS-PAGE suggest a number of possibilities concerning the relation of the ^{35}S-methionine labeled polypeptides, the 230,000 dalton molecule, and B antigen. One possibility is that the 230,000 dalton molecule represents B antigen while the 83,000 and 150,000 molecular weight polypeptides are virus-induced polypeptides reacting to antibody in the immune sera which is specific for viral proteins other than B antigen. However, this would imply that the different antisera with anti-B activity would, in every case, have this same antibody present. This interpretation can only be substantiated by comparative tryptic peptide analysis of the 83,000 and 150,000 dalton polypeptides and the polypeptide portion of the 230,000 dalton molecule.

Another interpretation of the different SDS-PAGE immunoprecipitation profiles of ^{35}S-methionine and ^{14}C-glucosamine labeled lysates involves the existence of glycosylated intermediates. Most viral glycoproteins, including herpesvirus glycoproteins, are membrane proteins (104) and current theories of membrane glycoprotein synthesis lend support to the existence of partially glycosylated intermediates. There also appear to be many biosynthetic and structural similarities among membrane glycoproteins of infected and uninfected cells (20,29). Current studies indicate that membrane glycoproteins are composed of a core carbohydrate chain linked to asparagine residues of a backbone polypeptide chain as depicted below (20):



Membrane glycoprotein structures tend to diverge from this point. Mannose-linked side chains containing varying amounts of mannose, galactose, n-acetylglucosamine, sialic acid, and fucose are added to this asparagine linked carbohydrate core (20,30,84).

Extensive studies concerning the biosynthesis of viral glycoproteins, such as the G glycoprotein of vesicular stomatitis virus (VSV), have been important in elucidating the important steps involved in the growth and maturation of membrane glycoproteins. The first step in the glycosylation of the VSV G glycoprotein occurs when the core carbohydrate chain is transferred from a lipid-linked oligosaccaride carrier to the G polypeptide being synthesized on polyribosomes of the rough endoplasmic reticulum. The partially glycosylated polypeptide is then inserted into smooth internal membranes of the Golgi apparatus where additional carbohydrate chains are both added and trimmed to yield the final virus-sized G glycoprotein (29,30,84). In addition, proteolytic cleavage of the VSV G polypeptide also appears to be associated with membrane insertion (37). The glycoprotein is then transported to the plasma membrane where it is involved in envelopment of the virion (37).

Although current knowledge of the structure and biosynthesis of herpesvirus glycoproteins is not as extensive as that of the VSV G glycoprotein, studies of herpes simplex virus indicate that the biosynthesis of herpesvirus glycoproteins and VSV G glycoprotein share many similarities. The initial site of glycosylation

of herpes simplex virus glycoproteins occurs in the rough endoplasmic reticulum, either before completion or immediately after synthesis of the polypeptide, while the bulk of carbohydrate addition is associated with smooth membranes (27). There appears to be a large pool of nonglycosylated precursor polypeptides in cells infected at high multiplicities (27). Immunoprecipitation analysis of herpes simplex type 1 infected cells with antisera specific for viral glycoproteins indicate that glycosylation of precursor polypeptides occur in two discreet steps, yielding partially glycosylated intermediates and fully glycosylated products (103). There is no existing evidence of proteolytic cleavage of the polypeptide chain, or of any processing or alteration of the carbohydrate chains.

In view of the current knowledge of glycoprotein synthesis, the 83,000 molecular weight polypeptide might represent the unglycosylated polypeptide backbone; the 150,000 molecular weight polypeptide might represent a partially glycosylated intermediate; and the ^{14}C-glucosamine labeled, 230,000 molecule could be the fully glycosylated antigen. Problems with this interpretation include the fact that the polypeptide portion of the 230,000 molecule should also be present in SDS-PAGE immunoprecipitation analysis of

^{35}S-methionine labeled lysates. In addition to the reasons cited above, the apparent nonexistence of a virus-induced 230,000 polypeptide in ^{35}S-methionine labeled lysates might be a consequence of extensive proteolytic cleavage of the polypeptide portion of the antigen during the course of glycosylation, which might subsequently reduce its intensity on SDS-PAGE. Following this same line of reasoning, if the 150,000 polypeptide actually is a partially glycosylated B antigen intermediate, it would be expected that this 150,000 molecular weight polypeptide show some incorporation of ^{14}C-glucosamine. The apparent absence of a 150,000 glycoprotein on SDS-PAGE analysis of ^{14}C-glucosamine labeled material might be a consequence of poor ^{14}C-glucosamine incorporation, and a much longer exposure of the gel might ultimately identify the 150,000 polypeptide as a glycoprotein. In addition, labeling with another carbohydrate may also be required.

The exact relationship between the 230,000 dalton molecule and the 83,000 and 150,000 polypeptides can ultimately be delineated by further characterization of both the protein and carbohydrate components of these molecules. Tryptic peptide analysis of the polypeptide chain can be accomplished by first removing the carbohydrate portion of the glycoprotein with

endonuclease H (108), while further characterization of the carbohydrate chain can be accomplished after removing the polypeptide chain with proteases. These experiments, however, are beyond the scope of this master's thesis.

APPENDIX

APPENDIX

ATTEMPTED IDENTIFICATION OF B ANTIGEN BY PURIFICATION WITH CONCANAVALIN A AFFINITY CHROMATOGRAPHY AND ISOELECTRIC FOCUSING

Materials and Methods

Concanavalin A (Con A) Affinity Chromatography

Isolation of glycoproteins from labeled cell extracts by Con A affinity chromatography was performed as described by Velicer, et al. (106). Sonically disrupted MDHV-infected cells, labeled with ^{35}S-methionine and clarified at 147,000 x g for 1 hr, were loaded at a protein concentration of 1 mg/ml onto a Con A column with a packed volume of 10 ml. Fractions of 0.5 ml each were collected and assayed for radioactivity, and peak fractions that eluted with α-methly-mannoside were pooled and dialyzed for isoelectric focusing.

Isoelectric Focusing

Pooled fractions containing labeled glycoproteins that eluted from Con A columns with α-methly-mannoside, were dialyzed and isoelectric focused, as described by Velicer, et al. (106) for the purification

of B antigen. At the end of the focusing period, 1 ml fractions were collected and assayed for pH and radioactivity. Fractions at the isoelectric point of B antigen (pH 4.5) were pooled and used for SDS-PAGE analysis.

Results and Conclusions

As an alternative approach, the identification of B antigen was attempted using the methods of B antigen purification involving Con A affinity chromatography and isoelectric focusing, as established by Velicer, et al. (106). The rationale was to determine which polypeptide(s) could be found at the pI of B antigen after isoelectric focusing. ^{35}S-methionine labeled cell extracts were applied to a Con A column and glycoproteins eluting with α-methly-mannoside were pooled and applied to an isoelectric focusing column. Profiles of both the Con A and the isoelectric focusing columns are shown in Figures 11 and 12, respectively. The major difficulty encountered with these procedures involved the loss of large amounts of labeled material with each step of the purification process so that an insufficient amount of labeled material was available for SDS-PAGE analysis. Approximately 55% of the total amount of labeled material that was applied to the Con A column remained irreversibly bound to the column

even after elution with 2 M NaCl. Only 2% of the labeled material applied to the column eluted as glycoprotein with α-methyl-mannoside and only 2% of this material eluted at the B antigen isoelectric point of 4.5. As a result, an insufficient amount of labeled protein remained for immunoprecipitation analysis on SDS-PAGE following these purification procedures, and no conclusions could be drawn concerning the nature of the B antigen.

In future attempts at the identification of B antigen by these methods of purification, the loss of large amounts of labeled protein might be minimized by the addition of unlabeled cell extracts with the labeled cell extracts. This should result in a decreased percentage of labeled protein lost at each step of the purification process, although it might reduce the specific activity of the B antigen in the mixture.

Figure 11.--Con A affinity chromatography of ^{35}S-methionine labeled cell sonicates. The indicated fractions eluting with α-methyl-mannoside were pooled, dialyzed and used for isoelectric focusing. The arrows indicate the addition of 0.1 M α-methyl-mannoside in PBS and 2.0 M NaCl in PBS, respectively.

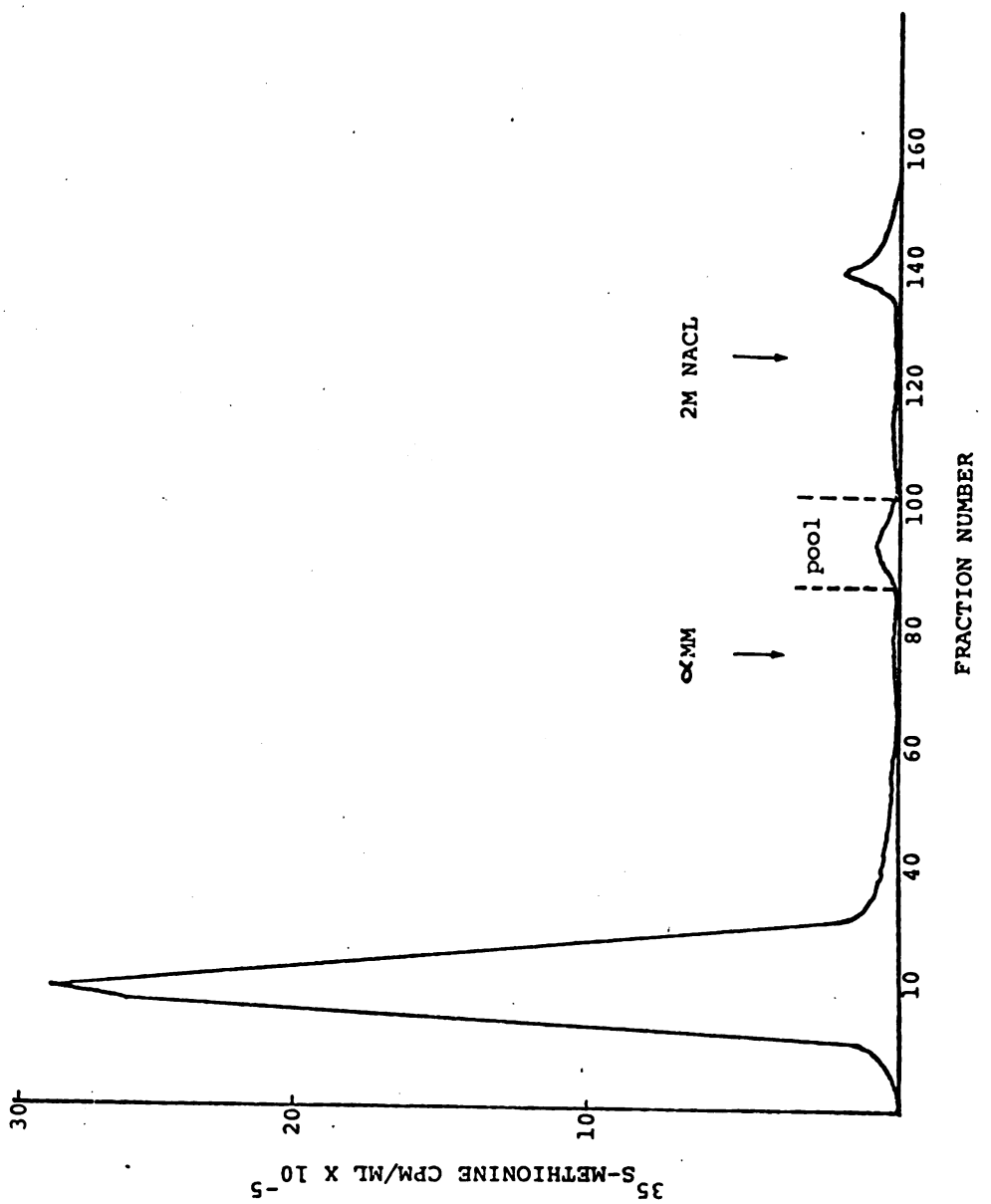
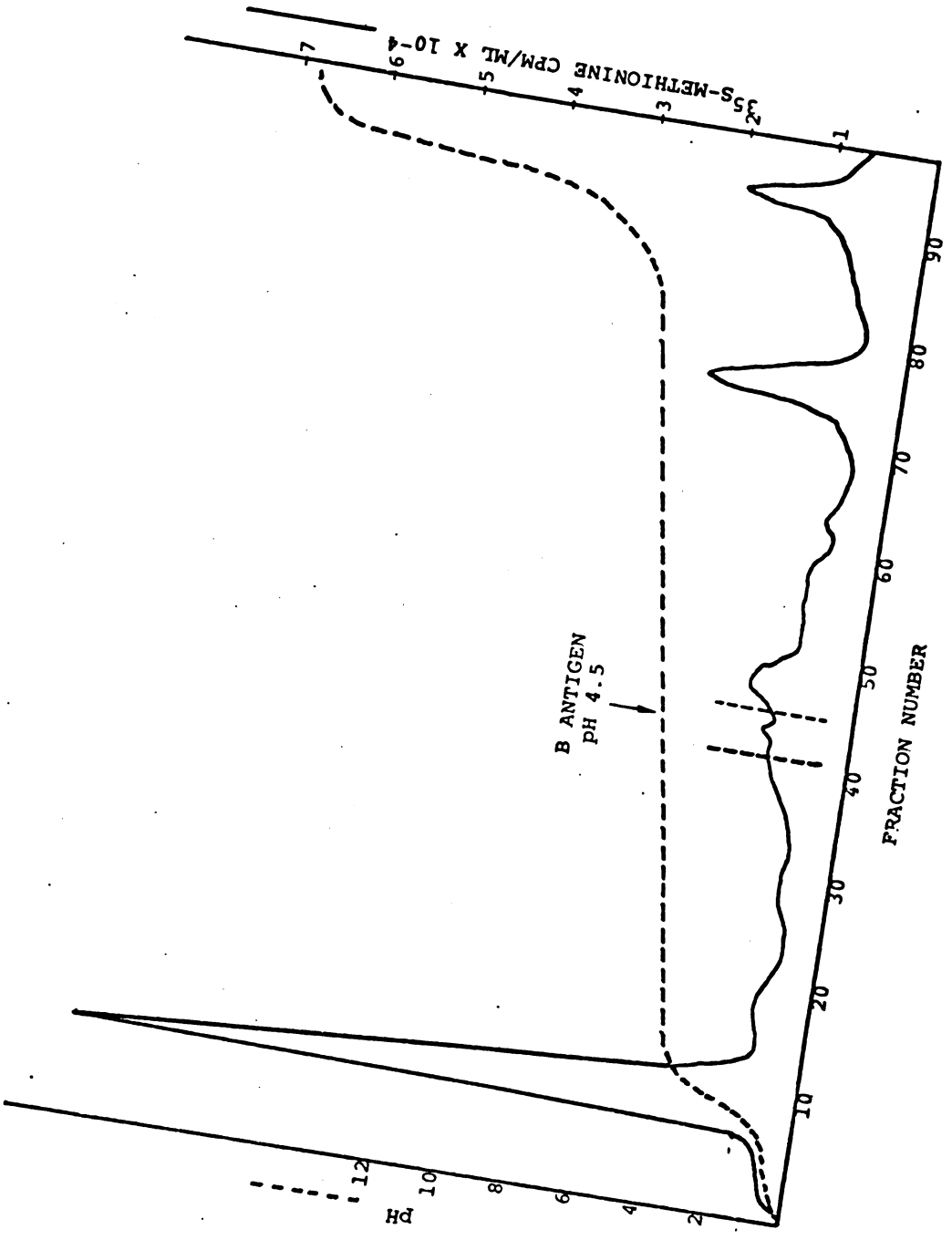

35S-METHIONINE CPM/ML X 10-5
30
20
10
10
40
60
80
100
120
140
160
FRACTION NUMBER
∝MM
pool
2M NACL

Figure 12.--Isoelectric focusing analysis. Pooled and dialyzed fractions eluting from a Con A column with α-methly-mannoside were subject to isoelectric focusing. Fractions at the isoelectric point of B antigen (pH 4.5) were pooled and used for immunoprecipitation analysis on SDS-PAGE.


^{35}S-METHIONINE CPM/ML X 10-4
PH
B ANTIGEN
PH 4.5
FRACTION NUMBER

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