JOURNAL OF VIROLOGY, Dec. 1987, p. 3783–3789
0022-538X/87/123783-07$02.00/0

Vol. 61, No. 12

Role of the Host Immune Response in Selection of Equine Infectious Anemia Virus Variants

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Received 1 June 1987/Accepted 21 August 1987

Equine infectious anemia virus was isolated from peripheral blood leukocytes collected during two early febrile cycles of an experimentally infected horse. RNase T_1-resistant oligonucleotide fingerprint analyses indicated that the nucleotide sequences of the isolates differed by approximately 0.25% and that the differences appeared randomly distributed throughout the genome. Serum collected in the interval between virus isolations was able to distinguish the isolates by membrane immunofluorescence on live cells. However, no neutralizing antibody was detected in the interval between virus isolations. In fact, multiple clinical cycles occurred before the development of a neutralizing antibody response, indicating that viral neutralization might not be the mechanism for selection of antigenic variants. The ability of early immune sera to recognize variant specific antigens on the surface of infected cells suggested that immune selection occurs through recognition and elimination of certain virus-infected cells. Alternately, the random distribution of the genomic differences observed between the two isolates may indicate that equine infectious anemia virus variants emerge as a result of nonimmunological selection processes.

Equine infectious anemia (EIA) is a naturally occurring, persistent disease of horses caused by EIA virus (EIAV), a member of the lentivirus subfamily of retroviruses (3, 16). The morphological (14, 26), genetic (4, 13, 33), and serological (2, 26) relatedness between EIAV and human immunodeficiency virus, the etiological agent of acquired immunodeficiency syndrome, has increased interest in understanding the pathogenesis of EIA, the mechanisms of viral persistence, and the host response to infection. Clinical characteristics of EIA include cyclical episodes of fever, weight loss, and anemia interspersed with periods of clinical quiescence. Febrile cycles are associated with the emergence of novel antigenic variants of EIAV which are thought to arise as a result of selective immune pressure (20, 22, 27, 34). Envelope glycoproteins of some lentiviruses are the targets recognized by neutralizing antibody (35), and it has been suggested that alterations in the EIAV envelope enable an antigenically novel virus to escape host immunosurveillance, resulting in a new round of viral replication and clinical disease (22, 27).

Antigenic variation of EIAV was first described by Kono and colleagues (20, 22), who found that viruses isolated from sequential febrile periods could be antigenically distinguished by autologous neutralizing antibody. Immune horse serum was able to neutralize virus isolated from previous, but not concurrent or subsequent, febrile periods. Recent studies by Montelaro and co-workers (27, 32, 34) indicated that variation was associated with biochemical alterations in the viral envelope glycoproteins, but not in viral core proteins, further implicating a role for immune pressure in the selection of EIAV variants. However, in some clinical EIA cases analyzed by Kono (19, 20), several cycles of fever and viremia occurred before the appearance of neutralizing antibody. This suggested that the emergence of antigenic variants which escape selective pressure by neutralizing antibody is not the cause of clinical cycles of EIA.

In visna virus infections there also appeared to be a correlation between the specificity of the neutralizing antibody response and the degree of genetic variation, suggesting a direct role for neutralizing antibody in the selection of visna viral variants (5, 6, 15, 29, 30). However, recent data indicate that antigenic variation may not be a major factor in the pathogenesis of visna virus. Although variants were frequently found in visna virus-infected sheep, their appearance did not correlate with clinical lesions, and the virus strain originally inoculated continued to be actively expressed during times when it should have been reduced or eliminated by neutralizing antibody (24, 38). Moreover, there was no correlation between the neutralizing antibody titer to the inoculum virus and the occurrence of antigenic variants (24). Therefore, neutralizing antibody appeared to have little or no role in immune selection of antigenic variants of visna virus.

An understanding of the role of neutralizing antibody in the selection of antigenic variants is an important consideration in the development and evaluation of effective lentivirus vaccines. In the present study, genetically distinct isolates of EIAV were recovered from two early cycles of clinical disease in an experimentally infected horse. Neutralizing antibody was not detected in the interval between viral isolations. However, early immune serum could antigenically distinguish the isolates by membrane immunofluorescence of infected cells. These data suggest that antigenic variation is due to an immune recognition and destruction of certain virus-infected cells rather than to pressure of specific neutralizing antibody.

MATERIALS AND METHODS

Clinical disease and virus isolation. A horse was experimentally inoculated intravenously with 100 ml of whole blood from an EIA-seropositive field-infected horse in Massachusetts. The experimentally inoculated horse was monitored daily for clinical signs of EIA, including pyrexia and

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anemia. Serum samples were collected, aliquoted, and stored at −20°C for further analysis.

Virus was isolated from horse leukocyte cultures established from whole blood collected during febrile periods (10, 28). Horse leukocyte culture supernatant was clarified and used to establish replication of field isolates of EIAV in the equine dermal (ED) cell line, ATCC-CCL57. ED cells grown in Dulbecco minimum essential medium supplemented with penicillin G (200 units/ml) and 20% fetal calf serum were seeded in 25-cm^2 tissue culture flasks and inoculated the following day with 2.0 ml of supernatant from horse leukocyte culture derived during clinical episodes of EIA. Infected cells were subcultured every 3 to 5 days and were tested at each passage for the presence of EIAV by direct immunofluorescence on methanol-fixed cells (9, 10). Supernatant collected at the first passage after EIAV-specific fluorescence was used as a source of cell-free EIAV.

Each isolate which successfully replicated on ED cells was biologically cloned by using a focal immunofluorescence assay on live cell monolayers (36) and employing a broadly reactive immune horse serum to avoid selection of a particular antigenic serotype during in vitro isolation. Briefly, ED cells were inoculated with serial 10-fold dilutions of cell-free EIAV. Five days postinoculation, the cells were tested for the presence of foci of infected cells by indirect immunofluorescence on live cell monolayers. Foci detectable at the highest dilution of virus inoculum were marked, picked from trypsinized cells with a Pasteur pipette, and seeded individually into wells of 24-well tissue culture plates. Cells in the original focus were expanded to a 25-cm^2 flask, and the supernatant was used as a source of cell-free virus for subsequent infections. All isolates used in this study were derived after three cycles of biological cloning from cells inoculated with limiting dilutions of virus.

RNase T_1-resistant oligonucleotide analysis. ED cells were infected with a biologically cloned stock of EIAV, passaged twice, and allowed to grow to confluency in 150-cm^2 tissue culture flasks. Metabolic labeling of virus, isolation of viral RNA, and oligonucleotide analyses were done as previously described (11, 12). The order of T_1-resistant oligonucleotides relative to the 3′ polyadenylate terminus of each isolate was determined as previously described (11, 12).

Immunofluorescence assays. Indirect immunofluorescence assays were done as previously described for live cell (36) and methanol-fixed cell (9, 10) monolayers. Reagents included, as the first antibody, a broadly reactive horse anti-EIAV serum diluted 1:100 in phosphate-buffered balanced salt solution (PBBS) supplemented with 2% fetal calf serum. In certain instances, serum from the experimentally infected horse was used as the first antibody. Fluorescein isothiocyanate-conjugated goat anti-horse immunoglobulin (Cooper Biomedical, Inc., West Chester, Pa.) diluted 1:200 in PBBS was used as the second antibody in all assays. Fluorescent foci were scored with a Leitz Orthoplan incident light fluorescent microscope. Twenty percent of the cell monolayer was examined, and the results were expressed as the number of foci per milliliter of inoculum.

For absorption experiments, immune horse serum was diluted 1:10 in PBBS and incubated with confluent monolayers of noninfected ED cells or with monolayers of ED cells chronically infected with EIAV. The serum was incubated with the cells for 1 h at 4°C, removed, and transferred to a second, and subsequently a third, monolayer. After the final absorption, the serum was clarified by centrifugation, heated at 56°C for 30 min to inactivate any residual virus, and used as the first antibody in the focal immunofluorescence assay.

Neutralization assays. A focus reduction assay with a constant amount of virus and various amounts of antibody was used to study the chronological development of serum neutralizing antibody during the course of EIA. Serum samples were heat inactivated at 56°C for 30 min and serially diluted in PBBS. Approximately 5×10^2 focus-forming units of virus in 0.1 ml were mixed with an equal volume of the antibody dilution, incubated for 1 h in a 37°C water bath, and brought to 1.0 ml with ice-cold PBBS. Preliminary experiments indicated that the addition of exogenous complement had no effect on the in vitro neutralization of EIAV (data not shown), and subsequent tests were done in the absence of complement. ED cells seeded the previous day at 10^5 cells per 60-mm tissue culture dish were overlaid with medium containing Polybrene (8 μg/ml) and inoculated with 0.5 ml of serial 10-fold dilutions of the virus-antibody mixture. The cells were incubated at 37°C in 5% CO_2 in air for 5 days, fixed in 70% methanol, and stained for the presence of EIAV-specific foci by indirect immunofluorescence as described above. Results were expressed as the serum neutralization titer, defined as the reciprocal of the highest serum dilution which gave a two-thirds or greater reduction in foci when compared with preimmune serum or diluent controls.

Immunoprecipitation. Confluent monolayers of ED cells chronically infected with EIAV were rinsed with warm PBBS, incubated for 1 h at 37°C in methionine-free Eagle minimum essential medium containing 1% dialyzed fetal calf serum, and pulsed for either 30 min or 4 h at 37°C in additional medium containing 50 μCi of [^{35}S]methionine (1,100 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml. Medium was aspirated, and the cells were rinsed in warm PBBS and harvested at a concentration of 10^6 cells per ml in lysing buffer containing 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 0.1% gelatin in TNE (0.05 M Tris hydrochloride [pH 8.0], 0.15 M NaCl, 0.001 M EDTA). Lysates were incubated for 30 min on ice and precleared with 10 μl of normal horse serum per ml and 100 μl of 10% (vol/vol) Formalin-fixed *Staphylococcus aureus* Cowan strain 1 per ml (18). Samples were centrifuged for 45 min at 35,000 rpm (type 65 rotor; Beckman Instruments, Inc., Fullerton, Calif.), and the supernatant was used for further analysis.

Immunoprecipitates were prepared by adding 10 μl of antiserum to approximately 10^6 cpm of precleared cell lysate in a minimum of 250 μl of lysing buffer. The samples were incubated overnight at 4°C, mixed with 0.1 volume of Formalin-fixed *S. aureus* Cowan strain 1, and incubated on ice for an additional 20 min. The precipitates were pelleted in a Beckman B Microfuge and washed three times in ice-cold lysing buffer. After the final wash, the pellet was drained of excess liquid and suspended in 25 μl of 62.5 mM Tris hydrochloride (pH 7.4) containing 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol. The samples were boiled for 5 min and were analyzed by SDS-polyacrylamide gel electrophoresis. Slab gel electrophoresis was performed by the method of Laemmli (23) with a 5% stacking gel and an 8% resolving gel. Gels were fixed, dried, and exposed to Kodak X-OMAT film as previously described (1).

RESULTS

Clinical disease and virus isolation. Experimental inoculation of the Massachusetts field isolate of EIAV resulted in a rapid onset of clinical disease characterized by frequent febrile cycles (Fig. 1A). Virus isolated from the supernatant of peripheral blood leukocyte cultures established during the

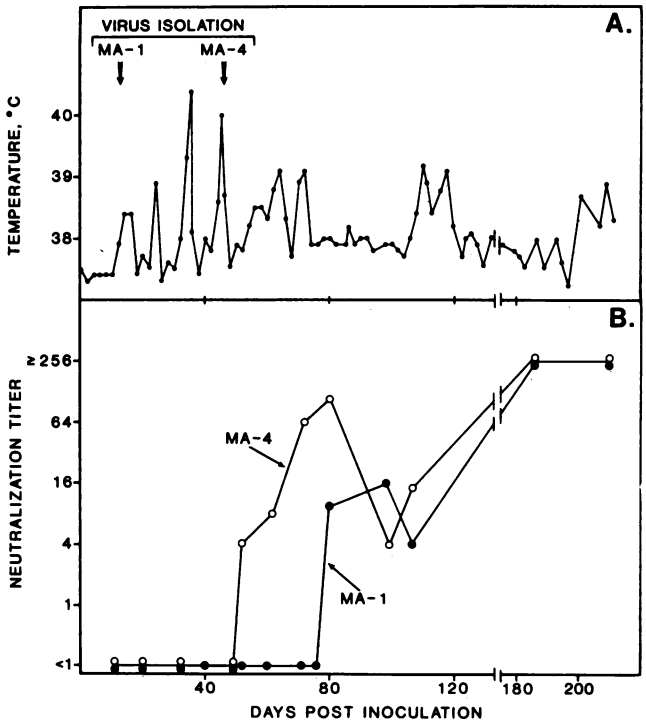


FIG. 1. (A) Clinical disease course in a horse experimentally inoculated with whole blood from a subclinical carrier of EIAV. (B) Development of neutralizing antibody to MA-1 and MA-4 during the course of clinical disease in a horse experimentally infected with EIAV. Virus isolates were recovered from peripheral blood leukocytes established at day 13 (MA-1) and day 46 (MA-4) postinoculation and were biologically cloned before their use in neutralization assays. Neutralization titers are expressed as the reciprocal of the serum dilution which neutralized at least 70% of viral infectivity in a focus reduction assay as described in the text.

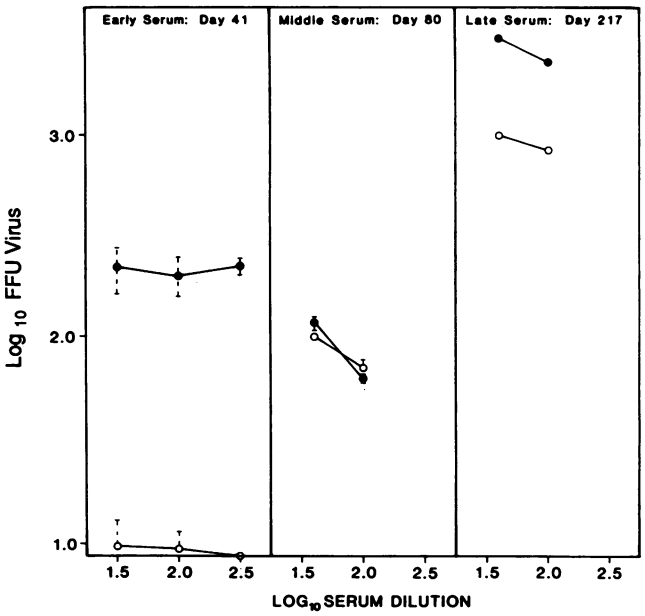


FIG. 2. Detection of antigenic variants of EIAV by membrane immunofluorescence on live cell monolayers. ED cells were infected with serial dilutions of biologically cloned stocks of MA-1 (●) or MA-4 (○). Immune horse sera collected on days 41, 80, and 217 postinoculation were serially diluted and used as the first antibody in a focal immunofluorescence assay as described in the text. Results are expressed as mean values ± the standard error of the mean pooled from three experiments in which the number of focus-forming units (FFUs) of virus detectable by a positive control sera has been standardized to 1,000.

first (day 13) and fourth (day 46) febrile periods of the experimentally infected horse successfully replicated in vitro on ED cells. These isolates are referred to as MA-1 and MA-4, respectively.

Serological characterization of MA-1 and MA-4. Previous studies have suggested that neutralizing antibody exerts immune pressure in vivo and thus plays a role in selection of antigenic variants of EIAV (22, 27, 34). If so, then neutralizing antibody to MA-1 should appear subsequent to the first febrile period but before the second febrile period. However, we observed that neutralizing antibody to MA-1, isolated on day 13, was not detected until day 80 (Fig. 1B). Moreover, during the interval between the isolation of MA-1 and the detection of neutralizing antibody to MA-1, the inoculated horse experienced five additional cycles of clinical disease. In contrast, neutralizing antibody to MA-4 was first detected at day 52, within a few days of the fourth febrile peak (day 46). Thus, neutralizing antibody to MA-4 appeared before neutralizing antibody to MA-1, and the emergence of these isolates of EIAV appeared to be independent of selective immune pressure of specific neutralizing antibody.

When membrane immunofluorescence was used to assay for MA-1- and MA-4-specific antibody, however, we found that antibody present in early immune serum collected in the interval between viral isolations (day 41) had a higher specific titer for MA-1-infected cells than for MA-4-infected cells (Fig. 2). The reactivity of day 41 serum to MA-1-infected cells remained high through a 2.5 $\log_{10}$ dilution, a point at which the initially low reactivity to MA-4-infected cells was no longer detectable. Immune serum collected after the fourth febrile episode (day 80) showed an increased reactivity to MA-4 without a concomitant increase in reactivity to MA-1. Thus, in the interval after the isolation of MA-4 there was an increase in MA-4-specific antibody but not an increase in MA-1-reactive antibody. The seroconversion of the experimentally infected horse from recognition of MA-1 at day 41 to recognition of both MA-1 and MA-4 at day 80 not only indicated that the two isolates were antigenic

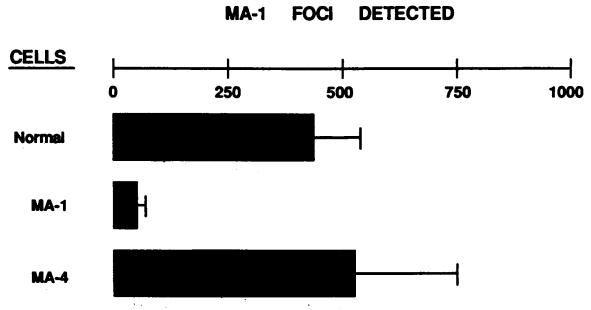


FIG. 3. Early immune serum (day 41) was absorbed with normal ED cells or with ED cells chronically infected with either MA-1 or MA-4 virus and used as the first antibody in the focal immunofluorescence assay on ED cells inoculated with serial dilutions of MA-1 virus. Results are expressed as the number of focus-forming units of MA-1 virus per 0.5 ml of undiluted inoculum ± the standard error of the mean from replicate dishes. Serum absorbed with MA-1-infected cells differed significantly ($P < 0.05$) from serum absorbed with normal cells and from serum absorbed with MA-4-infected cells.

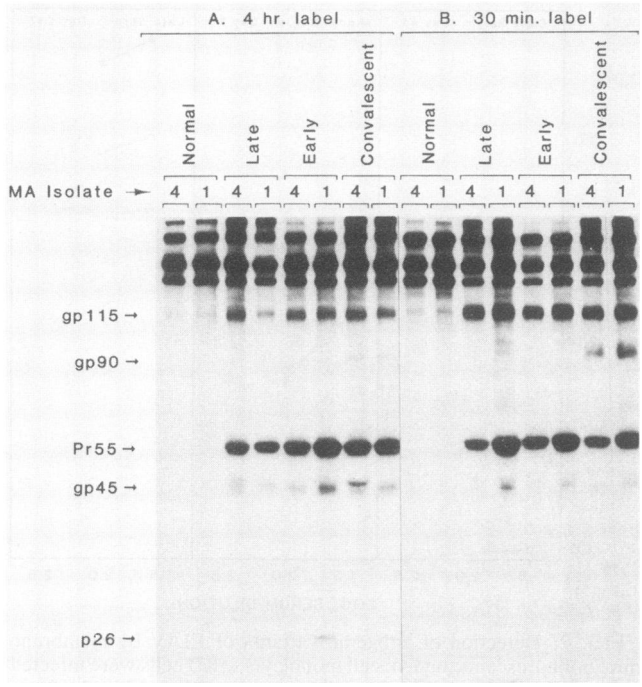


FIG. 4. ED cells chronically infected with MA-1 or with MA-4 were labeled with [^{35}S]methionine for 4 h (A) or 30 min (B), immunoprecipitated, and analyzed by SDS-polyacrylamide gel electrophoresis as described in the text. EIAV-specific proteins include the envelope glycoproteins gp115, gp90, and gp45 and the *gag*-specific proteins Pr55 and p26. Determination that individual proteins were *gag* or *env* specific was based on published results (17, 31) as well as on reactivity with rabbit anti-p26 (kindly provided by Kathryn O'Rourke, Washington State University, Pullman) and specific monoclonal antibodies (data not shown).

variants but also demonstrated that MA-1 and MA-4 represent biologically relevant populations of EIAV.

Further evidence that MA-1 and MA-4 were antigenically distinct was obtained from absorption experiments (Fig. 3). Absorption of early immune serum with cells chronically infected with MA-1 resulted in a significant ($P < 0.05$) reduction in reactivity to MA-1. In contrast, no decrease in reactivity to MA-1 was observed when early immune serum was absorbed with MA-4-infected cells or with uninfected cells. This demonstrated that the reactivity of early serum was due to a recognition of specific MA-1 determinants not found on MA-4-infected cells. These data are the first to demonstrate antigenic variation of virus-specific antigens on the surface of EIAV-infected cells.

RIP. Early (MA-1-specific) and late (MA-1- and MA-4-specific) sera were used in radioimmunoprecipitation (RIP) assays to determine which viral protein(s) expressed variant-specific epitopes. After metabolic labeling with [^{35}S]methionine, lysates of MA-1- and MA-4-infected cells were immunoprecipitated with early and late sera and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4). As expected, late immune serum, reactive with both MA-1 and MA-4 by the focal immunofluorescence assay was equally reactive with both isolates by RIP. Surprisingly, and in contrast to immunofluorescence results, early immune sera was not MA-1 specific by RIP: all detectable *env*- and *gag*-specific proteins of both MA-1 and MA-4 were immunoprecipitated by early serum as well as by late serum. These data indicated that cross-reactive epitopes were present on all viral proteins detected and, furthermore, that antibody to these epitopes was present early in the course of disease. Moreover, the broadly reactive epitopes appeared to be the predominant antigens present in detergent lysates of infected cells because we were unable to distinguish variant-specific epitopes by immunoprecipitation.

Structural analyses of MA-1 and MA-4. To determine the extent and location of genomic differences between MA-1 and MA-4, we analyzed the two virus isolates by RNase T_1-resistant oligonucleotide fingerprints. A total of five structural differences were found among approximately 100 randomly distributed large oligonucleotides resolved for each isolate (Fig. 5A and B). Three oligonucleotides were unique to MA-1, and two oligonucleotides were found in MA-4, but not in MA-1. Each of the large oligonucleotides averages 20 bases in length; thus, the analyses represented a direct comparison of approximately 2,000 bases. Since oligonucleotide differences were likely the result of a single base change, they reflected approximately 0.25% sequence divergence between MA-1 and MA-4 which, according to present estimates of the rate of retroviral mutation during replication (7), could occur after approximately 10 rounds of viral replication.

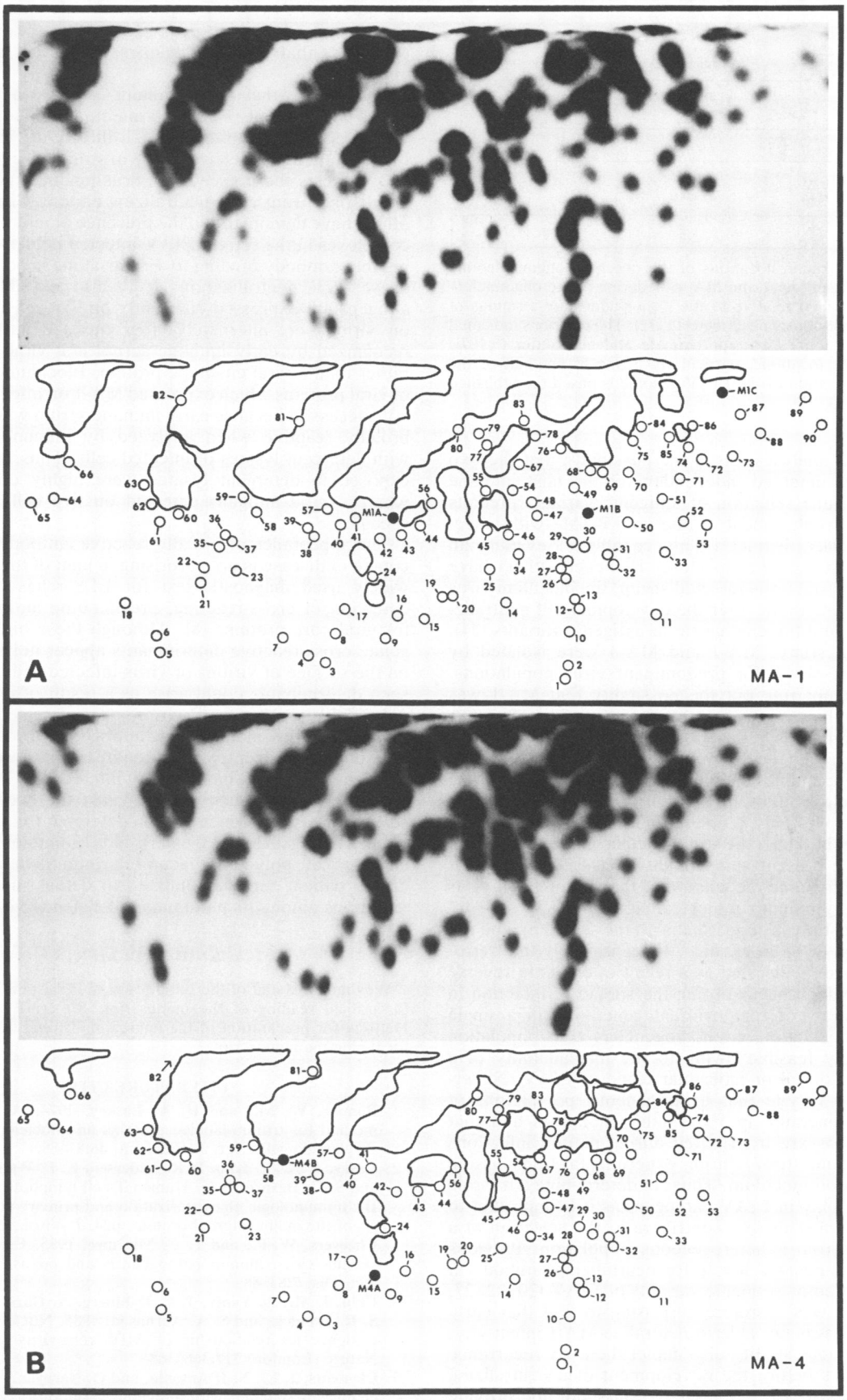
Mapping of the oligonucleotides (Fig. 6) relative to the 3′ end of the viral genome indicated that the observed differences were not clustered in the *env* gene, as might be expected for mutations which emerged as a result of selective immune pressure, but occurred randomly throughout the genome. This suggested that the variant-specific antibody response observed by the focal immunofluorescence assay was a result, rather than a cause, of the sequential appearance of MA-1 and MA-4.

DISCUSSION

Two in vivo isolates of EIAV, MA-1 and MA-4, were found to be genetic and antigenic variants as determined by RNase T_1-resistant oligonucleotide fingerprint analysis and membrane immunofluorescence, respectively. These data support the concept of antigenic variation of EIAV and the sequential development of antibody which specifically recognizes emerging viral variants. Moreover, the present findings are the first to indicate that antigenic variation of EIAV is expressed on the surface of virus-infected cells. It is possible, therefore, that immune selection of viral variants could occur through the destruction of certain variant-specific cells, mediated through strain-specific antibody or perhaps via direct T-cell cytotoxicity. Furthermore, antibody specific for variant antigens on the surface of infected cells was detected before virus-neutralizing antibody, suggesting that elimination of infected cells, rather than neutralization of cell-free virus, is a more critical immunological response in the selection of viral variants.

Interestingly, however, RNase T_1-resistant oligonucleo-

FIG. 5. RNase T_1-resistant oligonucleotides of MA-1 (A) and MA-4 (B). Open circles are used to designate oligonucleotides common to both isolates, while closed circles indicate oligonucleotides unique to that particular isolate. The 70S [^{32}P]RNAs of each isolate were digested with RNase T_1 and fingerprinted as previously reported (11, 12) and oligonucleotides were assigned numbers. Electrophoresis was from left to right, and homochromatography was from top to bottom.


A
MA-1
M1A
M1B
M1C
B
MA-4
M4A
M4B

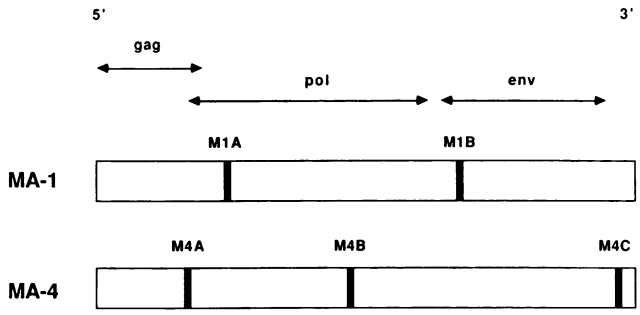


FIG. 6. Approximate locations of the observed oligonucleotide differences between MA-1 and MA-4. Ordering of the oligonucleotides was determined relative to the 3' polyadenylate terminus of each isolate as previously described (11, 12). The positions indicated for the oligonucleotides are approximate and may differ (±10%) from their actual locations, particularly in the 5' one-half of the genomes. Organization of the EIAV genome is based on published sequence data (33, 37).

tide fingerprint analyses raised the possibility that the two EIAV variants emerged independent of the host immune response. Immune selection of antigenic variants predicts the successive replacement of one viral isolate with a second, antigenically distinct isolate capable of evading an established host immune response. However, others have shown that in visna virus-infected sheep, the inoculum virus could be reisolated long after the development of neutralizing antibodies and the emergence of antigenic variants (24). In the present study, MA-1 and MA-4 were isolated by methods which select for predominant virus populations, and we could not rule out the possibility that MA-1 was present in vivo beyond the first febrile period. Therefore, we could not determine whether or not immunological elimination of MA-1 really occurred. However, if MA-1-specific antibodies were unable to eliminate MA-1 and influence the subsequent selection of further virus variants, antigenic variation may play only a minor role in EIAV persistence and pathogenesis. Thus, the strain-specific immune response detected in the present study might be a secondary event resulting from immune recognition of the predominant virus type selected by nonimmunological factors.

The present study is concerned with the selection, and not the generation, of virus variants. It seems likely that retrovirus variants are generated as a result of errors in reverse transcription which occur during the spread of infection in vivo (7). A study of rapid variant generation in a single animal after inoculation of a homogeneous virus population is not currently feasible owing to the unavailability of a pathogenic biological or molecular clone of EIAV. Nevertheless, it is probable that heterogeneous populations of variants are generated in EIAV-infected horses and that such populations are transmitted during natural infections or, in the present study, during experimental transmission with whole blood inoculum. Thus, in determining what role variation may play in EIAV pathogenesis it is important to consider what host factors contribute to the selection of a given variant from a heterogeneous population. Previous reports have implicated a role for neutralizing antibody in immune selection of antigenic variants of EIAV (20, 22, 27, 32, 34), and it was therefore surprising to find that early immune serum was not able to neutralize MA-1 infectivity. However, previous studies also depict the early recurrence of febrile cycles before the development of a neutralizing antibody response (19, 20). Our results do not provide unequivocable evidence that immune selection by neutralizing antibody does not occur in EIA, but they do raise the possibility that other mechanisms of variant selection are important.

The finding that early immune serum was specific for MA-1-infected cells by membrane immunofluorescence but was unable to neutralize MA-1 infectivity indicates that there is functional heterogeneity in strain-specific antibodies and suggests the presence of virus-binding epitopes which are distinct from virus-neutralizing epitopes. In this regard, others have demonstrated the presence of infectious immune complexes in the sera of EIAV-infected horses (25), indicating that antibody binding to virions alone is not sufficient to cause viral neutralization. It is also possible that viral antigens are expressed differently on live cell surfaces than on virions or, alternately, that the cell surface antigen recognized by early immune serum is a viral core protein rather than a viral envelope protein. Biochemical definition of viral proteins which expressed MA-1 on infected cells was not successful because early immune serum was found to be broadly reactive when analyzed by immunoprecipitation with detergent lysates of infected cells. Thus, viral antigens exposed in detergent lysates were highly cross-reactive, whereas viral antigens detected on live cells were strain specific.

The appearance of broadly reactive antibody early in the course of disease is not surprising in light of the fact that the widely used diagnostic test for EIA relies on the early detection of cross-reacting, precipitating antibody specific for viral core proteins (8). Although these highly immunogenic, cross-reactive determinants appear not to be located on the surface of virions or virus-infected cells, antibody to such determinants could arise as a result of in vivo immunological processing of virions and infected cells. The present findings indicate that antibody to broadly reactive determinants prevented detection by immunoprecipitation of antibody to more biologically interesting, strain-specific epitopes. These data caution against the exclusive use of serological assays which rely on detergent treatment of viral antigens. By analogy, Western blot (immunoblot) and RIP analyses of polyclonal acquired immunodeficiency syndrome patient sera are unlikely to detect subtle antigenic variations among human immunodeficiency virus strains.

ACKNOWLEDGMENTS

We thank the staff of the Laboratory of Persistent Viral Diseases for critical reading of the manuscript, Robert Evans and Gary Hettrick for preparation of the figures, and Irene Cook Rodriguez for typing the manuscript.

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