Antibody Patterns to *Herpesvirus saimiri*-Induced Antigens in Owl Monkeys

Gary R. Pearson, Thomas Orr, Harvey Rabin, John Cicmanec, Dharam Ablashi, and Gary Armstrong

SUMMARY—Antibody-response patterns to 3 major groups of *Herpesvirus saimiri* (HVS)-associated antigens (early antigens (EA), late antigens (LA), membrane antigens (MA)) in 10 owl monkeys infected with HVS were related to the clinical course of HVS-induced disease. Results are also presented which show that EA is produced 4-8 hours earlier than LA in HVS-infected cells providing further evidence that these were 2 distinct groups of antigens. In animals that developed neoplasms, antibodies against all 3 groups of antigens were found; however, the antibody response to EA was delayed, in general, 2-4 weeks compared with the responses to LA and MA. Two owl monkeys inoculated with HVS and one inoculated with HVS-induced tumor cells did not develop gross or clinical signs of disease; antibodies to LA and MA, but not EA, were detected in serum samples from these monkeys. These results provide additional evidence that the antibody response to EA may indicate lymphoproliferation (or cell transformation).—J Natl Cancer Inst 51: 1939-1943, 1973.

RECENT IMMUNOLOGIC STUDIES on cells infected with *Herpesvirus saimiri* (HVS) defined 3 groups of antigens by immunofluorescence (IF) procedures (1-3). These antigens were designated “LA” for HVS-associated intracellular antigens occurring late in the cycle of virus replication; “EA” for nuclear antigens produced in the presence of cytosine arabinoside (Ara-C) and which, therefore, represent early products of transcription of the virus genome; and “MA” for HVS-associated membrane antigens. LA and EA had these 2 groups of antigens (2). Some sera contained HVS-associated intracellular antigens occurring late in the cycle of virus replication, which placed them in the category of late antigens.

The initial findings from investigations of the immune responses to LA and EA in several species of HVS-infected nonhuman primates indicated that the response to EA was related to the presence and, to a lesser degree, the severity of malignant disease (2). Serum samples from adult squirrel monkeys (Saimiri sciureus) contained only LA antibodies, with one exception. In contrast, antibodies to both LA and EA were demonstrated in the sera of 8/8 marmosets (Saguinus fuscicollis, S. nigricollis) and 9/10 owl monkeys (Aotus trivirgatus) that developed malignant lymphoma and/or leukemia after infection with HVS. Four of 4 HVS antibody-free squirrel monkeys developed antibodies to both LA and EA after initial infection by HVS; however, in contrast to the findings with the other 2 species, antibodies to EA did not persist but diminished to undetectable levels approximately 9 months after infection.

In this study the antibody-response patterns to the major groups of HVS-associated antigens, including MA, in owl monkeys infected with HVS are described and related to the clinical course of HVS-induced disease. These results suggest that the response to EA reflects lymphoproliferation (or cell transformation).

MATERIALS AND METHODS

**Animals.**—Adult owl monkeys (*A. trivirgatus*) were obtained from commercial suppliers (Tarpon Zoo, Tarpon Springs, Fla., and South America Primates, Miami, Fla.). Both Peruvian and Colombian types were included. The monkeys were quarantined and conditioned in our laboratory for 3 months before the study began.

**Animal inoculations and monitoring.**—Intramuscular and subcutaneous inoculations were given in the thigh with approximately $1 \times 10^8$ plaque-forming units (PFU) of HVS, grown in early passage OMK cells, in a volume of 0.5 ml/inoculation site. The monkeys were observed daily and given thorough physical examinations weekly. At least once every 2 weeks blood samples were collected for hematologic determinations and serum analyses. Lymphoma was diagnosed if clinical examination revealed moderate to marked enlargement of more than one palpable lymph node. Lymphocytic leukemia was judged to be present if the total leucocyte count was greater than 20,000 cells/mm$^3$, the percentage of lymphocytes on differential count exceeded 85%, and, most importantly, atypical lymphocytes and prolymphocytes, appeared in peripheral blood.

**LITERATURE CITED**

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2. Supported in part by Public Health Service contract NIH-71-2025 within the Virus-Cancer Program of the National Cancer Institute.
5. Viral Leukemia and Lymphoma Branch, National Cancer Institute.
6. The comments and suggestions of Dr. Robert A. Manaker, National Cancer Institute, during these investigations are gratefully acknowledged.
Cell cultures.—Vero cells were grown in Falcon tissue culture flasks in RPMI 1640 medium containing 10% heat-inactivated (56°C, 30 min) fetal calf serum (FCS) supplemented with 100 U penicillin/ml and 100 μg streptomycin/ml. For weekly passage, the cells were detached with 0.25% trypsin and used to seed new flasks at a concentration of 5×10^5 cells/ml in fresh medium.

Production of LA, EA, and MA antigen-positive cells.—Vero cells (48- to 72-hour cultures) were exposed to 1 ml HVS (1×10^5 PFU/ml) for 2 hours at 37°C. After this incubation period, RPMI 1640 medium with 10% heat-inactivated FCS was added to half the cultures, and the same medium plus 20 μg/ml Ara-C was added to the remaining cultures. Culture flasks were incubated at 37°C in the presence of 10% CO₂. Cells from Ara-C-treated cultures were harvested 72 hours after infection by exposure to 0.25% trypsin for 5 minutes. Acetone-fixed (10 min) smears of the cells were prepared on glass slides pretreated with a Teflon-Epoxy coating (Roboz Surgical Instrument Co., Inc., Washington, D.C.). These smears were used to determine the presence of EA (1). Infected cultures without Ara-C were harvested 5–6 days after infection when cytopathology characteristic of HVS was evident and HVS-associated LA were present. Some of these cells were used in a viable state as a source for HVS-associated MA (1). The remaining cells were fixed in acetone for 10 minutes and served for the detection of intracellular HVS-induced LA (2). All acetone-fixed smears were kept at −70°C until used in the IF tests.

Immunofluorescence assays.—Indirect IF tests for membrane and fixed-cell antigens were performed as previously described (1, 2) with a fluorescein isothiocyanate (FITC)-conjugated caprine anti-human gamma globulin reagent (Hyland Laboratories, Los Angeles, Calif.).

RESULTS

Temporal Development of LA, EA, and MA

The temporal development of these 3 groups of antigens in productively infected cultures was studied in primary OMK cells and vero cells infected with HVS at a multiplicity of infection of <0.5. Cells were harvested at 4-hour intervals through 48 hours, and 5–6 days after infection when cytopathology characteristic Co., Inc., Washington, D.C.). These sera were titrated for anti-LA and fixed-cell antigens were performed as previously described (1, 2) with a fluorescein isothiocyanate (FITC)-conjugated caprine anti-human gamma globulin reagent (Hyland Laboratories, Los Angeles, Calif.).

production period. In contrast, the maximum percentage of EA-positive cells in the vero culture (EA-vero) in this experiment was 5–10%. This agreed with other studies demonstrating that primary OMK cells were more susceptible to HVS infection than were vero cells (4). LA were not detected in the OMK cultures (LA-OMK) until about 20 hours after infection and not until 24 hours post infection in the infected vero cells (LA-vero). In the vero cultures, approximately 1% of cells were LA-positive during the experimental period, whereas the percentage of LA-positive cells in the OMK cultures increased to nearly 20%. MA were detected on the surfaces of the HVS-OMK cells at around 48 hours after infection, and their number increased to about 25% of the total cells at 72 hours. Definite MA were not demonstrable on the HVS-vero cells.

Antibody Patterns to HVS-Associated Antigens in Owl Monkeys Infected With HVS

Serum samples were collected biweekly from 10 HVS-infected owl monkeys and titrated for antibodies to LA, EA, and MA. The monkeys were also monitored biweekly for the development of leukemia and/or malignant lymphoma by hematologic and physical examination.

Representative titration curves on sera taken from 2 owl monkeys that developed malignant lymphoma and leukemia are shown in text-figures 2 and 3. Detectable antibody activity to LA was usually present within 2–4 weeks post inoculation and persisted at high levels during the disease. The antibody responses to MA in these monkeys generally paralleled the LA response, though the titers were somewhat lower on OM 101 (text-fig. 3). As previously reported (2), antibodies to EA were detected 2–4 weeks later than those against LA. The response to EA preceded or coincided with the onset of disease, and the titers tended to increase with progressing disease. This was most apparent in OM 101 (text-fig. 3); antibodies to EA were not detected until approximately 10 weeks after virus inoculation at the time leukemia was initially diagnosed. Similar antibody patterns
were noted in 4 other owl monkeys with lymphoma and leukemia.

The antibody-response patterns of those monkeys with malignant disease after HVS inoculation differed markedly from those of 2 virus-inoculated owl monkeys that had no gross, clinical, or microscopic signs of neoplastic disease during the 8- to 12-week observation period. The patterns from one such monkey are presented in text-figure 4. Antibodies to LA and MA, but not EA, were detected in the sera of both monkeys. These animals died of massive hemorrhage due to a peculiar defect in the blood-clotting mechanism noted in owl monkeys (W. Loeb: Personal communication). A third owl monkey, OM 275, inoculated with lymph node cells from a lymphomatous monkey had persisting high antibody titers to LA during a 9-month observation period (text-fig. 5). MA antibodies also appeared at approximately the same time that LA antibodies were first detected, and these also persisted but at a lower level. EA antibodies were not demonstrated in any serum samples collected over the 9-month period. There were no gross or clinical signs of leukemia or lymphoma in this monkey, but histologic examination at necropsy revealed findings suggestive of early lymphoma in the lamina propria of the small intestine; examination of lymph nodes did not reveal evidence of lymphoma.

The importance of the antibody response to EA as an indicator of malignant lymphoma in the absence of leukemia is illustrated by the history of OM 981 shown in table 1. This animal had no symptoms of leukemia, as determined by its white cell (WBC) count and percentage of blood lymphocytes, during a 140-day observation period. However, high antibody titers to both LA and EA were demonstrated in serum samples starting at day 28 post inoculation. Because of the increasing EA titer noted in the serum, this

<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>WBC (percent lymphocytes)</th>
<th>Antibody titers</th>
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<tr>
<td>7</td>
<td>8100(88)</td>
<td>&lt;10</td>
</tr>
<tr>
<td>28</td>
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<tr>
<td>56</td>
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<td>84</td>
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<td>112</td>
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<td>140</td>
<td>11,600(79)</td>
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*Monkey was sacrificed on day 140 post inoculation. At that time a mass in the right axilla was diagnosed as malignant lymphoma.
†Not determined.
animal was monitored closely for signs of lymphoma or leukemia over the entire observation period. On day 98 post inoculation, enlarged lymph nodes were palpated in the left inguinal and right axillary regions, and the left inguinal lymph node was removed for histologic examination. The node was greatly enlarged and showed active germinal centers with no definitive evidence of neoplastic change. When this animal was sacrificed 5 weeks later, the enlarged mass in the right axillary region was diagnosed as malignant lymphoma.

Results from OM 993 (table 2) demonstrated that not all owl monkeys developing antibodies to LA and EA after infection with HVS presented with definite clinical or histopathologic signs of leukemia or lymphoma. Antibodies to LA were detected in the 28-day serum sample and antibodies to EA on day 42 post inoculation. Titers against both groups of antigens tended to increase during a 197-day observation period, though they remained fairly constant from day 42 through day 126. As shown in this table, there were no definite symptoms of leukemia in this animal as determined by hematologic examination; however, the percentage of lymphocytes in the blood was slightly elevated from day 140 through day 197. Physical examination on days 61–68 post inoculation, revealed moderately enlarged popliteal lymph nodes which could indicate lymphoproliferation, but microscopic examination of tissues taken at necropsy failed to detect lymphoma. Interestingly, peripheral blood samples drawn 42, 97, and 154 days post inoculation, and cells from the spleen, bone marrow, and peripheral blood taken at necropsy provided evidence for the presence of transformed cells in these tissues. Cultures of transformed lymphocytes, in which cells grew in clumps in suspension as reported in (5), were established from these tissues and found to contain HVS antigens.

DISCUSSION

Three groups of IF antigens, designated LA, EA, and MA, were recently demonstrated in cells infected with HVS (1–3). The results of these preliminary studies suggested that EA was an early gene product synthesized during the initiation of the virus replication cycle, whereas the production of LA and MA appeared later in the cycle and coincided with the synthesis of virus particles. The results of our time studies support this interpretation. EA was detected 4–8 hours earlier than LA in cultured HVS-infected cells grown under conditions that permitted the complete replication of the virus. Both the previously described "trabecular" and "punctate" staining patterns (2) were evident in all EA-positive preparations. These results suggest that, as with Epstein-Barr virus (EBV), LA may represent viral capsid antigens produced late in the viral replication cycle (6). MA were also produced late in the infection cycle and only in cultures containing a high percentage of LA-positive cells. This is in agreement with results reported for EBV (7) and Marek's disease herpesvirus (8) showing that synthesis of MA was a late event in the virus replication cycle. So far, an early membrane antigen similar to that described by Gergley et al. (9) for EBV has not been identified in the HVS system. The relationship of MA to LA is unclear. However, with other herpesviruses, MA is antigenically distinct from late intracellular antigens (10) and has been identified in the envelopes of infectious virus particles (7, 11–13).

Serial serum samples from 10 HVS-inoculated owl monkeys were examined for antibodies directed against these 3 groups of antigens; the correlation of these responses with clinical information supported the view that the antibody response to EA is related to lymphoproliferation. Every owl monkey with clinical signs of malignant disease produced antibodies against LA, MA, and EA. In general, but not always, the production of antibodies to EA was delayed compared with the responses to LA and MA and preceded or coincided with the manifestation of clinical disease. In contrast, the sera of 3 monkeys inoculated with HVS or HVS-induced tumor cells (in one case) but with no signs of disease contained antibodies to LA and MA but not EA. Two of these monkeys died of massive hemorrhage between 8 and 12 weeks post inoculation and might have developed leukemia and/or lymphoma had they lived longer; the third died 9 months post infection, and microscopic signs suggestive of early lymphoma were detected.

No clinical disease developed in OM 993, even though high antibody titers to LA and EA were measured. However, the successful establishment of lymphoid cells taken from this monkey in tissue culture provided evidence for the presence of transformed cells in some tissues; this supported the interpretation that antibodies to EA are produced only in response to virus-induced cell transformations (or HVS-induced lymphoproliferation). The cultures derived from lymphoid tissues of this monkey closely resembled cell cultures cultivated from monkeys with diagnosed malignant disease (3). The significance of this observation requires further study. Similar cells from 2 anti-LA-positive, anti-EA-negative owl monkeys could not be cultivated in vitro.

The results presented here, along with those previously reported (2), indicate that the antibody response to EA is indeed related to lymphoproliferation (or virus-induced cell transformation) in infected animals. The presence of antibody to LA and MA denotes prior exposure to the virus but not neces-

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<th>Days after inoculation</th>
<th>WBC (percent lymphocytes)</th>
<th>Antibody titers</th>
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<tr>
<td></td>
<td></td>
<td>LA</td>
</tr>
<tr>
<td>14</td>
<td>10,500(55)</td>
<td>&lt;10</td>
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<tr>
<td>42</td>
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<td>70</td>
<td>11,300(64)</td>
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<td>157</td>
<td>10,700(70)</td>
<td>640</td>
</tr>
<tr>
<td>197</td>
<td>14,500(84)</td>
<td>640</td>
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sarily the presence of disease. If this interpretation is true, then some lymphoproliferation must occur in squirrel monkeys, the natural host species for this virus (14), after initial exposure to HVS, since a transient EA antibody response is induced (2). It may be possible, therefore, to induce lymphomas in this species by either infecting newborn animals or by subjecting inoculated adult animals to immunosuppressive measures after virus infection. Information on the factors responsible for inhibiting the development of lymphoma in the natural host for HVS may provide insight into the development of lymphomas in humans.

The immunologic and biologic parallels between HVS in nonhuman primates and EBV in humans (15, 16) demonstrate the importance of this model system for investigating the function of herpesviruses in the etiology of lymphomas. As this parameter (antibody response to EA) may provide an early indication of disease, this model offers a unique opportunity for experimental chemotherapy studies as well as for investigating immunologic control studies which could potentially be applied to the control of herpesvirus-associated neoplastic diseases in humans.

REFERENCES


