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SPIDER MONKEY HERPESVIRUSES
AND MALIGNANT LYMPHOMAS

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### Background

Herpesviruses are known to induce neoplastic conditions in animal species. Lucke tumor virus, which causes adenocarcinoma of the kidney in leopard frogs, was the first herpesvirus for which oncogenicity was proved. This was elegantly demonstrated in 1969 by M. Mizell and co-workers. Other viruses for which oncogenicity has also been established are the Marek's disease virus and the cottontail rabbit herpesvirus. Marek's disease virus is the cause of neurolymphomatosis (malignant lymphoma) in chickens. The cottontail rabbit virus is latent in adult cottontails, but induces malignant lymphoma when inoculated into young cottontails.

Herpesviruses have also been associated with neoplasia in primates. One of the best known is the EB virus isolated by Epstein and co-workers from lymphoblasts of Burkitt's lymphoma patients. However, in spite of almost seven years of effort in many laboratories, no direct evidence has as yet been obtained to establish it as the etiological agent of Burkitt's lymphoma.

Another herpesvirus isolated from primates and for which direct evidence of oncogenicity in primates has been presented is Herpesvirus saimiri. This new herpesvirus was isolated at the New England Regional Primate Research Center from the squirrel monkey (Saimiri sciureus) by Meléndez and his associates. The same investigators demonstrated that

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H. saimiri is the etiological agent of malignant lymphoma in several non-human primates and in rabbits. Recently we have further demonstrated for the first time, that H. saimiri can produce leukemia in addition to malignant lymphoma in owl monkeys. We have also shown that H. saimiri may induce the development of solid tumors in black spider monkeys.

H. saimiri lymphoma is characterized by marked enlargement of the organs of the reticuloendothelial system (liver, lymph nodes, spleen, and thymus). This enlargement is due to an invasion and replacement of the normal cytoarchitecture of the affected tissues by reticulum cells. The invasion by these neo-elements into other tissues such as those of kidney, lung, testis, and choroid plexus, gave this disease the features of a neoplastic disease.

**History**

Dr. Hector Castellanos, Professor of Medicine at the University of San Carlos, Guatemala City, visited our laboratories in June 1970. Knowing our findings with H. saimiri, he suggested to us that perhaps spider monkeys also could be natural reservoirs for H. saimiri, since he had observed at least two patients developing lymphosarcoma after being in close contact with black spider monkeys (Ateles geofroyii). He was interested in obtaining our advice on culturing tissues derived from these animals in order to isolate a likely oncogenic agent.

The possibility that spider monkeys could also be carriers of H. saimiri was interesting. I therefore obtained from Dr. Felix Garcia the only available spider monkey at the Primate Center. This was spider monkey No. 810-69, originally obtained for us by Dr. Castellanos. This animal had been in the Primate Center for more than one year. Dr. Garcia obtained one kidney by nephrectomy and the renal cortex was cultured. The kidney culture yielded the viral isolate we will describe later as Herpesvirus atelis.

After this, several consultations were held with the National
Cancer Institute and the Department of Research Development and Coordination of the Pan American Health Organization to obtain support for travel to Guatemala. A request from the National Health Service of Guatemala to the Pan American Health Organization made possible my visit to that country in July 1970.

Dr. Castellanos and Dr. H. Ordoñez had prepared a tissue culture laboratory at the Medical School, University of San Carlos. Meanwhile in Guatemala, the following had occurred.

1. Mr. Ricardo Carrión (RC) was the only patient alive of the two who had developed lymphosarcoma. The contact pet spider monkey, AT-46, was part of a colony of monkeys established by Dr. Castellanos at El Petén, Guatemala.

2. A lymph-node biopsy from patient RC had indicated the presence of a mixed lymphosarcoma (lymphoblastic and lymphocytic).

Patient RC died of lymphosarcoma in March 1971.

3. In July 1970, a kidney was obtained by nephrectomy by Dr. A. Luna from spider monkey AT-46 and also from a small female baboon named Susie (PK). Cultures were initiated with renal cortex tissues from each animal.

4. A lymph-node biopsy was obtained from patient RC and cell cultures were prepared from it.

5. One week later, the kidney cultures presented early growing cell patches. At that time, I took all three cultures to our laboratories in Southboro, Massachusetts.

6. A complete lymph node from patient RC was received in our laboratories in October 1970. This lymph node is now growing in its 10th passage and has been named LN-RC cell line.
Purpose of these studies:

1. To determine whether *Herpesvirus saimiri*, a known oncogenic herpesvirus from squirrel monkeys, is also carried by spider monkeys.

2. To determine whether *H. saimiri*, if carried by spider monkeys, could induce malignant lymphoma in man.

First, kidney cultures were processed from spider monkey 810-69 housed at Southboro, and from animal AT-46 and baboon PK from Guatemala that had contact with patient RC.

Secondly, lymph-node cultures from patient RC were established. These human cultures when grown were to be employed for co-cultivation and cell fusion with owl monkey kidney cell lines maintained in our laboratories. These two tissue culture procedures are employed for viral rescue.

**Human lymph-node culture, RC cell line, LN-RC**

The first human lymph-node tissue culture did not grow well and after three months of cultivation, it was discarded. The second human lymph-node sample is known as LN-RC cell line. This culture was originally prepared from a cervical lymph node from patient RC. This lymph node was treated with 0.5% trypsin solution. The cells obtained were cultured in Roswell Park Memorial Institute 1640 medium with 40% fetal calf serum (RPMI-1640/40) following standard procedures.

Half of the lymph node was suspended in 8 ml of RPMI-1640/40, disrupted by sonic vibration with a Bronwill sonifier and then inoculated into owl monkeys. Three animals were inoculated each with 1 ml i.m. and three each with 1 ml i.p. These animals have not shown any detectable clinical symptomatology five months after inoculation. No alterations in the blood picture have been observed in studies done every other week.

The cell line LN-RC has reached the 10th passage and is now growing well in continuous culture. This culture is now being tested by fluorescent antibody techniques to detect the presence of viral antigens.
(H. saimiri, or spider monkey herpesvirus), and it will also be employed in co-cultivation and cell fusion studies.

Spider monkey kidney isolate AT-46 - Guatemala isolate

Kidney cultures were prepared with the renal cortex from spider monkey AT-46. These cells were obtained by trypsinization of the kidney tissues following standard procedures. The cells obtained were then grown in plastic flasks (Falcon) with Eagle’s minimum essential medium containing 10% heat inactivated fetal calf serum (MEM-10).

Two-day old cultures presented cytoplasmic vacuolization. Bizarre nuclei, and very ill-defined nuclear inclusions were observed at this time in hematoxylin eosin (HE) stained cultures. The cytoplasmic vacuolization had increased by the day 8. At this time secondary cultures were prepared, and on day 9 this culture presented a large number of polykaryocytes. Some of these cell cultures were scraped and pooled and collected as isolate E115F. This isolate was then stored at 4°C, -86°C and at -176°C (liquid nitrogen).

The type of lesion observed in HE stained cell cultures was characterized by disruption of the cell layer and the presence of a large number of polykaryocytes. Most of these polykaryocytes had more than 10 nuclei. Very small and poorly defined intranuclear inclusions were seen under immersion oil microscopic observation. A detailed description of this agent will be given elsewhere.

Cytopathogenicity of Guatemala isolate AT-46

Isolate AT-46 produced cell layer alteration in continuous cultures of rabbit kidney (RKL), owl monkey kidney (OMK-210) and in squirrel monkey fetus lung SMFL. Isolate AT-46 had a titer of $10^{3.5}$ TCID$_{50}$/ml in RKL, and $10^{5.0}$ TCID$_{50}$/ml in OMK, 21 and 23 days, respectively, after inoculation. The alteration in SMFL began by the appearance of large and dense spindle-shaped cells. These cells were observed to be polykaryocytes with small intranuclear inclusions in HE stained preparations.
These cells increased in number until the whole monolayer was affected. Cell lysis was the final result.

A similar type of cell layer alteration was observed in human embryonic lung (HEL) continuous culture.

**Spider monkey kidney isolate 810 - Herpesvirus ateles**

A primary kidney culture was prepared from the renal cortex of black spider monkey No. 810-69, as described above for isolate AT-46. A well-grown cell layer developed in 6 to 8 days. This cell layer presented a large number of bizarre cells and polykaryocytes on day 11. Most of these polykaryocytes shed from the surface of the plastic flasks. On day 16 the cell layer was scraped and together with the culture fluids was collected as isolate 810. Like isolate AT-46, it was stored at 4°C, -86°C, and at -176°C.

Undiluted isolate 810 inoculated in SMFL cultures produced the development of large spindle-shaped cells in greater number than in isolate AT-46. However, in OMK-210 cell line the cytopathogenic effect was characterized by scattered foci of swollen and rounded cells. In both cultures the development of intranuclear inclusions was observed in HE stained preparations, but these inclusions were better outlined in OMK-210 cell cultures.

**H. ateles cytopathogenicity**

_H. ateles_, strain 810 had a titer of $10^{4.5}$ TCID$_{50}$ per ml in OMK-210, $10^{4}$ TCID$_{50}$ per ml in SMFL, $10^{3.5}$ in HEL and $10^{2.0}$ TCID$_{50}$ per ml in RKL, 17, 14, 23, and 30 days, respectively, after inoculation. The type of cytopathogenicity that _H. ateles_ produces in SMFL varies depending upon the number of passages of _H. ateles_ in OMK or RKL cell cultures. The virus that was passed at least three times in OMK-210 cultures developed larger nuclear inclusions, more polykaryocytes, and was more virulent for SMFL than the virus that was passed in RKL continuous cultures.
H. ateles electron microscopy (EM)

HEL cultures inoculated with H. ateles contained typical herpesviral particles using electron microscopy. The same was observed in RKL cultures.

Spider monkey herpesvirus (SMHV) - Lennette-Hull isolate

This is another indigenous herpesvirus from the spider monkey. It was originally isolated by Lennette and characterized by Hull. SMV produced almost complete destruction of HEL cultures one day after inoculation with undiluted virus material and very slight alteration in SMFL five days after inoculation. A description of the cytopathogenic spectrum of these three herpesvirus from the spider monkey is summarized in Table 1. A comparative titration of these herpesvirus in spider monkey kidney cultures is given in Table 2. A detailed description of SMHV, H. ateles, and AT-46 spider monkey viruses will be presented at the First Symposium on Viruses of South American Monkeys, to be held in Mexico City, August 13, 1971. Only a brief description is given in this report.

Physicochemical reactions

All three spider monkey herpesviruses were destroyed by ether and heat. They were also inhibited by BDUR. Filtrates of 220 ml of each of the three viruses produced CPE in RKL cultures, but 100 ml filtrates did not (Table 3).

Antisera preparation

Goats were inoculated with SMHV, H. ateles, and AT-46 spider monkey herpesviruses, and New Zealand white rabbits with H. ateles and AT-46.

The immunization procedures followed for each animal group and the results obtained will be described elsewhere.
SMHV produced good levels of antibodies in goats. *H. ateles* developed antibodies only in rabbits and AT-46 produced antibodies in both species of animals. The antisera prepared against these viruses was able to neutralize only the CPE of its homologous agent. The cross-neutralization tests indicated that the above three spider monkey herpesviruses were distinct from each other. Data are summarized in Table 4.

**Plaque development of spider monkey herpesviruses**

*H. ateles* and AT-46 were tested for their ability to induce plaques in several *in vitro* cell cultures: RKL, OMK, Hamster heart (HH), and SMFL. Both agents were able to produce plaque development only in SMFL cultures (Table 5), though both viruses grew well in all the above-mentioned cell cultures.

Both agents formed heterogeneous plaque populations in a period of approximately 30 days. Large and small plaques have been plaque purified, but they do not breed true (Table 6). Both viruses differed from SMHV in that this agent produced large plaques in RKL in less than 3 to 4 days, whereas *H. ateles* and AT-46 did not.

**Inoculation of H. ateles and AT-46 isolate in non-human primates**

**Animals inoculated with AT-46.** Two owl monkeys and three cotton-top marmosets were inoculated intramuscularly each with approximately 100 TCID₅₀ of AT-46 isolate. These animals were bled every other week for antibody studies, clinical blood chemistry, and blood counts. The two owl monkeys died approximately 2-1/2 months postinoculation without presenting any previous symptoms or any histopathological changes. The cotton-top marmosets did not present any clinical symptoms or any alteration in their blood picture for a period of 5-1/2 months, at which time they were sacrificed.

**Animals inoculated with Herpesvirus ateles - isolate 810.** Two owl monkeys and three cotton-top marmosets were inoculated in the same way as indicated for AT-46, except that the viral inoculum was approximately
1,580 TCID$_{50}$ of $H$. ateles. Blood was also collected as indicated for AT-46 inoculated animals. Two of the marmosets died 28 days after inoculation, and one was sacrificed in moribund condition 40 days after inoculation. Each animal had malignant lymphoma with marked similarity to $H$. saimiri lymphoma. The proliferating and invading cells were predominantly large lymphoblasts or reticulum cells. Although no deviation of peripheral white blood cell count occurred, the animals developed terminal leukemia with up to 31% lymphoblasts in circulation.

The two owl monkeys were sacrificed 42 days after inoculation in a moribund condition. No significant lesion was seen in the first, however, in the second animal, a focal interstitial collection of reticulum cells, and a lesser number of large lymphocytes and eosinophils were present in the kidney and lung.

Conclusions

These preliminary findings indicate that the Ateles species (spider monkey) is a natural reservoir host for two new herpesviruses.

These two new spider monkey herpesviruses proved to be different from the spider monkey herpesvirus (SMHV) described previously by Hull. Therefore, three distinct herpesviruses are found in Ateles geofroyii: SMHV, Guatemala isolate AT-46 and Herpesvirus ateles. Of these three viruses the most conspicuous is $H$. ateles. This agent proved to have oncogenic capacity in owl and marmoset monkeys, since a disease resembling malignant lymphoma developed in these species after virus inoculation.

Each of the spider monkeys from which $H$. ateles and isolate AT-46 were obtained came from the same ecological niche in Guatemala. The patient RC was always in close contact with monkey AT-46, but this agent was non-oncogenic in owl and cotton-top marmosets. However, as of this date, no evidence has been obtained to implicate one of these agents as the etiological factor of the disease developed by patient RC.

Lymph-node cultures prepared from patient RC are now growing well in in vitro conditions and they will be employed in co-cultivation
and cell fusion procedures to attempt the rescue of *H. ateles*. Should these procedures be successful, they would provide some indirect evidence to consider *H. ateles* as the etiological agent of the lymphosarcoma of patient RC. However, this remains to be ascertained.

Another important consideration is that these studies have provided evidence of the existence of a second herpesvirus of primates with oncogenic capacity. The first *Herpesvirus saimiri* found by Meléndez et al. in 1969 was demonstrated by them to be able to induce malignant lymphoma and leukemia in various non-human primates. This undoubtedly is of great importance since EBV, another herpesvirus isolated from human tissues, is considered as the most likely etiological candidate for Burkitt's lymphoma, though a good number of studies remain to be done to prove this true. The fact that herpesviruses like *H. saimiri* and *H. ateles* can induce *in vivo* lymphoproliferation in non-human primates and that it proved to be oncogenic is of great importance. Primate model systems to study oncogenic processes, malignant lymphoma and leukemia, produced by herpesviruses are now available and ready to be unveiled.
ACKNOWLEDGMENT

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REFERENCES


### Table 1

Cytopathogenicity of spider monkey herpesviruses

<table>
<thead>
<tr>
<th>Cell cultures</th>
<th>H. atele</th>
<th>AT-46</th>
<th>SMHV</th>
</tr>
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<tbody>
<tr>
<td>Whole human embryo</td>
<td>+</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>Hamster heart</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Goat synovial bursa</td>
<td>+</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>Goat bursa capsule</td>
<td>+</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>Owl monkey kidney-210</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Squirrel monkey fetus lung</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rabbit kidney</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Human embryonic lung</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Not tested.*
Table 2

Comparative titrations of spider monkey herpes-viruses in homologous kidney cultures

<table>
<thead>
<tr>
<th>SMHV</th>
<th>H. ateres</th>
<th>AT-46</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0*</td>
<td>4.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*Titer log10/1.0ml
Table 3

Cytopathogenicity of spider monkey herpesviruses
in RKL\(^a\) after physicochemical treatments

<table>
<thead>
<tr>
<th>Treatments</th>
<th>SMHV</th>
<th>H. ateles</th>
<th>AT-46</th>
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</thead>
<tbody>
<tr>
<td>Heat</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ether</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>220 (\mu)(^b)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>100 (\mu)(^b)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\)Rabbit kidney continuous culture.

\(^b\)220 \(\mu\) and 100 \(\mu\) virus filtrates.
Table 4

Reciprocal neutralization indices of spider monkey herpesviruses

<table>
<thead>
<tr>
<th>Viruses</th>
<th>ANTISERA</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H. ateles</td>
<td>AT-46</td>
<td>SMHV</td>
</tr>
<tr>
<td>H. ateles</td>
<td>&gt;3.5(^a)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>AT-46</td>
<td>0.0</td>
<td>2.5</td>
<td>0.0</td>
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<tr>
<td>SMHV</td>
<td>0.0</td>
<td>0.0</td>
<td>2.5</td>
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</tbody>
</table>

\(^a\)Neutralization indices.
Table 5

Plaque-forming capacity of two new spider monkey herpesviruses

<table>
<thead>
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<th>Viruses</th>
<th>CULTURES</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>RKLa</td>
</tr>
<tr>
<td>H. ateles</td>
<td>-</td>
</tr>
<tr>
<td>AT-46</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\)RKL, OMK, HH, and SMFL are continuous cultures from rabbit kidney, owl monkey kidney, hamster heart, and squirrel monkey fetus lung, respectively.
Table 6

Plaque characteristics of spider monkey herpesviruses in SMFL cultures

1. Heterogenous plaque population, large and small plaques.
2. Long incubation period (30 days).
3. Plaques do not breed true.