The Incidence of Complement-Fixing Antibodies to Herpes Simplex and Herpes-Like Viruses in Man and Rhesus Monkeys (33061)

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Recent serologic studies by several workers (1–3) indicate the widespread distribution in primates of complement-fixing (CF) and immunofluorescent antibodies to herpes-like viruses (HLV) which were first detected in some cultures of Burkitt lymphoma cells (4). Subsequently, particles of similar morphology and antigenicity were detected in leukocyte cultures obtained from normal human donors (5,6). The identity of these viral agents and their possible relationship to malignancy in man remains to be determined. In the present study the incidence, age distribution, and duration of CF antibodies to HLV and herpes simplex virus in man were compared. Also presented are results of similar studies in rhesus monkeys.

Materials and Methods. Antigens. Raji and P_3 (jiyoye) cell lines of Burkitt lymphoma were grown in static suspension cultures in McCoy's medium (7) containing 10% fetal calf serum at the Pfizer laboratories, Maywood, New Jersey. Raji cells contain no detectable virus (8), while P_3 cells are a rich source of HLV particles. Both cell lines were free of detectable mycoplasma and other microorganisms. Cell pellets were washed with saline, sonically disintegrated and then fractionated on a linear sucrose gradient (20–60%) in a zonal centrifuge in a B-15 rotor at 22,000 for 30 min (9). Fractions containing maximal concentrations of virus at a sucrose concentration of 25–33% were determined by electronmicroscopic examination. In the case of virus-free Raji cells equivalent fractions were collected. These fractions were further purified by 1–2 cycles of Freon extraction. The final preparations contained considerable amounts of cellular material and had an average protein content of 2.5 mg/ml. The P_3 antigen contained 100–500 HLV particles per grid square.

Herpes simplex virus (HSV) antigen was prepared from baby hamster kidney cells (10) maintained with serum-free Eagle's medium and infected at high multiplicity with HSV, Kaplan strain. The cells were pelleted 18–20 hours after infection and washed in veronal-buffered saline, (VBS) pH 7.4. A 10% cell suspension in the same diluent was sonically disintegrated and clarified at 900*g* for 15 min. Control antigen was prepared from uninfected cells in a similar manner. All antigens were dialyzed extensively against VBS and stored at —70°C.

Sera. A series of 407 serum samples of healthy or hospitalized individuals without known malignant diseases were obtained through the courtesy of Drs. F. Robbins, A. Kapikian, and T. Tokumaru. In addition, Dr. Carleton Gajdusek kindly provided 154 sera collected from natives of isolated villages in New Guinea, Micronesian Islands, Australia, Turkey, and Paraguay.

Sera of normal rhesus moneys were collected in India in 1961 by Drs. H. Meyer and G. Van Hoosier within 1–4 days of the time of capture. These animals were then bled at periodic intervals during the first 4 months of quarantine at the National Institutes of Health.

Antiserum to herpes simplex virus was prepared by hyperimmunization of guinea pigs with HSV (Kaplan strain) propagated in rabbit kidney cell cultures in serum-free medium. This serum reacted specifically with HSV and did not cross-react with BHK control antigens. Antiserum to Raji cells was prepared by repeated injection of guinea pigs with 10% suspension of washed Raji cells in saline.

All sera were stored at —20°C and a 1:10 dilution in VBS was heated at 60°C for 20 min before use.

Complement fixation (CF) tests. A modi-

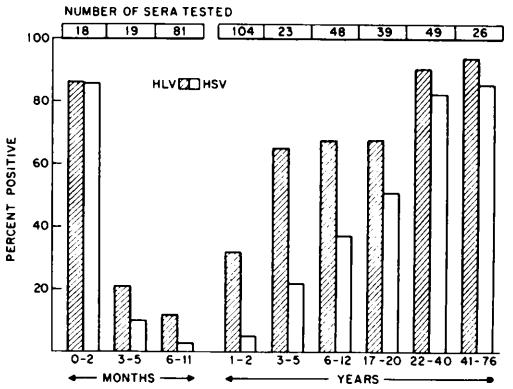

FIG. 1. Age distribution of complement-fixing antibodies to herpes-like viruses (HLV) and herpes simplex virus (HSV).

fied, quantitative CF test based on the technique of Wasserman and Levine (11) was used as follows: isotonic veronal buffer, pH 7.4, containing 0.1% bovine serum albumin, served as diluent. One-tenth ml of optimally diluted antigen, 0.1 ml of 1.1–1.2 50% hemolytic units of guinea pig complement, and 0.1 ml of serum were mixed and kept at 4°C overnight. Following addition of 0.2 ml of sensitized 0.25% sheep erythrocytes and incubation for 1 hour in a 37°C water bath, each tube received 1.0 ml of diluent and nonhemolyzed cells were removed by centrifugation. The optical densities (OD) of the supernatants were determined with a Beckman spectrophotometer at a wavelength of 418 mμ. The degree of fixation corresponded to the difference (ΔOD) between antibody control and antigen–antibody mixture and was expressed as percent fixation = (ΔOD × 100)/OD of Ab control. The serum dilution corresponding to 50% fixation was calculated by the method of Reed and Muench (12). The controls included in each test consisted of serum, antigen, and hemolytic controls, a known high-titered human serum reacting specifically with P_3 antigen, and guinea pig antiherpes serum. The lowest dilution at which sera were tested was 1:30. Sera failing to react at this dilution were considered negative. Under the experimental conditions employed the CF titers of the control sera were reproducible within ± 5%.

Results. Incidence of CF antibodies to HLV and HSV in various age groups. Sera obtained from 403 subjects comprising infants, children, and adults were tested for the presence of CF antibodies to HLV, HSV, and BHK; and Raji cell antigens. None of the sera reacted at a 1:30 dilution with Raji and BHK antigens. The results are illustrated in Fig. 1. A high incidence of both antibodies was found in infants up to 2 months of age. Subsequently the incidence of both antibodies decreased abruptly. The frequency of HLV antibody began to rise in the 1–2 year age group (32%) and reached a level of 65% in the 3–5 year age group. This level increased gradually and reached a peak in the adult population. By contrast, the frequency of HSV antibodies showed a marked age difference. Only 5% of the children in the 1–2 year age group had HSV antibodies. This percentage rose gradually with increasing ages and reached a peak (82–85%) in the adult groups. No significant sex difference in the incidence of antibodies was observed.

The distinct pattern of HLV and HSV infections becomes evident when the incidence of antibodies to both or either of these viruses is calculated within a given age group. For instance, analysis of the frequency of HLV and HSV infections in the 17–20-year age group (Fig. 1) consisting of 39 marine recruits revealed the following data summarized in Table I. Of the 26 recruits positive for HLV, 11 (42%) had no antibodies to HSV, and conversely, of the 20 HSV positive men, 5 had no evidence of past HLV infection. These results indicate that these viral infections are distinct entities and in many instances do not overlap.

Longitudinal studies. The cumulative data obtained in the population study suggested a significant increase in the incidence of HLV antibodies at 1–2 years after birth. In order to obtain additional evidence regarding this observation a longitudinal study was carried out on 10 infants where blood specimens were obtained at 0, 3, 6, and 12 months. These 10 children were selected from the population study on the basis of presence of HLV antibodies at 12 months after birth. The geometric mean values of the antibody titers are illustrated in Fig. 2 and the individual titers

TABLE I. Distribution of CF Antibodies to HLV and HSV among 39 Marine Recruits.[a]

HLV+		HSV+	
HSV+	HSV−	HLV+	HLV−
26/39 (67)		20/39 (51)	
15/26 (58)	11/26 (42)	15/20 (75)	5/20 (25)

[a] Numerator = number positive or negative; denominator = total of group; and nos. in parentheses indicate percentage incidence.

are summarized in Table II. The data indicate a sharp rise in titer of HLV antibodies between 6–12 months after birth following the disappearance of maternal antibodies. On the other hand geometric mean titers of herpes antibody showed only a slight increase at 12 months after birth. As shown in Table II only 2 infants had detectable HSV antibodies at 12 months.

Duration of CF antibodies. In order to obtain some information on the persistence of CF antibodies, paired sera of 26 normal individuals collected 10 years apart (1957–1967) were examined. Nine serum pairs were obtained from the Employees Health Unit at the National Institutes of Health and 17 pairs were obtained from the Heart Disease Epidemiology Study, Framingham, Massachusetts. The present age of these individuals ranged from 41 to 76 years. As seen in Table III, the geometric mean titers of CF antibody to both viruses remained constant during a period of 10 years. Twenty-four pairs (93%) were positive for HLV antibody and 21 pairs contained antibody to HSV. The paired sera of 2 individuals had no HLV antibody at 1:30 dilution, one of these obtained from one of the authors (PG) who has been exposed to material containing HLV in the laboratory during the past 2.5 years. The paired sera of these 2 individuals contained high levels of CF antibodies to HSV.

Distribution of CF antibodies in selected developing areas. A series of 154-serum samples collected by Dr. Carleton Gajdusek from natives of isolated communities in Micronesia, New Guinea, Australia, Turkey, and Paraguay were examined for antibody content to HLV and HSV. The results summarized in Table IV demonstrate the high incidence of both antibodies among these isolated population groups in widely dispersed locations. Among Paraguayan Indians antibodies to HSV were more frequent than HLV antibodies.

Serology of rhesus monkeys. Our initial report on the presence of CF antibodies to HLV in several subhuman primates (3) raised the question of the epidemiological significance of this finding. Do these animals acquire this infection during captivity or is this virus present in the simian population in nature? In an attempt to answer these ques-

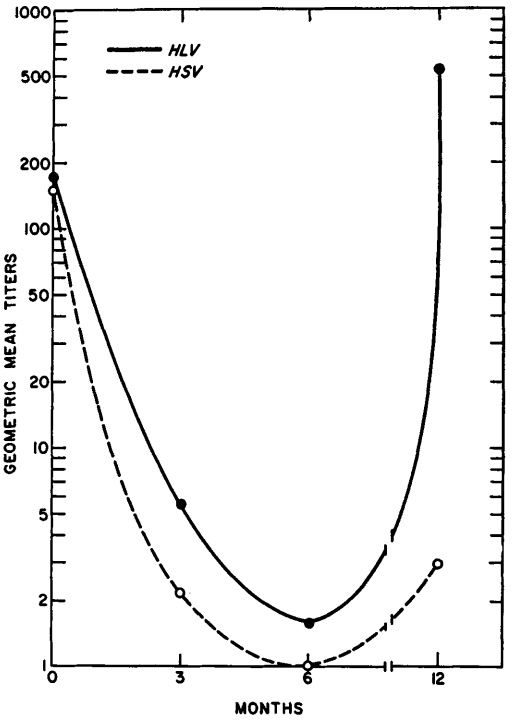

FIG. 2. Longitudinal antibody study in 10 subjects: geometric means of complement-fixing antibody titers to herpes-like viruses (HLV) and herpes simplex viruses (HSV).

TABLE II. Complement-Fixing Antibodies to HLV and HSV in Sequential Sera of 10 Infants.

Subj. no.	Months:	HLV 0	HLV 3	HLV 6	HLV 12	HSV 0	HSV 3	HSV 6	HSV 12
95		450	44	<30	400	90	<30	<30	<30
98		133	<30	<30	1100	350	50	<30	145
101		128	<30	<30	565	154	<30	<30	<30
102		180	<30	<30	1340	125	<30	<30	400
105		270	<30	<30	595	162	<30	<30	<30
127		360	50	<30	810	154	<30	<30	<30
132		140	NS[a]	90	170	182	NS[a]	<30	<30
163		90	30	<30	510	113	<30	<30	<30
186		175	30	<30	460	90	30	<30	<30
191		90	<30	<30	340	202	<30	<30	<30

[a] No serum sample available.

tions, sera collected from 99 rhesus monkeys within 1–4 days after capture in India were tested for their content of CF antibodies to HLV and HSV. The results are summarized in Table V. Fifty percent of the monkeys were found to contain HLV antibodies in their sera obtained within 1–4 days of the time of their capture. The serum titers ranged from 1:90–1:810. Only 3 sera had antibodies reacting with herpes simplex virus.

These monkeys came from 27 "family" groups each composed of approximately 10–120 members (13). Two to nine monkeys were captured from each "family" group. On the basis of body weight their ages were estimated to range from 1 year to more than 3 years. The frequency of HLV positive monkeys ranged from 0–75% in the captured members of a "family" group. No significant sex or age differences were observed.

Animals were individually caged and shipped to the National Institutes of Health

TABLE III. Persistence of CF Antibodies to HLV and HSV in Paired Sera of 26 Individuals.

Serum collected	HLV Titer range	HLV No. positive	HLV GMT[a]	HSV Titer range	HSV No. positive	HSV GMT[a]
1957	<30–1050	24	183	<30–690	21	210
1967	<30–1250	24	217	<30–570	22	201

[a] Geometric mean titer.

TABLE IV. Distribution of CF Antibodies to HLV and HSV in Selected Developing Areas.

Place of serum collection	Age range (years)	No. of sera tested	HLV positive	HSV positive
New Guinea	6–40	20	20 (100)[a]	20 (100)
Micronesian islands	3–56	20	15 (75)	20 (100)
Cape York (Australia)	3–30	20	19 (95)	17 (85)
Turkey (Black Sea coast)	11–20	20	13 (65)	17 (85)
Paraguay				
Sanapaná Indians	2½–55	38	19 (50)	34 (90)
Guayaqi Indians	25–45	16	11 (69)	16 (100)
Moro Indians	15–45	20	16 (80)	20 (100)

[a] Nos. in parentheses indicate percentage incidence.

TABLE V. The CF Antibodies to HLV and HSV in Rhesus Monkeys.

Bleeding time after capture	No. of sera tested	HLV			HSV		
		Positive			Positive		
		(No.)	(%)	Titer range	(No.)	(%)	Titer range
1–4 days	99	49	50	90–810	3	3	30–270
4 months	33	7	21	60–270	1	3	90

after a 1–4 weeks' stay in the exporter's compound. At the NIH the monkeys were housed 2/cage in 5 separate rooms. Sera collected 4 months after capture were available from 33 monkeys which were initially free of HLV antibodies. As shown in Table V, 7 animals (21%) acquired HLV antibodies during that period.

Discussion. The results of this sero–epidemiologic study in man indicate a marked difference in age distribution of CF antibodies to HLV and HSV. Approximately ⅓ of the 1–2 year old infants had HLV antibodies whereas only 5% showed evidence of past HSV infection. The data obtained in the longitudinal study demonstrate that HLV infection may occur in young infants and perhaps in some cases *in utero*. Experiments are in progress to study the occurrence of transplacental transmission of herpes-like viruses. The pattern of age distribution of HSV antibodies observed in this survey is in general agreement with the results reported by others (14).

The persistence of HLV antibodies during a 10-year period is probably a reflection of latent infection demonstrated by the detection of herpes-like viruses in cultures of leukocytes obtained from normal donors (5, 6, 15). Whether or not HLV may cause recurrent infections as seen in HSV remains to be determined.

The widespread distribution of HLV infections was demonstrated by the high frequency of CF antibodies among people living in remote areas. The lower incidence of HLV antibodies in comparison to HSV antibodies among certain Indian populations of Paraguay is of interest and its significance remains to be determined.

No attempt was made in this study to classify the subjects on a socioeconomic basis or to correlate serologic data with clinical observations. All sera from subjects older than 10 years were obtained from apparently healthy individuals while many sera of the younger age groups were obtained from patients hospitalized with various illnesses. The results of the serologic survey of rhesus monkeys reported here are of particular significance. These primates living in nature in "family" groups of relatively small size, have limited contact with other "family" groups or humans. The relatively high incidence (50%) of HLV infections among these free-living monkeys indicates enzootic maintenance of this infection. The fact that only 7 of 33 susceptible monkeys acquired HLV antibodies during 4 months of quarantine is probably a reflection of the strict isolation procedures employed. By contrast, monkeys held in gang cages showed a high incidence of HLV antibodies (3). The detection of HLV antibodies in nonhuman primates indicates that these viruses may infect other species than man. The high incidence of these antibodies limits the usefulness of these primates in experimental transmission of HLV infection.

At the conclusion of this study reports by Henle *et al.* (16) and Niederman *et al.* (17) came to our attention, which demonstrated serologic evidence (immunofluorescence) on the possible relationship of HLV to infectious mononucleosis. In the light of these new findings, if confirmed, the data presented in this report could be interpreted as the sero-epidemiology of infectious mononucleosis. Furthermore, this could suggest that antigenically related viruses are enzootically maintained in subhuman primates. On the other hand, the HLV groups may be composed of several members sharing common antigens but with distinct biologic properties.

Summary. The results of the comparative sero-epidemiology of herpes-like viruses (HLV) and herpes simplex virus (HSV) infections in man are presented. Sera from 573 subjects of various age groups were examined for the presence of complement-fixing antibodies to these viruses. Antibodies to HLV appeared at an earlier age than HSV antibodies and reached a frequency of 65% in the 3–5 year age group. Both antibodies were present in 85–90% of the adult population and were distributed on a worldwide basis. Both antibodies persisted for at least 10 years in sera of 26 normal individuals. Fifty percent of sera obtained from 99 rhesus monkeys within 1–4 days after time of capture contained antibodies reacting with HLV antigen.

Cultures of Raji and P_3 cells for antigen production were prepared by Dr. E. Jensen, Chas. Pfizer and Co., Inc., Maywood, N. J., who also checked these cells frequently for the presence of mycoplasma and other microorganisms. Mr. I. Toplin, from the same laboratory, prepared the partially purified antigens used in the present study. Production of cells and antigens was carried out under contract PH 43-66-98, Special Virus Leukemia Program, Etiology Area, National Cancer Institute.

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Characteristics of a Murine Hemagglutinin Induced by the M-P Virus (33062)

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The M-P virus (MPV) originally isolated from association with Ehrlich carcinoma cells in 1963 produces a marked lymphopenia of the lymph organs, bone marrow, and peripheral blood in all strains of mice accompanied by the development of peritoneal and pleural fluid in Swiss mice at high infection doses. Lymphopenia occurs also in guinea pigs, rats, and dogs. Some physical and chemical properties of the virus are: labile at 56°C for 30 min, unstable in 0.145 *M* NaCl, ether sensitive, and pH 3.0 sensitive, stable at −196°C in Hanks' essential basal medium with 5% fetal calf serum for