neutralization tests with paired human sera from clinical cases of rubella and with rabbit sera pre- and post-immunization with rubella virus which had been grown in RK-13 cells and had never been through a passage in Vero cultures.

Summary. Roller bottle cultures of Vero cells yield rubella virus fluids with infectivity titers in excess of 10^9 $TCID_{50}$/ml. The cells, after having produced maximal virus yields contain large quantities of HA and CF antigen which can be extracted by pH 9.0 glycine buffer.

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Attempts to Transmit Infectious Mononucleosis to Rhesus Monkeys and Marmosets and to Isolate Herpes-Like Virus (33478)

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The viral etiology of infectious mononucleosis (IM) has been postulated for many years but attempts to isolate the etiologic agent or to transmit the disease to experimental animals have been unsuccessful (1, 2). Recently published serologic evidence (3–5) suggests a possible etiologic relationship of a herpes-like virus (EBV) to IM. Our previous serologic studies demonstrated that several species of nonhuman primates had a significant incidence of complement-fixing antibodies to EBV or to a closely related virus (6). These findings suggested that immunity to infection could account for the failures to transmit IM to nonhuman primates. This report gives an account of renewed efforts to transmit the illness to rhesus monkeys and marmosets free of detectable EBV antibodies and to isolate in cell culture EBV from clinical materials obtained from IM patients during the acute stage of illness.

Materials and Methods. Design of experiments. A schematic presentation of the design of transmission experiments is presented in Fig. 1.

Clinical specimens. Patients with infectious mononucleosis were hospitalized at Kimbrough Hospital, Fort Meade, Maryland and at Walter Reed Army Hospital, Washington, D. C. The clinical diagnosis in each case was confirmed by the following laboratory findings: positive heterophile antibody test after guinea pig kidney absorption of the serum, lymphocytosis, presence of atypical lymphocytes, and elevation of serum transaminase levels.

A swab of the tonsillar region and a throat gargle in 10 ml of medium RPMI 1640 (7) containing 10% heat inactivated fetal calf serum were obtained. In most cases 50 ml of blood was collected in ACD anticoagulant solution (0.8% citric acid, 2.2% sodium citrate, 2.45% dextrose) and another 10-ml portion of blood was allowed to clot for the collection of serum which was separated and refrigerated. The citrated blood sample was centrifuged at 1500 rpm for 10 min, the layer of buffy coat cells and some of the plasma were transferred to 16 × 100-mm test tubes and incubated in upright position at 37° for 3 hr during which time most of the erythrocytes settled. The buffy coat cells were aspirated and counted in a hemocytometer. Depending on the total yield of cells available a

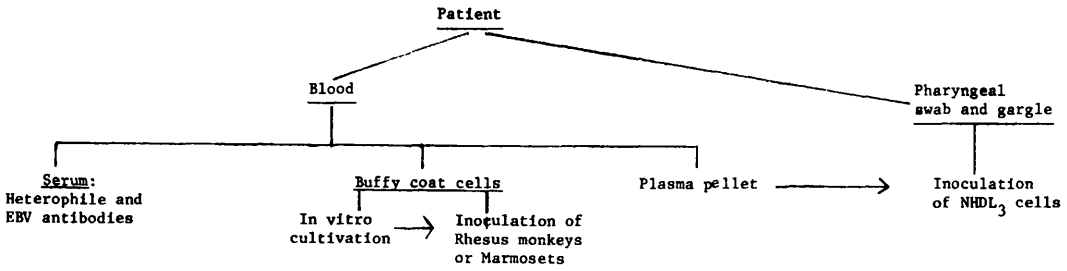

FIG. 1. Design of transmission experiments of infectious mononucleosis.

portion was pelleted and washed twice in phosphate buffered saline for cell culture studies.

The throat gargle and the swab material were treated with 400 units/ml of penicillin G, 200 μg/ml of streptomycin sulfate, and 100 μg/ml of amphotericin B at room temperature for 1 hr. The cells present in most samples were sedimented by centrifugation and resuspended in 2 ml of supernatant fluid.

Cell culture techniques. Approximately 5 $\times$ 10^6 buffy coat cells/ml were suspended in medium RPMI 1640 containing 20% heat inactivated fetal calf serum and 50 μg/ml of streptomycin sulfate. Six to 8 ml of the cell suspension were placed in 2-oz prescription bottles and these were incubated horizontally at 37° in a humidified CO_2 incubator. Partial changes of medium were made every 3–4 days.

Cultures of $NHDL_3$ cells, a leukocyte cell line derived from a normal human donor (8) which has been consistently free of detectable EBV, were grown in medium RPMI 1640 with 20% heat inactivated fetal calf serum and were maintained in the same medium.

Virus isolation experiments. Approximately 7 $\times$ 10^6 $NHDL_3$ cells were sedimented and resuspended in 2 ml of combined throat gargle and tonsil swab fluids and incubated for 2 hr at 37°. Throat gargle fluids from normal subjects served as controls. The cells were then resuspended in 15 ml of growth medium and placed in a milk dilution bottle at 37°. In a similar manner $NHDL_3$ cells were inoculated with a suspension of a patient's plasma pelleted for 1 hr at 112,000*g*. The cultures were maintained for a period of 2–3 months, the cell number was determined at weekly intervals and acetone-fixed smears were prepared for immunofluorescence tests.

Animal transmission experiments. The sera of adult or baby rhesus monkeys and cotton-top marmosets were tested for complement-fixing antibodies to EBV. Only those animals free of detectable antibodies to EBV were selected for these experiments. Rhesus monkeys were inoculated intravenously with 5.0 ml suspensions of buffy coat cells and contaminating erythrocytes of IM patients. In early experiments the cells were suspended in the patient's plasma, but in later studies the cells were washed and resuspended in serum-free Eagle's medium to remove the antibodies to EBV from the inoculum. The concentration of leukocytes ranged from 50–200 $\times$ 10^6. Marmosets received from 30–100 $\times$ 10^6 leukocytes in 1.0 ml plasma or washed and suspended in Eagle's medium. Some animals also received 200 $\times$ 10^6 cells of established leukocyte cultures obtained from 2 patients. All animals were observed and examined for a period of 2–3 months. Rhesus monkeys were bled at weekly intervals at which time a complete blood count was performed. Blood samples and blood smears were obtained from marmosets every other week.

Serological tests. Heterophile antibody titers were determined by Davidsohn's differential test (9). Complement-fixing (CF) antibodies to EBV were determined as previously described (6). The EBV antigen was extracted from Jiyoye cell cultures of Burkitt lymphoma and was partially purified by sucrose gradient centrifugation and Freon extraction. Similarly prepared fractions derived from Raji cells, a virus-free Burkitt lymphoma cell line, served as controls. The search for EBV antigen in buffy coat cultures of IM patients or in $NHDL_3$ cells inoculated with

TABLE I. Clinical and Laboratory Data of Infectious Mononucleosis Patients.

Case no.	Age	Day of illness specimen obtained	Cervical adenopathy	Splenomegaly	Exudative pharyngitis	Fever	WBC	Lymphs (%)	Atypical lymphs (%)	Heterophile Ab. GP kid. absorbed	Heterophile Ab. Beef cell absorbed	SGOT	SGPT	CF antibody to EBV
2	23	9	+	+	+	+	7600	83	50	112	<7	139	155	90
3	20	9	+	−	+	+	8800	64	40	56	<7	23	NT	660
5	22	17	+	+	+	+	10 300	57	40	896	<7	28	NT	217
8	18	8	+	+	+	+	18 700	80	60	3584	7	320	300	270
9	21	8	+	−	+	+	14 200	68	100	1792	<7	81	NT	270
11	15	8	+	+	+	+	15 500	68	95	56	<7	100	150	370
13	21	12	+	+	+	+	10 300	82	95	896	<7	79	85	410
14	22	17	+	+	+	+	11 300	80	45	224	<7	NT	NT	680
15	24	10	+	+	+	+	8200	78	50	56	<7	18	58	730
16	25	6	+	−	+	+	6500	83	75	224	<7	NT	NT	<30
17	19	9	+	+	+	+	9600	83	60	448	<7	350	540	90

patients' material was carried out by direct immunofluorescence (IF) tests. The fluorescein–isothiocyanate-labeled serum globulin fraction from a normal human donor containing high levels of CF, IF, and specific coating antibodies to EBV (6) was used throughout these tests.

Results. A total of 11 infectious mononucleosis patients were selected for this study. A summary of clinical and laboratory data pertaining to these patients is presented in Table I. The patients were military personnel or their dependents between 15 and 25 years old. The specimens for this study were obtained 6–17 days after onset of illness. All patients were febrile, had exudative pharyngitis and enlargement of the cervical lymphnodes. All except 3 patients had varying degrees of splenomegaly. The course of illness was uneventful in all except patient no. 2. This patient remained febrile, developed a maculopapular skin eruption 10 days after onset of illness and showed signs of liver involvement. He developed severe thrombocytopenia and lekopenia and expired on day 22 of illness.

Lymphocytosis with many atypical mononuclear cells and a positive Davidsohn test for heterophile antibodies were found in all cases. Several patients had elevated serum transaminase levels and all except patient no. 16 had CF antibodies to EBV at the time when the blood samples were obtained.

Detection of EBV in leukocyte cultures. Cultures of buffy coat cells were initiated from all but patients nos. 2, 3, 5, and 11. Leukocytes growing in continuous culture were successfully established from 5 of 7 patients (8, 9, 13, 15, 17) within 18–28 days after initiation.

Each of these established cell lines contained from 0.5–2% of IF positive cells. In no instance could we detect IF positive cells in preparations of fresh buffy coat cells.

Attempts to isolate EBV from pharyngeal washing and plasma pellets. The $NHDL_3$ cells inoculated with pharyngeal washing or suspensions of pellets of patient's plasma were maintained for 2–3-month periods. Weekly IF tests failed to reveal the presence of cells containing EBV antigens. Electronmi-

TABLE II. Appearance of Heterophile and EBV Antibodies in Rhesus Monkeys Inoculated with Plasma and Leukocytes of Infectious Mononucleosis Patients.

Rhesus monkey no.	Inoculum case no.	Patient's antibodies		Antibodies in monkeys		
		Heterophile[a]	EBV	Postinoc. (days)	Heterophile[a]	EBV
B 1576	5	896	217	0	<3.5	<10
				5	28	10
				12	14	10
				19	<3.5	<10
B 2009	5	896	217	0	<3.5	<10
				5	28	10
				12	7	<10
				19	<3.5	<10
B 1421	8	3584	270	0	<3.5	<10
				5	56	20
				12	28	10
				19	<3.5	<10

[a] After guinea pig kidney absorption.

croscopic examinations of representative samples were uniformly negative.

Animal transmission experiments. A total of 12 adult and 4 baby rhesus monkeys and 6 marmosets were inoculated with freshly obtained buffy coat cells suspended in 5 ml of patient's plasma (patients 2–8) or in 5 ml of serum-free medium (patients 9–17). In most instances the cells were inoculated either into 1 rhesus monkey or 1 marmoset. When sufficient numbers of cells were available 2 animals were inoculated. The established leukocyte cells of patients nos. 9 and 13 were inoculated intravenously each into one 2–3-week-old rhesus monkey at a concentration of 200×10^6 cells in 2.5 ml of serum-free medium. None of these animals developed lymphadenopathy or splenomegaly or any detectable signs of illness and no significant hematological changes were observed during a 2-month period of observation. None of the sera obtained at weekly (rhesus) or bimonthly (marmoset) intervals contained detectable heterophile or EBV antibodies with the exception of 3 rhesus monkeys each of which had received $100–200 \times 10^6$ buffy coat cells suspended in 5 ml of homologous plasma. As shown in Table II both of the donor patients had relatively high levels of heterophile and EBV antibodies in their plasma. Low titers of both antibodies appeared within 5 days after inoculation and disappeared in about 2 weeks. The early, transient appearance of these antibodies suggests a passive transfer rather than active immune response. Attempts to establish cultures of buffy coat cells from exsanguinated animals were unsuccessful.

Discussion. The experiments described here were initiated on the premise that EBV or a closely related virus is etiologically related to infectious mononucleosis in man. All animals selected for these transmission experiments were free of detectable CF antibodies to EBV and were therefore considered potentially susceptible to EBV infection. None of the inoculated monkeys in these experiments showed significant serological evidence of EBV infection nor did they develop any detectable signs of illness or significant hematological and gross pathological changes. Several factors could account for these negative results:

(i) Rhesus monkeys and marmosets may be resistant to experimental EBV infection or may carry other agents capable of interfering with EBV multiplication. Our previous studies (6) indicated that many nonhuman primates show serological evidence of infection with viruses antigenically closely related

to EBV. Henle and Henle (10) failed to detect antibodies to EBV in simians by indirect immunofluorescence tests but subsequently, Landon *et al.* (11) demonstrated the presence of herpes-like viruses related to EBV in leukocyte cultures of normal chimpanzees. Recent evidence indicated that these infections occur in nature and are maintained enzootically (12). It is likely that the herpes-like viruses may consist of a group of antigenically related agents exhibiting the narrow species specificity characteristic of the herpes virus group.

(ii) The inocula may contain insufficient amounts of infectious virus. All blood samples used for experimental transmission were obtained during the acute, febrile stage of illness when relatively high levels of circulating EBV antibodies were present in all patients with the exception of patient no. 16 whose specimen was collected on day 6 of illness. The consistent failure to detect EBV antigen by immunofluorescence tests in freshly isolated buffy coat cells of the patients in this study suggests that the quantity of complete EBV particles associated with peripheral leukocytes is below detectable levels during the acute stage of illness. Failure to respond to inoculation of cultured leukocytes of patients 9 and 13 may reflect the low levels of infectious EBV in these cells.

(iii) The intravenous route of infection employed in most cases may not be optimal. However, negative results were obtained also in 2 cases of nasal, oral, and intraperitoneal inoculation of marmosets.

(iv) Successful transmission of EBV infection to simians may not produce detectable signs of illness or development of heterophile antibodies. Nonhuman primates may either lack the clones of cells participating in the production of these macroglobulins or, if present, may not respond to the antigenic stimulation.

(v) The relationship, if any, of EBV to the pathogenesis of IM may involve the participation of other unknown factors, since the evidence for its possible etiologic role is based primarily on serological findings.

Our failure to isolate EBV from the throats of patients with IM may be due to insufficient amounts of virus shed during the acute phase of illness. The $NHDL_3$ cells employed in the isolation experiments appear to be potentially susceptible to EBV infection as determined in preliminary tests with concentrated preparations of EBV derived from a Burkitt lymphoma cell line.

Summary. Buffy coat cells obtained from the blood of 11 patients with infectious monucleosis were inoculated into selected rhesus monkeys and marmosets. These monkeys were free of detectable complement-fixing antibodies to a herpes-like virus (EBV) considered to be etiologically related to infectious mononucleosis. None of the inoculated animals developed detectable illness, or hematological and serological changes. Attempts to isolate a viral agent from the throats of the 11 patients in cultures of human leukocytes were unsuccessful.

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The experiments reported herein were conducted according to the principles enunciated in "Guide for Laboratory Animal Facilities and Care," prepared by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences, National Research Council.

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Effect of Electroshock and Pentamethylenetetrazol on Serum Cholesterol Levels in Monkeys* (33479)

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Apparent increases in serum constituents including cholesterol were previously reported to follow immediately after electroshock treatment (1, 2). A change in circulating blood volume was postulated as an explanation for these phenomena.

In previous studies we found that pentamethylenetetrazol (Metrazol) can lower serum cholesterol levels in mice (3) and in monkeys (4) as well as inhibit cholesterol synthesis in isolated mouse cells *in vitro* (5). In untreated monkeys weekly fluctuations in cholesterol expressed as mg/100 ml of serum were greatly minimized when recalculated as mg/10 g of serum solids, thereby eliminating apparent changes which were due to variations in serum water content (6).

In the present study convulsive seizures were induced in monkeys by electroshock as well as by parenteral Metrazol and changes in serum solids and cholesterol were compared to ascertain whether these altered values were due to *bona fide* changes in cholesterol metabolism in the organism or merely to changes in serum water content.

Methods. Twenty-two *M. mulatta* 3–6 kg, including 19 males and 3 females, were maintained on a standard diet consisting of bread, lettuce, and bananas offered at 4 p.m. Animals were caught by net and 1.5 ml of blood was obtained from the saphenous vein. All bleedings took place prior to feeding. Total and free serum cholesterol, serum solids, hematocrit, and proteins were determined as previously described (4). Animals were grouped according to body weight, and processed without knowledge of their initial cholesterol levels until after termination of the experiment.

For inducing electroshock seizures unilateral rectangular pulses of 0.3-msec duration, pulse repetition frequency of 120/sec for 1.3 sec were used. The preset current varied from 10mA average at 170 mA peak to 18 mA average at 300 mA peak. Convulsive or subconvulsive doses of pentamethylenetetrazol were administered as 10% Metrazol intramuscularly in dosages of 40–48 mg/kg.

In the first group of 7 monkeys, 2 received electroshock seizures, one received a convulsive dose of Metrazol (48 mg/kg), one received a subconvulsive dose of Metrazol (40 mg/kg), and 3 were injected with physiological saline i.m. to serve as controls. All monkeys were bled immediately prior to treatment. In the 3 monkeys in which seizures occurred, the first posttreatment sample was drawn 3 min after termination of the convulsion (which was 10 min after injection).

In a second group of 14 monkeys, 7 received a single subconvulsive dose of Metra-

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