JOURNAL OF VIROLOGY, Sept. 1976, p. 1090-1099

Vol. 19, No. 3
Printed in U.S.A.

Antigens and DNA of a Chimpanzee Agent Related to Epstein-Barr Virus

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Received for publication 28 March 1976

Biological and biochemical studies of the herpesvirus of chimpanzees previously demonstrated to be antigenically related to human Epstein-Barr virus (EBV) indicated that the agent is similar to EBV in that: (i) leukocyte culture of chimpanzees whose sera contained antibody against EBV capsid antigen could yield long-term lymphoblastoid cell lines (Ch-LCL) with B-cell characteristics; (ii) the DNA of Ch-LCL contained sequences homologous to approximately 35 to 45% of human EBV; (iii) Ch-LCL contained an intranuclear antigen, Ch-NA, that could be identified with some chimpanzee or orangutan serum in anticomplimentary immunofluorescence assays; and (iv) treatment of Ch-LCL with iododeoxyuridine resulted in expression of new antigenic activity that reacted with EA+ but not EA− human sera. Two lines of evidence indicate that the chimpanzee agent, although related to human EBV, is a distinct agent: (i) Ch-NA was antigenically distinct from EBV-related nuclear antigen (EBNA) and EBNA induced in chimpanzee cells by EBV infection although it cross-reacts to a limited extent with a minor component of EBNA; and (ii) Ch-LCL are missing 55 to 65% of the DNA sequences of human EBV.

Epstein-Barr virus (EBV) has been closely associated with Burkitt's lymphoma and nasopharyngeal carcinoma and is the causative agent of classical heterophile-positive acute infectious mononucleosis. Studies of the pathophysiology of EBV infection and of its possible role in malignant transformation have been limited by the narrow host range of the agent. Chimpanzee (17) and marmoset (21) lymphocytes can be infected with EBV. Immunosuppression of EBV-infected marmosets can lead to lymphoma (30). In general, however, herpesviruses behave differently in heterologous species. It would therefore be of importance to establish whether there is an EBV-related agent indigenous to non-human primates. This would offer an opportunity to study the pathophysiology of this virus infection in the natural species.

Previous reports have suggested that infection with an agent similar to EBV is prevalent among populations of several non-human primate species. Thus, the sera of chimpanzees and Old World monkeys (3, 5, 10), as well as the sera of marmosets and prosimians (6), frequently contain antibody to EBV that can be detected by complement fixation (CF) tests as well as by immunodiffusion and indirect immunofluorescence assays (11, 32). Long-term lymphoid cell lines can be established from chimpanzee blood (17). These cell lines have been shown to contain a herpes-type virus (17) and to react with EBV-positive human sera in immunodiffusion and indirect immunofluorescence assays (11, 32). A line of identity was observed between an antigen detected by immunodiffusion in extracts of a chimpanzee lymphoid cell line and an EBV-infected human lymphoid cell line using EBV-positive human sera, suggesting that chimpanzee cells contain an EBV-related antigen (32).

Several lines of evidence suggest that non-human primates can acquire infection with an EBV-related agent without human contact and that the agent is distinct from human EBV. (i) Fifty percent of rhesus monkeys bled at the time of capture had CF antibody to EBV. Furthermore, two of three wild chimpanzees living without human contact in the Budango Forest in western Uganda had antibody to EBV antigens detectable by indirect immunofluorescence (19). (ii) Absorption of EBV-reactive chimpanzee sera with EBV-infected human cells resulted in a serum that reacted well with chimpanzee lymphoid cell lines (Ch-LCL) in indirect immunofluorescence but poorly with EBV-infected human cells, suggesting that Ch-LCL contain distinct antigenic activity (11).

The experiments reported here were undertaken to further characterize the extent of anti-

genic and biochemical relatedness of the chimpanzee agent to human EBV.

MATERIALS AND METHODS

Chimpanzee cell cultures. Eight chimpanzees served as donors for leukocyte cultures. Six were imported adult animals, and two (888 and 889) were 3 months old and born at the International Center of Environmental Safety of Albany Medical College at the Holloman Air Force Base in New Mexico. The sera of all eight chimpanzees contain antibody to EBV viral capsid antigen (VCA) as determined by the technique of Henle and Henle (12).

The procedures used in the separation of chimpanzee leukocytes from heparinized whole blood and in leukocyte cultivation were those described previously for the separation and cultivation of human leukocytes (8). The leukocyte cultures were incubated at 37°C in a CO_2 incubator and maintained by periodic medium changes as long as viable cells were detectable. The emergence of long-term lymphoblastoid cell lines was heralded by the appearance of large floating cells usually in aggregates, increased metabolic activity, and cell growth. The continuous Ch-LCL were designated by the number of the animal of origin and were maintained for periods of 4 to 12 months.

Human lymphoid cell lines. The P3 HR-1 (HR-1) cell line (15) was used for determination of VCA and contained 5 to 10% VCA-positive cells. The Raji cell line (26) was used as a source of EBV-determined soluble CF antigens (24) and EBV-related nuclear antigens (EBNA) (29), and for induction of EBV-associated early antigens (EA) (7). The Vax cell line was derived from an ascitic tumor of an American Burkitt's lymphoma patient by Ian T. Magrath. These cells have B-cell marker characteristics and do not contain EBV DNA or EBNA (Magrath, personal communication).

Determination of T- and B-lymphocyte markers. Chimpanzee lymphoblasts were classified by three techniques: formation of sheep cell rosettes (18); determination of complement receptors on the cell surface using sensitized sheep erythrocytes preincubated with antibody and complement (2); and staining the surface immunoglobulin of viable cells with fluorescein-isothiocyanate (FITC)-conjugated horse anti-human immunoglobulin (27). Rabbit antibody against sheep erythrocytes was purchased from Difco Laboratories, Detroit, Mich. FITC-conjugated horse anti-human immunoglobulin was purchased from Roboz Co., Washington, D.C.

Virus isolation procedures. Two methods were used in an attempt to determine whether Ch-LCL contained an agent that could transform marmoset or human lymphocytes into cells capable of long-term growth in culture.

(i) Cell-free lysates of Ch-LCL prepared by three cycles of freezing and thawing of packed cells, or spent media concentrated 100-fold by centrifugation at 100,000 × *g* for 1 h, were filtered through 0.45-μm filters (Millipore Corp., Bedford, Mass.). These preparations were inoculated onto cultures of fresh cord blood leukocytes or marmoset leukocytes, which were then maintained and observed for 4 to 6 weeks.

(ii) In co-cultivation experiments, Ch-LCL derived from male animals were lethally irradiated with 5,000 R. Mixtures of 5×10^6 cord blood lymphocytes or 1×10^6 to 2×10^6 marmoset lymphocytes and 0.5×10^6 to 2.5×10^6 lethally irradiated chimpanzee lymphoid cells were centrifuged at 50 × *g* for 3 min to promote intimate cell contact. The mixed lymphocyte cultures and individual control cultures of cord blood and of marmoset and irradiated chimpanzee lymphocytes were incubated for 4 to 5 weeks at 37°C in 5% CO_2 with periodic medium changes.

Detection of other simian viruses such as cytomegalovirus, adenovirus, or foamy virus was performed by inoculation of cultures of rabbit kidney, African green monkey kidney, HeLa, and W138 cells with either cell-free lysates or cells and culture fluid of Ch-LCL.

Serological tests. The procedures used in the preparation of soluble antigens from lymphoblastoid cells and for assay for CF antibody activity have been described in detail previously (4). EBV-associated antigens were detected by indirect immunofluorescence (12) using iododeoxyuridine (IUdR)-treated Raji cells as the source of EA and HR-1 cells as a source of VCA.

Cytoplasmic fluorescence in Ch-LCL was detected by indirect immunofluorescence using a cell line (Ch 14, 23, or 765) in which 2 to 5% of the cells exhibited cytoplasmic fluorescence. The procedures used in induction of cytoplasmic antigens in chimpanzee lymphoblasts and for detection of the induced antigenic activity by indirect immunofluorescence were identical to those previously used for detection of EA in Raji cells (7).

FITC-conjugated goat anti-human globulin (Hyland Co., Los Angeles, Calif.) was used in indirect immunofluorescence assays with human and nonhuman primate sera. The EBNA and the intranuclear antigen in chimpanzee cells (Ch-NA) were detected using the three-step anticomplementary immunofluorescence (ACIF) test (13). The controls for the ACIF test included EBNA-negative Vax cells and EBNA-positive and -negative reference sera. EBV-associated membrane antigens (MA) were detected on the surface of viable cells by indirect immunofluorescence (23).

Serum antibody absorption experiments were performed with 30 to 40% (vol/vol) cell lysates. A 1:4 dilution of heat-inactivated serum was absorbed at 37°C for 1 h and at 4°C for 8 h. The absorption was carried out twice.

EBV preparations. Leukocyte-transforming virus was derived from the spent culture fluid B95-8 cells (21). The cell-free supernatant fluid of 5- to 6-day cultures was passed through 0.45-μm filter, and aliquots were stored at −70°C. These preparations contained 10^4 to 10^5 transforming units/0.25 ml as tested with human cord blood leukocytes (9). Nontransforming EBV was derived from HR-1 cultures kindly provided by the Research and Logistics Branch of the Special Virus Cancer Program of the National Cancer Institute. Cell-free supernatants were pelleted at 100,000 × *g* for 1 h, and the virus-

containing pellets were resuspended in 1/200 volume of the original volume. These preparations when titrated on Raji cells for their ability to induce EBV EA (14) had a titer of 10^3 to $10^{3.5}$/0.25 ml.

Sera. Human sera were obtained from normal donors, African Burkitt's lymphoma patients, and American patients with nasopharyngeal carcinoma. Chimpanzee and orangutan sera were kindly provided by the Yerkes Primate Center, Atlanta, Ga., and by L. Barker, Bureau of Biologics, Bethesda, Md.

Virus neutralization tests. Sera were inactivated at 56°C for 30 min and diluted in twofold steps in medium 1640. An equal volume of 80 to 100 transforming units of EBV or nontransforming EBV containing 100 EA-inducing units was added to each serum dilution. Virus and serum controls were included in all tests. The mixtures were held at 4°C for 18 h. Transforming activity was determined in cord blood lymphocytes. The neutralization end point was read 7 to 10 days after the appearance of cell transformation in the virus control group. Neutralization of EA-inducing activity was assayed in Raji cells. The 50% neutralization end point was calculated by the method of Reed and Muench (28).

Preparations of cellular DNAs. Cell pellets were resuspended in a 40-fold volume of a solution containing 0.05 M Tris-hydrochloride, 0.1 M NaCl, and 10 mM EDTA, at pH 7.4. The suspension was made 1% (wt/vol) in sodium dodecyl sulfate and heated at 60°C for 5 min. Proteins were extracted at 60°C with equal volumes of phenol and chloroform containing 2% (vol/vol) isoamyl alcohol until the aqueous phase was clear and the interphase free of debris (20). The nucleic acid was precipitated overnight at −20°C after the addition of 2 volumes of ethanol. RNA was removed by digestion of the nucleic acid in a solution consisting of 0.5 N KOH for 18 h at 37°C. The DNA solution was brought to neutral pH by the addition of 5 N HCl, and the DNA was precipitated by the addition of 2 volumes of ethanol at −20°C. The absence of residual RNA was determined by the orcinol reaction (1).

Preparation of labeled viral DNA. EBV was purified from extracellular fluid of HR-1 cells as previously described (25). DNA was prepared from purified EBV and labeled in vitro with [^{3}H]thymidine triphosphate (specific activity, 50 Ci/mmol; New England Nuclear Corp., Boston, Mass.) using *Escherichia coli* DNA polymerase 1 (Boehringer Corp., New York, N.Y.) (25). The specific activity of the resultant DNA ([^{3}H]EBV HR-1 DNA) was approximately 3×10^6 cpm/μg.

DNA-DNA hybridization. ^{3}H-labeled EBV HR-1 DNA (0.016 μg/ml, final concentration) was mixed with cell DNA that had been sonically treated at 100 for 30 s with an Artek 300 sonic oscillator (Artek Corp., New York, N.Y.). The DNA mixture was denatured in 0.2 N NaOH, neutralized with HCl, sealed in 50-μl capillary tubes in a solution containing 1.25 M NaCl, 0.0025 M EDTA, and 0.02 M Tris-hydrochloride (pH 7.4), and incubated in a water bath at 68°C for various intervals up to 72 h. The samples from each experiment were stored at −30°C and analyzed at the same time. Single-stranded DNA was differentiated from double-stranded DNA by using a single-strand-specific nuclease, S1, prepared from α-amylase of *Aspergillus oryzae* (type IV α-amylase obtained from Sigma Biochemical Corp., St. Louis, Mo.) (33). Under the conditions used, the preparation of S1 digested less than 1% of native [^{3}H]EBV HR-1 DNA and 95% of denatured [^{3}H]EBV HR-1 DNA. The data were corrected for the efficiency (95%) with which the S1 nuclease digested an aliquot of the hybridization mixture that was frozen at −30°C immediately after neutralization.

The curves drawn in the figure were established by nonlinear, nonweighted least-square regression (31) of the data to an equation $C/C_0 = 1/(1 + k\,C_0 t)$, where k is the reaction rate constant, C_0 is the starting concentration of labeled single-stranded DNA, and C is the concentration of labeled single-stranded DNA remaining at each time of incubation (t) of the hybridization mixture. The concentration of EBV-homologous DNA in the cell DNA was determined from the ratio of $k\,C_0$ observed with probe DNA hybridized in the presence of lymphoblast cell DNA to the $k\,C_0$ observed by the hybridization of probe DNA in the presence of an equal concentration of DNA prepared from cells that do not contain EBV-homologous sequences (calf thymus or chimpanzee lung).

RESULTS

Leukocyte cultures of five of eight chimpanzees showed evidence of increased metabolic activity after 20 to 34 days in culture, whereas leukocytes obtained from three other chimpanzees, numbers 22, 783, and 784, degenerated within 30 days of culture. At the time of the third attempt at establishment of long-term cultures of leukocytes from animals 22, 783, and 784, aliquots of the leukocytes were inoculated with 0.2 ml of EBV derived from B95-8 lymphoblasts. The inoculated cultures showed signs of increased growth after 12 to 16 days. The lymphoblastoid cell lines that grew spontaneously (Ch 14, 23, 765, 888, and 889) and those that were infected with EBV (Ch-EBV 22, 783, and 784) had a doubling time of 24 to 36 h and have been maintained in culture for periods of 4 to 12 months. From less than 1% up to 5% of the cells in these cultures contained cytoplasmic fluorescence when tested with reference EBV chimpanzee sera containing antibodies to VCA (Table 1). Cytogenetic analysis, kindly performed by W. E. Peterson, Jr., at the Child Research Center of Michigan and J. Peng Whang at the National Cancer Institute, confirmed the chimpanzee origin of these cell lines.

None of the cells of the eight Ch-LCL formed rosettes with sheep erythrocytes or with sheep erythrocytes that had been preincubated with antibody to sheep erythrocytes. From 20 to 30% of the cells of each culture had detectable surface immunoglobulin, and 85 to 95% had C3

TABLE 1. *Origin of long-term chimpanzee lymphoid cell lines*

Culture no.	Date initiated	Inoculation of leukocytes	Transformation after days	Percent[a] immunofluorescence-positive cells
14	2/25/75	None	30	1-3
23	6/22/72	None	20	2-5
765	6/5/75	None	26	2-5
888	7/3/75	None	34	<0.1
889	7/3/75	None	30	<0.1
22	9/11/72	EBV	12	<0.1
783	10/2/73	EBV	16	<0.1
784	10/2/73	EBV	16	<0.1

[a] Average percentage of cytoplasmic fluorescence with EBV-reactive chimpanzee sera in indirect immunofluorescence.

receptors as indicated by rosette formation with erythrocyte-antibody complement complexes. These results indicate that all eight Ch-LCL contained cells with B-cell characteristics.

An attempt was made to isolate virus from each of the Ch-LCL. The Ch 14, 23, and 765 cell lines were most extensively assayed for leukocyte-transforming agents since these cultures contained the highest percentage of cells with cytoplasmic fluorescence. Cell lysates or 100-fold-concentrated spent culture media were inoculated into fresh leukocyte preparations derived from human cord blood or from marmoset blood or into monolayer tube cultures of rabbit and African green monkey kidney, HeLa, or WI38 cells, as described in Materials and Methods. No long-term lymphoblastoid cell lines resulted. Co-cultivation of lethally irradiated chimpanzee cells with human cord blood leukocytes containing the chromosome marker of the opposite sex was without effect.

Lysates of two Ch-LCL (14 and 784) produced multinucleated syncytia in rabbit kidney and WI38 cells. The syncytia-causing agent was identified as chimpanzee foamy virus type 2 by indirect immunofluorescence with type-specific antisera provided by the Resources and Logistics Branch of the Special Virus Cancer Program of the National Cancer Institute.

Relationship between EBV-associated antigens and the antigens of Ch-LCL. The extent of antigenic cross-reactivity of EBV and the EBV-related agent in CH-LCL was investigated by (i) comparing the reactivity of selected human sera, VCA+ EBNA+, VCA+ EBNA−, VCA+ EA+, VCA+ EA−, or VCA− EA− EBNA−, with antigens present in EBV-infected human and Ch-LCL and in spontaneously performed lymphoblastoid cells; and by (ii) comparing the reactivity of non-human primate sera with antigens present in Ch-LCL and EBV-infected human and chimpanzee lymphoblastoid cells. Nuclear, cytoplasmic, IUdR-induced cytoplasmic, membrane, and neutralizing antigens of these cell lines were compared. The results were as follows.

(i) Intranuclear antigens detectable by ACIF. One hundred and six human and nonhuman primate sera were assayed for antibody to EBV VCA and for activity against EBNA or Ch-NA in ACIF tests (Table 2). None of the sera reacted with the EBV genome-negative Vax cells and were all therefore considered to be free of nonspecific antinuclear antibodies. The sera of 30 humans and 4 orangutans were negative for VCA and failed to react in the ACIF test with Raji or chimpanzee cells. The 39 human sera with antibody to VCA were positive in ACIF tests against EBNA and nuclear antigens of EBV-transformed Ch 784 cells. Seven of these 39 VCA antibody-positive human sera also reacted with Ch-NA of spontaneously transformed chimpanzee cells in ACIF.

By contrast, none of 20 chimpanzee sera with VCA antibodies detected EBNA in Raji or EBV-transformed Ch 784 cells, but 55% (11/20) reacted strongly with Ch-NA in spontaneously transformed Ch 888 cells.

Figure 1 illustrates ACIF staining of Ch-NA in Ch 888 cells. Generally 90 to 95% of the cells were positive, and a similar staining pattern was observed with the other spontaneously transformed Ch-LCL.

A comparison of CF and ACIF reactivity of VCA antibody-positive human and chimpanzee sera (Table 3) indicated a consistent qualitative correlation: EBNA-positive human sera had antibodies to EBV-soluble CF antigens, and, similarly, chimpanzee sera positive in ACIF tests with Ch-NA had antibodies to soluble CF antigens derived from Ch 888 cells. Five human sera reacted with both EBNA and Ch-NA in the ACIF test and had antibodies to soluble CF antigens from Raji as well as Ch 888 cells. The consistent correlation between ACIF and CF reactivity of human and chimpanzee sera agrees with similar observations with human sera (16). There was no correlation between the

TABLE 2. *Reactivity of human and primate sera in ACIF tests with nuclear antigens in lymphoid cell lines*

Serum source	EBV VCA titers	No. of positive sera/no. tested			
		Human cells[a]		Chimpanzee cells[a] transformed:	
		Vax	Raji	Spontaneously (Ch 888)	By EBV (Ch 784)
Human	<4	0/30[a]	0/30	0/30	0/30
	32-2,048	0/39	39/39	7/39	39/39
Chimpanzee	32-256	0/20	0/20	11/20	0/20
Orangutan	<4	0/4	0/4	0/4	0/4
	16-128	0/13	0/13	6/13	0/13

[a] Target cells.

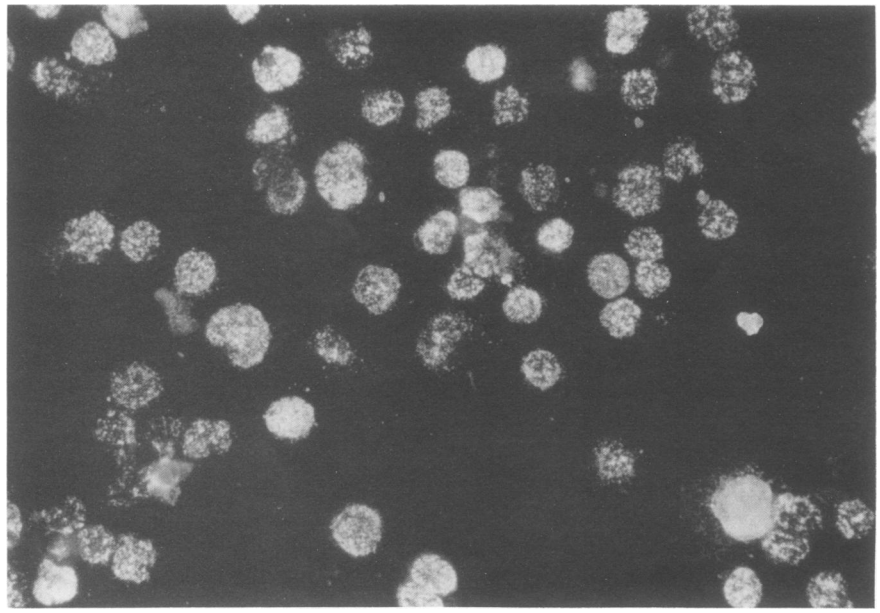

FIG. 1. *Anticomplement immunofluorescence staining of nuclear antigens in Ch 888 cells with chimpanzee serum 198. ×130.*

quantitative antibody titer of the human sera to EBNA or CF antigens from Raji cells and the antibody titer to nuclear and CF antigens of chimpanzee cells. This suggests that major components of EBNA and EBV-soluble CF antigens were not present in spontaneously transformed Ch-LCL. None of the VCA antibody-positive chimpanzee sera, some of which contained high antibody titers against intranuclear ACIF antigen and soluble CF antigens of spontaneously transformed Ch-LCL, had detectable antibody against EBNA or EBV-soluble CF antigens from Raji cells. Absorption of two human sera (Table 4) with Raji cell extracts eliminated the ACIF reactivity against Ch-NA, whereas absorption with Ch 888 cell extract had no detectable effect on the anti-EBNA titers, further suggesting that Ch-LCL contain an antigen that cross-reacts with a minor component of EBNA.

(ii) Induced cytoplasmic antigens. Treatment of Raji cells with halogenated pyrimidines has been previously shown to induce the synthesis of EA (7). Twelve of the 39 VCA+ human sera had antibody to EA (Table 5). All 12 VCA+ EA+ human sera reacted with cytoplasmic antigens induced by IUdR in 1 to 2% of Ch 888 and 889 cells. None of the VCA+ EA− human sera reacted with the induced antigens in chimpanzee cells. Similarly, of 20 VCA+ chimpanzee sera, 8 reacted with IUdR-induced antigens in Ch-LCL and had EA antibodies, whereas 12 EA− chimpanzee sera failed to react with IUdR-induced antigens in chimpanzee cells.

(iii) Cytoplasmic antigens. Twenty-eight of

TABLE 3. *Comparison of CF and ACIF titers of EBV-positive human and chimpanzee sera*

Sera		Diagnosis[a]	ACIF titers to nuclear antigen in:		CF titers to soluble antigens in:	
Source	Code		Raji	Ch 888	Raji	Ch 888
Human	E294	Normal	32	8	64	8
	E296	Normal	4	<4	32	<4
	E297	Normal	4	<4	64	<4
	E298	Normal	8	<4	32	<4
	E312	Normal	32	<4	256	<4
	E321	Normal	4	<4	32	<4
	E323	Normal	16	<4	64	<4
	E340	Normal	64	8	128	16
	E352	NPC	256	<4	512	<4
	K136	BL	<4	<4	<4	<4
	K296	BL	16	4	512	32
	K321	BL	16	<4	512	<4
	K322	BL	32	4	256	32
	K333	BL	16	8	128	8
Chimpanzee	14	Normal	<4	16	<4	128
	23	Normal	<4	32	<4	256
	70	Normal	<4	<4	<4	<4
	91	Normal	<4	<4	<4	<4
	98	Normal	<4	<4	<4	<4
	99	Normal	<4	4	4	16
	198	Normal	<4	64	<4	128
	765	Normal	<4	<4	<4	<4
	783	Normal	<4	4	<4	32
	784	Normal	<4	32	<4	128

[a] Abbreviations: NPC, nasopharyngeal carcinoma; BL, Burkitt's lymphoma.

TABLE 4. *Absorption of human and chimpanzee sera reactive in ACIF test with lymphoid cell lines*

Target cells	Serum											
	Human								Chimpanzee			
	294				340				(198)			
	None[a]	Raji	Ch 888	Vax	None	Raji	Ch 888	Vax	None	Raji	Ch 888	Vax
Raji	32[b]	<4	32	32	64	4	64	64	<4	<4	<4	<4
Ch 888	8	<4	<4	8	8	<4	<4	8	32	32	<4	32
Vax	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4

[a] Cells with which sera were absorbed.
[b] Reciprocal of ACIF titer.

TABLE 5. *Reactivity of human and chimpanzee sera with antigens in human and chimpanzee lymphoid cells activated by IUdR*

Serum	IUdR-treated Raji cells		IUdR-treated Ch 888 cells	
	Positive	GMT[a]	Positive	GMT
Human	12/39	10	12/39	4
Chimpanzee	8/20	4	8/20	4

[a] Geometric mean titer.

39 VCA+ human sera with a geometric mean titer of 60 reacted weakly with an antigen present in the cytoplasm (Ch-CyA) of 1 to 5% of the cells of the Ch 14, 23, and 765 cell lines with a geometric mean titer of 4 (Table 6) in indirect immunofluorescence assay. After absorption of the human sera with virus-producing HR-1 cells, the human sera failed to react with Ch-

CyA (Table 7). All 20 VCA+ chimpanzee sera reacted strongly with VCA and with Ch-CyA with geometric mean titers of 40 and 45, respectively (Table 6). Preabsorption of the reactive chimpanzee sera with HR-1 cell extracts reduced the titer of activity against VCA and against Ch-CyA.

(iv) EBV-neutralizing and anti-MA activity of chimpanzee sera. Live cells of Ch-LCL exhibited only weak and irregular membrane fluorescence with human and chimpanzee sera and could not be used for quantitative cross-reaction experiments. Table 8 summarizes the results of EBV neutralization tests and MA-staining reactions. Seven of ten chimpanzee sera had neutralizing antibodies to transforming (B95-8) and nontransforming (HR-1) strains of EBV. Neutralizing antibodies correlated with staining reactivity of MA on HR-1 cells.

DNA-DNA hybridization studies. To determine the extent of relatedness of the putative chimpanzee virus to human EBV, the extent to which chimpanzee lymphoblasts contain DNA sequences homologous to EBV HR-1 DNA was determined by hybridization of labeled purified EBV DNA with excess unlabeled cell DNA. The results of a typical experiment are shown in Fig. 2 and indicate the following. (i) As expected, DNA extracted from HR-1 cells contained sequences homologous to more than 95% of EBV HR-1 DNA. (ii) The hybridization of denatured [^{3}H]EBV HR-1 DNA was not specifically increased by incubation with calf thymus DNA or with DNA extracted from chimpanzee lung tissue, indicating that chimpanzee lung cells do not contain sequences homologous to EBV DNA. (iii) DNA extracted from Ch-LCL initially increased the hybridization of the denatured [^{3}H]EBV HR-1 DNA to a rate indicating approximately three genome equivalents of EBV-homologous DNA per diploid cell genome. After 35 to 45% of the [^{3}H]EBV HR-1 DNA was hybridized, the reaction rate was similar to that of denatured [^{3}H]EBV HR-1 DNA alone, suggesting that the Ch-LCL DNA does not contain sequences homologous to the entire EBV HR-1 genome.

DISCUSSION

All of the chimpanzee sera investigated in this study contained antibody to VCA. Some of the chimpanzee sera contained antibody to EBV-determined early antigen and membrane antigen, suggesting that these animals had been infected with an EBV-related agent. The

TABLE 8. *Neutralization of EBV and reactivity with EBV-associated membrane antigens by chimpanzee sera*

Chimpanzee serum	Neutralization of:		Antibody titer to EBV-associated MA
	Transforming EBV strain B95-8	Nontransforming EBV strain HR-1	
14	20[a]	10	4
22	<10	<10	<4
23	40	20	8
70	<10	<10	<4
91	<10	<10	<4
98	40	20	8
198	320	80	16
783	60	40	16
784	10	10	4
A01	60	20	8

[a] Reciprocal of 50% serum neutralization titer.

TABLE 6. *Reactivity of EBV-positive human and chimpanzee sera in immunofluorescence tests with lymphoid cells*

Serum	No. tested	HR-1 cells		Ch 23 cells	
		Positive	GMT[a]	Positive	GMT
Human	39	39	60	28	4
Chimpanzee	20	20	40	20	45

[a] Geometric mean titer.

TABLE 7. *Absorption of human and chimpanzee sera with extracts of Raji or HR-1 cells*

Target cells	Human serum B76 absorbed with:			Chimpanzee serum 913 absorbed with:		
	None	Raji	HR-1	None	Raji	HR-1
HR-1	256[a]	256	<8	256	256	32
Ch 23	4	4	<4	256	256	64

[a] Reciprocal of highest dilution of serum with 2+ fluorescence.

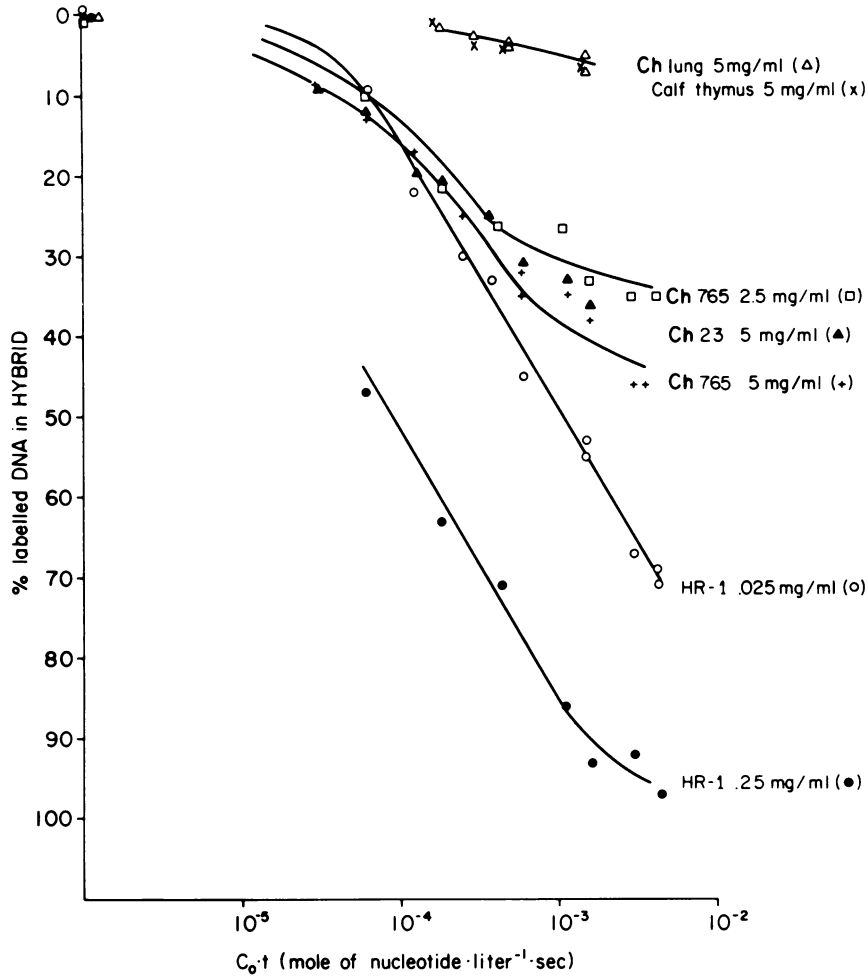

FIG. 2. *Reassociation of [^{3}H]EBV HR-1 DNA (0.016 μg/ml, 1.3 × 10^4 cpm) in the presence of Ch 765 lymphoblast DNA (2.5 mg/ml; 5.0 mg/ml), Ch 23 lymphoblast DNA (5.0 mg/ml), HR-1 lymphoblast DNA (0.25 mg/ml; 0.025 mg/ml), and chimpanzee lung DNA (2.5 mg/ml). The conditions of reassociation and the methods of analysis are described in Materials and Methods.*

objective of these experiments was to determine the relationship of the agent that infected these animals to EBV. A characteristic of human infection with EBV is that viral antigens can be demonstrated in long-term lymphoblastoid cell lines derived from peripheral blood. Our data indicate that the chimpanzee agent is biologically and biochemically similar to human EBV in the following respects. (i) Leukocyte cultures of chimpanzees whose sera contain anti-VCA activity frequently will yield long-term lymphoblastoid cell lines that possess surface markers associated with immunoglobulin-producing cells. (ii) The DNA of cells in the Ch-LCL contains sequences homologous to at least 35% of EBV DNA. (iii) Some EBNA-positive human sera react with an intranuclear antigen in spontaneously transformed Ch-LCL. The reaction can be blocked by preabsorbing the sera with cells containing EBNA suggesting that Ch-LCL contain an intranuclear antigen that cross-reacts with a component of EBNA. (iv) Treatment of Ch-LCL with IUdR results in expression of new antigenic activity that reacts with EA+ but not EA− human sera.

Two lines of evidence emerge from these studies which indicate that the chimpanzee agent is not EBV but a distinct EBV-related agent. (i) Chimpanzee sera contain antibody to an intranuclear antigen (Ch-NA) of spontaneously transformed Ch-LCL. These same sera have no activity against EBNA, and the activity against Ch-NA cannot be removed by absorption of the sera with cells containing

EBNA. Although the intranuclear antigen of the Ch-LCL has some reactivity with human EBNA+ sera, some human sera with high anti-EBNA titer do not contain activity against Ch-NA. The failure of Ch-LCL to contain major components of EBNA and the presence in these cells of a new nuclear antigen cannot be attributed to a difference in expression of EBV in human and non-human primate lymphocytes, since chimpanzee lymphocytes infected with EBV contain a nuclear antigen identifiable as EBNA by its reactivity with all EBNA-positive human sera (many of which lack activity against Ch-NA) and by its lack of reactivity with Ch-NA-positive chimpanzee sera. (ii) The DNA-DNA hybridization data suggested that spontaneously transformed Ch-LCL do not contain all the sequences of EBV. Because of the small number of copies of EBV-homologous DNA in spontaneously transformed Ch-LCL, we cannot precisely determine the point at which hybridization of Ch-LCL DNA with EBV DNA reaches a plateau. However, the fact that the hybridization with EBV DNA reaches a plateau at approximately 35 to 45% hybridization is unlikely to be due to an artifact in that hybridizations of labeled EBV DNA with unlabeled DNA containing even fewer copies of EBV DNA than are present in Ch-LCL proceeds at a slower initial rate, but does not reach a plateau (Fig. 2). Quantitation and precise determination of which sequences are missing from Ch-LCL will require hybridization with specific sequences of EBV DNA such as can be obtained after treatment with restriction enzymes.

In initial experiments, we have been unable to detect an agent in the spontaneously transformed Ch-LCL that is capable of transforming human cord blood or leukocytes of EBV seronegative marmosets. This may be due to the restricted host range of this agent. The prevalence of antibody to the agent in the sera of chimpanzees and other non-human primates suggests that the agent is highly infectious. Attempts at demonstrating the biological activity of this EBV-related virus are therefore continuing.

ACKNOWLEDGMENTS

Part of this work was supported by the following grants: Public Health Service grants NIH 1 F22 CA00341, 5 P30 CA14599, and 1 R01 CA17281, all from the National Cancer Institute, ACS VC1133A, The Leukemia Research Foundation, and the Schweppe Foundation.

We wish to thank Elizabeth Kim for her excellent technical assistance.

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