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Two Epstein–Barr viral nuclear neoantigens distinguished by gene transfer, serology, and chromosome binding

(chromosomes/nuclear antigen)

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ABSTRACT **We recently identified, by means of cotransformation of LTK⁻ cells, a region of the Epstein–Barr virus (EBV) genome (the *Bam*HI K fragment) that encodes or induces an EBV nuclear neoantigen (EBNA) serologically related to the EBNA found in lymphoid cells carrying the entire EBV genome. We now find that a second EBV DNA fragment, *Bam*HI M, is also able to give rise to cotransformed LTK⁻ cells with stable expression of a nuclear antigen. The *Bam*HI K and M fragments have no apparent DNA homology. Many human sera that are reactive to EBNA in Raji cells detect both antigens; however, certain anti-EBNA-positive human sera are discordant and react only with the *Bam*HI M or only with the *Bam*HI K nuclear antigen. Every Raji cell appears to express both "M" and "K" antigens; D98 Raji cells, a somatic cell hybrid, express only "K" antigen. The K antigen is found on metaphase chromosomes of LTK cells and Raji cells. The M-induced antigen is not located on chromosomes when the cells are in metaphase but is present as granules within the nucleus.**

A nuclear neoantigen (EBNA) is found in Epstein–Barr virus (EBV)-associated tumors such as Burkitt lymphoma, polyclonal B-cell lymphoma, and nasopharyngeal cancer (1). EBNA is located on chromosomes in lymphoid cell cultures and in mitotic circulating B lymphocytes in infectious mononucleosis (2). During the process of immortalization of lymphocytes *in vitro*, EBNA appears within a few hours after infection by the virus (3). Therefore, EBNA is thought to play an important role in the immortalization reaction. The biochemical definition of EBNA is still unclear; a number of different molecular weights and other properties of EBNA have been described (4–7). By cotransformation of mouse LTK⁻ cells with the herpes simplex thymidine kinase (*tk*) gene and cloned EBV DNA, we recently mapped one gene that encodes or induces EBNA to the leftmost 2,900 base pairs of the *Bam*HI K fragment of EBV DNA (8). In the course of these studies, we found that LTK⁻ cells also expressed a nuclear antigen transiently after exposure to the *Bam*HI M EBV DNA fragment (about 4.7 kilobase pairs). We have now stably introduced the *Bam*HI M fragment into mouse LTK⁻ cells by cotransformation. The nuclear antigen (M) that such cells express is also an EBNA but is serologically distinct from the *Bam*HI K nuclear antigen. Furthermore, both the K and M EBNAs are present in Burkitt lymphoma lines such as Raji and HR-1 and in cells transformed *in vitro*. The two EBNAs differ in their binding to metaphase chromosomes.

MATERIALS AND METHODS

Plasmids. The *Bam*HI M fragment of EBV DNA, strain FF41, was cloned on pBR322 and propagated in *Escherichia coli* LE392. Plasmid DNA was purified on sucrose gradients as described (9).

Cotransformation. These results are based on two cotransformation experiments. In the first, LTK⁻ cells in 20-cm^2 Petri dishes were exposed to calcium phosphate precipitates containing 1 μg of plasmid DNA from pBR-*Bam*HI M, 0.1 μg of DNA from pXI, plasmid pBR322, which carries the herpes simplex virus thymidine kinase (*tk*) gene, and 8 μg of carrier DNA from LTK⁻ cells (10–12) for 48 hr. Then, transformants were selected for the tk⁺ phenotype by addition of MAGGT medium [0.6 μM methotrexate/50 μM adenosine/50 μM guanosine/0.1 μM glycine/16 μM thymidine (13)]. Transformants that survived selection were examined for antigen expression as pools of transformed colonies. In the second experiment, the transformation was repeated with 3.2 μg of pBR-*Bam*HI M DNA, 0.3 μg of pXI DNA, and 8 μg of carrier DNA. Twenty-five transformed colonies that survived MAGGT selection were then isolated by using cloning rings and were tested individually for antigen expression.

Immunofluorescence. Nuclear antigen was detected by anticomplement (C3) immunofluorescence (14). Cells were fixed in methanol and frozen at −20°C overnight or longer. Sources of antibody were various human sera with or without antibody to EBNA present in Raji cells. Fluorescein isothiocyanate-conjugated goat anti-human β_1-globulin, purchased from Atlantic Antibodies (Scarborough, ME), was used at a dilution of 1:30.

Two-Color Immunofluorescence for Detection of Antigen Location on Chromosomes. Cotransformed mouse LTK⁺ cells or Raji cells were incubated at 37°C for 2 hr with Colcemid (0.1 μg/ml; GIBCO). They were then held for 10 min at 37°C in a hypotonic solution consisting of phosphate-buffered saline/1% sodium citrate/1 mM $CaCl_2$/1 mM $MgCl_2$ (1:4). Coverslips were then processed for detection of nuclear antigen by anticomplement immunofluorescence. After the reaction, the cells were counterstained for 5 min at room temperature with propidium iodide at 0.5 μg/ml (15). The coverslips were washed twice with phosphate-buffered saline and mounted in buffered glycerol. Green (fluorescein isothiocyanate) immunofluorescence was detected with a blue excitation filter (Zeiss 487709) and a red suppressor (BG38); for red (propidium iodide) immunofluorescence, we used a green excitation filter (Zeiss 487714). Cells were photographed with Kodak Tri-X film.

Examination of Transformed LTK⁺ Cells for EBV DNA Sequences. Intracellular DNA was prepared from LTK⁻ and var-

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Abbreviations: EBV, Epstein–Barr virus; EBNA, EBV-encoded nuclear neoantigen; *tk*, herpes simplex virus thymidine kinase gene; C3, third component of human complement.

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ious LTK$^+$ cells by the method of Wahl *et al.* (16). About 8 μg of intracellular DNA was digested with *Bam*HI, electrophoresed through 0.5% agarose gels, and transferred to nitrocellulose. Filters were probed with pBR322 labeled with ^{32}P by nick translation, or with pBR322 containing EBV *Bam*HI M fragment. Because the sizes of pBR322 and the *Bam*HI M fragment are so similar, it was not possible to excise *Bam*HI M in pure form from a gel.

RESULTS

Nuclear Antigen Expressed in LTK$^-$ Cells Cotransformed with *Bam*HI M. In our initial experiment, 48 tk$^+$ colonies that survived selection were pooled and examined for nuclear antigen by anti-C3 immunofluorescence with, as antibody, a serum from a patient with nasopharyngeal carcinoma that contained high antibody titers to all EBV-associated antigens. A few clusters of nuclear antigen-positive cells (<5% of the cells) were seen. From this pool, we attempted to derive cell clones that were antigen positive in 100% of the nuclei but, among 60 clones examined, none displayed the antigen. This result contrasts with the ease of deriving cell clones that express the K antigen (8). When the transformation was repeated, 2 of the 25 individual colonies isolated by using cloning rings expressed the antigen; 50–75% of the nuclei reacted by anticomplement immunofluorescence. One clone, designated C20, was used for subsequent studies. The M antigen was more granular in appearance than that seen in L cells with the EBV *Bam*HI K fragment, and the M antigen was barely detectable by antiimmunoglobulin immunofluorescence, a technique that readily stains the K antigen. The M antigen remained in C20 cells cultured *in vitro* for a period of 8 months, but the fraction of antigen-positive nuclei gradually drifted downward to 20–40%.

***Bam*HI M DNA in the Cotransformed LTK$^+$ Cells.** LTK$^+$ cells that arose following cotransformation with the HSV *tk* gene and the EBV *Bam*HI M fragment appear to retain several copies of the EBV sequences, probably linked to high molecular weight cellular or carrier DNA (Fig. 1). Total cellular DNA from clone C20 contained five *Bam*HI fragments that were specifically detectable on a Southern blot with pBR-*Bam*HI M fragment as probe. These fragments were not hybridizable with pBR322 alone (Fig. 1). In three experiments, there were no differences in the patterns of hybridization when pBR322 or pBR-*Bam*HI M was used as the probe in Southern blots containing LTK$^-$ cells, LTK$^+$ cells, or LTK$^+$ cells cotransformed with EBV *Bam*HI K. One copy of EBV DNA was present as a *Bam*HI fragment of approximately the same size as the *Bam*HI M fragment of EBV DNA. Three of the homologous fragments were larger and one was smaller than *Bam*HI M (Fig. 1). On the basis of the intensity of the hybridization, there is less than one copy per cell of the sequences with homology to *Bam*HI M. At the time DNA was prepared from the C20 cells, about 40% of the cells expressed nuclear antigen.

Serologic Comparison of the *Bam*HI K and M Nuclear Antigens. A panel of 53 human sera was used to characterize the nuclear antigen in C20 cells by anti-C3 immunofluorescence (Table 1). The ability of these same sera to recognize the neoantigen in L cells (clone D3) harboring the EBV *Bam*HI K fragment was studied in parallel. The M nuclear antigen was detected with 24 of 37 anti-EBNA-positive sera; 30 of these same 37 sera reacted with the K antigen. None of 16 human sera lacking antibody to EBNA recognized either the K or M nuclear antigen. Thus, on serologic grounds, the nuclear antigens found in LTK$^+$ cells cotransformed by either EBV DNA fragment are "EBNAs."

The two antigens are serologically distinct, however. A number of human sera were discordant in their ability to detect the M and K antigens in cotransformed LTK$^+$ cells. Six sera (M$^+$/K$^-$) contained antibody to the M antigen and lacked antibody to the K antigen. The antibody titer to the M nuclear antigen in one of these six sera, from a patient with a chronic EBV infection, was >1:320; the same serum was unable to detect the K antigen at a dilution of 1:2. The other five M$^+$/K$^-$ sera contained anti-EBNA in titers of 1:2 to 1:4. Human sera with the

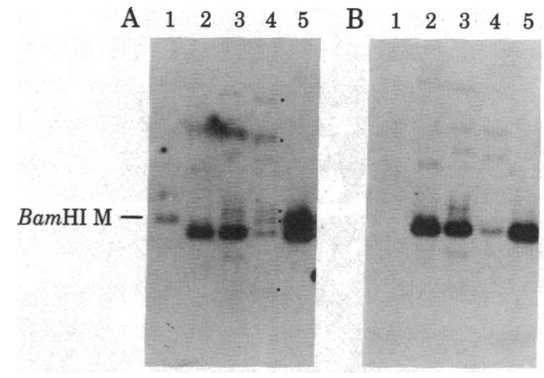

FIG. 1. Southern blot showing multiple copies of the EBV *Bam*HI M sequences in cotransformed LTK$^+$ cells. (*A*) Hybridization probe containing both *Bam*HI M and pBR322 sequences. (*B*) Hybridization probe containing only pBR322 sequences. Lane 1: 100 ng of intracellular DNA from a lymphoid line, FF467, that contains ≈40 EBV DNA copies per cell. The signal is equivalent to about one copy of the *Bam*HI M fragment per cell. Lane 2: 8 μg of intracellular DNA from a LTK$^+$ cell line cotransformed with the *Bam*HI K EBV DNA fragment and the herpes simplex virus (HSV) *tk* gene. Lane 3: 8 μg of intracellular DNA from LTK$^+$ cells cotransformed only with the HSV *tk* gene on plasmid pXI. Lane 4: 8 μg of intracellular DNA from LTK$^+$ cells (clone C20) cotransformed with EBV *Bam*HI M and the HSV *tk* gene. Lane 5: 10 pg of plasmid pBR322 containing the *Bam*HI M fragment. All DNA samples were digested with *Bam*HI M. Note: in this and in two other replicate experiments, the hybridization patterns with probes containing pBR322 or pBR322 and *Bam*HI M were similar with DNA from LTK$^+$ cells cotransformed with *Bam*HI K (lane 2) or HSV *tk* alone (lane 3). By contrast, in lane 4, several *Bam*HI DNA fragments (black dots) detected by a probe containing *Bam*HI M sequences are not seen when the comparable sample is probed with pBR sequences alone. In other experiments (data not shown), neither pBR322 nor pBR322/*Bam*HI M detects homology with LTK$^-$ cells.

Table 1. Serologic comparison of EBNAs in LTK$^-$ cells stably transformed by EBV DNA fragments *Bam*HI K and *Bam*HI M

Serum category	Antibody to VCA	EA	EBNA	No. of sera	No. positive on *Bam*HI K	on *Bam*HI M
1	−	−	−	7	0	0
2	+	−	−	2	0	0
3*	+	+	−	7	0	0
4	+	+	+	20	13	18†
5	+	−	+	17	17‡	6
Total				53	30	24

* The seven sera in this category, with antibody to EA but not to EBNA, come from six patients with recent infectious mononucleosis and one patient with pneumonia.

† The five sera that contain antibody to the M antigen and lack antibody to the K antigen are from patients with pneumonia, current infectious mononucleosis, chronic EBV infection, and recent EBV infection (two patients).

‡ Eleven sera that contained antibody to the K antigen and lacked antibody to the M antigen were obtained from patients with past histories of infectious mononucleosis (3), suspect mononucleosis (1), pneumonia (3), influenza (1), American Burkitt lymphoma (1), and encephalitis (1) and one with an unknown past history.

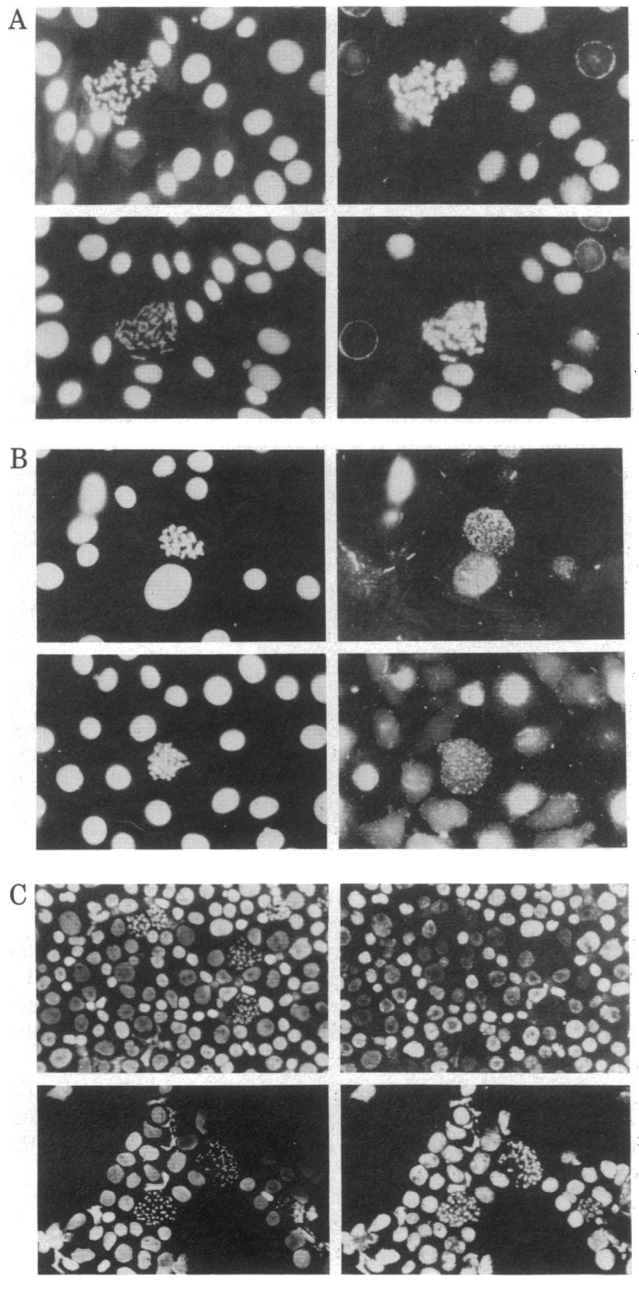

FIG. 2. (*A*) The *Bam*HI K nuclear neoantigen is associated with metaphase chromosomes. The D3 clone of cotransformed LTK$^+$ cells was examined by two-color immunofluorescence. A polyvalent human serum containing antibody to both the M and K nuclear antigens was used in indirect anti-C3 immunofluorescence with fluorescein (FITC)-conjugated goat anti-human C3. After the immunofluorescence reaction, the cells were counterstained with propidium iodide. The same field of propidium iodide-stained cells (*Left*) or FITC anti-C3-stained cells (*Right*) was photographed. (*B*) The *Bam*HI M nuclear antigen is not found on metaphase chromosomes. The C20 clone of cotransformed LTK$^+$ cells was processed as described above. (*Left*) Propidium iodide-stained cells. (*Right*) The same cells were examined by anti-C3 immunofluorescence. As a source of antibody, the same serum was used as in *A*. (*C*) Differences in chromosomal binding of two nuclear antigens in Raji cells. Eighteen hours after their last subculture, Raji cells were treated with Colcemid for 2 hr and then for 5 min with "swelling buffer." Slides were prepared with a cytocentrifuge and stained for EBNA determination by anti-C3 immunofluorescence either with a 1:10 dilution of human serum (W.C.), which has antibody only to the *Bam*HI M nuclear antigen (*Upper Right*) or with a similar dilution of a serum (R.M.) that is reactive only with the *Bam*HI K nuclear antigen (*Lower Right*). Slides were counterstained with propidium iodide at 0.25 μg/ml (*Left*). Note that both sera detect nuclear antigen in all the Raji cells. However, with the M$^+$/K$^-$ (W.C.) serum, the metaphase chromosomes are not stained but there are faint antigen granules in the cell. The K$^+$/M$^-$ serum (R.M.) detects EBNA on the chromosomes.

capacity to detect the M nuclear antigen usually contained antibody to early antigens as well as EBNA; however, six sera lacking antibody to early antigens also detected the M antigen in cotransformed LTK$^+$ cells. Among 12 sera which were K$^+$/M$^-$, 11 lacked antibody to early antigen (Table 1).

In our earlier study, a few human sera with low titers of antibody to EBNA in Raji cells failed to react with the nuclear antigen induced by *Bam*HI K in LTK$^-$ cells. Three of the EBNA$^+$/K$^-$ sera studied previously have now been found to have antibody to the *Bam*HI M nuclear antigen. However, one serum, with antibody to EBNA at a titer of 1:32, reacts with neither the M nor K antigen in L cells. This result suggests that there is yet a third serologic variant of EBNA.

Expression of Two Serologic Variants of EBNA in Human Lymphoid Cells. The availability of sera that were discordant in their reactivity to the K or M nuclear antigen in cotransformed L cells allowed us to ask whether more than one EBNA variant was expressed in lymphoid lines harboring EBV. Both K$^+$/M$^-$ and M$^+$/K$^-$ sera detect EBNA in the Raji and HR-1 Burkitt lymphoma lines and in X50-7, a line of umbilical cord lymphocytes transformed *in vitro* (17). However, only sera with anti-K reactivity detect nuclear antigen in the D98/Raji somatic cell hybrid line (18). The inability of M$^+$/K$^-$ sera to detect EBNA in D98/Raji cells is further evidence for the distinctiveness of the two antigens. By Southern blotting, we found that D98/Raji cells have a *Bam*HI M fragment of standard size. In a transient expression assay a nuclear antigen that reacted with an M$^+$/K$^-$ serum was found after the D98/Raji cells were transfected with 3 μg of pBR-*Bam*HI M DNA. Therefore D98/Raji cells do not block expression of the M nuclear antigen from a plasmid.

Chromosome Location of the *Bam*HI M and K Nuclear Antigens. To study the location of the two nuclear antigens during metaphase in their respective cotransformed LTK$^+$ clones, we used two-color immunofluorescence with propidium iodide to reveal DNA in all nuclei and metaphase chromosomes and fluorescein-labeled anti-C3 to detect the nuclear antigens (15) (Fig. 2 *A* and *B*). At least 50 metaphase cells in each preparation were counted. The fraction of mitotic LTK$^+$ cells that contained each antigen was the same as the proportion of nondividing nuclei that were antigen positive—i.e., ≈55% of C20 cells (M antigen) and ≈75% of D3 cells (K antigen). However, the K antigen was invariably found on the chromosomes (Fig. 2*A*); in metaphase cells, the M nuclear antigen was present in granular form but not attached to chromosomes (Fig. 2*B*).

We then asked whether this difference in chromosome binding of the two antigens seen in LTK$^+$ cells was also manifest in Raji cells. For this experiment, we again used the two-color immunofluorescence technique and human sera that were able to detect only the K or the M antigen (Fig. 2*C*). Five sera that were K$^+$/M$^-$ reacted with antigen on Raji cell metaphase chromosomes. By contrast, three sera that were M$^+$/K$^-$ detected faint granular antigen that was not associated with individual chromosomes in the same cell. The single serum with antibody to EBNA that reacted with neither the M nor the K antigen in cotransformed LTK$^-$ cells detected an EBNA in Raji cells that at metaphase resembled that of *Bam*HI M; it is not bound to chromosomes.

DISCUSSION

The availability of LTK$^+$ cells stably transformed by two different EBV DNA fragments, each of which induces the expres-

sion of nuclear antigens with different serologic and cell biologic properties, should be useful in future studies of the structure and other biochemical properties of the EBV nuclear antigen complex. Such cells should aid in the preparation and screening of monoclonal antibodies to EBNA and in dissecting the human immune response to these important products of the latent life cycle of the virus. Since the products of both the *Bam*HI K and *Bam*HI M EBV DNA fragments return to the nucleus, but to different locations, the two EBV genes may ultimately prove useful in exploration and definition of signals that influence the transport and binding of proteins to chromosomal or nonchromosomal nuclear sites.

The identification by gene transfer of at least two different EBNAs may help to account for the heterogeneity in the molecular weights and avidity for chromatin of EBNAs extracted from different lymphoid lines (4–7). Furthermore, we have found, in our efforts to characterize the proteins responsible for the two antigens, that the K antigen is readily solubilized from the transformed LTK$^+$ cells by simple sonication, whereas the M antigen cannot be solubilized by sonication alone (D. K. Fischer, personal communication). This should help in the purification of the two EBNAs from each other.

The DNA sequences in the genes responsible for the two antigens are distinct. Part of the *Bam*HI K DNA fragment shows homology to a repetitive DNA sequence present in normal mouse and human DNA (19). The *Bam*HI M fragment has no apparent homology to cellular DNA nor does it hybridize to the *Bam*HI K fragment (unpublished data). *Bam*HI M, but not *Bam*HI K, is represented in heterogeneous EBV DNA (20). Transcripts from *Bam*HI K have been found in Raji and IB4, nonproducer lines that express EBNA (21, 22). Weigel and Miller (23) found a transcript from *Bam*HI M during latency in a nonproducer subclone of P3J HR-1 cells. This subclone expresses M antigen.

The *Bam*HI K EBNA is located on metaphase chromosomes, a property of EBNA first described by Reedman and Klein (14). However, in other respects, the *Bam*HI M antigen also fulfills the criteria for EBNA. The M antigen in L cells is reactive with human sera that have anti-EBNA antibodies and no anti-early antigen (EA) antibody; furthermore, seven sera with anti-EA activity but lacking anti-EBNA did not detect the antigen in L cells harboring *Bam*HI M sequences (Table 1). Most important, human sera that lack antibody to the *Bam*HI K nuclear antigen detect antigen in 100% of cells in two Burkitt lines and in cells transformed *in vitro* (Table 2). By definition, antigens that are expressed in 100% of nuclei, in the absence of entry into the viral replicative cycle, are EBNAs. We did observe, however, that anti-M reactivity was more common among sera with anti-EA antibody (Table 1). Pearson *et al.* (24) have recently identified, by using a monoclonal antibody to EA, two antigenic polypeptides that can be translated *in vitro* from a mRNA selected by the *Bam*HI M fragment (24). This fragment may thus code for both an EA and an EBNA. There may ultimately prove to be antigenic relatedness between some components of these two antigen classes.

Because we have used polyvalent human sera, which are still the only source of antibody to EBNA, we cannot yet conclude that the antigen detected in C20 LTK$^+$ cells cotransformed with *Bam*HI M and the antigen found in lymphoid cells are identical. This will require comparison of the two antigenic polypeptides.

Nonetheless the evidence suggests the existence of two or more components of the nuclear antigen complex that potentially play a role in the immortalization phenomenon or in maintaining the latent EBV plasmid.

Table 2. Detection of nuclear antigen in different cells with human sera discordant for reactivity with the EBV *Bam*HI K and *Bam*HI M nuclear antigen

			Human serum			
			K$^+$/M$^-$		M$^+$/K$^-$	
Cell line	EBV genome	Type	R.M.	82-1110	W.C.	82-1423
Raji	+	Burkitt lymphoma	+	+	+	+
HR-1*	+	Burkitt lymphoma	+	+	+	+
X50-7	+	Umbilical cord lymphocytes immortalized *in vitro*	+	+	+	+
D98/Raji	+	Somatic cell hybrid	+	+	−	−
Bjab	−	Burkitt lymphoma	−	−	−	−

*Clone HH514-16 (20).

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