RE-EXPOSURE OF HUMAN LYMPHOBLASTOID CELL LINES TO EPSTEIN-BARR VIRUS

by

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Human lymphoblastoid lines of various origins which harbour Epstein-Barr virus (EBV)-specific nucleic acid were re-exposed to EBV. Following infection, cells of the non-virus-producing lines, Raji and S 95, predominantly synthesized EBV-specific early antigens (EA), whereas only a small percentage of cells revealed viral capsid antigens (VCA). In Raji cells, the number of VCA-producing cells was paralleled by the percentage of virus-specific DNA-synthesizing cells. In S 95 cells, however, viral DNA-synthesizing cells exceeded the number of VCA-producing cells by a factor of more than 10. Induction of EA in Raji cells was dose-dependent and inversely related to cell growth. Irradiation of the virus by ultraviolet light prior to infection led to reduced infectivity. This reduction seemed to follow single-hit kinetics. Raji cells, previously re-exposed to EBV, showed reduced EA induction after re-infection with EBV, as compared to Raji control cells not previously exposed. Of 10 lines which spontaneously synthesize EBV-specific antigens, seven lines proved to be refractory to re-infection, whereas three were as susceptible as the Raji and S 95 controls. From three of the refractory lines infectious virus could be recovered from the culture medium prior to infection.

These results permit the following interpretations: (1) the response of human lymphoblastoid cells after re-infection with EBV results from the infecting virus and not from stimulation of endogenous genomes; (2) cells demonstrating EA synthesis ultimately die; (3) re-exposure to EBV increases the resistance to re-infection of the surviving cells; and (4) cell lines producing infectious EBV are refractory to re-infection. It is suggested that the spontaneous synthesis of infectious virus favours the selection of resistant cells.

Epstein-Barr virus (EBV) specific antigens have been observed in all human lymphoblastoid cell lines which were carefully examined for their presence (reviewed by Epstein, 1970; Klein, 1972; zur Hausen, 1972). Usually a small number of cells synthesize EBV, whereby the rate of virus production seems to be cell-line-specific. Cloning experiments (Zajac and Kohn, 1970; Maurer *et al.*, 1970), induction of viral particle synthesis by chemical inducers (Hampar *et al.*, 1972; Gerber, 1972), and nucleic acid hybridization experiments (zur Hausen and Schulte-Holthausen, 1970; Nonoyama and Pagano, 1971; zur Hausen *et al.*, 1972*b*) revealed that every one of these cells carries the viral genetic information. There exists also some evidence that uptake of viral genomes is a prerequisite for their continuous proliferation in tissue culture (Pope *et al.*, 1968; Nilsson *et al.*, 1971; Chang *et al.*, 1971). Transformation of human lymphocytes *in vitro* is quite easily achieved by infecting these cells with EBV (Henle *et al.*, 1967; Pope *et al.*, 1968; Chang *et al.*, 1971).

Despite a number of efforts, however, EBV cannot be grown in primary human or animal cells. Neither epithelial nor fibroblastic cells proved to be susceptible for this agent and attempts to produce virus in primary human leukocytes were also unsuccessful. Since a relationship of this virus to infectious mononucleosis has been postulated (Henle *et al.*, 1968; Niederman *et al.*, 1968), still undiscovered permissive cells (permissive for virus production) must exist in order to guarantee the continuous transfer of viral infections.

In recent years it has been shown that some lymphoblastoid cell lines of human origin which already harbour EB viral genomes (reviewed by zur Hausen, 1972) are susceptible to re-infection by EBV (Horosciewicz *et al.*, 1970; Durr *et al.*, 1970; Pearson *et al.*, 1970). These cells, although probably previously transformed by the same virus, represent at the present time the only subject for studying the time course of EB viral macromolecule synthesis in lytic infections. Most of these cells become abortively infected: they synthesize EBV-specific antigens without viral particle production (Henle *et al.*, 1970; Henle *et al.*, 1971). The susceptibility of cells from lymphoblastoid lines to re-infection varies from line to line. This seems to depend in part on the presence or absence of virus-specific receptors on the cell membrane (Klein *et al.*, personal communication).

The following report tries to analyze some of the aspects of re-infection of selected lymphoblastoid cell lines by EBV. Special attention is focused on the question whether the alterations of the host cells, induced upon re-exposure, are due to stimulation of endogenous or to newly penetrated viral genomes. The synthesis of viral DNA in the abortive cycle, as well as the re-infectibility of EBV-synthesizing cells, in comparison to cells of non-virus-producing lymphoblastoid lines, are also examined.

MATERIAL AND METHODS

Cell lines

The origin of cell lines used in these experiments is summarized in Table I. The Table also contains the percentage of cells which reveal EBV-specified antigens as detected by immunofluorescence. The cells were maintained in medium RPMI 1629 supplemented with 10 or 20% foetal bovine serum, penicillin and streptomycin (Diehl *et al.*, 1968).

EBV preparations

EBV was obtained from P3HR-1 cells by two procedures: (1) 700 ml of supernatants from P3HR-1 cells (1.5-2.2×10^6 cells/ml), grown for 10-12 days at 34° C, were clarified by low-speed centrifugation (10 min, $1,000 \times g$). This was followed by centrifugation at $45,000 \times g$ over a

TABLE I

ORIGIN OF CELL LINES AND PRESENCE OF EBV-SPECIFIC ANTIGENS WITHIN THEIR CELLS

Cell line	Origin	References	EBV-reactivity percent fluorescent cells	
			EA and VCA	VCA
P3HR-1	Burkitt's lymphoma-clone of P3J	Hinuma *et al.* (1967)	7.5%	4.5%
HK LY-28	Nasopharyngeal carcinoma (tumor)	de Thé *et al.* (1969)	1.6%	0.4%
Kaplan	Infect. mononucleosis (per. blood)	Diehl *et al.* (1968)	1.75	0.6%
Di I	Healthy individual (per. blood)	Diehl, unpublished results	10.7%	9.6%
Di II	Healthy individual (per. blood)	Diehl, unpublished results	1.8%	0.5%
Di IV	Healthy individual (per. blood)	Diehl, unpublished results	0.6%	0.4%
Di V	Healthy individual (per. blood)	Diehl, unpublished results	0.6%	0.4%
Raji	Burkitt's lymphoma (tumor)	Pulvertaft (1964)	<0.001%	<0.001%
S-95	Hodgkin's disease, sarcoma type (pleural effusion)	Diehl and Johansson, in preparation	<0.001%	<0.001%
S118	Hodgkin's disease, granul. type (per. blood)	Diehl and Johansson, in preparation	0.5%	0.1%
2003 (P₃J)	Burkitt's lymphoma (tumor)	Pulvertaft, unpublished results	0.1%	0.1%
SHO I	Healthy individual (per. blood)	Diehl, unpublished results	4.5%	2.5%

period of 110 min in the 21 rotor of the Beckman preparative centrifuge. The resulting sediment was resuspended in a small volume in order to obtain a 30- to 70-fold concentrate. The pH of these preparations was adjusted to 7.0. They were stored at 4° C until used. Concentrates could be kept at this temperature up to 17 days without detectable loss of infectious EBV. (2) Supernatants of P3HR-1 cells, kept for 6-8 days in the same medium, were clarified by two centrifugations at $1,000 \times g$ for 10 min each. The supernatants were carefully removed with a Pasteur pipette, their pH was adjusted and they were stored at 4° C until used.

In addition to P3HR-1 cells, concentration of EBV was attempted from five other EBV-producing cell lines (Di-I, Di-II, Kaplan, HK LY-28, S118). Supernatants from a non-virus-producing line (Raji) were concentrated in the same way and used as controls.

Infectivity tests

Infectivity was assayed by infecting 3×10^6 cells with 1 ml of the virus suspension. The virus was allowed to absorb for 2 h at 37° C. Thereafter, 8 ml of fresh culture medium were added. After additional incubation for 48 and 72 h at 37° C, the cells were acetone-fixed and examined for EBV-specific antigen production by direct immunofluorescence.

Immunofluorescence tests

Infected cells were monitored for induction of EBV-associated capsid (VCA) and early antigens (EA) by methods described by Henle et al. (Henle and Henle, 1966; Henle et al., 1970). For VCA tests, fluorescein-isothiocyanate (FITC) conjugated human serum (anti-VCA titre 1:640), derived from a healthy individual, was used in a dilution of 1:30. At this concentration no reactivity against EA could be detected.

A FITC-conjugated serum from a patient with Burkitt's lymphoma " Nathan " was kindly supplied by Dr. George Klein (Karolinska Institutet, Stockholm), and used for the detection of EA in a dilution of 1:20-30. The difference in fluorescence-positive cells, resulting from the separate incubation of infected cells with both sera, indicated the EA reactivity.

Cell counting

The number of viable cells was determined by trypan-blue exclusion tests in the Neubauer haematocytometer chamber.

Irradiation of EBV by ultraviolet light (UV)

Five ml of the virus suspension (in RPMI 1629 with 10% calf serum in a 6 cm Petri dish) were UV-irradiated (Thomas UV-lamp) at a distance of 10 cm for different periods of time, during which they were continuously stirred with a magnetic stirrer.

Membrane and in situ hybridizations

The techniques used for membrane and in situ hybridizations have been described before (zur Hausen and Schulte-Holthausen, 1970; zur Hausen and Schulte-Holthausen, 1972a). EBV-specific complementary RNA (cRNA), of high specific radioactivity (about 2×10^7 cpm/μg) synthesized in vitro with the aid of E. coli RNA polymerase, was used for all tests.

RESULTS

Induction of EBV-associated antigens and virus-specific DNA synthesis in Raji and S 95 cells

After infection of Raji cells with concentrated EBV, derived from P3HR-1 supernatants, the time course of virus-specific macromolecule synthesis was studied. After various periods post infection, cells were removed for immunofluorescence and in situ hybridizations. In addition, DNA was extracted for filter hybridization with EBV-cRNA. The results are shown in Figure 1.

EBV-specific early antigens appeared first about 12 h after infection, as previously described by others (Henle et al., 1970; Gergely et al., 1971; Horosciewicz et al., 1970). The number of fluorescent cells increased up to 72 h PI. Thereafter it rapidly declined, and only an occasional cell was still found to be positive for EA 30 days after infection. VCA was first detected about 24 h after exposure to EBV but only in a small number of cells (1.5%). After 6 additional days no VCA synthesis was discernible.

The number of VCA-synthesizing cells was paralleled by the counts of labelled nuclei after in situ hybridizations. Thus, EBV-DNA synthesis after re-infection of Raji cells was correlated to VCA production. Additional confirmation for
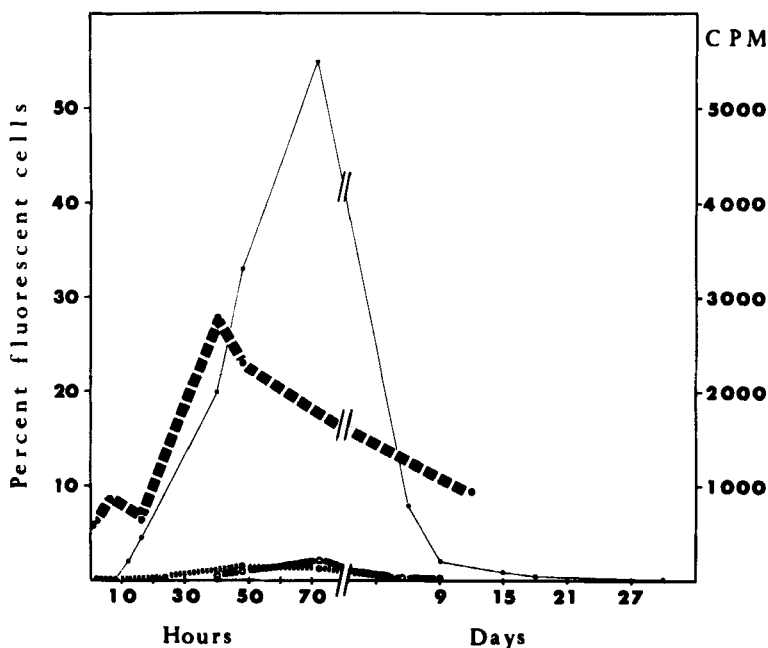
FIGURE 1

Induction of EBV-specific antigens and viral DNA synthesis after infection of Raji cells with EBV. Early antigen synthesis (●———●) starts after about 12 h and reaches a peak about 72 h post infection. Only a small number of cells demonstrate VCA production (·▪▪▪▪▪▪·). Approximately the same number of cells synthesize viral DNA (O▬▬O), as shown by *in situ* hybridization with EBV-cRNA. A significant increase of virus-specific DNA synthesis after infection with EBV is also indicated by hybridization on nitrocellulose filters of DNA derived from infected cells after various periods of infection with EBV-cRNA (·▪▪▪·).

virus-specific DNA synthesis originated from membrane hybridizations. Hybridizability of Raji DNA with EBV-cRNA increased approximately 15-fold about 40 h post infection.

In S 95 cells, infection with EBV concentrates provoked a similar response as far as EA and VCA synthesis were concerned (Fig. 2). The decline in EA synthesis after 3 days PI, however, was slower than in Raji cells. Despite a low percentage of VCA-synthesizing cells (1.8% 48 h after infection), there was a surprisingly high number of EBV-DNA producing cells as revealed by *in situ* hybridizations (Fig. 3). Three days after infection they exceeded the counts for VCA-positive cells 22-fold, approaching the number of EA-reactive cells. This increased EBV-DNA production in S 95 cells as compared to Raji cells was also indicated by an approximately 30-fold increase in hybridization with

EBV-cRNA 40 h after infection as compared to values of uninfected S 95 cells. The discrepancy in the number of VCA-producing and EBV DNA-synthesizing cells after EBV-re-infection was repeatedly observed in this line and remains as yet unexplained.

Dose-response after re-exposure of Raji and S 95 cells to EBV

Induction of EBV-specified antigens (EA and VCA), as well as survival of the infected cells, was studied in Raji and S 95 cells by the use of different dilutions of EBV concentrates for infection. The result is shown in Table II.

This Table demonstrates that EBV antigen reactivity was inversely related to cell survival. When non-diluted viral concentrates were used as input, only a fraction of the original cell population was still alive 72 h after infection.
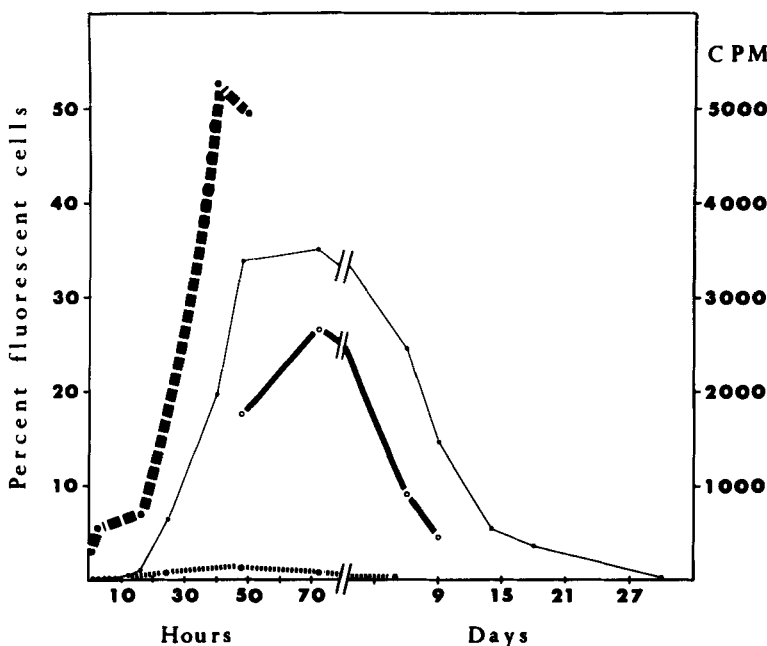
FIGURE 2

Induction of EBV-specific antigens and viral DNA synthesis after infection of S 95 cells with EBV. The increase in early antigen synthesis (●────●) is paralleled by an increase in viral DNA synthesis, as revealed by *in situ* hybridizations (O■■■O) and hybridization on nitrocellulose filters (·■ ■ ■·). Only a small number of cells produce EBV-VCA (·■■■■■·).

This indicated that the abortive infection of Raji and S 95 cells resulted in killing of the cells. Apparently induction of early antigens without viral DNA synthesis (at least in the case of Raji cells) led to cell destruction and death.

Although the data shown in Table II did not reveal a linear decrease in the number of fluorescent cells after infection with various dilutions of EBV-concentrates, they indicated the dependence of virus-specific antigen synthesis on the multiplicity of infection. A similar dependence on the EBV-input for virus-specific antigen synthesis and cell survival, as indicated in Table II, has been observed 48 and 96 h after infection.

EBV-specific antigens after infection of Raji cells with UV-irradiated virus

Raji cells were exposed to EBV, irradiated for various periods prior to infection. A representative experiment is shown in Figure 4.

UV-irradiation reduced the number of viral antigen-synthesizing cells corresponding to the time of exposure. Whereas 28.5% of the cells showed viral antigens 72 h after infection with non-irradiated virus, almost no specific fluorescence was detectable after 8 min of irradiation prior to infection. Here again cell survival was inversely related to the dose of infectious virus. The decrease in the induction of virus-specific immunofluorescence after UV-irradiation of the virus seems to follow single-hit kinetics.

Re-exposure of EBV re-infected cells to EBV

To determine whether Raji cells surviving infection with EBV-concentrates remain susceptible to EBV, these cells were re-exposed to the virus 33 and 75 days after the first infection. Uninfected Raji cells served as controls. The result is shown in Figure 5.
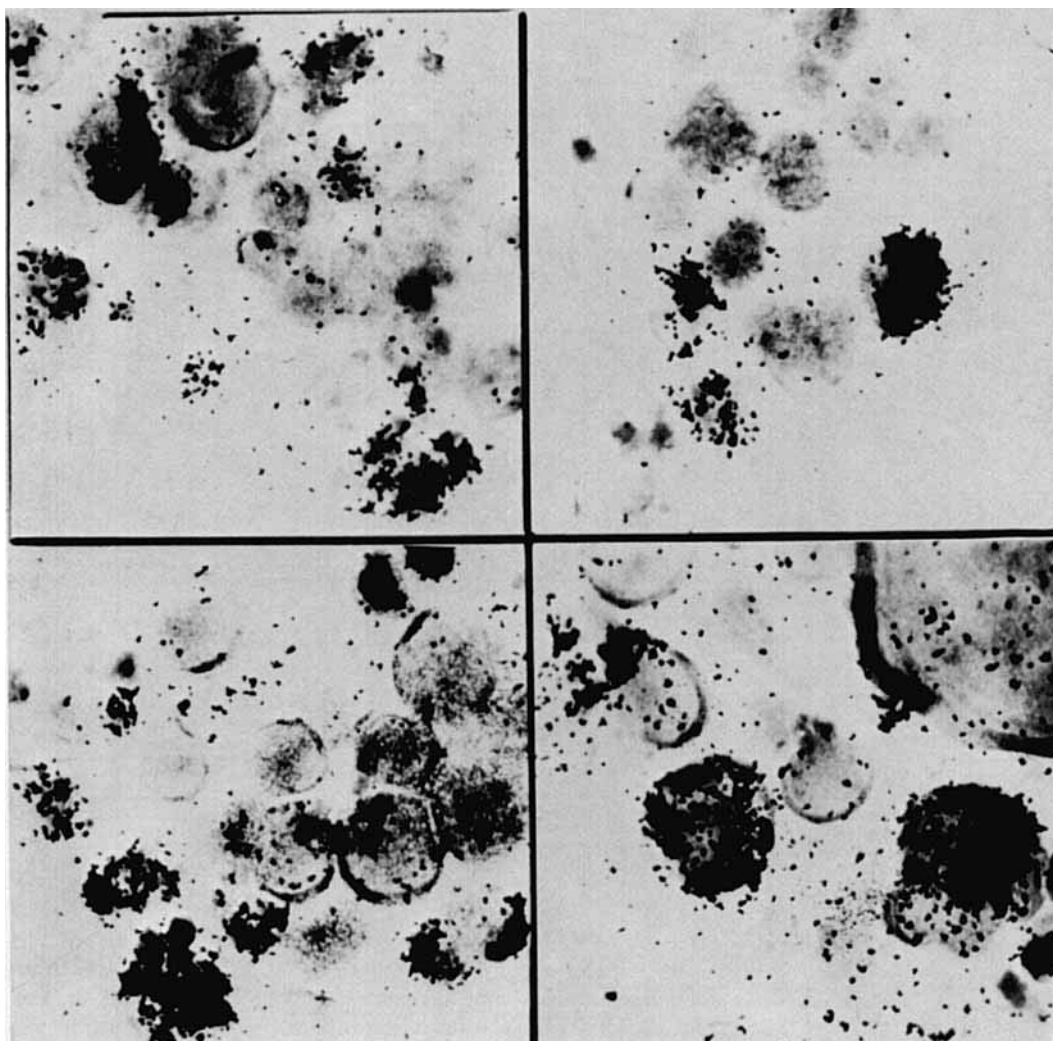
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FIGURE 3

In situ hybridization of ³H-labelled EBV-cRNA with S 95 cells 72 h after infection with EBV. Note the heavily labelled nuclei in contrast to some almost unlabelled cells.

The pre-infected Raji cells were less susceptible to re-infection. This was especially pronounced in the Raji line which had been infected 75 days earlier.

By annealing DNA from both pre-exposed Raji lines with EBV-cRNA, these hybridized slightly above the values of DNA from non-infected Raji cells (1.32 and 1.38 times more than the controls). This could suggest that exposure of Raji cells to EBV increased the viral genome content of the surviving cells.

Re-infection of cells from EB virus-synthesizing lines with EBV

Cells of EBV-synthesizing lymphoblastoid lines were infected with EBV concentrates. Raji and S 95 cells served as controls. Figure 6 shows the EA and VCA synthesis in five recently isolated virus-synthesizing lines after infection with EBV. Four of these lines (Di-II, Di-IV, Di-V, and SHO, all derived from healthy donors) did not show any increase in virus-specific antigen

TABLE II

INDUCTION OF EBV-ASSOCIATED ANTIGENS 72 H
AFTER INFECTION OF RAJI AND S 95 CELLS
WITH VARIOUS CONCENTRATIONS OF EBV

Cell line	Dilution of EBV-concentrate	% EA and VCA reactive cells 72 h PI	Number of surviving cells/ml 72 h PI $\times 10^6$
Raji	non-dil.	45	0.05
,,	1:3	36	0.14
,,	1:9	14	0.29
,,	1:27	10	0.65
,,	no virus	0	1.31
S 95	non-dil.	25	0.10
,,	1:3	21	0.21
,,	1:9	9	0.25
,,	1:27	6	0.34
,,	no virus	0	0.59

FIGURE 5

Re-exposure of EBV re-infected cells to EBV 33
(O●●●●●O) and 75 days (●▬▬●) after the first
exposure to EBV. (·▬▬·) represents the control
which was not pre-exposed to EBV.

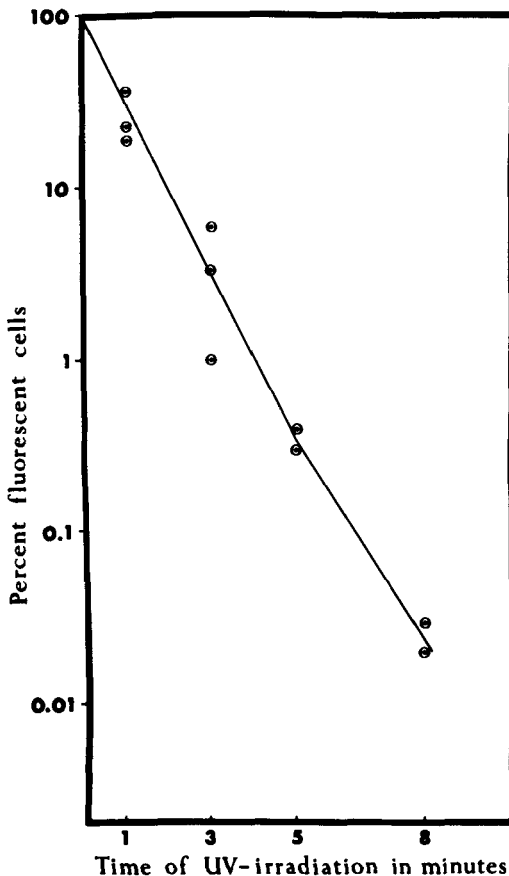FIGURE 4

Induction of early antigens in Raji cells after
infection with EBV irradiated for various periods by
ultraviolet light.

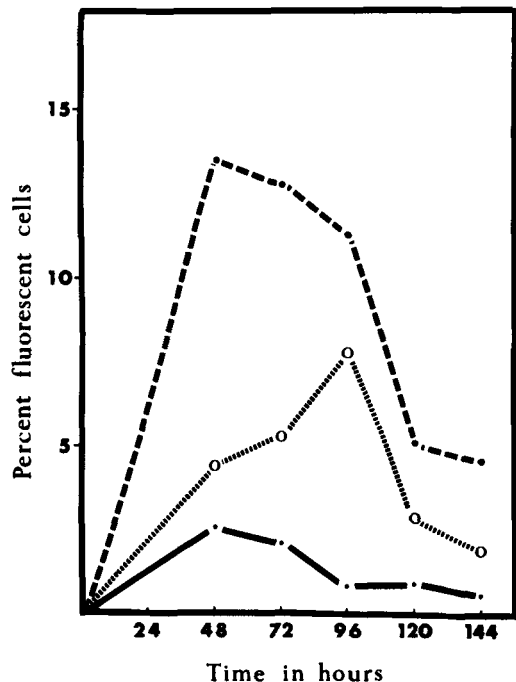synthesis. The fifth one, however, S 118, derived
from the peripheral blood of a patient with
Hodgkin's disease, was as sensitive to re-
infection as Raji and S 95 cells.

Following infection of cells of five additional
virus-producing lines, most of which had been
in culture for several years, three of these
(P3HR-1, 2003, and Di-I) proved to be refractory
to re-infection (Fig. 7). The other two, Kaplan
and HKLY-28, however, showed a considerable
increase in early antigen synthesis. To a lesser
extent, an increase in viral capsid antigen positive
cells was also noted in these two lines.

*Recovery of infectious virus from virus-producing
cell lines*

Supernatants of cells from six EB virus VCA-
synthesizing lines were concentrated one hundred
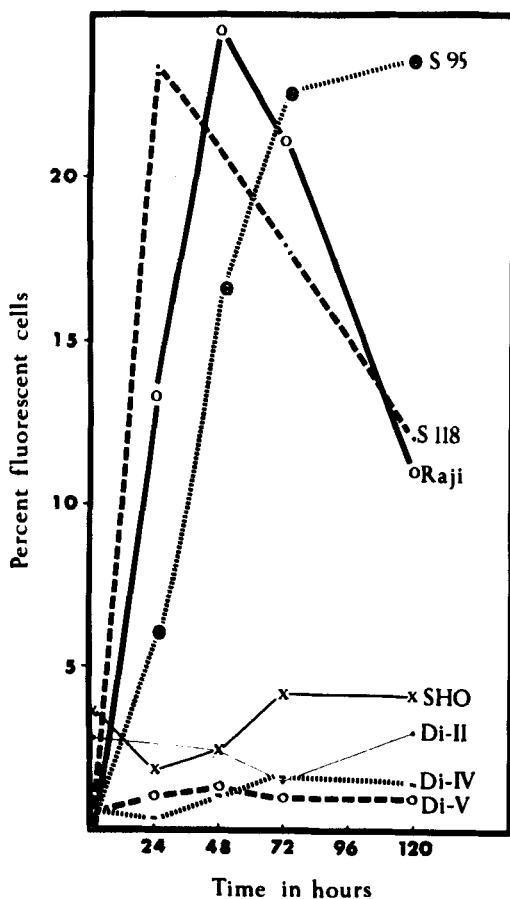fold and used for infection of Raji cells. The

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FIGURE 6

Induction of early antigen synthesis in various cell lines after infection with EBV. Except for the Raji control, all other lines represent recent isolates.
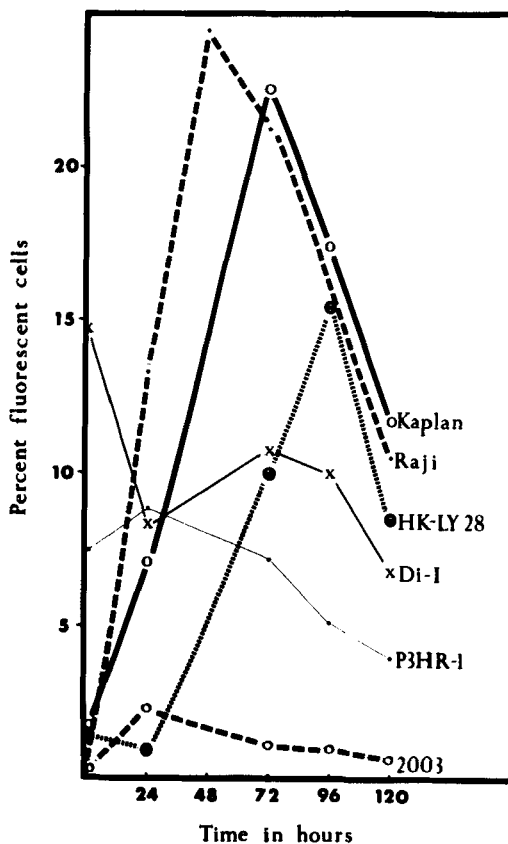
FIGURE 7

Induction of early antigen synthesis, in various cell lines after infection with EBV. Except for the Di-I line, all other lines have been kept in tissue culture for several years.

induction of EA in these cells after various periods of infection is shown in Table III. The two lines with a rather high VCA-reactivity (P3HR-1 and Di-I) induced EA synthesis in Raji cells 72 h post infection in 30.1 and 22.4% respectively. A third line which was also refractory to re-infection, Di-II, induced virus-specific antigens in a very small percentage of cells. Concentrates derived from the three VCA-producing lines which were susceptible to re-infection (HKLY-28, Kaplan, and S 118), did not induce detectable amounts of EBV-specific antigens after infection of Raji cells.

TABLE III

INFECTION OF RAJI CELLS WITH 100 FOLD CONCENTRATES OF VARIOUS VIRUS PRODUCING CELL LINES

Virus-donor lines	Percent VCA reactivity	Percent VCA and EA positive cells after		
		48 h	72 h	96 h
HR1K	5.3	29.9	30.1	19.7
Di-I	8.4	20.2	22.4	22.1
Di-II	0.3	0.02	0.01	0
Ly-28	0.4	0.01(?)	0	0
Kaplan	0.5	0	0	0
S118	0.2	0	0	0

DISCUSSION

Infection of primary human lymphocytes with EBV results in the establishment of lymphoblastoid cell lines (Henle *et al.*, 1967; Diehl *et al.*, 1968; Pope *et al.*, 1968; Nilsson *et al.*, 1971; Miller *et al.*, 1970; Chang *et al.*, 1971). Usually a small percentage of these continuously proliferating cells is induced to synthesize EBV which leads to death of the involved cell. No lytic response, however, has been observed thus far after exposure of primary cells to EBV.

It is therefore rather surprising that cells of several transformed lines which contain a considerable load of persisting EBV genomes (zur Hausen and Schulte-Holthausen, 1970; Nonoyama and Pagano, 1971; zur Hausen and Schulte-Holthausen, 1972*b*; zur Hausen *et al.*, 1972) react upon re-exposure to EBV with virus-specific antigen synthesis and cell death. In this study, the two non-virus-producing lymphoblastoid cell lines, Raji and S 95, proved to be susceptible to re-infection. At least in the majority of the infected cells virus replication was abortive: in Raji cells, early antigen synthesis was predominant. Only a small number of cells revealed viral DNA and VCA production. In S 95 cells, a similar situation was encountered, except for a much higher number of viral DNA-synthesizing cells. Thus far it is not understood why in this case the number of viral structural antigen producing cells, as visualized by immunofluorescence, did not correspond to the number of viral DNA-replicating cells. In both lines, abortive infection resulted in death of the affected cells.

The described reaction of EBV genome-carrying lines to EBV re-infection could be explained by two alternatives: either the infecting genome is responsible for the synthesis of EBV-specific proteins and DNA, or a virus-specific product, transferred to the recipient cell during infection, derepresses and stimulates the endogenous genomes. Our experiments provide evidence for the first alternative, since UV-inactivation kinetics point to a direct involvement of the genome of the infecting virus. It is possible that the endogenous EBV genomes in Raji and S 95 cells cannot be reactivated under these conditions due to genomic defects—this in spite of the recent demonstration that Raji cells contain viral DNA of the size of complete

genomes (Nonoyama and Pagano, 1972). The reactivation of these genomes after 5-bromodeoxyuridine (BUdR) treatment (Hampar *et al.*, 1972; Gerber, 1972) could be explained by assuming reverting point mutations due to BUdR incorporation.

The response of cells from EBV-synthesizing lines to EBV re-exposure varies from line to line. Whereas seven lines tested in this study proved to be refractory to re-infection, three others were almost as susceptible as the Raji and S 95 controls. In these experiments we did not test whether resistance to re-infection, as observed in the majority of virus-synthesizing lines, was due to lack of viral adsorption or to an effect on penetration and uncoating of the virus. It has been shown by Klein *et al.*, 1972 (personal communication), however, that some of the resistant lines do not adsorb virus whereas in others viral adsorption was not inhibited at all.

Since we have been able to isolate infectious virus only from three lines which were resistant to re-infection, these data suggest that resistance to re-infection and infectious virus synthesis might be correlated. It is tempting to speculate that spontaneous induction of infectious virus must result in the selection of cells which are resistant to re-infection with their own virus. The inhibition of the infectious process could occur at various levels before irreparable damage alters the host cell.

This interpretation is supported by the re-infection experiments of Raji cells which survived a previous EBV re-exposure. These cells were more resistant to EBV as compared to the non-exposed control culture.

The abortive response of persistently EBV-infected human lymphoblastoid lines to re-infection, in addition to the non-lytic infection of primary lymphocytes, clearly indicates that the postulated permissive cells which guarantee the continuous transfer of EBV infections, still remain to be discovered.

RÉEXPOSITION DES LIGNÉES CELLULAIRES LYMPHOBLASTOÏDES HUMAINES AU VIRUS D'EPSTEIN-BARR

Des lignées lymphoblastoïdes humaines d'origines diverses, qui contiennent de l'acide nucléique spécifique du virus d'Epstein-Barr (VEB), ont été réexposées au VEB. Après l'infection, les cellules des lignées Raji et S 95, qui ne produisaient pas de virus, ont surtout synthétisé des antigènes précoces (EA) spécifiques du VEB, alors qu'un faible pourcentage de cellules contenait des antigènes des capsides virales (VCA). Dans les cellules Raji, on a observé un parallélisme entre le nombre de cellules productrices de VCA et le pourcentage de cellules synthétisant l'ADN spécifique du virus. Par contre, dans les cellules S 95, les cellules synthétisant l'ADN viral étaient au moins dix fois plus nombreuses que les cellules produisant des VCA. L'introduction d'EA dans les cellules Raji dépendait de la dose et était inversement proportionnelle à la croissance cellulaire. L'irradiation du virus par les ultraviolets avant l'infection réduit le pouvoir infectieux. Ce phénomène semble relever d'une cinétique " à un coup ". Les cellules Raji, préalablement réexposées au VEB, induisent moins d'EA après la réinfection par le VEB que les cellules témoins Raji qui n'ont pas déjà été exposées au virus. Lors de la réinfection des cellules provenant de dix lignées qui synthétisent spontanément les antigènes spécifiques du VEB, sept lignées se sont avérées réfractaires, alors que trois lignées étaient aussi sensibles que les témoins Raji et S 95. Dans trois lignées réfractaires, on a récupéré du virus infectieux dans le milieu de culture avant l'infection.

Ces résultats permettent de formuler les interprétations suivantes: 1) la réponse des cellules lymphoblastoïdes humaines après la réinfection par le VEB provient du virus infectant et non d'une stimulation des génomes endogènes; 2) les cellules qui synthétisent les EA finissent par mourir; 3) la réexposition au VEB accroît la résistance à la réinfection des cellules survivantes; et 4) les lignées cellulaires produisant du VEB infectieux sont réfractaires à la réinfection. Les auteurs pensent que la synthèse spontanée du virus infectieux favorise la sélection des cellules résistantes.

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