the accumulation of this substance in washed suspensions of *C. thiosulfatophilum*. It is possible that this pathway involves one or more reductive carboxylations (*9*). There is good evidence (*10*) for a novel mechanism of biosynthesis of isoleucine (of which α-keto-β-methylvalerate is the immediate precursor) in *C. thiosulfatophilum*, and the present system offers a good opportunity for investigating this.

On the basis of the results of this investigation, it is suggested that the fixation of CO_2 occurs largely via a reversed TCA cycle, resulting in the formation of acetate which can then be converted via pyruvate and a reversal of the conventional Embden-Meyerhof glycolysis pathway to hexose. The acetate can also be converted to α-keto-β-methylvalerate by an unknwn mechanism, or it can reenter the cycle after conversion to oxaloacetate (*3*). While the reductive pentose phosphate cycle cannot be excluded as a mechanism of CO_2-fixation under these conditions, its operation appears to be of minor quantitative significance.

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Differential Reactivity of Human Serums with Early Antigens Induced by Epstein-Barr Virus

Abstract. *Inoculation of 64-10 or Raji cultures with Epstein-Barr virus derived from the HRI-K clone of the P3J Burkitt's lymphoma line caused abortive infections in most of the lymphoblastoid cells with synthesis of "early antigens" but few, if any, capsids. Antibodies to early antigens were detected by indirect immunofluorescence in serums of many patients with infectious mononucleosis, Burkitt's lymphoma, or nasopharyngeal carcinoma. These antibodies were rarely present in other serums even though some of them showed high titers of antibodies to Epstein-Barr virus when assayed on EB3 Burkitt tumor cells; they also prevented synthesis of early antigens, provided the serums were mixed with the virus prior to inoculation. Antibodies to early antigens possibly reflect current or recent disease processes that are associated with the virus.*

The Epstein-Barr virus (EBV), a member of the herpes group, has been detected with high frequency in blastoid cell lines derived from Burkitt's lymphomas (BL) or from leukocytes of various donors (*1*). As a rule, only a small fraction of cells in such carrier cultures is overtly infected, as shown by electron microscopy (*2*) or by EBV-specific immunofluorescence (*3*). It has not been possible to transmit EBV to cultures of cells other than leukocytes (*4*) or blastoid cells of EBV-free lines (*5*). As reported here, inoculation of EBV-free cultures with EBV may lead, in the majority of the exposed cells, to abortive infections that are detectable by indirect immunofluorescence with certain human serums but not with others, even though these serums may have equally high titers of antibody to EBV when tested on cell smears of the EB-3 line (*2*) of BL cells ("anti-EBV test").

Concentrated suspensions of EBV were prepared (*5*) from culture media of the HRI-K subline of P3J (BL) cells (*6*). Stock cultures, initially furnished by J. S. Horoszewicz, were maintained at 37°C by addition every 3 to 4 days of fresh medium (30 percent); the medium was RPMI-1640, supplemented with fetal calf serum (10 percent) and antibiotics. Lots (1 to 2 liters) were then incubated at 32°C for 10 to 12 days without further feeding. The cells were sedimented at 10,400*g* for 20 minutes and the supernatants were centrifuged at 27,300*g* for 90 minutes. The sedimented virus was suspended in 1 to 4 ml of medium (500- to 1000-fold concentration). This concentrate was passed through a Millipore filter (pore size, 0.8 μm).

For infection, 1 to 2 × 10^7 cells of the Raji (Burkitt's lymphoma) or 64-10 (myelogenous leukemia) lines (*7*), both free of EBV antigens, were sedimented at 1000*g* and resuspended in 1 to 3 ml of virus suspension. After 1 to 3 hours at 37°C, medium was added or the cells were washed and resuspended in fresh medium to yield 5 to 10 × 10^5 cells per milliliter. Infected and uninfected control cultures were kept at 37°C and viable cell counts and acetone-fixed cell smears were obtained at intervals as described (*3*). For assay of EBV-infected cells by immunofluorescence (IF), human γ-globulins conjugated with fluorescein isothiocyanate were used in the direct IF test, and various human serums and fluorescein isothiocyanate-conjugated goat antibodies to human immunoglobulin G (IgG) (Hyland Laboratories, Los Angeles, California) were used in the indirect IF test. The human serums were limited initially to serums obtained from seemingly healthy blood donors ("donor reagents"), one "positive," having a titer of 1: 640 on EB-3 cell smears and the other "negative" with a titer of $< 1:5$. Later, serums from patients with apparently EBV-associated diseases (that is, infectious mononucleosis, Burkitt's lymphoma, or nasopharyngeal carcinoma) were also used. The antibody titers to EBV of these patients' serums ranged from 1 : 160 to 1 : 1280 (*8*).

Exposure of Raji and 64-10 cells to EBV yielded similar numbers of IF-positive cells in early experiments, when human IgG conjugates were used for the direct test and when the serums from positive donors were used for the indirect IF test. The 64-10 cell line became increasingly resistant to EBV, to the extent that no IF has been elicited in recent tests by the donor reagents, although inocula of concentrated EBV continued to cause substantial reductions in the rates of cell growth. However, when patients' serums were used (Table 1) numerous fluorescent cells were noted in 64-10 cultures as well as in Raji cultures exposed to EBV which were not stained by the positive donor reagents. Both sets of reagents stained similar numbers of cells in EB-3 cell smears. In the examples, none of the 64-10 cells exposed 4 days previously to a 10^{-1} dilution of EBV stained with the donor reagents; with the patients' serums, fluorescent cells became detectable even after exposure to the 10^{-4} dilution. The same virus preparation yielded

titers of 10^{-2} and 10^{-4} in Raji cells when tested with the donor or patients' reagents, respectively. The percentages of stained cells declined roughly in tenfold steps in parallel to the dilution of virus inoculated. The numbers of stained cells reached peaks usually within 1 to 3 days and declined thereafter more or less rapidly with few, if any, stainable cells remaining 3 to 4 weeks later. Figure 1 shows the differential emergence of antigen-containing cells in Raji cultures exposed to virus diluted tenfold, as measured by the two sets of IF reagents. Positive cells detectable by the patients' serums appeared about 4 hours earlier than cells stainable by the donor reagents.

These results suggested that EBV infections of Raji and of 64-10 cells were largely or totally abortive; that is, "early antigens" were synthesized but not viral capsid antigens. This suggestion was supported by (i) limited, if any, second cycling of the infection and (ii) failure to detect intracellular virus particles by electron microscopy in 64-10 cells, more than 30 percent of which were stainable by the patients' serums. In contrast, intracellular virus particles were found in infected Raji cells when 5 to 10 percent were stainable by human IgG conjugate and the positive donor serum, both containing antibodies to EB viral capsids as shown by the antibody-coating technique (*9*).

Immunofluorescence exhibited by early antigens appeared to be intracytoplasmic; because of its brilliance, intranuclear antigen could well have escaped detection. The pattern of immunofluorescence did not differ detectably from that elicited by the donor reagents in EB3 cells that contained virion antigen. The specific relationship of the staining to EBV seems assured because no reactions were observed when the virus, prior to inoculation, was heated to 56°C, exposed to ultraviolet irradiation, or mixed with the positive donor serum (Table 2), unlabeled human γ-globulins, or serums from patients with infectious mononucleosis, Burkitt's lymphoma, or nasopharyngeal carcinoma. The development of early antigens was not prevented when the virus was mixed with serums from donors without antibodies to EBV, including six taken well in advance of onset from infectious mononucleosis patients (*8*) who later provided neutralizing serums during the acute and convalescent stages. Serums from four patients with primary cytomegalovirus infections that occurred after

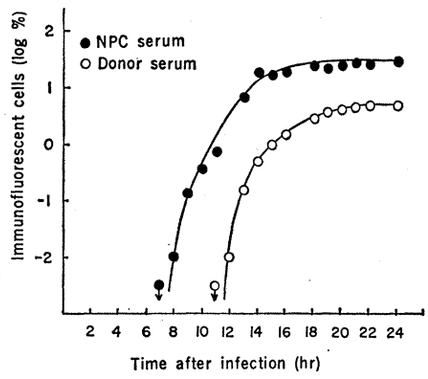

Fig. 1. Differential appearance of early and virion antigens in EBV-infected Raji cells.

open-heart surgery failed to react with the early antigens.

Antibodies to the early antigens have thus far been found only in serums with relatively high antibody titers to EBV; that is, mainly in serums from many, but not all, patients with infectious mononucleosis, Burkitt's lymphoma, or nasopharyngeal carcinoma. In contrast to the prolonged persistence of detectable antibodies to EBV, antibodies to the early antigens usually disappeared rapidly after recovery from infectious mononucleosis. In serums of some Burkitt's lymphoma patients in prolonged remission these antibodies were absent or present in low titers. These observations, if confirmed in a large series of patients, suggest that the antibodies to early antigens may reflect extensive, current, or very recent EBV-associated disease processes and that separate antibody determinations for early and viral capsid antigens might be of diagnostic and possibly prognostic value.

Present data indicate that early antigens are distinct from antigens induced by EBV in the cell membrane (*10*) or precipitating antigens (*11*) since serums can be found which react in one but not the other tests. Arrest of cellular infections at the early antigen stage has been observed also in blastoid cell lines derived from Burkitt's lymphoma biopsies, leukocytes of patients with infectious mononucleosis, or leukocytes of healthy donors exposed in vitro to lethally x-irradiated, EBV-carrying cells or to EBV suspensions (*4*). Several of these lines contained appreciable numbers of cells that could

Table 1. Titration of EBV in RPMI 64-10 and Raji cells. NPC, nasopharyngeal carcinoma; BL, Burkitt's lymphoma; IM, infectious mononucleosis; +, positive; and −, negative.

Cells	Virus dilution	Cellular growth (% of control)	Immunofluorescent cells (%) on day 4			
			Direct test	Indirect test: serum from		
				Donors		Patients
			Human γ-globulin	EBV +	EBV −	NPC, BL, IM
RPMI 64-10	10^{-1}	0	0	0	0	>70
	10^{-2}	54	0	0	0	40–50
	10^{-3}	82	0	0	0	3–5
	10^{-4}	98	0	0	0	0.1–0.4
	10^{-5}	106	0	0	0	0
	None	100	0	0	0	0
Raji	10^{-1}	7	4	5	0	>80
	10^{-2}	42	0.4	0.6	0	15–20
	10^{-3}	95	±	±	0	2–3
	10^{-4}	108	0	0	0	0.1–0.3
	10^{-5}	96	0	0	0	0
	None	100	0	0	0	0
EB-3	None		10	10	0	10

Table 2. Neutralization of EBV by EBV positive donor serum prior to inoculation of RPMI 64-10 cells.

Inoculum: mixture of EBV and	Dilution of serum	Cellular growth (% of control)	Immunofluorescent cells (%) on day 3	
			Direct test	Indirect test
			Human γ-globulin	NPC serum
EBV positive donor serum	Undiluted	95	0	0
	1 : 4	91	0	6
	1 : 16	51	0	13
	1 : 64	32	0	≧ 30
EBV negative donor serum	Undiluted	28	0	≧ 30
Saline control		29	0	≧ 30

be stained by serums from patients with infectious mononucleosis, Burkitt's lymphoma, or nasopharyngeal carcinoma but they contained fewer, or none at all, that reacted with the positive donor reagents. Thus EBV may be associated with lymphoblastoid cells of carrier cultures in several ways: (i) in a few cells complete replicative cycles occur resulting in death of the cell; (ii) in additional cells, viral replication is arrested at the early antigen stage; (iii) in still other cells (as observed also in tumor biopsy cells) only EBV-induced cell membrane antigens are synthesized (*10*); and (iv) in the remaining cells the viral genome is present but wholly repressed, as indicated by the fact that clones derived from singly picked cells under conditions preventing external infection all were shown to harbor EBV (*12*).

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Bacterial Flagella: Polarity of Elongation

Abstract. *Newly synthesized regions of* Bacillus subtilis *flagella were labeled with fluorophenylalanine or [^{3}H]leucine. The flagella were then examined for altered gross morphology or by radioautography. Results of both experiments indicate that flagella elongate in vivo by polymerization of flagellin subunits onto the distal end of the filament.*

Bacterial flagella are primarily composed of a single protein, flagellin, which polymerizes in vitro and in vivo to form filaments with a characteristic wave. Asakura *et al.* (*1*) demonstrated that polymerization in vitro is polar and proceeds only at the distal end of seed fragments. Although subunits can be assembled onto flagella fragments in vitro, the fact that it has not been possible to polymerize subunits onto flagellar stubs in vivo (*2*) suggests that the mechanism of elongation may be different in vivo and in vitro. However, using the amino acid analog fluorophenylalanine, Iino performed experiments which implied that *Salmonella* flagellar growth in vivo also occurred at the distal end (*3*). Incorporation of the analog into flagellin resulted in a curly flagellum displaying half the normal wavelength (*4*, *5*). If flagella were partially synthesized in medium containing phenylalanine, then completed in fluorophenylalanine, heteromorphic flagella containing both normal and curly waves on the same filament were produced. If subunits were assembled proximally, the basal region should have been curly, whereas if assembly were distal, the curly region should have been located at the tip. Iino's experiments with *S. typhimurium* showed that the heteromorphic flagella were curly at the tip. We have independently performed similar experiments with *Bacillus subtilis*.

Flagella were removed from cells requiring phenylalanine (*6*) by shearing in a blender. Short flagella were allowed to regenerate at 37°C in minimum salts medium containing 0.1 mg of phenylalanine per milliliter (*7*). After 15 minutes, the bacteria were washed and reincubated at 37°C in the same medium containing 0.1 mg of fluorophenylalanine per milliliter. Two hours later growth was terminated by addition of formaldehyde to 0.5 percent and the bacteria were stained with Leifson's flagella stain (*8*). These incubation periods were necessary for complete regeneration, since the rate of flagellar elongation appears to decrease with increasing flagellar length (*3*). Examination of the stained flagella by light microscopy demonstrated that the heteromorphic flagella were curly on the distal end, suggesting that flagellin is polymerized at the tip (Table 1).

A major criticism of this interpretation is that it assumes that fluorophenylalanine alters the morphology of the flagellum at the site of incorporation of the subunit. It is possible that subunits are added to the base but that the filament assumes the curly form at the free tip which might not be as conformationally restricted as the basal region. It is, therefore, important to do the reverse experiment. Flagella should first be regenerated in fluorophenylalanine, then in phenylalanine, and should contain the curly region at the base. This experiment was attempted repeatedly but the results were uninterpretable since the curly portion appeared at either end and heteromorphs were too rare to permit statistical evaluation. Furthermore, we found that the incorporation of fluorophenylalanine alone does not necessarily cause the formation of curly flagella. When flagella were completely regenerated at 32°C in either fluorophenylalanine or phenylalanine they had the normal wavelength. If the cells were fixed with formaldehyde and incubated at 4°C overnight, curly flagella appeared. Both samples contained some flagella that were normal, curly, or heteromorphous. The curly region can appear at either end of the flagellum (Table 1). The partial regeneration experiments were repeated at 32°C, and the flagella were scored after fixation and incubation at 4°C. Incubation with phenylalanine and then with fluorophenylalanine resulted in a nonrandom distribution of the curly region among the heteromorphs, confirming the initial results, but the reverse experiment led to a random distribution (Table 1). These data illustrate the difficulties in interpreting the results of analog incorporation. It is therefore necessary to test for the direction of flagellar growth by an independent method. Incorporation of isotopically labeled normal precursor into flagella can be measured directly by radioautography and should produce unambiguous results. If the cells are allowed