Some Recent Developments in the Molecular Epidemiology of Epstein-Barr Virus Infections

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We have applied two different recombinant DNA techniques to the study of the epidemiology of Epstein-Barr virus infections. In the first application, cloned subfragments of viral DNA were used as probes to detect EBV DNA in a variety of lymphoproliferative disorders and in lymphoid cell lines. Patients who are epidemiologically unrelated harbor EBV genotypes which can readily be distinguished from each other. Patients who are epidemiologically related (such as mothers and infants) have similar EBV genotypes. Some patients, especially those who are immunocompromised, are infected with two distinct genotypes.

In the second application, we have examined the immune response to specific EBV antigens expressed from small cloned viral DNA subfragments. We have identified a group of patients with presumed chronic EBV infection who *selectively* fail to recognize one subcomponent of the EB nuclear antigen complex.

INTRODUCTION

Many epidemiologic mysteries about Epstein-Barr virus (EBV) remain unsolved. There is the mystery of geographic pathology. The virus is associated with Burkitt lymphoma, with endemic pockets in East Africa, and nasopharyngeal carcinoma, a disease with high incidence in persons of Southern Chinese descent [1,2]. There is the mystery of variation in clinical expression in normal individuals in our country and other industrially developed countries. Some experience inapparent infection; others have severe mononucleosis. There are further mysteries about pathogenesis of the infection in abnormal hosts, such as those immunosuppressed to maintain organ grafts or by acquired immunodeficiency syndrome, who develop progressive lymphoproliferative disease associated with EBV [3]. There are marked effects of age on disease expression: the infected younger individuals more often have an inapparent infection. There are, as so often emphasized by Alfred Evans, dramatic effects of socioeconomic class and geography on virus transmission [4]. In the poor and in the Third World, the virus seems to be acquired at an early age. All of these epidemiologic mysteries are, for the present, unsolved.

MOLECULAR APPROACHES TO SOLVE THE EPIDEMIOLOGIC QUESTIONS

Molecular biology, particularly the technology of recombinant DNA, offers the promise of partial solution of some of these mysteries. The DNA probe technology is

particularly suitable for comparison of the genetic make-up of different individuals, whether they be different virus strains or different populations of hosts. Furthermore, recombinant DNA permits the cloning and expression of individual gene products. The herpes viruses, of which EBV is one, are large, complex organisms, which possess more than 100 genes. Recombinant DNA permits analysis of the immune response to individual gene products.

We hope to illustrate the application of this powerful technology to some epidemiologic questions concerning EBV. We shall describe experiments which concern variations in genotype among different isolates of EBV. We shall also summarize seroepidemiologic studies in which we measure the immune response to *one individual* EBV gene product defined by gene transfer technology.

AN INFANT AND MOTHER INFECTED WITH TWO EBV GENOTYPES

We began by studying several EBV isolates which were obtained from a child with AIDS who developed a central nervous system lymphoma [5]. There was a difference between the genotype of EBV found in an isolate from a lymph node and the EBV genotype seen in the peripheral blood and brain. We next isolated a lymphoblastoid cell line from the patient's mother, a drug addict who was HTLV III-seropositive, and compared EBVs found in the mother and the child. The mother was also infected with two genotypes of EBV. This fact was evident by the presence of doublet restriction fragments (Fig. 1). Furthermore, the two viruses harbored by the mother were similar in genome structure to the two viruses found in the infant. The next question was whether every cell in the cell line from the mother's blood contained two EBVs or whether some cells were infected by one variant and other cells by another. A single cell clone grown from the mother's cell line contained only one EBV. This finding suggested that the mother's blood probably contained one population of cells with one EBV genotype and another with the other.

This small experiment in clinical epidemiology raised a number of interesting questions. Is infection with multiple genotypes seen in immunologically normal hosts? Are genotype variants regularly found in different sites of the body? Are the multiple genotypes the result of simultaneous infection with more than one EBV strain, sequential infection with several EBV strains, or do they arise during virus multiplication within the infected individual?

To attempt to address some of these questions, we studied 13 more patients; seven had uncomplicated infectious mononucleosis (IM) and six were immunocompromised children, two immunocompromised as the result of liver transplantation and four because of HIV infection. We studied two or more EBV isolates from each patient. Sometimes they were separate isolates from the peripheral blood, separated by time; more frequently they were isolates from different body sites. (These data have been presented in preliminary form [Katz BZ, Miller G: Infection with multiple genotypes of Epstein-Barr virus in immunocompromised and normal hosts. Pediatr Res 20:313A, 1986], and a manuscript describing these experiments in detail has been submitted for publication.) When three epidemiologically unrelated infectious mononucleosis patients from Yale were studied, each was shown to have a distinguishable genotype of EBV. Furthermore, multiple isolates from the same individual, for example saliva and blood, contained similar genotypes. This finding was evidence that genotype variation did not arise as the result of propagating the virus *in vitro*. The presence of the same EBV genotype in saliva and lung of a child with AIDS and interstitial pneumonitis
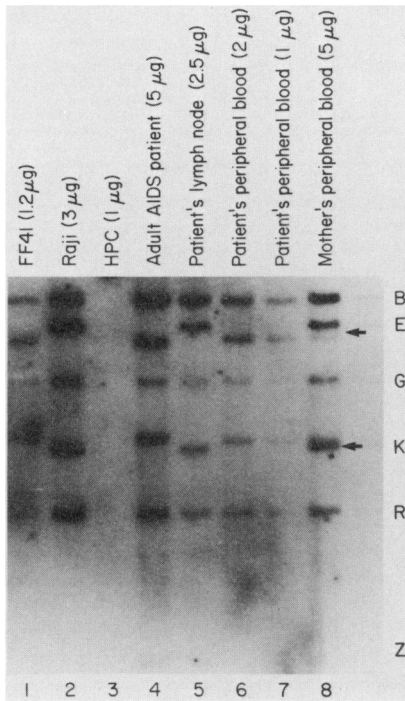
FIG. 1. Evidence for simultaneous infection with two genotypes of EBV. Cell lines were established from the lymph node and peripheral blood of a child with AIDS and from the peripheral blood of the child's mother. Cellular DNA from these cells was digested with *Bam*HI, electrophoresed, and transferred to nitrocellulose by Southern's method. The blot was hybridized with a probe prepared from a plasmid carrying the *Eco*RI B fragment of EBV DNA [17]. The *Bam*HI subfragments recognized by this probe are indicated with letters at the right. Two prototype EBV variants, **FF41** and **Raji**, are shown in lanes 1 and 2. A negative control consisting of human placental cell DNA and lacking EBV genomes is found in lane 3. The patient's two cell lines (lanes 5–7) contain different genotypes of EBV as distinguished by variations in the size of the *Bam*HI E and *Bam*HI K fragments. The cell line from the mother's peripheral blood (lane 8) contains two genotypes; *Bam*HI fragments E and K are doublets *(arrows)*. Reprinted from [5] with permission of the *Journal of Infectious Diseases*.

demonstrated that immunocompromised patients do not invariably harbor different genotypes in different anatomical sites.

Epidemiologically related patients, again a child with AIDS and her mother, demonstrated the same genotype. This finding was further evidence that EBV is probably transmitted between mother and child. When the same patients were studied sequentially, in one instance one month apart, in another case six months apart, the same EBV genotypes were found on both occasions.

We did encounter two mononucleosis patients who harbored two EBV genotypes in the peripheral blood. In one patient the cell line established by co-cultivating the patient's blood lymphocytes with umbilical cord lymphocytes contained a doublet restriction fragment. One of these variants was found in isolates from the saliva of the same patient. Once again a single cell clone contained one variant, although another cell clone apparently carried both variants.

Overall, simultaneous infection with multiple EBV genotypes could be identified in five of seven immunocompromised patients and two of seven infectious mononucleosis patients. Thus both healthy and immunocompromised patients may harbor multiple genotypes; however, the phenomenon probably occurs more often in immunocompromised than in normal hosts. In general, epidemiologically unrelated patients harbor distinct genotypes and epidemiologically related patients harbor similar genotypes. This fact should permit some tracking of transmission. We have preliminary evidence for transmission between mother and child, which has often been assumed but never documented. Usually multiple isolates from the same patient are of similar genotype whether obtained from different sites or at different times. These findings imply that genotype variation is not an artifact of *in vitro* propagation.

Here are some implications of this work and, it is hoped, material for further discussion and experimentation. Do genotype variants represent viruses with different tissue tropisms or pathogenic potential, or are the DNA variations biologically meaningless? Do the different genotypes vary in their antigens, particularly their surface antigens? Would an individual infected with one genotype be immune to infection with a different genotype? If so, does this process operate via classical humoral antibody, cellular immunity, or perhaps at the level of the infected target cell? When multiple genotypes are identified, do they arise as the result of simultaneous or sequential infection?

IDENTIFICATION OF AN EBNA GENE

We have begun to undertake epidemiologic studies using a single defined gene product of EB virus: one of the nuclear antigens, EBNA. In collaboration with Wilma Summers, our group mapped a gene for an EBNA to the BamHI K fragment of the EBV genome. This mapping was done by putting smaller and smaller pieces of EBV DNA into mouse cells, using the technique of herpes simplex thymidine kinase co-transformation. We eventually ended with a piece of EBV DNA, about 3 percent of the length of the entire genome, which caused the expression of a nuclear antigen that, like classical EBNA, bound chromosomes which were in metaphase [6]. This gene encoded a polypeptide with a molecular weight of about 78 kilodaltons, which was similar in size and antigenicity to EBNA found in cells with the whole EBV genome [7]. To be certain that the gene we had identified encoded an EBNA, we used a large panel of human sera with known reactivity to different EBV antigens. Sera which contained antibody to EBV capsid and early antigens but which lacked anti-EBNA did not react by immunofluorescence with the mouse cells containing the BamHI K fragment. Only sera with anti-EBNA reacted; however, we did find five of 35 sera which were EBNA-positive, when used on Raji cells, but which did not react with the K antigen [6]. This result was evidence that Raji cells contained more than one EBNA gene product, and recent studies have shown that there are at least four different EBNA gene products in EBV-transformed cells [8].

SOME PATIENTS WITH CHRONIC EBV INFECTION SPECIFICALLY FAIL TO RECOGNIZE THE BamHI K EBNA

We went back to find out more about the five patients in our original series who failed to detect the K component of EBNA (which is now also called EBNA 1). Recall that these sera *did* react with other EBNAs. Two of them were patients who had recently been infected with the virus; Dr. Niederman has subsequently discovered that, during acute infection, antibodies to some of the other EBNAs appear before antibody to K [9]. Three of the five patients had most unusual EBV serology. These three patients were carrying a diagnosis of "chronic EBV infection." They had persistent fever, pneumonitis, and hematologic abnormalities after mononucleosis [10]. Their serum antibody response was unique because they had extremely high titers to EBV replicative antigens (viral capsid and early antigens) and they reacted very strongly with another nuclear monoclonal antigen M, also defined by gene transfer, which we know now to be a component of the early antigen complex [11]; however, they specifically failed to make antibody to the K antigen [12] (Fig. 2).

We next asked whether one of these three patients had a mutation in her virus which prevented it from expressing the K EBNA. Dr. Schooley of Massachusetts General
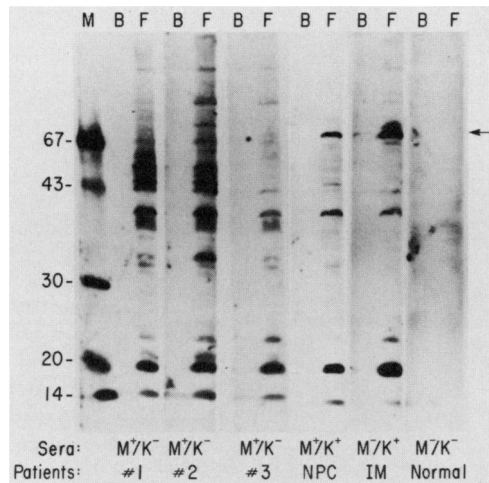
FIG. 2. Three patients with putative chronic EBV infection whose sera lack antibody to the *Bam*HI K component of Epstein-Barr nuclear antigen (EBNA 1). Shown is a Western immunoblot using six human sera—three from patients with chronic EBV infection (numbers **1, 2,** and **3**) and one each from a patient with nasopharyngeal carcinoma (**NPC**), infectious mononucleosis (**IM**), or normal. Reactivity with the M antigen (a component of the diffuse early antigen) or the K antigen (EBNA 1) is shown above each serum. Each serum was reacted on a nitrocellulose strip to which had been transferred EBV polypeptides from a virus producer cell line FF41 (**F**) or, as a negative control, an EBV genome negative line BJAB (**B**). The sera from three patients with presumed chronic active EBV infection did not detect the prominent 78,000 dalton polypeptide *(arrow)* which was seen by the patients who had anti-K. Those three patients, however, did react with many other viral-specific polypeptides. **M** in lane 1 denotes molecular weight markers. Reprinted from [12] with permission of the *New England Journal of Medicine.*

Hospital established a lymphoid line from the patient's blood. We found (Fig. 3) that her cells contained a normal-sized K DNA fragment, so her virus contains the gene for K EBNA. Furthermore, her lymphoid cells, when cultivated *in vitro,* expressed the K polypeptide product, as assayed with a human serum (RM) containing anti-K. Her own serum, however, did not recognize the K-EBNA in her cells. This finding suggested that the defect in this patient lay not in her ability to express the antigen, but in her capacity to recognize it.

RESPONSE TO K DURING UNCOMPLICATED INFECTIOUS MONONUCLEOSIS

In order to interpret the significance of the specific lack of anti-K, we have studied the appearance of anti-K antibodies during uncomplicated mononucleosis (Fig. 4).
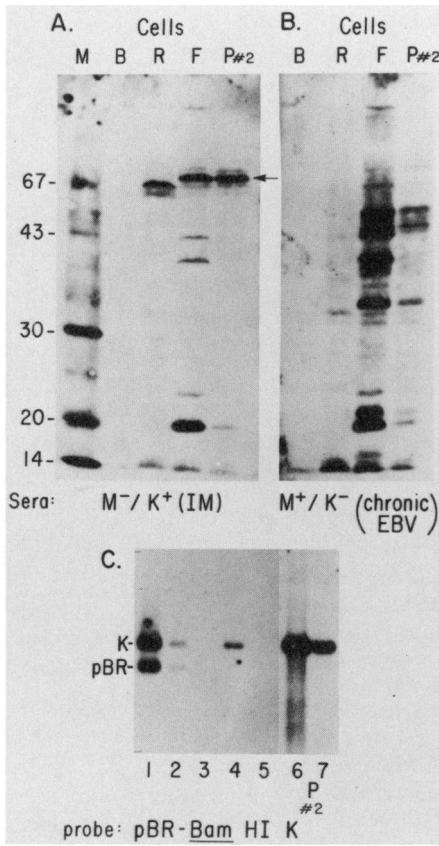
FIG. 3. A lymphoid line from a patient with presumed chronic active EBV infection who lacks antibody to EBNA 1 contains the *Bam*HI K (EBNA 1) nuclear antigen.   Panels **A** and **B** show immunoblots with molecular weight markers (**M**), BJAB (**B**), Raji (**R**), FF41 (**F**), and a lymphoid line from the patient (**P#2**). In **A**, the immunoblot was reacted with a human serum containing antibody to EBNA 1 (**K+**), and, in **B**, a replicate immunoblot was reacted with the patient's own serum (**K−**). Note that the K+ serum recognizes EBNA 1 in the EBV-positive Raji and FF41 cells, and in the patient's cell line. The patient's serum does not react with EBNA 1, although it does recognize many EBV replicative polypeptides.   Panel C is a Southern blot in which lanes 1–3 show 100 pg, 10 pg, and 1 pg of a recombinant plasmid (pBR322) containing the K DNA fragment. Lanes 4–5 show 500 ng and 50 ng of cell DNA containing 4 and 0.4 EBV copies per cell, respectively; lanes 6–7 have 500 ng and 50 ng of cellular DNA from the lymphoid line established from patient 2. All DNAs were digested with *Bam*HI before electrophoresis, blotting, and hybridization with the cloned *Bam*HI K fragment probe. The patient's cells contained many copies of the K DNA fragment. Reprinted from [12] with permission of the *New England Journal of Medicine*.
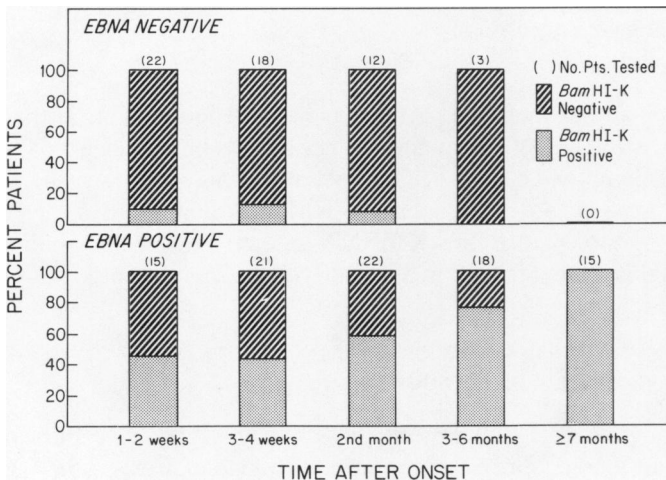

FIG. 4. Development of *Bam*HI K nuclear antigen antibody in relation to EBNA antibody presence in 43 infectious mononucleosis cases. Numbers in parentheses are number of patients tested; hatched areas, percentage of patients negative for *Bam*HI K; stippled areas, percentage positive for *Bam*HI K. Reprinted from [9] with permission of the *Journal of Infectious Diseases*.

TABLE 1
Serologic Response to the *Bam*HI K Component of EBNA in Different EBV
Seropositive* Patient Populations

Patient Category	No. Tested	Anti-K Antibody Status		
		Positive	Negative	(%)
Healthy convalescent mononucleosis	15	15	0	(0)
Nasopharyngeal carcinoma	30	30	0	(0)
Other cancer	18	18	0	(0)
Healthy adults	33	33	0	(0)
Neuropsychiatric disease	44	44	0	(0)
Burkitt lymphoma	43	39	4[a]	(9.3)
"Chronic mononucleosis"—Fatigue syndrome	39	35	4	(10)
"Chronic mononucleosis"—Severe syndrome	25	17	8	(32)
Children with AIDS	15	3	12	(80)
Total	262	234	28	(11)

*All have antibody to viral capsid antigen.
[a]Two African and two European B.L.; the latter were not EBV-associated.

Early after onset, sera tended to react with EBNA, but not with K. This suggests that EBNA antigens, other than K, are preferentially recognized early after infection; however, by seven months after onset of clinical IM, all the patients we studied had developed anti-K [9].

LACK OF ANTI-K IN CHRONIC EBV INFECTION

We have recently embarked on a seroepidemiologic study, on a larger scale, of the usefulness of the selective lack of anti-K as a diagnostic marker in chronic EBV infection. Patients who carry the diagnosis of chronic EBV infection fall into two categories—the common and the severe. The common form consists of chronic fatigue, fever, sore throat, and other constitutional symptoms which persist for more than one year following infectious mononucleosis. In the severe form, there are more objective clincial findings, which include thrombocytopenia, neutropenia, abnormalities in the serum immunoglobulins, and chronic pneumonitis. Altogether, we have studied 64 patients with this diagnosis whose serum was sent to us. Thirty-nine had the common and 25 the severe form of the syndrome. We have identified 12 patients who lack anti-K. Eight of 25, or about one-third of those with the severe form of the clinical syndrome, lack anti-K. Four out of 39, or about 10 percent with the common form of the syndrome, are K-negative. We do not see lack of anti-K in most other patient groups, including EBV seropositive normals, and patients with various EBV-associated cancers (Table 1). We have noted, however, that children with AIDS and EBV-associated lymphoproliferative disease only rarely mount an antibody response to K [3].

Clinical and Epidemiologic Features of the Twelve Patients Who Lack Anti-K

The 12 patients (refer to Table 2) varied in age between ten days and 32 years at onset of symptoms. Five developed symptoms when they were children. Two children developed disease in the perinatal period, one of them possibly as the result of *in utero* infection. The patients were born in various parts of the world, including India, France, Nova Scotia, and Holland. None of the patients were blood relatives. All patients were

TABLE 2
Patients with Chronic Active EBV Infection Who Lack Antibody to the *Bam*HI K
Component of EBNA

Patient No.[a]	Age[b]	Sex	IM[c]	Major Clinical Manifestations
1	18	F	+	Fever, anemia, pneumonitis
2	17	F	−	Fever, leukopenia, vision loss, pneumonitis (died)
3	8	F	−	Hyper IgG, thrombocytopenia
4	27	M	+	Hyper IgE, neutropenia, Job's syndrome
5	5	M	+	Hyper IgG, ascites, pneumonitis
6	2 months	F	−	Fever, leukopenia, thrombocytopenia
7	5	F	+	Sinusitis, cartilage destruction, vision loss
8	10 days	F	−	Fever, anemia, leukopenia, hepatosplenomegaly
9	31	M	+	Lymphadenopathy, fatigue
10	32	M	+	Recurrent fever, sore throat, fatigue, myalgia
11	20	F	−	Fever, incapacitating malaise
12	15	M	+	Fever, pharyngitis, myalgia

[a]Place of birth: number 2, India; number 3, France; number 5, Nova Scotia; number 8, Holland; all others, United States
[b]At onset of symptoms; in years, unless indicated differently
[c]IM, past history of infectious mononucleosis

HIV-seronegative, and none had an identifiable congenital immune deficiency. Thus the syndrome of chronic EBV infection occurs in epidemiologically unrelated patients.

Response to EBV Replicative Antigens

Another potential serologic marker for chronic EBV infection is persistent elevation of antibody to the capsid and early antigens of the virus [13,14]. All eight patients with the clinically severe syndrome who lacked anti-K had high titers to capsid and early antigens. Their response included high titer antibodies to monoclonal early antigens encoded by *Bam*HI M, *Hind*III G, and *Bam*HI Z. Only one of the four patients with the common form of the syndrome who lacked anti-K recognized early antigen, and none reacted with the monoclonal early antigens M, G, and Z. This finding raises the possibility that a panel of antigens will be needed in order to develop diagnostic serologic techniques for the chronic EBV syndrome. In the fatigue syndrome, in particular, it may turn out that EBV is not etiologically related.

Response to Other EBNAs

It has sometimes been argued that failure to make antibody to EBNA is the result of a defect in cellular immunity which prevents lysis of EBV-immortalized cells [12]. Since cells containing EBNA are healthy, they can grow *in vivo*, unlike cells making early nuclear antigens, which are lysed by virus-induced cytopathic effects. Virus products in cells which are lysed by virus replication would be readily released for immune recognition. Immune recognition of EBNA would, however, require intact cell-mediated cytotoxicity to break open the cell and display the nuclear antigens.

Therefore it was of considerable interest to know whether these 12 patients made antibody to other EBNAs. Only four of 12 patients whose sera lacked antibody to K failed to recognize EBNA in Raji cells. The remaining eight patients detected EBNA

in Raji cells and were shown to make antibody to EBNA 2, which was expressed by gene transfer techniques. This finding was confirmed by Western blotting experiments, which demonstrated recognition of the EBNA 2 antigen, but not the EBNA 1 antigen, by eight patients. This evidence increases the specificity of the finding of lack of antibody to EBNA 1. This lack is evidently *not* the result of failure to lyse EBV-immortalized cells or to release the virus-encoded nuclear antigens to the immune system, since antibody is made to EBNA 2. There is evidently some very specific problem in the recognition of the EBNA 1 product.

GENOTYPE VARIATION IN THREE CELL LINES FROM CHRONIC EBV PATIENTS

Thus far we have been able to study viral DNA in lymphoblastoid cell lines which were grown from five of the patients. In confirmation of our earlier results, all five cell lines contain a *Bam*HI K EBV DNA fragment without grossly evident deletion. Furthermore, various probes indicate that each of these patients was infected with a distinct genotype of EBV. This would be expected because they are epidemiologically unrelated.

CONCLUSIONS AND IMPLICATIONS FROM USING THE *Bam*HI K EBNA IN SEROEPIDEMIOLOGIC STUDIES

We have found that the antibody response to *Bam*HI K EBNA develops slowly. Nonetheless, all EBV-seropositive individuals who are healthy have developed anti-K by seven months after infection. Furthermore, patients with Burkitt lymphoma, nasopharyngeal carcinoma, or cancers not associated with EBV, have made an anti-K response (Table 1). Some patients with symptoms and serologic evidence suggestive of chronic active EBV infection specifically lack anti-K (Table 2). These patients do make antibodies to other latent products (e.g., EBNA 2) and early antigens which are found in the nucleus.

At the very least, these findings provide a serologic marker for some patients with chronic EBV infection. Among the 25 patients with severe disease, in addition to the eight patients who had *no* demonstrable antibody to K, were five others whose antibody titers to K were only 1:10, though their antibody to viral capsid antigen was 1:1,000 or higher. This finding suggests that "relative" lack of anti-K antibody may also be useful in the serologic definition of chronic EBV infection.

We do not know what, if any, bearing the lack of antibody to K has on the pathogenesis of EBV infection. It is possible that an appropriate response to K is required for successful control of the infection. Alternatively, lack of anti-K may be a marker for another, more fundamental defect.

Nor do we yet understand how the specific lack of anti-K comes about. The studies done so far indicate that the K product can be made by cultured cells from four patients, but we do not have evidence that K is expressed *in vivo*. For five patients we now know that a K DNA fragment is present, but finer analysis, including DNA sequence, will be needed before we can say that this gene is entirely normal.

More likely the defect lies in one of two other areas. The patients may be unable to mount an immune response to K, even though they are not globally immunodeficient. They may be selectively immunodeficient. It is known, however, that EBNA 1 has two antigenically distinct unrelated epitopes [15,16]. Thus failure to make anti-K must represent an inability to recognize *both* epitopes.

Perhaps the most likely explanation lies in the area of virus/cell interactions. The lack of antibody to K may result from extensive viral replication. In the presence of replicating virus, the K antigen may not be made or may not be positioned properly in the cell. Alternatively, the patients may be unable to present the antigen to the immune system in the proper context. Perhaps recognition of K requires it to be in association with cellular histocompatibility antigens or viral products. The patients may be unable to generate this association.

Exploring molecular epidemiology quickly leads to questions of molecular pathogenesis. This result comes as no surprise, however—as Alfred Evans and his mentor John Paul have taught, epidemiology and pathogenesis are always intimately related.

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