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Immune Regulation in Epstein-Barr Virus-Associated Diseases

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INTRODUCTION

The herpesviruses represent a very large, clearly defined group of viruses of considerable medical importance and uniqueness. Of these, Epstein-Barr virus (EBV) is the best-known and most widely studied member because of its clinical and oncogenic importance. EBV is the etiological agent of infectious mononucleosis (IM) and has been implicated in the pathogenesis of an increasing number of human malignancies. The best characterized are endemic Burkitt's lymphoma, nasopharyngeal carcinoma (NPC), and polyclonal lymphomas in immunocompromised individuals.

Since the discovery of EBV 30 years ago (54), an immense body of information has been accumulated on this agent (reviewed in references 53, 114, 145, 146, 182, 184, 188, and 244). Denis Burkitt, an English surgeon working in Africa in the 1950s, was the catalyst for the discovery of this virus. He became progressively interested in an unusual form of childhood lymphoma, which was unknown outside Africa. This lymphoma, later to be known as Burkitt's lymphoma (BL), was first encountered in Uganda but proved to be unusually common throughout equatorial Africa (22, 23). Although fresh tumor biopsy specimens obtained from patients with BL by conventional virus isolation techniques were uniformly negative, Epstein and colleagues successfully established a number of cell lines from BL biopsy specimens (55), and examination of these cell lines by electron microscopy showed clear evidence of herpesvirus-like particles in a small proportion of the cells (52, 56). It soon became clear that the virus present in BL cells was serologically distinct from other members of the human herpesvirus family (86, 87, 90) and would not grow in cells known to be able to support the replication of other herpesviruses. Experimental evidence supporting a role for EBV in human cancers soon emerged with the discovery that EBV caused primary human B cells to be immortalized as lymphoblastoid cell lines (LCLs) (88, 172) and displayed an ability to induce malignant B-cell proliferation in vivo when inoculated into certain species of nonhuman primates (53).

Infection of individuals with EBV is widespread in all human populations, as shown by the high proportion of individuals with specific antibodies in their serum (86, 89–92). Furthermore, the virus can be rescued in vitro from the circulating

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lymphocytes of seropositive individuals many years after primary infection, indicating that EBV is carried for life in the immune host (162). These two features (the ubiquity of the agent and the persistent nature of its infection) are indeed characteristic of the herpesvirus family as a whole. EBV has two major target tissues in vivo, B lymphocytes and squamous pharyngeal epithelium. In B cells, the infection is predominantly nonproductive (latent), although clearly there are situations in vitro when latently infected B cells can be triggered into the virus-productive lytic cycle (162, 185, 257). In oropharyngeal epithelium, the infection is predominantly lytic, with complete replication of the virus linked to ordered squamous epithelial differentiation (73, 216). EBV is distinct from the other human herpesviruses in its potent growth-transforming ability for B-lymphoid target cells in vitro and its strong association with a range of lymphoid and epithelial tumors in vivo.

The present review addresses broad features of the biology of the virus, both in cell culture systems and in the natural host, before turning to the role of the immune system in controlling EBV infection in healthy individuals and in EBV-associated diseases.

VIRUS AND GENOME STRUCTURE

Classification and Genome Organization

EBV is classified in the family *Herpesviridae*, which comprises a large number of enveloped icosahedral viruses of eukaryotes. These viruses replicate in the nuclei of infected cells and have a single, linear, double-stranded DNA genome. Herpesviruses have been classified on the basis of biological characteristics, features of virus replication, and certain aspects of genome structure into three subfamilies: the *Alpha*-, *Beta*-, and *Gammaherpesvirinae*. The members of the *Gammaherpesvirinae* have a narrow host range and tend to produce chronic infections, particularly of the lymph-related cells.

EBV has a protein core which is wrapped with DNA, a nucleocapsid with 162 capsomeres, a protein tegument between the nucleocapsid and envelope, and an outer envelope with external glycoprotein spikes typical of other herpesviruses (49, 54, 56, 173). The viral genome is a double-stranded DNA molecule of 172 kb composed of 60 mol% guanine plus cytosine (11, 40, 69–71). EBV was the first herpesvirus to be completely sequenced. The genome structure of EBV and other lymphocryptoviruses includes a single overall format and gene arrangement, 0.5-kbp terminal direct repeats, and 3-kbp internal direct repeats (11, 32, 43–45). These repeats divide the genome into short and long sequence domains. EBV isolates differ in their tandem repeat reiteration frequency (82, 105). Although most of the early and late lytic-infection genes show consistent homology in different herpesviruses, the EBV genes expressed during latent infection in B lymphocytes and a few lytic-infection genes show no homology to other herpesvirus genes. It is interesting that some of these genes may have arisen in part from cellular DNA. Consistent with this hypothesis is the finding that irregular GGGGCAGGA repeat motifs (84, 95), which are part of EBV nuclear antigen 1 (EBNA1), are also present in cellular DNA (83, 94). Other EBV genes, such as BZLF1 (immediate-early gene formed by the first leftward open reading frame in the *Bam*HI Z restriction fragment), BHRF1 (early gene), and BCRF1 (late gene) also display distant but collinear and functional homology to either *jun/fos*, *Bcl-2*, and the interleukin-10 gene, respectively (85, 140, 170).

Viral Genotypes

At least two EBV types have been identified in most human populations (2, 20, 34, 41, 42, 204, 210, 217). These EBV subtypes are designated as EBV-1 and EBV-2, so as to parallel the herpes simplex virus type 1 and 2 (HSV-1 and HSV-2) nomenclature. An alternative nomenclature is also used in the literature to designate these subtypes as type A and type B (158). In contrast to HSV-1 and HSV-2, both EBV subtypes show extensive homology except for the genes that encode nuclear antigens (EBNAs) and small polyadenylated RNAs (EBERs) in latently infected cells (4, 5). EBNA2, EBNA3, EBNA4, and EBNA6 of EBV-1 differ in amino acid sequence from those of the EBV-2 subtype by 16 to 47% (2, 41, 204). Fewer differences occur in EBNA5, since it is encoded by repeating exons from the 3-kb internal repeat which is conserved in the EBV-1 and EBV-2 subtypes. The sequence variation in EBERs is less consistent with respect to subtypes but shows consistent differences in EBV strains.

Earlier studies have shown that EBV-1 is more commonly detected in individuals from western societies, while African isolates are evenly distributed between EBV-1 and EBV-2 (264). Almost half of the African BL cell lines are infected with EBV-2. Although EBV-2 DNA is frequently detected in oropharyngeal secretions from individuals in western societies (209, 217, 261), isolation of this subtype from peripheral blood is unusual. The failure to isolate EBV-2 from peripheral blood of healthy individuals from western societies may reflect less aggressive growth of this subtype in blood lymphocytes (189).

Stages of Infection

There are two distinct stages of the EBV life cycle. In latent infection, the genome is maintained at a constant copy number and a limited number of regions of the genome are expressed. In the other stage of infection (lytic cycle), viral DNA is amplified and a variety of viral antigens (lytic antigens) are produced prior to virion maturation. It has generally been assumed that many of the characteristics of lytic and latent infection in vivo may be studied in vitro with LCLs. These cell lines can be established by exogenous EBV infection of B lymphocytes (86, 91, 172).

Sixbey et al. have provided evidence to suggest that most EBV infections occur by the oral route and that the primary site of viral replication is not in B lymphocytes but in oropharyngeal epithelium (218). Here, the virus establishes a persistent infection of epithelial cells, presumably through continued cycles of infection, virus replication, cell death, virus release, and infection of new cells (218).

Latent infection. Entry of EBV into B cells is initiated by an interaction between the major viral envelope glycoprotein gp350 and the B-lineage-associated C3d complement receptor CR2 (otherwise designated CD21) (103). Infection of B lymphocytes with EBV results in persistent latent infection and immortalization of the cells to perpetual proliferation. Specifically, EBV infection induces RNA and DNA synthesis, immunoglobulin (Ig) secretion, expression of B-cell activation markers, and cell division (6, 72, 112, 240). EBV-transformed B lymphocytes differ from B lymphocytes stimulated in vitro with polyclonal B-cell activators or surface IgM cross-linking, since the latter cells proliferate for a short time only.

Most of the details of latent infection in B lymphocytes have been drawn from studying EBV gene expression in LCLs. At least 11 EBV genes are expressed during latent infection in LCLs. These include two small polyadenylated RNAs (EBER-1 and EBER-2), six nuclear antigens (EBNA1 through EBNA6), and three integral membrane proteins (LMP1, LMP2A, and

TABLE 1. Significant features of the humoral response to EBV replicative and latent antigens

Status	Reference(s)	Serum antibody to:	
		Replicative antigens	Latent antigens
IM	89, 92, 93	IgM VCA positive, IgA VCA positive (38%), EA-D and EA-R positive (90%)	EBNA2 positive (at 1–3 mo), EBNA1 positive (at 2–12 mo)
EBV positive, healthy	199	VCA IgG positive, IgA and IgM negative (95%)	EBNA1 positive EBNA2 negative
XLPS	197	EA and VCA response variable in XLPS survivors	EBNA negative
BL			
Endemic	88, 211	IgA VCA positive (28%), IgG VCA positive, EA-R positive prognostic	EBNA2 positive (38–76%)
AIDS	17	IgG VCA positive	EBNA2 positive (70%)
NPC	92, 165, 211	IgA VCA positive prognostic EA-R positive prognostic	EBNA2 positive (45–62%)
PTLD	233	VCA positive, EA positive	EBNA2 positive (30%)

LMP2B). An alternative nomenclature for the EBNAs (EBNA1, EBNA2, EBNA3a, EBNA3b, EBNALP, and EBNA3c) is also used by some authors (157, 158). Sequential analysis of latent-gene expression following EBV infection in B lymphocytes in vitro suggests that EBNA2 and EBNA5 are the first nuclear antigens expressed (3). EBNA2 *trans* activates cellular genes such as CD21, CD23, c-*fgr* and viral genes, including those encoding LMP1 and LMP2 (1, 3, 37, 58, 59, 246, 251, 252). Within 48 h of primary B-cell infection in vitro, all EBNA proteins have reached the level that is maintained consistently through latent infection (4, 152). However, the expression of EBERs does not reach substantial levels until 70 h postinfection (3).

Lytic infection. Two different approaches have been used to induce lytic antigens in LCLs. First, expression of lytic antigens can be induced with phorbol esters or by cross-linking of surface IgM or treatment with a calcium ionophore (125, 129, 265). By this approach, a few cell lines (e.g., Akata) can be induced to lytic virus replication in 10 to 50% of cells (229, 230). The second approach involves superinfection of the Raji cell line with a latent antigen-defective strain of EBV from the P3HR-1 cell line (16, 36, 154). Superinfection of Raji cells complements this defect in late-gene expression (81).

Following induction, cells which have become permissive for virus replication show changes characteristic of herpesvirus cytopathology, including margination of nuclear chromatin, synthesis of viral DNA, assembly and nucleation of nucleocapsids, envelopment of virus by budding, and inhibition of host macromolecular synthesis (66, 231). In contrast to the latent phase of infection, when only a limited number of proteins are expressed, activation of the EBV lytic cycle is characterized by the appearance of as many as 80 virus-specific RNA species (230). On the basis of their time of appearance postinduction, these transcripts are designated early (immediate early and delayed early) or late. Early genes are expressed independently of new protein synthesis, after switching the virus from latency into replication (93). The early antigens (EAs) are composed of a series of polypeptides, such as BZLF1, a replicon activator; BMRF1 and BMLF1, which are both *trans* activators; BALF5, a DNA polymerase; BORF2 and BARF1, ribonucleotide reductases; BXLF1, a thymidine kinase; and BHRF1, a cytoplasmic antigen (230). Early immunological studies divided the EA response into restricted (EA-R) and diffuse (EA-D) components. The response to EA-R and EA-D is frequently of diagnostic significance (93) (Table 1).

The components of the EBV late-antigen complex, which are mostly structural viral proteins, are required for successful packaging of the viral DNA and formation of the virion. The viral capsid antigen (VCA) is abundantly expressed in cells undergoing productive infection, and it has polypeptide and glycoprotein components ranging in size from 26 to 200 kDa, with a 143-kDa polypeptide (BNRF1 reading frame) being the major component (239). The membrane antigen complex is found on the virion envelope and consists of at least three major EBV-induced glycoproteins: gp350/220, gp110, and gp85. gp350/220 is produced by alternative splicing from BLLF1 and is involved in mediating virus binding to the B-lymphocyte receptor, CR2. gp110, encoded by BALF4, is found in abundance in the cell nucleus and in the endoplasmic reticulum (ER), but it is not a major structural protein of the virion. BXLF2 encodes the minor virus component, gp85, which participates in viral fusion. An unglycosylated component of the envelope, p140, has also been identified (230). It is likely that EBNA1 is also a component of the lytic cycle in the epithelium, given its expression in productively infected B cells (119). There is no firm evidence for the expression of other latent antigens during the productive cycle.

In vivo, there is compelling evidence of a productive EBV infection in stratified squamous epithelium in immunocompromised individuals with oral hairy leukoplakia (73). In normal stratified epithelium, the least differentiated epithelial layers express low levels of the EBV receptor (216), whose significance at this site remains unresolved. Fusion of circulating EBV-positive B cells with oropharyngeal epithelium or uptake of IgA-virus complexes by the epithelium (219) are two alternative explanations for the mechanism of EBV infection of oral epithelium. It seems likely that these cells release virus as differentiation proceeds and BZLF1 expression increases. Subsequently, free virus and virus-infected epithelial cells are released and become available for infection of susceptible individuals.

CLINICAL ASPECTS AND PATHOGENESIS OF EBV-ASSOCIATED DISEASES

EBV causes a wide spectrum of acute and chronic infections in normal and immunocompromised individuals. The paradigm for acute EBV infection is classic heterophile-positive IM (208). EBV has been implicated as the causal agent of BL and plays a role in the pathogenesis of NPC. In recent years, EBV has also been potentially associated with a wide variety of additional clinical syndromes, such as chronic mononucleosis, fatal IM (X-linked lymphoproliferative syndrome [XLPS]/Duncan's syndrome), certain B- and T-cell lymphomas such as the lymphoproliferative syndromes including lymphomas in immunodeficient hosts (35), and other epithelial malignancies including carcinoma of the parotid gland (104, 181, 203, 225). It has also been postulated that this virus contributes to

TABLE 2. Viral gene expression and phenotypic characteristics of EBV-associated malignancies

Tumor	% EBV positive	EBV antigen expression	Phenotypic characteristic		Reference
			Characteristic	Presence	
Polyclonal lymphomas			Adhesion molecules	++++	233, 234
PTLD	100	EBNA1–EBNA6, LMP1, LMP2	CD23 and CD40	++++	
AIDS	100	EBNA1–EBNA6, LMP1, LMP2	MHC expression	++++	
XLPS	100	EBNA1–EBNA6, LMP1, LMP2	Antigen-processing genes (TAP1 and TAP2)	?	
BL			Adhesion molecules	−/+	74, 106, 133, 201, 242
Endemic	>95	EBNA1	CD23 and CD40	−/+	
Sporadic	15–25	EBNA1	CD10 and CD77	+++	
AIDS	25–40	EBNA1	MHC expression	−/+	
			Antigen-processing genes (TAP1 and TAP2)	−/+	
NPC	100	EBNA1, LMP1, LMP2	Adhesion molecules	−/+	30, 31
			MHC expression	++	
			Antigen-processing genes (TAP1 and TAP2)	?	
HD[a]			Adhesion molecules	++	50, 96
MC/LD	80–90	EBNA1, LMP1, LMP2	MHC expression	++	
NS	30	EBNA1, LMP1, LMP2	Antigen-processing genes (TAP1 and TAP2)	?	
T cell	10	EBNA1, LMP1, LMP2	Adhesion molecules	?	46, 77
			MHC expression	?	
			Antigen-processing genes (TAP1 and TAP2)	?	

[a] MC, mixed cellularity; LD, lymphocyte depleted; NS, nodular sclerosing.

Hodgkin's disease (HD) (7, 97, 254). In addition, a wide variety of other diseases including chronic fatigue syndrome and autoimmune disorders (for example, Sjögren's syndrome and rheumatoid arthritis) have been indirectly associated with EBV (61, 102, 148, 256), although a causal role for the virus in the pathogenesis of these last conditions seems less than convincing.

Infectious Mononucleosis

It is now clear that a proportion, in some studies up to 50%, of serologically confirmed primary EBV infections occurring in adolescence or early adult life are manifested as IM. It has been suggested that IM reflects the events of asymptomatic infection, albeit in exaggerated form. IM is characterized by the appearance of heterophile antibodies in the serum and an atypical lymphocytosis. The general clinical symptoms range from mild fever to prolonged debilitating illness. Typically, the disease presents as pharyngitis, lymphadenopathy, and general malaise, fever, and splenomegaly (91). Transmission of EBV is oral, and most immune individuals secrete the virus in saliva (183, 258). The primary site of virus replication is in pharyngeal epithelium (65, 216, 218), and recirculating B lymphocytes can presumably become infected at this primary site of virus replication, thereby generalizing the infection. EBV can be isolated in two types of target cells from IM patients, oropharyngeal epithelium, in which virus replication occurs (73), and mature B lymphocytes, in which the infection appears to be nonproductive (115, 116, 172).

X-Linked Lymphoproliferative Syndrome

A number of studies have shown that X-linked immunodeficiency selectively predisposes affected individuals to EBV-associated disease (177, 180). After acute infection, approximately 75% of males with XLPS develop fatal IM within a few weeks, while the small proportion of such individuals who survive are subsequently at an increased risk of developing hypogammaglobulinemia and/or lymphoma (178, 179, 232). The pathogenesis of XLPS can vary from uncontrolled immunoblastic lymphoma to widespread tissue destruction and the infiltration of lymphoid organs by phagocytic cells.

The occurrence of malignant B-cell lymphoma in XLPS emphasizes the importance of immunosurveillance in the control of proliferation of B cells immortalized by EBV in vivo during primary infection. Although the exact nature of the immune defect is not yet clear, XLPS carriers or patients have defective IgM-to-IgG switching, fail to develop EBNA-specific antibodies (75, 163), and have significantly reduced production of gamma interferon by T cells, which suggested that these individuals might have an underlying defect in the cytokine cascade responsible for maintaining the activity of EBV-specific T cells (164). Substantial progress has been made in mapping this genetic defect, and a tight linkage has been demonstrated to two chromosome markers, DXS42 and DXS37 (205, 220). This should ultimately lead to a molecular explanation of the defect.

Burkitt's Lymphoma

There are now three recognized forms of BL: endemic, sporadic, and AIDS-associated BL (Table 2). In spite of their clinical heterogeneity, all bear one of three reciprocal translocations between chromosome 8, near the site of the c-*myc* locus at 8q24, and either the Ig heavy-chain locus on chromosome 14 (the common translocation seen in up to 80% of tumors) or one of the light-chain loci on chromosome 2 or 22 (the variant translocations) (13, 39, 123, 130, 131).

Endemic BL accounts for approximately half of all childhood lymphomas occurring in equatorial Africa and Papua New Guinea and has an unusually high incidence of 8 to 10 cases per 100,000 people per year. The association of BL with EBV is based on two observations: (i) the demonstration of EBV genome in the majority of tumors from areas where the disease is endemic (68, 122, 132, 160) and (ii) the finding that BL patients have an unusually high titer of antibody to EBV

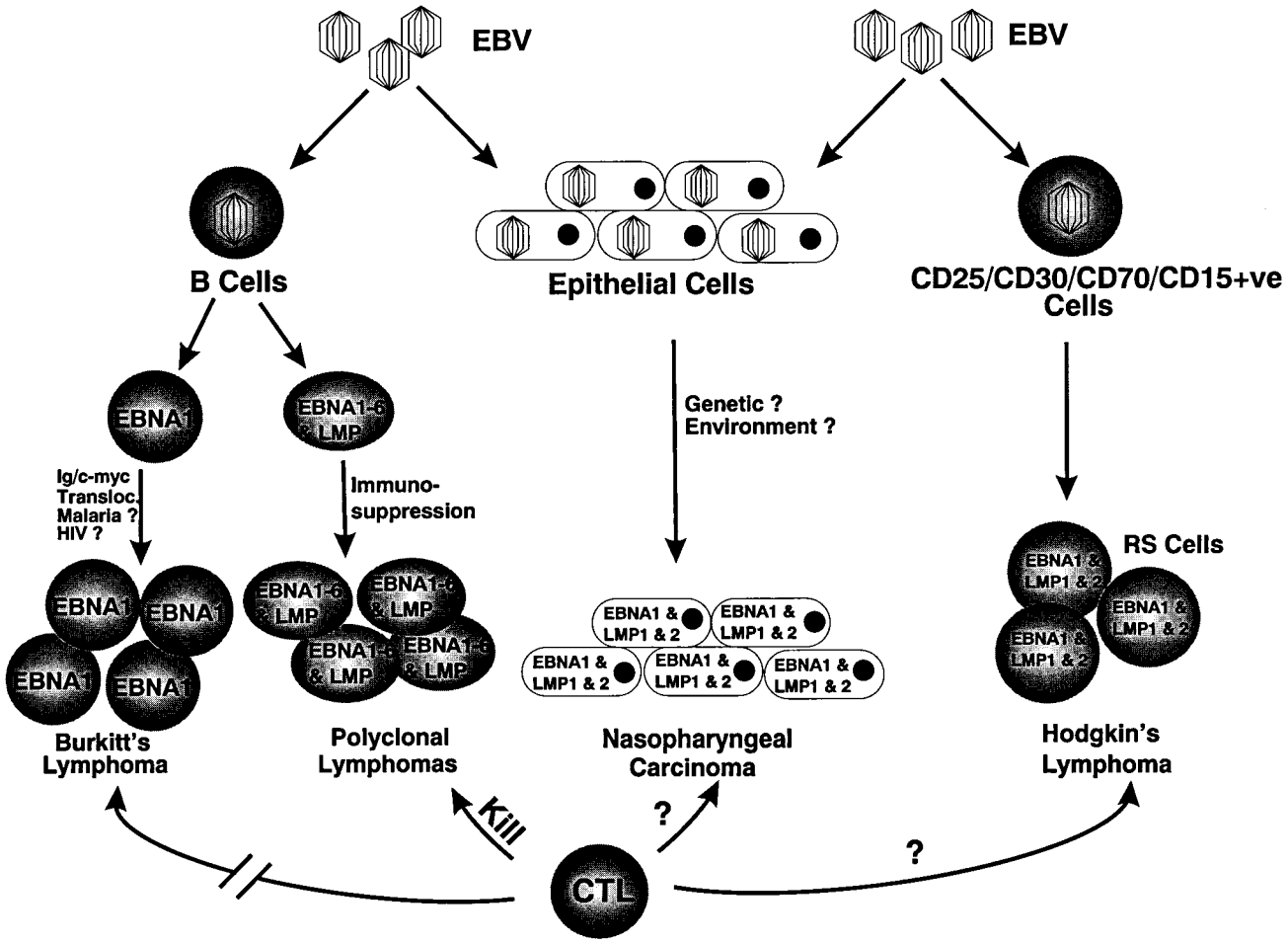

FIG. 1. Diagrammatic representation of the development and immune control of EBV-associated malignancies.

about 5 years before the clinical appearance of tumors (67, 88). The clinical features of the endemic form of BL usually involve the jaw in association with molar tooth development, while sporadic BL and AIDS-associated BL more frequently occur in the abdomen and bone marrow. Endemic BL shows the strongest and most consistent association with EBV (122, 132) (Table 2), although the exact role which the virus plays in the etiology and pathogenesis of the tumor is still controversial. More than 95% of endemic BL tumors are EBV genome positive, and each tumor carries a monoclonal EBV episome (160). There is no indication that either EBV type occurs preferentially in BL, but the presence of the two virus types appears to reflect their relative prevalence within the population (261, 264). In BL occurring outside the area of endemicity (sporadic and AIDS-associated BL), EBV DNA can be detected in only approximately 20 to 40% of the tumors (Table 2).

Various models for the role of EBV in the pathogenesis of BL have been proposed (113, 114, 124). It is conceivable that endemic BL develops in three stages (Fig. 1). The first stage involves primary EBV infection, resulting in virus-induced immortalization of B lymphocytes, which are arrested in an early stage of differentiation. Stage 2 involves risk factors, most probably holoendemic malaria or AIDS, which act as potent stimulators of B-cell hyperplasia or mediators of T-cell suppression (118, 144). The third and final step involves the translocation of the distal part of chromosome 8 to chromosome 14 (or chromosome 2 or 22), leading to the constitutive deregulation of the c-*myc* oncogene and subsequent monoclonal B-lymphocyte proliferation, resulting in BL (124). Indeed, there is now increasing evidence to suggest that EBNA1, the only EBV antigen expressed in BL, can participate in the deregulation of c-*myc* and may increase the chance translocation by interacting with Ig enhancers (132). On the other hand, studies carried out by George Klein suggest that EBV might not play a direct role in the pathogenesis of BL but may simply increase the risk of development of BL by virtue of its ability to immortalize B cells (113). This hypothesis is also consistent with the lack of expression in BL cells of those EBV latent genes (e.g., EBNA2 through EBNA6 and LMP) known to be necessary for transformation of B cells (198, 201). In fact, the only latent gene invariably expressed in EBV-positive BL cells is EBNA1, an antigen with no proven transforming function or ability to influence the expression of any cellular gene (3, 255).

Cell lines established from BL biopsy specimens in vitro initially display the cellular phenotypic characteristics of the original biopsy specimen and grow as a single-cell suspension with down-regulated intracellular adhesion molecule expression (188, 190). On serial passage, the majority of these BL cell lines retain this group I (biopsy cell) phenotype, showing either no detectable change in surface profile or relatively minor increases in adhesion molecule expression, usually accompa-

nied by a tendency to grow in small loose clumps. However, during prolonged culture in vitro, some BL cell lines show a dramatic phenotypic drift with increased expression of B-cell activation antigens and adhesion molecules and the appearance in the culture of clumps of more lymphoblastoid-like cells (referred to as the group III phenotype). These cells are clearly derived from the group I tumor cell population and are not contaminants of normal B-cell origin, since they retain the cytogenetic and Ig isotype markers of the tumor. As the group III phenotype cells dominate the culture, they frequently lose expression of CD10 and CD77 BL markers while other LCL-associated markers such as CD23, CD40, intracellular adhesion molecules and Bcl-2 are up-regulated (85, 188, 191, 201). These contrasting features of the group I and group III cellular phenotypes are an important tool in understanding the immune escape mechanisms of BL.

Nasopharyngeal Carcinoma

NPC is a tumor of epidermoid origin with endemic distribution among well-defined ethnic groups; it is prevalent in several regions around the world. It is one of the most common cancers in southern China and occurs less commonly in North African Arabs. An association between EBV and NPC was first described by Old et al. in 1966 (165). This association was based on the observation that IgA antibodies directed against the EBV early antigens and VCA were elevated in patients with NPC compared with those in matched controls (92, 263). Moreover, EBV genome can be uniformly found in every anaplastic NPC specimen (161). Early studies on EBV latent-gene expression in NPC tissue demonstrated the presence of EBNA1 in nearly all cases while, LMP1 and LMP2A could be detected in approximately 75% of EBV-positive NPC specimens (58, 260) (Fig. 1; Table 2).

The high incidence of NPC among southern Chinese suggests that, other than EBV, genetic or environmental factors may also contribute to the development of NPC (Fig. 1). As with many other diseases, early evidence for a genetic determinant among Chinese was a human leukocyte antigen (HLA)-associated risk for NPC (214). The presence of HLA-A2 and HLA-B*Sin*2 major histocompatibility complex (MHC) alleles in Chinese individuals was shown to be associated with an increased risk (relative risk, 2.35) of NPC. In addition, other MHC class I alleles, Aw19, Bw46, and B17, are associated with an increased risk of developing NPC, whereas HLA-A11 is associated with a decreased risk (128, 214, 215). It is important to mention here that so far this genetic susceptibility is based on familial rather than population studies. Among the environmental or cultural factors, it has been suggested that ingestion of Cantonese-style salted fish, especially during childhood, correlates with increased risk for NPC (10, 99, 101, 262). This hypothesis is supported by experiments with laboratory animals; rats fed with salted fish can develop an NPC-like tumor. Moreover, epidemiological studies have suggested that reduced consumption of salted fish might decrease the incidence of NPC in southern China (99). Thus, the etiology of NPC is multifactorial and includes virological, genetic, and environmental factors, providing a model system for understanding the interactions of these factors in oncogenic transformation.

Posttransplant Lymphoproliferative Disease

The association of EBV with lymphoproliferative diseases in transplant recipients was first noted in 1980 by Crawford et al. (38). EBV-associated posttransplant lymphoproliferative disease (PTLD) is a potentially fatal complication of chronic immunosuppression following solid-organ or bone marrow transplantation. Histological analysis of PTLD shows a quite complex clonal diversity. This can range from polymorphic B-lymphocyte hyperplasia to malignant monoclonal lymphoma.

Analysis of the cellular phenotype of PTLD by immunohistochemical techniques has revealed that in most cases the lymphoma cells have phenotypic characteristics similar to those of EBV-transformed B cells. PTLD cells express high levels of CD23 and intracellular adhesion molecules such as ICAM and LFA3 (234) (Table 2). It has generally been assumed that these lymphoma cells express a full array of EBV latent genes (EBNA1 through EBNA6 and LMP), as seen in immortalized B-cell lines (234) (Fig. 1). However, a number of reports have shown that a complex heterogeneity is often seen in these tumor cells with respect to viral gene expression. EBV latent antigens such as EBNA2 and LMP are expressed in only a subpopulation of EBNA1-positive cells (233). PTLD cells with a BL-like phenotype have also been identified in transplant recipients (127). Prevention of PTLD and successful management of these lymphomas continue to present a daunting task for clinicians and immunologists. However, recently some encouraging success has been achieved in controlling these lymphomas by adoptive immunotherapy (168, 195). The role of cytotoxic T lymphocytes (CTL) in this immune system-mediated control is discussed below in the section on cell-mediated immune response to EBV infection.

EBV-Associated Hodgkin's Disease and Non-Hodgkin's Lymphoma

Hodgkin's disease. An association between EBV infection and HD was first suggested by serological studies. Subsequently, analysis of tumor tissue showed the presence of EBV DNA, RNA, and protein in pathologic specimens from HD patients. HD is seen worldwide but is particularly common in the Western world, where it is the most common malignant lymphoma. A statistically significant association between age and HD has been reported by one group (76), with a peak in incidence at 25 to 30 years and a second rise beyond the age of 45 years. It has been suggested that those individuals who show clinical manifestations of acute IM have a three- to fourfold-increased risk of developing HD (155, 196). The histological examination of HD shows a disrupted architecture of the affected lymph node and the presence of characteristic mononuclear Hodgkin and multinuclear Reed-Sternberg (RS) cells. Nodular sclerosing and mixed-cellularity types of HD are frequently EBV positive (up to 90% or more), whereas the lymphocyte-depleted and lymphocyte-predominant histological types are rarely positive for EBV (7, 166) (Table 2). RS cells constitute the malignant population but are greatly outnumbered by various nonmalignant infiltrating cells (primarily $CD4^+$ T cells), which typically make up more than 98% of the tumor mass. The exact lineage of RS cells is still unknown. However, a number of features common to most, if not all, cases of HD are well established (50, 96) (Fig. 1). These include expression of lymphocyte activation antigens, such as CD25, CD30, and CD70, and MHC class II antigens on RS cells. These cells are also usually positive for various adhesion molecules (51) (Table 2) and for a carbohydrate marker, CD15, originally thought to be myeloid cell specific but now recognized on a variety of cell lineages (96). The present evidence suggests that if HD is a tumor of lymphoid origin, it frequently appears to be derived from an immature cell not yet committed to either the B- or T-cell lineage.

The latent-gene expression in EBV-associated HD is usually very similar to that seen in the majority of NPC cases. RS cells

are EBER positive and EBNA1, LMP1, LMP2A, and LMP2B positive but EBNA2 through EBNA6 negative (96) (Table 2). Interestingly, the levels of LMP expression seen in RS cells are usually toxic in normal cells (78), suggesting either an acquired abnormality of RS cells to tolerate high levels of LMP or the presence of mutations within this oncogene (117) that affect its transcription or toxicity (117). Evidence for the lytic cycle in RS cells is rarely seen and is often restricted to the expression of the immediate-early antigen BZLF1 in a very small number of RS cells (167).

Non-Hodgkin's lymphoma. The discovery of EBV involvement in lymphomas of T-cell lineage first raised the possibility that EBV, long considered a strictly B-lymphotropic agent, can also infect the T-cell compartment under certain circumstances. Some of these lymphomas show geographical focusing, notably the peripheral T-cell lymphomas seen in Chinese populations (225) and the midline granulomas of the Japanese (79). A recent study showed that as many as 10% of peripheral T-cell lymphomas are EBV positive by immunohistochemical techniques (77) (Table 2). It is unclear how EBV gains access to T cells in vivo. Although a low level of CR2/CD21 expression has been detected on a subpopulation of immature thymocytes and circulating T cells (247, 253), the relevance of this to human T lymphomas remains controversial.

Other non-Hodgkin's lymphomas associated with EBV are primary central nervous system lymphoma, angioimmunoblastic lymphadenopathy, and CD30-positive anaplastic large cell lymphoma (46, 64, 156). A large proportion of these lymphomas (between 20 and 70%) are consistently positive for EBV DNA or RNA by in situ hybridization and/or PCR.

HUMORAL IMMUNE RESPONSE TO EBV INFECTION

The presence of EBV in epithelial cells and in B cells provokes an intense immune response consisting of antibodies to a large variety of virally encoded antigens. During the acute phase of IM, the humoral response is directed primarily toward viral antigens of the lytic cycle, notably membrane antigen complex, EA, and VCA (92, 93), while the antibody response to EBNA1 and EBNA2 is delayed (Table 1). The anti-MA response in IM is directed toward the gp85 component rather than gp350/220 (92). The antibody response to the latter component is known to be the more potent source of neutralizing antibodies (238). The overall effect of this lack of gp350/220 reactivity during acute IM is that the humoral response is largely ineffective. Acute IM is also characterized by a pronounced IgM antibody response to autoantigens and heterophile antigens presumably associated with the well-documented role of the virus as a polyclonal B-cell activator. As IM patients recover from clinical symptoms, the IgM response reduces significantly while the IgG response in serum plateaus at a reduced level and is maintained throughout the persistent infection. It has also been suggested that antibody to gp350/220 binds to productively infected cells, rendering them susceptible to antibody-dependent cellular cytotoxicity-mediated lysis (169). The anti-EBNA and anti-VCA responses, although persisting for life, appear to have little if any protective role (145). It is interesting that while healthy EBV-seropositive individuals have low or undetectable antibody to EBNA2, a positive antibody response to this antigen is a common feature of EBV-associated disease. The major features of the EBV humoral response in different EBV-associated diseases are summarized in Table 1.

CELL-MEDIATED IMMUNE RESPONSE TO EBV INFECTION

The marked lymphocytosis during acute IM provoked an early suspicion that a cellular response might be important in controlling EBV infection. This was reinforced by the observation that these atypical lymphocytes are not virus-infected B cells but lymphoblasts of thymic origin, apparently reactive to the viral infection (213). This population includes cells expressing CD45RO (138), an antigen which is considered to be a marker for memory T cells. Current evidence suggests that these memory T cells are important in limiting the expansion of latently infected B cells and may also reduce the load of infectious virus by targeting productively infected cells expressing lytic antigens.

Lytic Antigens as Targets for T Cells

Although considerable information has been generated recently on T-cell responses to viral antigens expressed during the latent cycle (see below), much less is known about the contribution of cellular immunity against antigens synthesized during the viral replication cycle. This is mainly because there is no known in vitro grown cell that can serve as the target for a fully permissive EBV infection. Early studies by Thorley-Lawson et al. demonstrated that T cells inhibit the proliferation of EBV-infected B cells (236, 237). This observation has been extended in recent years, and there is reason to believe that the effect may be mediated by gamma interferon released by $CD4^+$ T cells (8, 80). Although it was originally inferred that this inhibition of proliferation was directed toward latent antigens, the kinetics of the effect (236) suggests that replicative antigens may be involved (Fig. 2). Indeed, more contemporary studies with a related system have verified a role for T cells in inhibiting cells expressing replicative antigens, including gp350 (14, 15). Since all of these studies have involved B cells rather than epithelial cells, which are the likely site of EBV replication, they can best be regarded as models for T-cell control in the oropharynx.

Significantly increased shedding of virus in the oropharynx of immunosuppressed individuals provides further evidence in support of an important role for T cells in controlling the replicative EBV infection. Reports by Bejarano et al. (14, 15) indicate that recognition of processed virion antigens elicits T-cell-mediated inhibition of EBV-induced B-cell transformation. They further showed that the same effect could be achieved with purified virus envelope antigen, gp350 iscoms. These gp350 iscoms were also shown in a separate study to be the target of EBV-specific MHC class II-restricted proliferating clones generated by stimulation of lymphocytes from EBV-seropositive individuals with UV-inactivated virus (249). More recently, a number of $CD4^+$ gp350 T-cell epitopes have been identified (170, 171, 250). It has been suggested that at the site of virus replication, $CD4^+$ T cells might be activated by viral antigens and play an important role in modulating a local inflammatory response through lymphokine release. In addition, lytically infected cells have an increased sensitivity to lysis by natural killer cells (19) and to antibody-dependent cellular cytotoxicity mediated by antibodies against gp350 molecules on the surface of cells undergoing replicative infection (111) (Fig. 2).

The T-cell response to other virus replication-associated antigens, particularly to the EA complex, has been examined by Pothen et al. (174, 175). They found that the proliferation of memory T cells from EBV-infected individuals could be induced by the affinity-purified protein gp350, two viral capsid antigen polypeptides (p160 and gp125), and three major EA

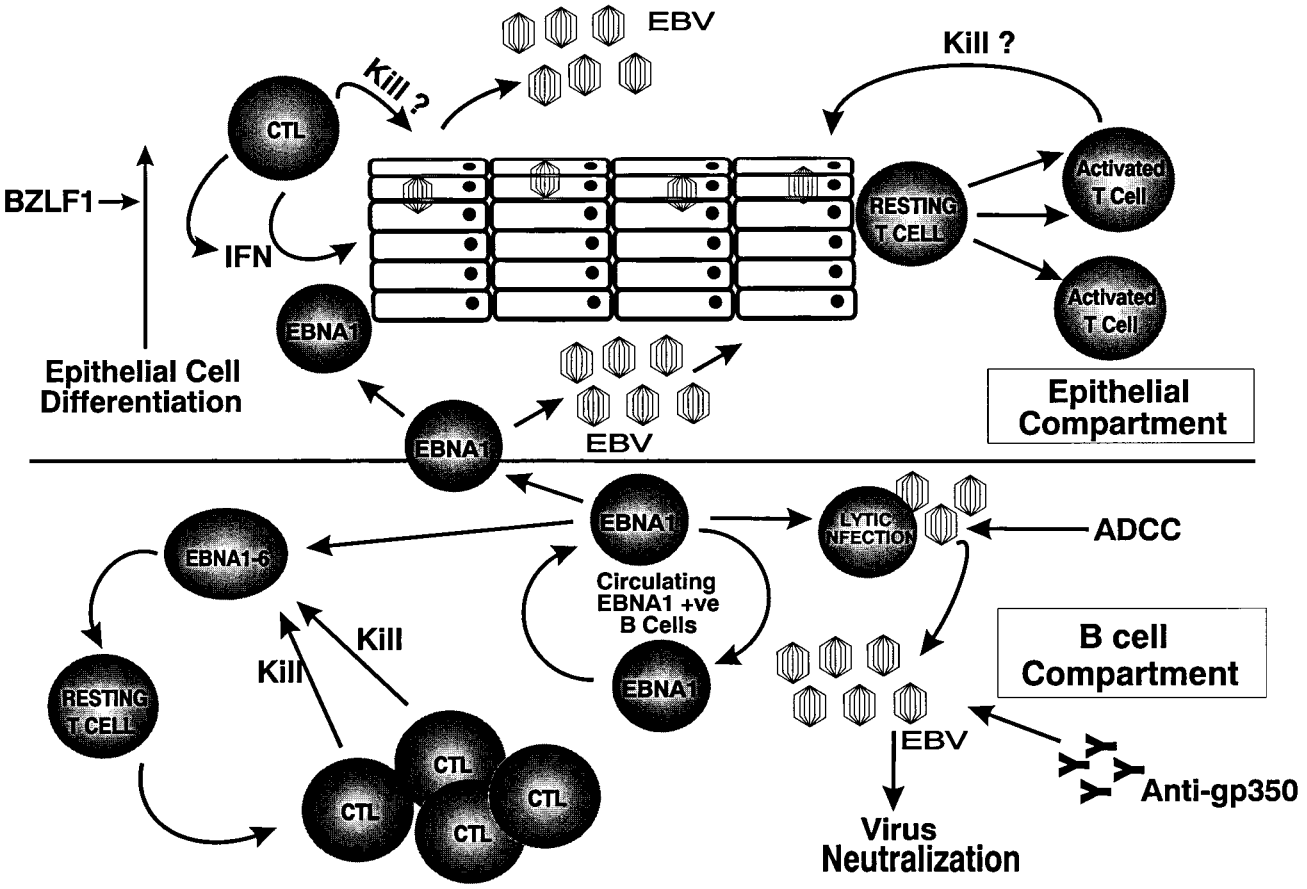

FIG. 2. Model for the immune control of EBV infection in the epithelial cell and B-cell compartment. Different aspects of this model are discussed in the text (see the sections on humoral immune response to EBV infection and on cell-mediated immune response to EBV infection).

proteins (p17 = BHRF1, p85 = BORF2, and p50 = BMRF1). Cell lines established against p17 or p50 were composed primarily of $CD4^+$ T cells, although $CD8^+$ cells also persisted. However, there was no evidence suggestive of a cytotoxic effector function. A later study further analyzed the immune response to p17 (BHRF1) and defined both a $CD4^+$ and $CD8^+$ proliferative T-cell epitope, as well as three B-cell epitopes (175). Thus far, however, evidence for CTLs specific for antigens produced during the EBV lytic cycle has not yet been reported in the literature. These CTLs could conceivably function in vivo to eliminate cells producing new virus progeny and thus prevent virus spread (Fig. 2).

Role of Cytotoxic T Cells

Role in acute infectious mononucleosis. The lymphocytosis during acute IM includes both $CD8^+$ and $CD4^+$ T cells which express activation markers such as HLA-DR (241). The exact role of these T lymphocytes in restricting the proliferation of EBV-infected B lymphocytes is unclear. These atypical lymphocytes are seen not only in the peripheral blood but also infiltrating many of the tissues (33). The specificity and function of these activated T cells have been difficult to assess. Interpretation of early studies on the function of these cells has been complicated following the observation that most of them die by apoptosis within hours of culture (18, 143, 248). The induction of these "anergic" T cells during acute IM is reminiscent of a response mediated by superantigen. Early reports described an EBV-specific, Fcγ receptor-depleted population in acute IM that showed broad-ranging lysis of a wide panel of non-HLA-matched LCLs (202, 227). While subsequent studies challenged the EBV specificity of this population (226), they sustained the view that lysis was non-HLA restricted and similar to that seen in experimental situations in which T cells were activated in vitro.

With hindsight, it seems likely that the uncertainties surrounding these early studies were compounded by the fact that the HLA type of the patient was not known at the time effector function was being defined and that the target panel did not include the autologous LCL. Subsequent studies involved a protocol for the cryopreservation of acute IM effector cells at the same time as the patient's HLA type was being determined. The cryopreserved effector cells (already depleted of $Fc\gamma^+$ cells) revealed the presence of two distinct $CD8^+$ specificities in fresh T cells from patients with acute IM: (i) a population of EBV-specific, HLA class I-restricted cells which could be expanded in vitro and (ii) a population with apparent specificity toward alloantigens (224, 241). When considered in the light of experiments which have demonstrated that EBV-specific CTL bulk cultures (62) and clones (25, 206) cross-react with alloantigens, it is entirely feasible that the $CD8^+$ CTL response during acute IM is dominated by EBV-specific, MHC class I-restricted CTLs and that alloresponses previously reported represent a population of EBV-specific CTLs that cross-react with MHC alloantigens (25). Indeed, the polyclonal CTL response to another viral infection has been shown to include a

large population of T cells with dual specificity for allogeneic and virus-infected autologous cells (159). It seems likely that the function of these $CD8^+$ T cells is to limit the expanding pool of latently infected B cells (EBNA1 through EBNA6, LMP1, and LMP2) (Fig. 2).

A class II-restricted, $CD4^+$ CTL response may also play an important role in controlling primary EBV infection. Indeed, this was predicted in early in vitro experiments in which a primary EBV-specific, $CD4^+$ CTL response was generated by cocultivating unfractionated mononuclear cells with the irradiated autologous LCLs from EBV-seronegative individuals (137). In more recent experiments, EBV-specific $CD4^+$ CTL activity has been demonstrated in fresh lymphocytes from patients with acute IM, and clones established from such individuals possessed a similar phenotype and recognized a sequence within the EBV early antigen, BHRF1 (207a). This result raises the possibility that an important component of the EBV-specific CTL response during primary infection is directed toward antigens associated with the replicative phase of the viral cycle. Whether these $CD4^+$ CTLs play any role in limiting EBV replication in epithelial cells is not known at this stage. However, it would not be entirely surprising if $CD4^+$ cells recognize viral particles and structural antigens following processing by the class II pathway.

Thus, the picture is emerging that a significant EBV-specific, $CD4^+$, and $CD8^+$ CTL response is activated in acute IM. It will be important to establish the role of these effector cells in defining the progress of the primary infection toward either silent EBV seroconversion or acute IM.

Role in healthy virus carriers. There is now convincing evidence for a specific memory T-cell response against the largely nonproductive EBV infection in B cells in healthy individuals, although the degree to which T cells control the latently and permissively infected epithelial cells is uncertain. The basis of this evidence was in vitro observations revealing the existence of EBV-specific memory T cells through their capacity to regulate the course of virus-induced transformation of B cells (150, 185). Thus, when adult donor lymphocytes (including T cells) are exposed to the virus and placed in culture, the proliferation of virus-infected B cells, which occurs within the first 2 weeks postinfection, is followed by a complete regression of growth brought about by CTLs reactivated in vitro. These CTLs are present only in lymphocyte cultures from EBV-seropositive individuals and are specific for MHC-matched EBV-infected cells since they do not kill autologous mitogen-activated blasts (151, 153, 186). This CTL response is a classic virus-specific response ($CD8^+$, class I restricted) (187), although EBV-specific $CD4^+$, class II-restricted CTLs have also been described (134, 135, 137). The relative frequency of class I- and class II-restricted CTLs that can be cloned from activated cultures from seropositive donors can, to a degree, be manipulated by the conditions of activation (unpublished observations). It is particularly important to consider this point when ascribing effector activity in vivo which conceivably might vary in different phases of the infection depending on the load of virus-infected cells (Fig. 2).

Target Antigens for EBV-Specific Cytotoxic T Cells

In 1981, studies carried out in our laboratory suggested that the EBV-specific CTL response is directed to an EBV-associated lymphocyte-detected membrane antigen, LYDMA, whose existence was first postulated to explain the apparently virus-specific CTL function in blood from IM patients (152). The molecular identity of LYDMA, however, remained unresolved until fairly recently, in spite of extensive research (158). Given what we know about presentation and processing of endogenously synthesized proteins to small peptide fragments in association with HLA class I molecules and by analogy with the influenza virus model in which nuclear antigens are frequently the site of CTL epitopes (12), attention was turned to EBNA proteins as a source of CTL epitopes. Indeed, early studies involving leukocyte migration inhibition suggested that EBNAs may play a role in immune control of EBV (228). The nature of antigen processing and presentation by class I molecules suggests that all nine EBV antigens constitutively expressed in LCLs are potential sources of immunogenic peptide epitopes irrespective of the native location of these proteins within the cell.

The key observation in the identification of target antigens was that for certain donors, it was possible to exploit the allelic polymorphism in the EBNA proteins between EBV-1 and EBV-2 by isolating EBV-1-specific CTL clones (149). These clones provided an opportunity to define CTL epitopes by screening selected EBNA peptides for reactivity on B-type transformants (28). This led to the definition of the first EBV-specific CTL epitope which was presented by EBV-1-transformed B cells but not EBV-2 transformants. To define multiple epitopes including those conserved on both EBV-1 and EBV-2 transformants presented a more difficult problem. However, two recent technical advances have facilitated this study: (i) the construction of recombinant vaccinia viruses capable of expressing individual EBV latent antigens (158) and (ii) the establishment of an EBV-negative host cell (B-cell blasts) for these recombinant vaccinia viruses (109, 110). These EBV-negative B-cell blasts are an ideal target since they also express high levels of adhesion molecules, are not plastic adherent, and, most importantly, are efficiently infected with vaccinia virus. With these two important tools, it was shown that most healthy seropositive individuals have a strong CTL response to more than one EBV latent antigen (107, 157). The results of these studies are summarized in Table 3. This work led to several important conclusions. (i) CTL reactivity against EBNA3, EBNA4, LMP1, and EBNA6 latent proteins appears to form a substantial part of the EBV-specific CTL response in a high proportion of individuals. (ii) More than 30 distinct CTL specificities restricted through eight different class I alleles were defined, and HLA class I alleles such as HLA-A2, HLA-B7, HLA-B8, HLA-B35, HLA-B40, and HLA-B51 were each shown to present more than one distinct CTL epitope derived from different latent antigens (Table 3). It is surprising, given the high frequency of the HLA-A1 allele, that no CTL reactivity restricted through this allele has been detected. (iii) The specificity of a large number of EBV-specific CTL clones (68%), using the panel of vaccinia virus constructs encoding most of the latent antigens, could not be defined. A possible explanation for this result is that antigens associated with the EBV replicative cycle could also include CTL epitopes. These antigens are detected in only a small proportion of latently infected cells by conventional techniques. However, the exquisite sensitivity with which CTLs recognize peptide fragments from viral antigen raises the possibility that low levels of replicative antigens are processed and presented as target epitopes on LCLs. (iv) No CTL response against EBNA1 was detected (Table 3). The lack of detectable CTL epitopes within EBNA1 has two important implications with regard to the immunobiology of EBV infection in peripheral B cells and EBV-associated tumors. First, it provides a mechanism by which long-lived, nonreplicating, EBNA1-expressing normal B cells (200) can maintain a nonimmunogenic phenotype by not expressing the critical latent proteins needed for CTL recog-

TABLE 3. Summary of the MHC class I- and class II-restricted CTL reactivity against EBV latent antigens in healthy individuals[a]

HLA type	Reactivity against:							
	EBNA1	EBNA2	EBNA3	EBNA4	EBNA5	EBNA6	LMP1	LMP2A
MHC class I								
HLA-A2	−	+	−	−	−	−	+	+
HLA-A3	−	−	+	−	−	−	−	−
HLA-A11	−	−	−	+	−	+	−	−
HLA-A23	−	−	−	−	−	−	−	+
HLA-A24	−	−	+	−	−	−	+	+
HLA-B7	−	+	+	−	−	+	+	−
HLA-B8	−	−	+	−	−	−	+	−
HLA-B27	−	−	−	−	−	+	−	+
HLA-B35	−	−	+	+	−	−	−	−
HLA-B40	−	−	+	−	−	−	+	−
HLA-B44	−	−	−	−	+	+	−	−
HLA-B51	−	+	+	−	−	−	+	−
HLA-B57	−	−	−	+	−	+	−	−
MHC class II								
HLA-DR1	+	−	−	−	−	−	−	−
HLA-DR/DQ	−	+	−	−	−	−	−	−

[a] This summary is based on the data published in references 63, 107, 146, and 157, while HLA-DR/DQ-restricted EBNA2 reactivity is based on unpublished observations.

nition. Second, it provides a mechanism whereby BL cells (which express only EBNA1) proliferate in vivo, in the face of a potent EBV-specific CTL response (192–194, 198).

The peptide determinants recognized by EBV-specific CTL clones restricted through a number of HLA alleles were identified by reconstitution experiments with synthetic peptides derived from the primary sequence of the relevant viral antigen. To date, 19 CTL epitopes have been described at the peptide level and are included in EBNA1 through EBNA4, EBNA6, and LMP2 (21, 24, 26, 27, 29, 63, 98, 107, 121, 207) (Fig. 3). It is interesting that precursor frequency analyses demonstrate that HLA-B allele-restricted CTL epitopes are

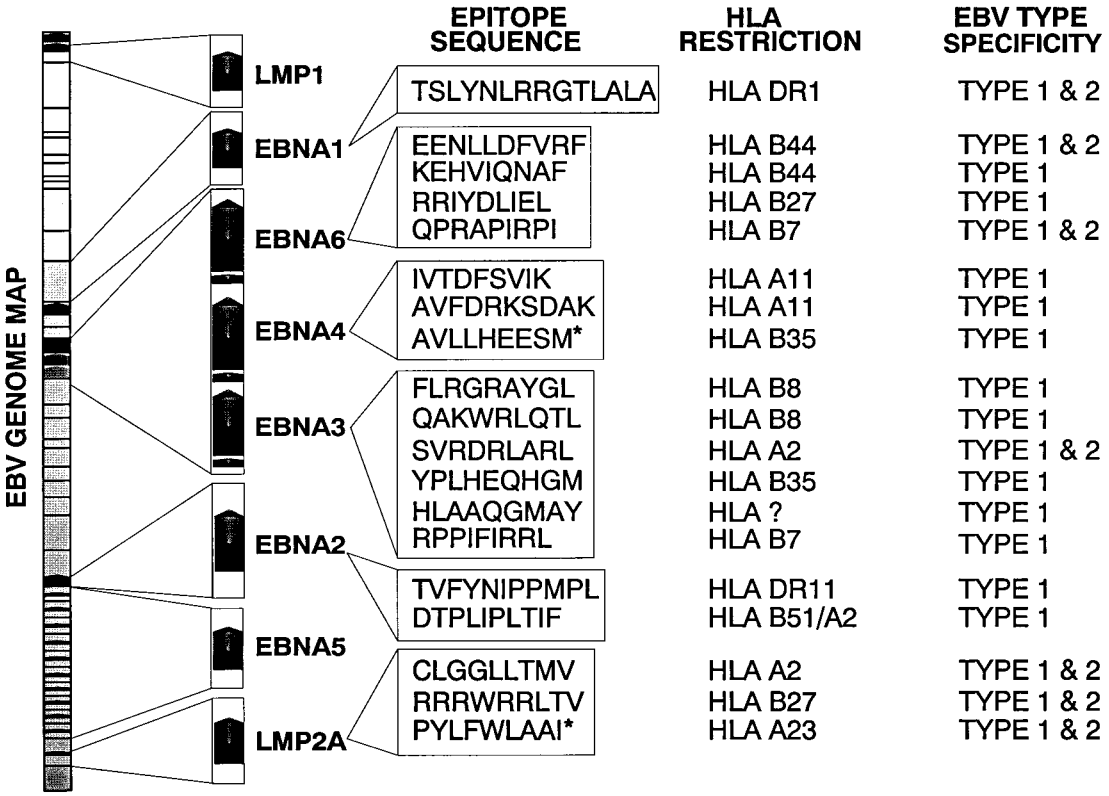

FIG. 3. MHC class I- and class II-restricted CTL epitopes from EBV latent antigens defined at the peptide level. This list of epitopes has been compiled from the data published in references 21, 24, 26–29, 63, 98, 107, 121, and 207. The asterisks denote unpublished epitopes.

often more dominant than HLA-A allele-restricted epitopes (105a). Although the precise reason for such a phenomenon is not known, it is possible that HLA-B alleles are more efficient in peptide loading, maturation, and surface translocation. Another interesting feature of the EBV-specific CTL response is the immunodominance of a single peptide epitope restricted through a single HLA allele. Thus, the majority of HLA-B8-seropositive individuals show a dominant response to a peptide epitope derived from the EBNA3 protein (FLRGRAYGL) and those who are HLA-B*4405 positive show a dominant response to an epitope from EBNA6 (EENLLDFVRF) (105a). In spite of the focused nature of these responses through a single epitope, these HLA alleles can present more than one epitope (Table 3).

Although some of the epitopes defined thus far are conserved in both EBV-1 and EBV-2 transformants, a significant number are specific for EBV-1 (Fig. 3). Since it is well established that the majority of single amino acid substitutions within CTL epitopes can result in loss of recognition (27), the common isolation of type 1 specific CTL epitopes is not unexpected when the degree of latent antigen sequence variation between the two types is compared (204).

The possibility of CTL-driven epitope mutations in natural isolates of EBV is an important consideration in relation to the role of EBV in disease. This issue was first addressed by Appoloni et al. (9). Studies on a panel of type 1 EBV isolates derived from different geographical locations indicated that two CTL epitope sequences (HLA-B8-restricted epitope FLR GRAYGL and HLA-B44-restricted epitope EENLLDFVRF) were widely conserved. However, a polymorphism within the HLA-A11-restricted CTL epitope, IVTDFSVIK, derived from EBNA4, has recently been reported (47, 48). Although the epitope sequence is conserved in most EBV-1 strains worldwide, all of the EBV-1 strains isolated from individuals from Southeast Asia and Papua New Guinea (coastal region), where the HLA-A11 allele frequency is very high, consistently carried an altered sequence with specific mutations in one of the anchor residues (48). These mutant peptide sequences do not bind to HLA-A11 molecules and are thus not recognized by HLA-A11-restricted EBV-specific CTLs. This raises the interesting possibility that viral strains lacking the dominant A11 epitope have enjoyed a selective advantage in populations in which an unusually high proportion (>50%) of individuals possess this allele. Accordingly, these viruses may have more opportunity to establish a persistent infection and/or to establish a large pool of EBV-carrying cells from which infectious virus can be reactivated and transmitted. However, extension of these observations to West African populations, in which the HLA-B35 allele is relatively common, indicated that the HLA-B35-restricted target epitope sequence (24) in the EBNA3 protein is largely conserved (120). Furthermore, a recent study from our laboratory detected identical mutations in the HLA-A11-restricted epitope (similar to those seen in virus isolates from coastal Papua New Guinea) in virus isolates from the highlander population of Papua New Guinea, in which the HLA-A11 frequency is very low (unpublished observations). These two studies raise questions on the importance of HLA distribution and CTL responses in influencing virus evolution. Thus, it is possible that the presence of mutations within CTL epitope sequences in virus strains isolated from Papua New Guinea and Chinese individuals represents a founder effect rather than the result of any selective pressure exerted by the immune system.

T-Cell-Mediated Control of EBV-Associated Tumors

Polyclonal lymphomas. There are strong indications that the outgrowth in vivo of EBV-infected polyclonal tumors is modulated by (i) the pattern of viral antigens expressed by that tumor and (ii) the immunocompetence level of the host. For instance, the role of immunesurveillance in limiting the proliferation of EBV-infected B cells in vivo is clearly illustrated by the development of EBV-positive polyclonal lymphomas in immunosuppressed individuals. Polyclonal lymphomas frequently present as multifocal lesions within lymphoid tissue and/or in the central nervous system and are often seen in individuals with PTLD or with human immunodeficiency virus infection. A number of studies have demonstrated that phenotypically polyclonal lymphomas are quite similar to EBV-transformed LCLs and represent an opportunistic tumor growing out as result of immunosuppression. Indeed, regression of polyclonal lymphomas has been reported in transplantation patients after relaxation of immunosuppressive therapy (38, 192). Characterization of these lymphomas has shown that a full spectrum of EBV latent genes are expressed, with high surface expression of cellular adhesion molecules such as ICAM1 and LFA3 as well as cellular activation antigens such as CD23 (233, 234). Clearly, the expression of all EBV latent antigens provides a wide range of potential targets for immune system recognition of these tumors. Much of what is known about the role of EBV latent antigens as targets for CTL has been derived by studying the response in healthy EBV-seropositive individuals (see above). Thus, it is assumed that the sensitivity of polyclonal lymphomas to virus-specific CTLs is similar to that for LCLs, since the phenotype of each is identical. Evidence supporting this assumption has recently been provided by adoptive transfer experiments in which donor lymphocytes were used as a source of virus-specific CTL to treat polyclonal lymphomas in bone marrow transplantation patients (168, 195).

Burkitt's lymphoma. BL provides a most amenable model for understanding the mechanism(s) of immune system evasion by tumor cells. The model draws strength from the fact that BL cells carrying the relevant translocation have been established in culture while LCLs have been independently derived from the normal circulating B cells from the same patient by infection with EBV in vitro. Thus, it is possible to compare and contrast the sensitivity to immune system lysis of tumor-derived and non-tumor-derived tissue from the same patient. It is now well established that EBV-positive group I BL cell lines are highly resistant to virus-specific CTL lysis and are poorly immunogenic in their ability to stimulate an alloresponse (190). It has been postulated that these factors are important in the pathogenesis of EBV genome-positive BL. Studies by Rooney et al. identified a number of BL patients who retain detectable EBV-specific T-cell surveillance, indicating that CTL dysfunction is an unlikely cause of the outgrowth of these tumors in vivo (193). However, when matched sets of LCL and BL cell lines from the same patient are compared, the group I BL cells are not lysed in vitro by polyclonal EBV-specific T-cell lines or CTL clones (108, 194). This result is interesting in light of previous data that have indicated a depressed delayed-type hypersensitivity in BL patients (60). The lack of CTL lysis of BL cells is not due to any inherent resistance of these targets to cytolysis, since such lines are killed by both allospecific CTL clones (243) and non-MHC-restricted lymphokine-activated killer cells (136). It is interesting that as EBV-positive BL cell lines switch from a group I to a group III phenotype, they show increased susceptibility to EBV-specific CTL recognition (194).

A number of possible mechanisms have been suggested to explain the resistance of group I BL cells to virus-specific CTL lysis in vitro: (i) very low expression of adhesion molecules, especially LFA3 and ICAM1 (74), and (ii) selective down-regulated expression of certain HLA class I alleles (133, 242). However, recent studies from our laboratory have demonstrated that while the virus-specific CTL recognition of LCL is dependent on an intact LFA3/CD2 pathway, BL cell lysis could be achieved by peptide epitope sensitization, which was critically dependent on the LFA1/ICAM2 pathway (108). As there is consistently high expression of ICAM2 on all BL cell lines, it appears that down-regulation of LFA1, LFA3, and/or ICAM1 expression on BL cells does not provide an absolute barrier to tumor cell recognition by virus-specific CTLs.

The viral phenotype of BL cells is also likely to be a very important factor in reducing tumor susceptibility to EBV-specific CTL surveillance, since viral antigen expression in EBV-positive BL cells in vivo is restricted to a single protein, EBNA1 (201). It has generally been considered that this antigen does not include CTL epitopes. Thus, BL cells fail to express viral antigens that have been shown to be common targets for CTL recognition in healthy virus carriers. Although earlier studies have demonstrated that no CTL response to EBNA1 is detectable in healthy EBV-immune individuals (107, 157), we have recently identified an MHC class II-restricted CTL epitope within EBNA1 (107a, 146). One of the most important features of this epitope is that EBV-transformed LCLs are unable to process and present this epitope endogenously. However, this epitope is recognized by CTLs after exogenous sensitization of autologous or HLA-DR1-positive B cells. Although the precise reason for the inability of B cells to process this antigen is not known, it is possible that the EBNA1 protein is not processed by the class II pathway in such a way that allows the loading of the HLA-DR molecules endogenously. It is interesting that this epitope is included in the EBNA1 DNA-binding region, which may limit transport of the epitope into the class II processing compartment. Moreover, it has been suggested that EBNA1 bound to DNA is highly resistant to degradation by proteases (212). An effective method of testing this hypothesis will be to determine whether translocation of EBNA1 or the epitope sequence alone, in a different compartment of the cell (other than the nucleus), facilitates endogenous processing of the epitope.

Another possible mechanism whereby BL cells escape CTL recognition involves a defect in the endogenous antigen-processing function in these cells. Well before the molecular identity of LYDMA was resolved, Rowe et al. proposed that the insensitivity of EBV-positive BL cell lines to virus-specific CTLs might reflect a cellular defect in the ability to process and present LYDMAs to the T-cell system rather than an absence of the antigens per se (201). Very recent results from our laboratory have demonstrated that the class I antigen-processing pathway, which delivers peptide epitopes from endogenously synthesized proteins for presentation on the cell surface in association with HLA class I molecules, appears to be very much less active in BL cells than in LCLs (106, 110a). This is reflected in very low expression of the transporter proteins TAP1 and TAP2 in a number of BL cell lines. These proteins are involved in peptide transport from the cytosol into the ER (176, 221–223). Given what is now known about class I processing, these observations suggest that BL cells are unable to transport peptide epitopes from the cytoplasm into the ER. Deficiencies in this antigen-processing pathway are generally associated with reduced stability and surface expression of MHC class I molecules in laboratory-generated antigen-processing mutant cell lines (such as RMA-S and T2) (126, 139). This reduction is apparently due to a deficient supply of peptide epitopes into the ER for MHC stabilization. Indeed, BL cells show a similar phenomenon, with a very low proportion of the MHC class I molecules expressed in the cytoplasm being transported onto the cell surface. Moreover, the endogenous presentation of CTL epitopes on BL cells can be restored by transfection with an expression vector encoding a CTL epitope fused to an ER translocation signal sequence (106). However, in contrast to the RMA-S and T2 cell lines, in which the TAP genes are deleted, BL cell lines show a transcriptional deficiency with significantly reduced levels of TAP1 and TAP2 mRNA and protein expression (106, 110a). Thus, down-regulated expression of viral antigens and defective endogenous processing function may contribute to immune system evasion from the normal EBV-specific CTL response in BL patients.

Nasopharyngeal carcinoma and Hodgkin's disease. NPC and HD, like BL, are seen in relatively immunocompetent individuals. Very limited information is available on the role of specific T cells in controlling these tumors, although IgA-positive NPC patients show a significantly depressed EBV-specific CTL response (147). However, it is unlikely that this diminished T-cell response is solely the basis for tumor outgrowth, since a proportion of the IgA-positive NPC patients showed a normal response.

The effectiveness of the T-cell response is presumably also limited by the pattern of viral gene expression, which is restricted to EBNA1 and in some cases to LMP1 and LMP2 as well. It is surprising that the expression of the highly immunogenic antigens LMP1 and LMP2 does not result in rejection of these tumors in vivo. A possible explanation is provided by a murine model used by Trivedi et al., which showed that the LMP1 gene from NPC cells, when expressed in mouse carcinoma cells, was completely nonimmunogenic, while the LMP1 gene from the B95.8 cell line was highly immunogenic (245). Interestingly, LMP1 encoded by NPC isolates shows numerous amino acid changes compared with the B95.8 sequence (100). More recently, similar amino acid changes in the LMP1 gene have also been found in EBV isolates from HD (117). These observations strongly suggest that mutations in the LMP1 sequence might render tumor cells nonimmunogenic for CTLs. Although a number of CTL clones specific for LMP1 have been isolated from healthy EBV-immune individuals (107), the peptide specificity for these clones has not yet been defined. It will be interesting to see whether the mutations within the LMP1 sequence of NPC isolates are encoded within epitope sequences that are relevant for CTL recognition. These observations therefore provide an interesting focus for future work intended to delineate the role of the EBV-specific CTL response in the control of these tumors.

VACCINE DEVELOPMENT

There are two relevant issues when considering an EBV vaccine. First, in Western societies, where the aim of vaccination is to prevent the symptoms of IM, it may not be imperative to induce sterile immunity or prevent the establishment of a latent EBV infection, because a reduction in the EBV load during primary infection may avert clinical symptoms. On the other hand, any vaccine which aims to prevent BL, NPC, or HD must be capable of preventing the establishment of a latent EBV infection, since all of these malignancies develop years after the primary infection with the virus. Two broad approaches are currently being considered to design an effective vaccine for controlling EBV-associated diseases.

gp350 as a Target Antigen

In the last few years, vaccine development efforts have concentrated on the use of a subunit preparation of gp350 (recombinant and affinity purified) and have been directed toward blocking virus attachment to the target cell in the oropharynx. The general approach has been to immunize cottontop marmosets with gp350 and determine their ability to restrict the outgrowth of EBV-positive lymphomas in these animals. Indeed, when highly purified gp350 was administered subcutaneously in conjunction with adjuvants (muramyl dipeptide or immune-stimulating complexes), it induced high levels of serum-neutralizing antibodies and inhibited tumor formation in cottontop tamarins (141). A number of recombinant vectors including vaccinia virus gp350 and adenovirus type 5 gp350 have also been successfully used in these animals to block tumor outgrowth (142). The precise mechanism by which gp350 affords protection from lymphomas in cottontop tamarins remains unclear. The fact that development of neutralizing-antibody titers in vaccinated animals does not always correlate with protection (57) indicates that gp350-specific T-cell-mediated immune responses may also have an effector role. Furthermore, Yao et al. (259) showed that very low levels of neutralizing anti-gp350 antibodies are present in the saliva of healthy EBV-immune donors, which suggests that such antibodies are unlikely to be the basis of long-term immunity in healthy seropositive individuals. It seems unlikely that a vaccine based solely on gp350 will be completely effective in preventing infection of every single epithelial or B cell; however, this kind of vaccine might have the potential to significantly reduce the load of infectious virus and thus limit the long-lived EBV-positive B-cell pool.

Latent Antigens as Potential Vaccine Candidates

Since latently infected B cells do not express gp350 and are controlled by CTLs specific for EBV latent antigens, an alternative approach is based on a subunit vaccine that incorporates EBV CTL epitopes restricted through dominant HLA alleles. This vaccination strategy involves the generation of the $CD8^+$ T-cell repertoire with formulations of synthetic peptides which mimic immunodominant epitopes known to be recognized by the EBV-induced CTL response. Indeed, our laboratory is currently conducting a phase I human trial with a CTL epitope (FLRGRAYGL) from the EBNA3 protein (Fig. 3), restricted through HLA-B8. The ultimate aim of this trial is to establish whether such a vaccination strategy can protect against IM and PTLD. Obviously, one of the major obstacles of this approach is the HLA polymorphism, since each HLA class I allele presents different epitopes. However, this obstacle can be overcome by simply mixing multiple peptides; alternatively, minimal CTL epitopes can be fused to construct a recombinant or synthetic polyepitope protein. In recent experiments with a recombinant vaccinia virus that incorporates many of the CTL epitopes listed in Fig. 3, we found that each $CD8^+$ CTL epitope within the construct was efficiently presented to its restricting allele (235). Thus, it seems that amino acid sequences flanking the $CD8^+$ CTL epitope may not be required when designing a subunit vaccine which incorporates multiple CTL epitope from either a single pathogen or several human pathogens. It should be mentioned here that any vaccine design based on CTL epitopes from latent antigens will be directed against EBV-transformed latently infected B cells and might also be used in EBV-seronegative transplant recipients because of their increased susceptibility to virus-induced polyclonal lymphomas. However, it should be stressed that the vaccine based on CTL epitopes is aimed at reducing the load of virus-infected B cells rather than inducing sterile immunity. This reduction in viral load may be sufficient to subdue the clinical manifestations of acute IM and PTLD but are unlikely to afford protection against other EBV-associated malignancies such as BL and NPC.

If the apparent inability of virus-infected cells to process and present CTL epitopes from EBNA1 is substantiated, any vaccine design based on EBV latent antigens is unlikely to afford any protection against EBV-associated tumors such as BL and NPC, in which EBV latent-gene expression is often restricted to EBNA1. However, the possibility of modification of certain regions of the EBNA1 protein might render it immunogenic and open up the possibility of endogenous processing of CTL epitopes from EBNA1 sequences. Potentially, this would open up the possibility of mounting an effective response not only against the latent EBV infection but also against EBNA1-bearing tumors. Realistically, if a suitable delivery system that is capable of achieving long-lasting immunity is developed, it might be possible to attain a level of immunity that is at least able to modify the primary infection.

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