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(54) Title: RECOMBINANT EPSTEIN-BARR VIRAL CAPSID ANTIGEN USEFUL IN DIAGNOSTIC METHOD

(57) Abstract

Recombinant EBV-gp125 protein is made in a *baculovirus* expression system in sufficient quantity and purity to enable its use in assays such as ELISAs for quantitative detection of EBV VCA-IgG and VCA-IgM antibodies. The recombinant gp125 protein is similar in size to EBV-gp125 viral capsid antigen isolated from EBV-infected human cell lines, and has similar sensitivity and specificity in immunological assays for VCA-Ig antibodies.

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RECOMBINANT EPSTEIN-BARR VIRAL CAPSID ANTIGEN USEFUL IN DIAGNOSTIC METHOD

Background of the Invention

Technical Field

This invention relates to diagnosis and treatment of Epstein-Barr virus (EBV) infection.

Background Art

Epstein-Barr virus is a member of the herpesvirus family, which infects most humans. Most persons are infected with the virus in early childhood and then carry the virus for life. The majority of primary infections in young children are asymptomatic. In adolescents and young adults, infectious mononucleosis (IM) is the most common manifestation of primary infection with EBV (G.H. Roberts, Diagnostics and Clinical Testing 27:16-21, 1989). EBV has also been implicated in rheumatoid arthritis, chronic fatigue syndrome, Burkitt's lymphoma, nasopharyngeal carcinoma, aplastic anemia, aggressive lymphoproliferative disorders, and rupture of the spleen. EBV is known to be an opportunistic pathogen, often developing in immunosuppressed subjects, such as for example patients with allografts, or persons having acquired immune deficiency syndrome ("AIDS").

Diagnosis of IM is often based on the presentation of characteristic symptoms including primarily fever, pharyngitis, and cervical lymphadenopathy. However, IM may be complicated by splenomegaly, hepatitis, and myalgia, as well as headache and further nonspecific symptoms (S.H. Cheeseman, Semin. Hematol. 25:261-268, 1988). Moreover, other pathogens, mainly cytomegalovirus but also adenovirus, rubella virus, human

immunodeficiency virus (HIV), and *Toxoplasma gondii*, can cause a similar syndrome. At present, testing for heterophil antibodies is frequently used to relate IM to EBV infection. Such test results are not necessarily conclusive for EBV, because heterophil antibodies can be induced by viruses other than EBV and because the incidence of heterophil antibodies among patients with IM is, at most, 90% (A.S. Evans, et al., J.Infect.Dis. 132:546-553, 1975). In particular, young children often lack a heterophil antibody response (C.V. Sumaya and Y. Ench, Pediatrics 75:1011-1019, 1985).

Serological diagnostic methods have progressed from testing for the presence of non-specific heterophil antibodies to EBV specific serodiagnosis (W. Henle, et al., Hum. Pathol. 5:551-565, 1974). To date, this is performed predominantly by indirect immunofluorescence (IF) or immunoperoxidase (IP) staining of EBV-infected cells. Standard assays make use of fixed cells derived from selected EBV-infected cell cultures which specifically express EBV-associated antigens such as the viral capsid antigens (VCAs), the set of early antigens (EAs, which include EA diffuse and EA restricted), and EBV nuclear antigens (EBNAs). These antigens correspond to the late lytic phase, the early lytic phase, and the phase of latency of EBV infections, respectively. Qualitative and quantitative determinations of immunoglobulin IgM and/or IgG antibodies against individual antigens or combinations of these antigens then allows discrimination among primary, past, and reactivated EBV infections. However, IF assays are time-consuming and are not suitable for large-scale testing. Because of variability of antigen-producing cells as well as subjective reading of results, these tests are also difficult to standardize. Moreover, IgM-IF tests may suffer from non-specific reactions when rheumatoid factors are present in the serum (G. Henle, et al., Clin. Exp. Immunol. 36:415-422, 1979).

Various attempts have been made to facilitate specific serodiagnosis of EBV infections by developing microtiter plate assays based either on native antigens purified from EBV-infected cells (G.R. Pearson, J. Virol. Methods 21:97-104, 1988) or on synthetic peptides selected empirically or by computer analysis from the amino acid sequences of presumptively relevant EBV-associated antigens. (R.I. Fox, et al. J. Clin. Lab. Anal. 1:140-145, 1987; J.E. Geltofsky, et al. J. Clin. Lab. Anal. 1:153-162, 1987; J.M. Middledorp and P. Herbrink, J. Virol. Methods 21:133-146, 1988).

One of the best indicators of active EBV infection is the presence of antibody to viral capsid antigens, structural proteins necessary for replication of the virus (G.R. Pearson and J. Luka, Characterization of the virus-Determined Antigens. In The Epstein-Barr Virus: Recent Advances, Epstein and Achong, Eds. Wiley: New York, pp. 47-73, 1986). Viral capsid antigens are present in every cell infected with EBV. VCA-IgG is the first antibody to appear at the onset of the primary EBV-infection, and it peaks within four to six weeks. VCA-IgG gradually declines during convalescence but remains detectable for life (G.H. Roberts, Diagnostics and Clinical Testing 27:16-21, 1989). Almost all patients are positive for VCA-IgM by the third week following onset and can be expected to become VCA-IgM negative 4 to 8 weeks later. Detection of VCA-IgM antibodies remains the most sensitive procedure for mononucleosis because an absence of VCA-IgM from serum in which VCA-IgG is detected excludes a diagnosis of primary EBV infection (ibid.).

Two major polypeptides associated with the VCA complex have been identified. These are a glycosylated protein of 125,000 daltons size, designated gp125, and a non-glycosylated protein of 160,000 daltons size, designated p160. Studies have shown that gp125 is the most dominant antigen in the VCA complex and therefore the strongest immunogen (G.R. Pearson *et al.* (1986),

supra). gp125 has already been found to be most useful for diagnosing a recent or past EBV infection. 100% of sera tested from individuals having confirmed infectious mononucleosis have been positive for IgM antibodies to this glycoprotein. gp125 has also served as a reliable substrate for measuring IgA antibodies to VCA (G.R. Pearson *et al.* (1986), *supra*), which have been found to be a useful marker for nasopharyngeal carcinoma (G.H. Roberts (1989), *supra*).

Conventionally, gp125 has been isolated and purified from EBV-infected cells. The cell culture system is very expensive and time consuming, however, and yields only small quantities of the protein. Further, suitable culture systems for bulk production of gp125 are lacking, and use of gp125 for routine diagnosis has therefore been unsupported.

M. Gong *et al.* (1987), Jour. Virol. 61:499-508, describes expression of an EBV open reading frame BALF4 in *E. coli*.

Recently, four diagnostically useful recombinant EBV proteins, representative of the viral capsid antigen (p150), the diffuse early antigen (p54), the major DNA-binding protein (p138), and the EBV nuclear antigen (p72), were used to set up individual enzyme-linked immunosorbent assays (ELISAs) for detection of immunoglobulin IgM and IgG antibodies (M. Gorgievski-Hrisoho, et al. J. Clin. Microbiol. 28:2305-2311, 1990). In direct comparison with results obtained by standard IF or IP assays, the recombinant EBV ELISAs were shown to provide a means for specific and sensitive serodiagnosis of infectious mononucleosis (IM).

Summary of the Invention

Disclosure of Invention

In one aspect, in general, the invention features pure recombinant EBV-gp125 protein having sufficient selectivity and sensitivity to be useful in an

immunoassay for EBV VCA antibodies, and methods for producing recombinant gp125 protein in sufficient amount and purity to enable its use in assays such as enzyme-linked immunosorbent assays (ELISA) for the qualitative and quantitative detection of EBV VCA-IgG and VCA-IgM antibodies, which would facilitate the diagnosis of primary and past EBV infection. A recombinant gp125 protein according to the invention "has sufficient selectivity and sensitivity to be useful in an immunoassay for EBV VCA antibodies" if, when used in an immunoassay of serum for EBV VCA antibodies, the results indicate substantially the same pattern of seropositives and seronegatives as are indicated in a similar immunoassay using an EBV gp125 viral capsid antigen conventionally isolated and purified from EBV-transformed cells.

The terms "recombinant gp125 protein" and, alternatively, "recombinant EBV-gp125 protein", as used herein, mean and include the full-length recombinant gp125 polypeptide, whether or not partially or fully glycosylated; and mean and include any recombinant polypeptide fragment of the gp125 polypeptide, whether or not partially or fully glycosylated, that is immunoreactive with VCA antibodies with which the EBV gp125 viral capsid antigen is immunoreactive. Thus, a recombinant gp125 protein according to the invention can be a polypeptide, glycosylated or not, having a length less than that of the full-length gp125 viral capsid antigen; and, for example, I have shown that a purified recombinant gp125 protein according to the invention, made by expression in a *baculovirus* system of a DNA sequence corresponding to that portion of the EBV DNA sequence between a start codon at 159,322 and a stop codon at base 156,755, can be as effective as EBV gp125 protein isolated and purified from EBV-infected cells in an ELISA for VCA antibodies.

Preferred recombinant gp125 protein is made by expression of the EBV viral capsid protein-encoding DNA

sequence in a *baculovirus* expression system, as the resulting expression product can have substantially better specificity and sensitivity than product obtained by expression of the same EBV viral capsid protein-encoding DNA in, for example, *E. coli* or yeast. Moreover, the recombinant gp125 protein according to the invention can be expressed in high yield in a *baculovirus* system.

Recombinant gp125 protein according to the invention can be obtained in greater quantities at lower cost, and can be purified to higher degrees of purity, than obtainable for gp125 obtained by conventional purification from EBV-infected cell culture.

Preferably, the method for making recombinant EBV-gp125 protein in high amounts and purity includes steps of cloning and expressing the EBV-gp125 protein in a *baculovirus* expression system, and isolating and purifying the resulting protein by metal affinity chromatography. In another general aspect, the invention features recombinant gp125 protein made by such a method.

In another general aspect, the invention features a serologic method for diagnosing present or past primary EBV infection in a subject by using recombinant gp125 protein in an immunoassay to detect gp125 protein-binding antibody in serum from the subject; and apparatus for carrying out the method. The apparatus preferably includes the recombinant gp125 protein immobilized on a solid support and blocked for nonspecific antibody; and is provided with means for contacting the blocked immobilized recombinant gp125 protein with the sample (such as serum) to be tested; and is provided with a secondary antibody conjugated with IgG and/or IgM having a detectable label; and is provided with means for detecting the label.

Description of Preferred Embodiments

Best Mode of Carrying out the Invention

Purified EBV-gp125 Protein.

Purified recombinant gp125 protein can be made in high quantity according to the invention generally by inserting a DNA fragment containing a gp125-encoding sequence into an expression vector that is capable of expression in a suitable host, transforming host cells with the vector, and culturing the cells. Recombinant gp125 can then be purified from lysates of the cultured cells. A preferred protocol follows.

Expression of EBV-gp125 Protein in insect cells.

The complete (172,282 base pairs) nucleotide sequence of Epstein-Barr virus has been established (R. Baer, et al. Nature 310:207-211, 1984). The gp125 protein has been mapped to a *Bam*HI A DNA fragment. A plasmid DNA, designated as pTZ19-110, encoding gp125 was obtained from Dr. Frederick Wang (Harvard Medical School, Boston, MA). In this recombinant plasmid, a 3.5 kb *Bam*Hi-*Eco*RI fragment encoding gp125 was cloned in a plasmid vector pTZ19R (Pharmacia, Piscata Way, NJ). This EBV sequence represents nucleotides 159,469 to 155,969 on the EBV genome. From the DNA sequence, it was found that the protein start codon ATG (methionine) is at nucleotide 159,322 and the stop codon is at base 156,755. The size of the protein was predicted from the DNA sequence as 95,640 daltons. Because gp125 is a glycosylated protein, the size of the protein isolated from EBV-infected cells was determined to be 125,000 daltons.

Preferably the gp125 protein is expressed in a *baculovirus* system.

For expression of the protein in *baculovirus*, the plasmid pTZ19-110 was digested with restriction enzyme *Nhe*I, at a site located 33 bp downstream from ATG of gp125 coding sequence. The coding sequence was then released from the vector by digesting with restriction enzyme *Eco*RI. This *Nhe*I-*Eco*RI fragment coding gp125 was

isolated from low melting 0.7% agarose gel using spindBind DNA extraction unit (FMC, Rockland, ME). In order to obtain the correct reading frame in *baculovirus* transfer vector pBlueBacHis C (Invitrogen, San Diego, CA), the DNA fragment was cloned into the *Nhe*I-*Eco*RI site of *E. coli* vector pTrcHis C (Invitrogen, CA). Then the *Nco*I-*Hind*III coding gp125 was subcloned at the corresponding site of pBlueBacHis C. Since this vector is derived from *E. coli* pUC vector, all the manipulations were done in *E. coli* Top 10 strain (Invitrogen, CA).

The pBlueBacHis C vector is designed for efficient protein expression and purification from recombinant *baculovirus* clones in insect cells. High levels of expression of DNA sequences cloned into the pBlueBacHis C vector is made possible by the presence of the polyhedrin promoter. The polyhedrin protein is the major structural component of the *baculovirus* occlusion bodies and accounts for more than 50% of the total "stainable" protein of infected *Spodoptera frugiperda* on SDS-polyacrylamide gels. This vector contains the natural polyhedrin leader sequence followed by a sequence which codes for (in 5′ to 3′ direction from N-terminal to C-terminal) and ATG translation initiation codon, a tract of six histidine residues that function as a metal binding domain in the translated protein, a transcript stabilizing sequence from gene 10 of phage T7, and an enterokinase cleavage recognition sequence. A multiple cloning region positioned downstream of this sequence allows insertion of the foreign gene in the correct reading frame relative to the initiation codon. This vector also allows co-expression of β-galactosidase upon transfection enabling rapid identification of the recombinant plaques in the presence of the substrates X-gal or Blue-gal.

Sequences which flank the polyhedrin gene in the wild-type *baculovirus* genome are positioned 5′ to 3′ to the expression cassettes on the pBlueBacHis C transfer vector. Following co-transfection with the wild-type

viral DNA, homologous recombination between these sequences results in a recombinant virus with the gene of interest expressed under the control of the viral polyhedrin enhancer/promoter elements. The BaculoGold linearized *baculovirus* DNA (Pharmingen, San Diego, CA) was used for co-transfection of pBlueBacHis C. This DNA contains a lethal deletion, and thus the transfected virus DNA cannot make infectious virus particles in insect cells unless the deletion is complemented by co-transfected polyhedrin-based pBlueBacHis C. Transfection of insect cell Sf9 and production and purification of recombinant virus was done according to the method described in the Invitrogen manual.

Expression of a gp125-encoding DNA fragment in pBluBacHis C was confirmed by western blot analysis. About 1 ml insect cells infected three days earlier with the recombinant virus, and 1 ml of a EBV-transformed human B cell line (Raji cell line) were pelleted and dissolved in 100 µl of Laemmli buffer. The sample was boiled for 2 minutes and then loaded on a 7.5% SDS-polyacrylamide gel and electrophoresed overnight at 70 volts. The protein was transferred electrophoretically to a nitrocellulose membrane. Nonspecific binding of the protein was blocked by treating the membrane with 5% nonfat dry milk in TBST (10 mM Tris, pH 8.0), 1 mM EDTA, 0.05 % Tween-20). Monoclonal antibody to gp125 was obtained from DuPont Company (Cat # NEA 9243) and added to the membrane-bound protein and incubated for 1 hour, washed 3 times with TBST, and then incubated with anti-mouse IgG-alkaline phosphatase conjugate for 30 minutes. The membrane was washed again 3 times with TBST, and the color was developed with NBT (nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate). A single protein band that reacted with the monoclonal antibody in the extract of recombinant virus transfected cell line Sf9 migrate close to that of the monoclonal antibody-reacting band of the EBV-gp125 viral capsid protein from the EBV-transformed Raji cell line. This result confirms

expression of gp125 protein using the *baculovirus* system in the insect cells.

Purification of Recombinant gp125 Protein.

Recombinant gp125, expressed in transfected insect culture Sf9 for example as described above, can be purified from the culture by using affinity column chromatography methods, for example.

The vector pBluBacHis C contains DNA sequence below the translation start codon which produces 6 histidine peptide at the N-terminal region of the expressed protein. This region has the affinity to bind to Ni^2 charged ProBond resin (Invitrogen, CA) and thus allows one-step purification. About 50 ml of insect cell Sf9 was grown at a density of 2 x 10^6 cells/ml in a 100 ml spinner flask. Cells were infected at the multiplicity of infection of 6 and grown at 27 °C for 3 days. The cells were pelleted by centrifugation and suspended in 20 mM sodium phosphate and 500 mM NaCl and lysed by sonification and centrifuged to remove cell debris. The recombinant protein was purified from the supernatant through ProBond resin by the method described by Invitrogen. The N-terminal fusion peptide was removed from gp125 protein by enterokinase cleavage and subsequent purification through the ProBond resin. This method gave about 1 mg of protein from 50 ml of culture, and the purity of the protein was confirmed on 7.5 % SDS polyacrylamide gel.

Industrial Applicability

ELISA Using Recombinant gp125 Protein.

The following ELISA demonstrates by way of example the use of recombinant gp125 protein according to the invention in an assay for EBV serological parameters in sera from patients having confirmed EBV infection.

About 100 µl of a recombinant gp125 protein solution (1 µg/ml of the purified recombinant gp125 protein in PBS buffer, pH 7.5) made as described above was added to Maxiabsorb Nunc microwell strips and incubated overnight

at 4° C. Each plate was washed 2 times with 200 µl of TBST buffer (10 mM Tris pH 8.0, 1 mM EDTA, 150 mM NaCl and 0.05% Tween-20) at room temperature for 5 minutes. Each plate was blocked overnight at 4 °C with 300 µl of 0.1% nonfat dry milk in TBST buffer. A 100 µl aliquot of diluted test serum (1:21 dilution in TBST buffer) was added and incubated for 1 hour at 37 °C, and then the wells were washed 3 times for 5 minutes at 37 °C with TBST. A 100 µl aliquot of alkaline phosphatase-conjugated anti-human IgM and/or IgG was added and incubated for 30 minutes at 37° C. The wells were washed 3 times with TBST for 5 minutes at 37 °C, and then 100 µl of alkaline phosphatase substrate (prepared by adding 5 mg p-nitrophenylphosphate and 1 ml of 5x diethanolamine buffer (Kierkegaard and Perry, Gaithersburg, MD) to 4 ml of distilled water) was added and incubated at 37 °C for 30 minutes. Optical density was measured at 405 nm.

The results are shown in the Table. The Table shows a comparison of an ELISA method ["TTI"], using recombinant gp125 protein according to the invention, with the commercially available Gull's Immunofluorescence and ELISA kit, using gp125 isolated and purified from EBV-infected cells. The results for the Gull's kit and for the method of the invention are similar. Owing to the small quantity of protein obtainable by isolation from EBV-infected cells, and owing to the expensive cell culture and isolation method used in so obtaining the material, the Gull's kit is very expensive. The method of the invention provides for producing large quantities of protein relatively inexpensively, providing for a kit that can be produced in quantity and very cost effectively. Conventional methods, using for example immunofluorescence, are more time-consuming and labor-intensive, and yield less reliable determinations, owing to substantial cross-reactivity.

Table

Comparison of EBV serological parameters determined by standard immunofluorescence techniques (IF) and by Gull and TTI recombinant ELISAs in sera from patients with confirmed recent or acute EBV infection.

Patient #	IF		ELISA			
			Gull		TTI	
	VCA-IgG	VCA-IgM	IgG	IgM	IgG	IgM
IM						
1	+	+	1.432	0.631	1.550	0.792
2	+	+	1.341	0.827	1.466	0.927
3	+	+	1.263	0.498	1.629	0.619
4	+	+	1.172	1.020	1.429	1.495
5	+	+	1.265	0.396	1.779	0.500
6	+	+	1.486	0.440	1.783	0.540
7	+	+	1.192	0.344	1.232	0.430
8	+	+	1.622	1.058	1.841	1.120
9	+	+	1.634	0.766	1.764	0.786
N						
1	-	-	0.068	0.075	0.112	0.115
2	+	-	1.885	0.047	1.958	0.049
3	+	-	1.351	0.110	1.526	0.090
4	-	-	0.083	0.052	0.076	0.036
5	+	-	1.455	0.048	1.581	0.052
6	-	-	0.094	0.039	0.086	0.028
7	+	-	1.646	0.051	1.773	0.041
8	+	-	1.383	0.072	1.456	0.037
9	+	-	1.788	0.061	1.858	0.056
10	-	-	0.096	0.045	0.082	0.078

Other Embodiments

Other embodiments are within the following claims. For example, the recombinant gp125 protein can be made using an expression system other than the *baculovirus* system described above. Expression in a *baculovirus* system is much preferred, according to the invention, over expression in, for example, *E. coli* or yeast or mammalian cells, because the *baculovirus* system provides for production of high-purity recombinant gp125 protein in higher yield. Moreover, the configuration (folding, glycosylation) of the recombinant gp125 protein made in a *baculovirus* system is sufficiently like that of the EBV gp125 viral capsid antigen from EBV-transformed human cells to provide for a high degree of specificity and selectivity in an immunoassay system. Recombinant gp125 protein expressed in *E. coli* or in yeast apparently lacks some conformational characteristics that are required for high specificity and selectivity. Moreover, yields of recombinant gp125 protein are lower in yeast expression systems. Mammalian expression systems are difficult and costly to maintain, and purification of recombinant gp125 expressed in mammalian cells is costly and time-consuming.

I have found that recombinant gp125 expressed in *E. coli*, for example, is unsatisfactory in an immunoassay for EBV VCA antigens, as it is not sufficiently selective or sensitive.

For expression of the protein in *E. coli*, plasmid pTZ19-110 was modified as follows. A sequence of about 183 bp was deleted by digesting plasmid pTZ19-110 with restriction enzyme *Nhe*I, at a site located 33 bp downstream from ATG, and *Bam*HI, at a site located 150 bp upstream from ATG. Both sites were blunt-ended with T DNA polymerase and ligated, and the resulting circular plasmid was transformed into *E. coli* NM 522. This manipulation resulted in formation of a new *Bam*HI site. The plasmid was then double digested with *Bam*HI and *Eco*RI

and the fragment encoding gp125 was cloned into the same translational reading frame of the expression vector pTrHisC (obtained from Invitrogen, San Diego, CA). This vector is designed for high level expression of recombinant protein in *E. coli* under the control of the inducible *tac(trp-lac)* promoter. This vector also contains the translation start codon, the polyhistidine metal binding domain, and enterokinase cleavage sites.

Expression of a gp125-encoding DNA fragment in pTrcHisC was confirmed by Western blot analysis. In this procedure, transformants were grown in LB to OD_{600}0.5 at 30 °C, and then IPTG was added to final concentration of 1 mM to induce expression of gp125. After induction the cells were grown for 3 hours at 30 °C. About 200 µl of the culture was spun down and resuspended in SDS buffer. The sample was boiled for 2 minutes and then loaded on a 7.5% SDS-polyacrylamide gel and electrophoresed overnight at 70 volts. The proteins were transferred electrophoretically to a nitrocellulose membrane. Nonspecific binding of the protein was blocked by treating the membrane with 5 % nonfat dry milk in TBST (10 mM Tris pH 8.0, 1 mM EDTA, 0.05 % Tween-20). Sera containing VCA-IgM, previously confirmed by IF test, were added to the membrane-bound protein and incubated for 1 hour, washed 3 times with TBST, and then incubated with anti-human IgM-alkaline phosphatase conjugate for 30 minutes. The sera were washed again 3 times with TBST, and the color was developed with NBT (nitro blue tetrazolium) and 5-bromo-4-chloro-3-indolyl phosphate. A single protein band having a molecular weight of approximately 95 kd reacted with the VCA-IgM sera, suggesting that gp125 is produced as a 95 kd protein in *E. coli*, but the size of the gp125 molecule isolated from EBV-infected cells was 125 kd. This size difference is most likely due to a lack of protein glycosylation in *E. coli*.

Recombinant 95 kd gp125 protein, expressed in culture in *E. coli* for example as described above, can be

purified from the culture for example by using affinity column chromatography methods. By way of example, recombinant 95 kd gp125 was purified from *E. coli* transformants made as described above, according to the following protocol.

About 1.2 liter of LB broth + glucose and ampicillin was inoculated with 12 ml of an overnight culture of cells containing the fusion plasmid. The cells were grown for 3 hours at 30 °C, and then IPTG to 1 mM final concentration was added to induce expression. The cells were incubated for a further 3 hours at 30 °C, and then were harvested and suspended in lysis buffer (10 mM sodium phosphate pH 7.0, 30 mM NACl, 0.25 % Tween-20, 10 mM β-mercaptoethanol and 1 mM PMSF (phenylmethylsulfonyl fluoride). The cells were lysed by sonication and centrifuged.

The recombinant gp125 protein expressed in *E. coli* was found to be insoluble, and was present in the pellet obtained from the crude fraction obtained as described above. The pellet was described in a denaturing buffer containing 8 M urea, 20 mM sodium phosphate pH 7.8, and 0.5 M NaCl. This was then passed through a ProBond metal affinity chromatography resin column (Invitrogen, San Diego, CA). The column was washed and the protein was eluted using procedures set out in the Invitrogen manual.

As noted above, the recombinant gp125 protein expressed in *E. coli* is unacceptable for use in an immunoassay for EBV VCA antibody because it is not sufficiently selective and sensitive to yield consistent and reliable results. Its poor performance in such an immunoassay may owe in part to the fact that it is denatured during processing, so that it no longer has a configuration presenting the appropriate antigen binding sites.

There are apparently post-translation modifications that result in differences between the recombinant gp125 protein expressed in *E. coli* and that expressed in the

baculovirus system. Recombinant gp125 protein expressed in *E. coli* as described above has a molecular size about 94 kd, corresponding to the molecular size that would be predicted for a non-glycosylated polypeptide product of expression of the DNA sequence. Recombinant gp125 protein made according to the invention in a *baculovirus* system has, as noted above, much better specificity and sensitivity than that made in *E. coli*; this may be owing to the substantial glycosylation in the *baculovirus*-expressed protein (which has a molecular size comparable to that of the EBV gp125 viral capsid antigen as isolated from EBV infected cells). There may also be differences in folding between the *E. coli*-expressed gp125 protein and the *baculovirus*-expressed gp125 protein, that may affect the conformation of antibody-binding sites and compromise the specificity or the sensitivity of antibody-binding at the sites.

As I have demonstrated, recombinant gp125 protein resulting from expression according to the invention of fractions less than the whole length of the EBV gp125 viral capsid antigen-encoding DNA can display antibody-binding specificities and sensitivities comparable to those of EBV gp125 viral capsid antigen isolated from EBV-infected cells. Fragments smaller than that described in the examples are within the claims, and can be found as a matter of routine, for example by the following protocol. First, the amino acid sequence can be examined for hydrophilic (and hydrophobic) regions that are more (or less) likely to appear on the surface of the immunologically active molecule, and therefore are more (or less) likely to make up part of an antibody-binding domain. Then sequences that appear less likely to make up part of a binding domain can be excised using standard techniques, and the resulting candidate fragment or fragments can be expressed, purified, and isolated and then used in an assay generally as described above to determine their VCA-Ig-binding specificities and sensitivities.

Alternatively, candidate polypeptide fragments can be constructed synthetically according to well-established and automated techniques, and then used in an assay generally as described above to determine their VCA-Ig-binding specificities and sensitivities. As will be appreciated, however, to the extent that post-translational modification of the recombinant gp125 protein (or of any fragment of it) can have an effect on the specificity and selectivity of the resulting molecule, as suggested above, synthetically-generated polypeptide fragments may not be as effective in an immunoassay for EBV VCA antibodies as the recombinant gp125 protein (or recombinant fragments of it) as expressed in, for example, the *baculovirus* system, as detailed above.

Claims

1. Purified recombinant EBV-gp125 protein, having sufficient selectivity and sensitivity to be useful in an immunoassay for EBV VCA antibodies.

2. Purified recombinant EBV-gp125 protein, made using a *baculovirus* expression system.

3. A method for making purified EBV-gp125 protein, comprising steps of producing recombinant EBV-gp125 protein in a *baculovirus* expression system and purifying the resulting product by affinity chromatography.

4. A method for diagnosing present or past EBV infection in a subject, comprising determining whether recombinant EBV-gp125 protein-binding antibodies are present in a sample of serum from the subject.

5. The method of claim 4 wherein said determining step comprises an enzyme-linked assay.

6. The method of claim 5 wherein said enzyme-linked assay comprises an ELISA.

7. The method of claim 4 wherein said determining step comprises an immunofluorescence assay.

8. Apparatus for carrying out serological diagnosis of present or past EBV infection in a subject, comprising recombinant EBV-gp125 protein affixed to a support.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/01775

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/70; C12P 21/06; C07K 3/00
US CL :435/5, 69.3; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 7.92, 69.3; 436/518, 518, 543; 530/350, 413

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS; Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Epstein et al., "The Epstein-Barr Virus: Recent Advances," published 1986, John Wiley & Sons, New York, pages 47-73, especially pages 59-63.	1-8
Y	Bio/Technology, Volume 6, issued January 1988, V.A. Luckow et al., "Trends in the Development of Baculovirus Expression Vectors," pages 47-55, especially page 48.	1-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 18 MAY 1994	Date of mailing of the international search report JUN 14 1994
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