PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 7/02, 7/00, 7/06
C07H 21/04, C07K 15/28

A1

(11) International Publication Number: **WO 92/20787**

(43) International Publication Date: 26 November 1992 (26.11.92)

(21) International Application Number: PCT/US92/04314

(22) International Filing Date: 22 May 1992 (22.05.92)

(30) Priority data:

704,814	23 May 1991 (23.05.91)	US
763,039	20 September 1991 (20.09.91)	US

(60) Parent Applications or Grants
(63) Related by Continuation

US	704,814 (CIP)
Filed on	23 May 1991 (23.05.91)
US	763,039 (CIP)
Filed on	20 September 1991 (20.09.91)

(71)(72) Applicant and Inventor: MARTIN, William, John [AU/US]; 1634 Spruce Street, South Pasadena, CA 91030 (US).

(74) Agents: WARBURG, Richard, J. et al.; Lyon & Lyon, 611 West Sixth Street, 34th Floor, Los Angeles, CA 90017 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), BG, BR, CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC (European patent), MG, MW, NL (European patent), NO, PL, RO, RU, SD, SE (European patent), US.

Published
With international search report.

(54) Title: STEALTH VIRUS DETECTION IN THE CHRONIC FATIGUE SYNDROME

(57) Abstract

The present invention relates generally to methods for diagnosing chronic fatigue syndrome and certain other neurological, psychiatric, rheumatological and other stealth virus associated diseases in humans and in animals. Tissue culture and molecular probe based methods for the screening of stealth viral infection are described. The methods are applicable to the diagnosis of stealth virus infection in patients with chronic fatigue syndrome and with various atypical neurological, psychiatric, rheumatological, liver, testicular, salivary gland and other diseases. The methods are also applicable to the detection and the monitoring of naturally infected and experimentally infected animals. Isolates obtained by culture from infected human and animal sources can be used in the development and testing of therapeutic modalities to help in the treatment and prevention of spread of viral infection. The viral detection assays can be applied to the pre-clinical and clinical moinitoring of potential therapy and also to the detection of possible sources of infection, including human to human contact, blood products, domestic pets, farm animals, uncooked foods, vaccines and environmental sources. The isolates can also be used to improve upon the present detection methods, principally through the construction of synthetic antigens based upon the nucleotide sequences of the virus. Antigens produced either synthetically or by recombinant DNA technology, can be used as vaccines to prevent infection and as reagents to monitor immunological responses. A toxin associated with stealth virus, an antiviral composition comprising the toxin, and methods of monitoring disease state based on detecting the level of toxin or its toxic activity are described.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		
ES	Spain	MG	Madagascar		

DESCRIPTION

Stealth Virus Detection in the Chronic Fatigue Syndrome

This application is a continuation-in-part of copending United States patent applications Serial No. 07/704,814, filed May 23, 1991; and Serial No. 07/763,039, filed September 20, 1991 and which were entitled "Spumavirus detection in the chronic fatigue syndrome". These prior submissions are incorporated by reference in their entirety herein.

1. Field of Invention

The present invention relates generally to methods for diagnosing the presence of a specific type of virus (termed stealth viruses) in patients with symptoms associated with the chronic fatigue syndrome (CFS) and with various neurological, neuromuscular, rheumatological, psychiatric, auto-immune and liver diseases. More particularly, it relates to the detection of disease-associated stealth viruses, by tissue culture and by molecular probe and immunological techniques, in blood and other samples derived from patients and from animals. It also relates to the detection of these viruses in potential sources of human and animal infections, including blood transfusions, unpasteurized milk, undercooked or uncooked meat and seafood, viral vaccines produced in tissue cell lines, domestic pets, farm animals and environmental sources. The present invention is based on the discovery that stealth viruses can be isolated and cultured in vitro from a number of patients with CFS and from some patients with more severe neurological, neuromuscular, psychiatric, liver and auto-immune diseases. Infection by stealth viruses in these patients can also be demonstrated using molecular probe assays. Virus can also be isolated from domestic pets, including the pets belonging to viral infected

patients. Virus cultivation is enhanced by reducing the level of a virus infected cell derived toxin which otherwise acts to suppress the in vitro development of the virus. The stealth viral isolates can be further characterized using molecular techniques. The isolates and the viral infected animals can be used to help develop and evaluate antiviral therapies including the development of chemotherapeutic agents and anti-stealth viral vaccine.

2. Background of the Invention

The chronic fatigue syndrome (CFS) refers to an illness whose major characteristic is an unexplained fatigue lasting beyond 6 months which results in greater than 50% reduction in an individual's normal level of activity (Holmes GP et al., 1988, "Chronic fatigue syndrome: A working case definition," Ann. Intern. Med. 108:387-389; Holmes GP, 1991 "Defining the chronic fatigue syndrome". Rev. Inf. Dis. 13 (Suppl. I): S53-5. Shafan SD. 1991. "The chronic fatigue syndrome". Am. J. Med. 90: 731-7). To establish a clinical diagnosis, the patients should show evidence of suffering at least eight of the following minor symptoms: fever, sore throat, myalgia, muscle weakness (which may be exacerbated by exercise), arthralgia, lymphadenopathy, sleep disturbance, headaches, acute or subacute onset, and neuropsychological symptoms. In many patients, the neuropsychological symptoms are the most disabling. They include a difficulty in thinking, dysnomia, confusion, forgetfulness, irritability, depression, photophobia and transient visual scotomata. Because of evidence of immune dysregulation, this syndrome has also been referred to as chronic fatigue immune dysregulation syndrome (CFIDS). A term in widespread use in England and Australia, is myalgic encephalomyelitis (ME). Other terms include epidemic diencephalomyelitis, epidemic neuromyasthenia, post-viral fatigue syndrome and chronic Epstein-Barr virus syndrome.

The subjective nature of many of the clinical criteria on which the diagnosis of CFS is made, and the overlap of many of the symptoms with those associated with common neurological, rheumatological, endocrinological and psychosomatic (non-organic) syndromes, including such illnesses as multiple sclerosis, systemic lupus erythematosus, fibromyalgia, hypothyroidism, reactive depression, psychiatric disease, etc., has resulted in considerable uncertainty in making the diagnosis and controversy as to even the existence of this syndrome. (Shepherd, 1989 "Myalgic encephalomyelitis-is it a real disease?" Practitioner 233:41-46; Kendall, 1991, "Chronic fatigue, viruses and depression," Lancet 337:160-161; Palca J. 1991. "On the track of an elusive disease," Science 254; 1726-1728.)

Various hypotheses have been suggested as to the cause of the chronic fatigue syndrome. These can be broadly categorized as i) a primary metabolic dysfunction for example due to underproduction of a specific hormone such as corticotrophin releasing factor (CRF) (Demitrack MA, Dale JK, Straus SE, et al., 1991. "Evidence for impaired activation of the hypothalamic-pituitary-adrenal axis in patients with chronic fatigue syndrome". J. Clin. Endocrin. Met. 73; 1224-1234); ii) a secondary metabolic dysfunction due to the overproduction of cytokines resulting from immune overactivity to various antigens including viral antigens, bacterial antigens, fungal antigens and autoantigens (Landry Al, Jessop, C, Jennette ET, et al., 1991. "Chronic fatigue syndrome: clinical condition associated with immune activation". Lancet 338; 707-712; Chao CC, Janoff EN, Hu SX, et al., 1991. "Altered cytokine release in peripheral blood mononuclear cell culture from patients with the chronic fatigue syndrome". Cytokine 3; 297-298); and iii) a specific infectious disease which may directly involve the brain and, possibly also the peripheral muscles.

Serological assays and, more recently, studies using the polymerase chain reaction (PCR), have provided some support for chronic persistent viral infection in patients diagnosed as having CFS. Suggested pathogens have included Epstein-Barr virus (EBV), human herpesvirus-6 (HHV-6), human enteroviruses and human T cell lymphocytotropic virus type II (HTLV-II) related virus. (e.g., Schooley, 1989, "Chronic fatigue syndrome: A manifestation of Epstein-Barr virus infection," Curr. Topics Inf. Dis. 126-146; Buchwald D, Cheney PR, Peterson DL, et al., 1992 "A chronic illness characterized by fatigue, neurologic and immunologic disorders, and active human herpesvirus type-6 infection." Ann. Int. Med. 116: 103-113, Archard et al., 1988, "Postviral fatigue syndrome: persistence of enterovirus RNA in muscle and elevated creatine kinase," J. Roy. Soc. Med. 81:326-329; Palca J, 1990, "Does a retrovirus explain fatigue syndrome puzzle?" Science 249:1240-1242; De Freitas E, et al., 1991. "Retroviral sequences related to human T lymphocytotropic virus type II in patients with chronic fatigue immune dysfunction syndrome," Proc. Nat'l. Acad. Sci. U.S.A. 88: 2922-2926). More detailed serological studies in which CFS patients have been compared with control patients have failed to confirm a consistent and/or a significant association between CFS and infection with several of these suggested pathogens and have helped to exclude other potential pathogens (e.g., Dale et al., 1988, "The Inoue-Melnick virus, human herpesvirus type 6 and the chronic fatigue syndrome," Ann. Int. Med. 110:92-93; Dale et al., 1991 "Chronic fatigue syndrome. Lack of association with hepatitis C virus infection". J. Med Virol. 34; 119-121). Similarly, published data based on the PCR assay have been difficult to replicate in different laboratories. Even if an association were to be found, the issue of whether the viral infection actually caused the illness or was merely a secondary coincidental manifestation occurring, for example, as a result of an underlying immune dysfunction,

would remain uncertain (Lloyd A, Hickie C, Dwyer C. et al., 1992. "Cell mediated immunity in patients with chronic fatigue syndrome, healthy controls and patients with major depression Clin. Exp. Immunol. 87; 76-79).

Although, the majority of specialist clinicians probably doubt the existence of the chronic fatigue syndrome, they are frequently faced with patients who appear to have an organic illness with features that overlap some of those described for the chronic fatigue syndrome. Depending upon the sub-specialty interest of the clinician, these patients may be classified as having an atypical neurological, psychiatric, rheumatological, infectious or otherwise unspecified atypical disease. Laboratory testing is generally unremarkable and both the clinician and the patient become frustrated with the lack of a confident diagnosis on which to suggest appropriate therapy. Alternatively, the patients may be classified as having one or more of the generally established disease categories such as multiple sclerosis, lupus erythematosus, post viral encephalopathy, depression, manic-depressive disorder, schizophrenia, migraine, hepatitis, pelvic inflammatory disease, etc., etc. Less widely accepted diagnoses may also be applied such as "sick building syndrome", "seasonal affective disorder"; exposure to environmental toxins, chronic candidiasis, chronic ciguatera poisoning, etc.

Uncertainty with the diagnoses of human diseases also extends to the diagnosis of veterinary illnesses. Many CFS patients remark that their pets appear to suffer from lethargy which may be accompanied by balance difficulties, mood swings and even seizures. Other patients from rural areas have cited an association of their illness with an apparent infectious disease among farm animals. In both situations, the veterinarians usually cannot offer a diagnosis, even after the animals have been autopsied. Still, patients have wondered if an animal infection such as African Swine Fever virus could be involved in human CFS

(Ostrom N. 1991, "What really killed Gilder Radner? Frontline reports on the chronic fatigue syndrome epidemic". That New Magazine, Inc., P.O. Box 1475 Church Street Station New York, NY 10010 Lib. Congress 90-70516). Similarly, the issue of potential contamination of viral vaccines produced in animal cell lines has been considered as a possible cause of human CFS.

The infectious agents described in this patent application have cleverly avoided being detected in prior studies by other investigators. This is mainly because the type of virus discovered does not evoke the usual inflammatory response that normally alerts the clinician to the presence of an infectious agent. Nor is it easy to culture the agent without an awareness of the production of a viral growth inhibitory toxic factor which suppresses in vitro viral growth. Because of these features, I have designated the viruses described in this application as stealth viruses. At present this term is preferable to foamy cell inducing virus, spumavirus (spuma=Latin term for foam), atypical herpesvirus, chimeric virus, etc., which relate to some but not all features of the virus and which tend to have restrictive connotations not meant to be implied in the present application.

In addition to assisting with human and veterinary diagnostic medicine and with the development and monitoring of the efficacy of specific therapy; the discovery of stealth viruses has important Public Health implications in preventing disease transmission. The virus is resilient to freezing and can survive simple drying. Potential modes of disease transmission could include both direct and indirect human-human and human-animal contacts, ingestion of foods contaminated with stealth viruses, inoculation of vaccines containing these viruses, etc. These topics are covered in this application.

3. Summary of Invention

The present invention provides methods for diagnosing stealth virus infection in patients with symptoms associated with the chronic fatigue syndrome and with atypical neurological, psychiatric, rheumatological and various other illnesses due to infection with this type of virus. In particular, the present invention enables definitive diagnostic assays for detecting the presence of stealth virus in a specimen from a patient being considered as possibly having various types of illnesses, including those listed above. The diagnosis of stealth virus infection can also be made in animals. The presence of stealth virus in human and animal samples can be detected by in vitro culture assay, molecular probes and immunoassay techniques. Stealth virus can also be detected in foods, including unpasteurized milk, undercooked or uncooked meat or seafoods. Virus may also be detected in blood transfusion products and in viral vaccines produced in primary cell cultures. Diagnostic kits for detecting disease-associated stealth virus are also provided.

The present invention further provides an isolated stealth virus which has been associated with a patient with the chronic fatigue syndrome. It provides methods of culturing disease-associated stealth virus by removing and/or inhibiting the activity of a stealth virus-associated toxin which normally suppresses the in vitro expression of the virus. The invention provides nucleic acids sequence encoding stealth virus proteins, and stealth virus-specific molecular probes. Stealth viral protein antigens are also provided. Such protein antigens are used to develop anti-stealth viral vaccines and immunological diagnostic probes for detecting the presence of virus. in vitro cultures and animals either naturally infected with or experimentally inoculated with stealth viruses, provide further assays for evaluation of potential anti-stealth viral therapeutic agents. The present

invention provides therapeutic agents and methods for treatment of stealth virus infection.

A toxin associated with stealth virus infection is also provided. In one instance, toxin may be provided therapeutically, at sub-toxic levels, to suppress an acute viral infection. In another embodiment, chronic stealth virus-associated toxic activity may be inhibited, thereby alleviating symptoms associated with stealth virus-infection. The invention provides a method and a kit for detection of the level of toxin in a patient, which can be used diagnostically to detect the presence of a stealth virus or to monitor disease progress. The similarity in antigenicity and biological activities of stealth virus-associated toxin and a polyether toxin such as ciguatera toxin, suggests a relationship between stealth viral infection and the chronic fatigue-like illness which occasionally follows an acute ciguatera toxin-associated food poisoning. Common diagnostic methods and common therapies for both polyether marine toxin poisoning and stealth viral infection are provided.

4. Description of the Figures

Figure 1. Dot blot hybridization assay showing reactivity of PCR amplified products with a 32P-labeled probe representing a region of the HTLV-I tax gene. The sequences of the primers used were based on those used to amplify the tax genes of HTLV-I and HTLV-II (Section 7.1.). Dot blot positions A and B are positive controls in which DNA from HTLV-I infected cells was used in the PCR. Position C is supernatant and D is the cell pellet from the patient's CSF. Control dots are E (negative DNA) and F (reagent control). Exposure time was 14 hours. This sample was obtained from a young adult (initials P.M.) with suspected herpes encephalitis.

Figure 2. Dot blot hybridization assay as described in Figure 1. Positions A6 to F6 are positive controls, corresponding to A and B in Figure 1. Position A2 is a

hybridization control in which previously amplified products were blotted. Positions G6 is CSF cell pellet undiluted; H6 is cell pellet diluted 1/10. Position E7 is CSF supernatant; F7 is supernatant diluted 1/10. G7 is negative DNA and H7 is distilled water. The sample was obtained from a six-month old baby (initials B.U.) with unexplained encephalopathy.

Figure 3. Dot blot hybridization assay showing reactivity of PCR products from five patients diagnosed with CFS. The hybridization was performed as described in Figure 1. Position A is an HTLV-positive control; positions B, C, D, E and F are patient samples. All five patients tested were sero-negative for HTLV. Positions G and H are negative controls corresponding to G and H in Figure 2.

Figure 4. Photomicrograph of MRC-5 early cytopathic effect in cells infected with stealth virus-X, after a few days in culture. The cells were cultured with virus-X after the virus had been passed in vitro. Note the intranuclear inclusions and intracytoplasmic vacuoles.

Figure 5. Photomicrograph of the MRC-5 cells in Figure 4 at a more advanced stage of the CPE. Note the large "foamy cell" syncytia.

Figure 5'. A. Normal MRC-5 cells stained with hematoxylin and eosin. Note the orderly formation of the elongated cells. B. Phase contrast of MRC-5 cells demonstrating the typical rounding and clumping of cells. The adjacent cells show evidence of toxicity. C. Hematoxylin and eosin stained of a "foamy" cell present in a virus infected MRC-5 culture. Note the multiple nuclei and vacuolated cytoplasm.

Figure 6. Electron micrograph of MRC-5 cells infected with the virus isolated from D.W., a patient with CFS. Enveloped viral particles with spherical capsids are conspicuously seen within cytoplasmic vacuoles. Immature viral particles are also present within the nucleus and elsewhere in the cytoplasm. Magnification x 5700.

Figure 7. Enlarged view of the intracytoplasmic vacuoles containing virus in Figure 6. Note also the dense inclusions in the vacuoles and the cytoplasm.

Figure 7A. Electron micrograph of viral infected cells obtained from an independently derived culture from patient D.W. The region selected demonstrates the appearance of incompletely formed (or more likely, partially degraded) virions in cell cultures. These virions either lack a central nucleocapsid core, or the core region appears fragmented. In addition, there is often a conspicuous lack of a coating (tegument) around the region of the core.

Figure 8. Ethidium bromide-stained agarose gel of the PCR product of virus-X. The PCR method is described in Section 6.1. A. Lane 1 -- PCR product; lane 2 -- positive control HTLV-I product; lanes 3 and 4 -- size markers (MspI pBR322 in lane 3, Hind III - digested k DNA in lane 4). B. Gel A after the X-virus product bands have been cut out.

Figure 9. Dot blot hybridization assays with PCR product as probe. The 1.5 kb product of the PCR reaction on culture containing virus from patient D.W. was excised from an agarose gel and ^{32}P labeled. This labeled product was used to probe DNA extracted from the following sources: Stealth virus culture from patient D.W. in position A1. Stealth virus culture from patient B.H. B1. CMV culture C1. Positions E2 and F2 are blots of PCR products previously amplified from cultures of patients D.W., and B.H., respectively. H2 contain an extract from control uninfected cells.

Figure 10. Southern blot hybridization of uncut and EcoRI digested DNA from infected and uninfected cultures. The right panel shows ethidium bromide stained gel of uncut DNA from infected culture (lane 1, second from right next to lane showing DNA size markers), and from uninfected culture (lane 2). The respective smear patterns showing completer EcoRI digestion are shown in lanes 3 and

4 of the right panel. Following transfer to nitrocellulose, the DNA was hybridized with ^{32}P-labeled PCR product of 1.5 kbp generated from the stealth virus culture. Hybridization is seen only to DNA from infected culture. The EcoRI digested DNA gave 3 bands corresponding to 9-10 kbp, 2.5-3.5 kbp and 1.5 kbp.

Figure 11. Result of Southern blot hybridization performed on PCR products from six independently derived stealth virus infected cultures (positions 7-12) obtained from patient D.W. The primer set and detecting probe was based on the sequences shown in Table 2 which are described in an Example included in Section 9. Well number 4 contains DNA extracted from the plasmid 15-5-4 used for sequencing.

Figure 12. Result of Southern blot hybridization performed on PCR products from seven blood samples obtained from patient D.W. Each independently obtained blood sample gave a strong positive response. The blood samples had been processed for culture as described in Section 8. As described in the Example, only 0.6 of 1.0 ml of the combined granulocyte/lymphocyte fraction are normally used for culture. The remaining material was stored at -80°C for 2-18 months before being tested in this experiment. Negative PCR results were obtained in identically processed and stored blood samples from normal individuals. The primer set and detecting probe was based on the sequences shown in Table 2 which are described in an Example included in Section 9.

Figure 13. Electron micrograph of foamy cell syncytia developing in a culture from a CFS patient. Although viral particles are not present, the CPE shows the marked changes characteristic of stealth virus infection. Upon further passage, this culture did show the production of viral particles.

Figure 14. Electron micrograph of MRC-5 cells infected with stealth virus from patent B.H. Note the similarity to Figure 6.

Figure 15. Electron micrograph of cellular material derived in a fine needle aspirate (FNA) of the submandibular gland of patient G.P.

Figure 16. Higher power electron micrograph of the cell shown in Figure 15. Viral like particles and disrupted mitochondria can be identified.

Figure 17. Electron micrograph of MRC-5 cells infected with stealth virus from CFS patent L.B. Note the similarity to the viruses found in patients D.W. and B.H. in Figures 6 and 14.

5. Detailed Description of the Invention

The present invention provides methods for diagnosis of stealth virus infection in patients, animals, foods and biological products. Patients with stealth virus infection may present with symptoms associated with the chronic fatigue syndrome or with symptoms associated with various neurological, neuromuscular, psychiatric, rheumatological, auto-immune, glandular and liver diseases. In addition to disease diagnosis, the invention is useful for prevention of disease transmission by identifying potential sources of infection. Such potential sources include unpasteurized milk, undercooked or uncooked meat or seafoods, viral vaccines produced in primary cell cultures, farm animals, household pets an environmental objects. Furthermore, the present invention provides for development and preclinical evaluation of anti-viral therapy using viral isolates from patients, and development of anti-stealth viral vaccines in human and animal hosts. Therapeutic and diagnostic products for treatment of patients and animals with stealth virus infection are also provided.

The present invention further relates to the recognition of a toxin associated with stealth virus infection, herein referred to as a "stealth virus-associated toxin" or "toxin." As used herein the term "stealth virus-associated toxin" or "toxin" refers to the molecu-

lar entity (or entities) that mediates the toxic activity observed in stealth virus cultures in vitro, and which appears capable of suppressing stealth virus growth. The toxic activity is detectable in serum and cerebrospinal fluid from stealth virus infected patients and may mediate certain symptoms associated with the patient's illness. Use of agents that neutralize toxic activity of the toxin and methods of inhibiting its toxic activity provide methods for improved in vitro culture of virus by neutralization of the toxin, and therapy for toxicity associated with viral infection. Agents to detect the presence of toxic activity are also provided. Methods of diagnosis and monitoring of stealth virus infection in vivo which are based on detection of toxin, and kits therefore, are provided.

The present invention is based on the discovery that stealth viruses can be isolated from a significant number of patients with CFS and can account for many of the symptoms associated with this disease entity. Stealth viruses can also be isolated from patients diagnosed as having more severe neurological illnesses, including encephalopathies and multiple sclerosis; psychiatric diseases, including schizophrenia and manic depressive illnesses; various neuromuscular and rheumatological diseases including fibromyalgia; auto-immune diseases including systemic lupus erythematosus; chronic pain syndromes; migraine headaches; depression; parotid and testicular swelling; fatty changes in the liver; and more. Until this discovery, the diagnosis of and understanding the pathogenesis of stealth virus infection in these patients had posed considerable difficulties, especially in distinguishing whether the illnesses were, in fact, due to viral infection or due to psychosomatic (nonorganic) or non-infectious causes. Stealth viral infected patients have not been able to benefit from therapy directed at the cause of their illness. There was no approach to preventing disease transmission. The

discovery of the association of stealth viral infection with the chronic fatigue syndrome and with other disease entities has made it possible to more confidently diagnose these illness; to develop and target therapy directly aimed at eradication the underlying stealth viral infection; and to help prevent disease transmission by identifying potential sources of infection and by developing a vaccine for both human and animal use. The additional discovery of stealth viral infection in symptomatic animals and evidence for viral infections among members of the same family highlight the need for specific diagnostic, therapeutic and disease prevention efforts against this class of virus as provided herein.

As used herein, the term "atypical neurological disease" include a variety of illnesses that affect cognitive or ether neurological functions which appear outside of the range of the normally accepted major neurological disease categories such as Alzheimer's disease, multiple sclerosis, herpesviral and enteroviral encephalopathies, AIDS dementia complex, acute and chronic drug toxicity, and others. "Atypical psychiatric disease" include depression especially when accompanied with evidence of cognitive disorder, unexplained memory loss, certain cases of manic depressive illnesses and of schizophrenia in which the patients do not appear to conform to the generally accepted pattern of illness, attention deficit disorders and atypical autism in children, alcohol and substance abuse in which these habits appear to be secondary to an underlying sense of loss of control of mental function, and others. "Atypical rheumatological diseases" include patients with features suggestive of systemic lupus erythematosus often with low titers of anti-nuclear antibodies, Sjogren's syndrome with salivary gland enlargement, polymyositis with painful atrophied muscles, fibromyalgia. Characteristically, the intensity of the illness will show significant fluctuations over time and when tissue biop-

sies are performed a striking lack of an inflammatory reaction will be noted. Other diseases when have been identified in stealth viral infected patients have, in addition to chronic fatigue syndrome, included migraine and cluster headaches and a well documented case of pseudotumor cerebri (headaches accompanied by a marked elevation in cerebrospinal fluid pressure). Examples of elevated liver transaminase enzymes suggestive of a chronic persistent or a chronic active hepatitis have been seen. On biopsy the liver may show evidence of fatty change and even Mallory bodies which are generally considered a marker of alcoholic hepatitis. The inflammatory reaction of viral hepatitis due to hepatitis B virus or hepatitis C virus is normally not seen. Pelvic and testicular chronic pain syndromes have been noted. It should be noted that with many of these atypical diseases, the interpretation of clinical and usual laboratory parameters varies among different clinicians and it is not always clear to the clinician if the patient has a typical or an atypical illness. The ability to detect stealth virus infection should help resolve many of these diagnostic uncertainties. As shown in the Example sections, infra, patients mis-diagnosed with many of the above listed illnesses have, in fact, an active stealth virus infection.

It is to be understood that symptoms that indicate a particular disease may mask a secondary concurrent illness. Additionally, certain diseases create conditions for opportunistic infection by other infectious organisms. For example, patients with AIDS, congenital immunodeficiency, or undergoing immunosuppressive therapy may suffer a multitude of opportunistic infections, including stealth virus infections. Thus, the present invention is directed to diagnosis and treatment of stealth virus infection in a wide range of diseases, and is not intended to be limited to any particular disease, or to be excluded in patients with other known diseases.

Stealth virus infection is to be viewed as a primary diagnosis in a patient. The actual clinical manifestation of the infection can differ in different patients and the diagnosis in any particular patient nay be qualified by referring to the symptoms exhibited by the particular patient. Since therapy and disease prevention relate directly to the detection of the stealth virus, a clinically convenient term has been introduced to cover the multiple clinical expressions seen in viral infected patients. This term is Multisystem stealth virus infection (MSVI).

The above consideration of the clinical spectrum of human diseases associated with stealth virus infection in humans apply equally well to animals.

As used herein, the term "stealth virus" is intended to include a virus having one or more of the following properties: i) Ability to induce a cytopathic effect in fibroblast cultures which is characterized by the production of foamy appearing cells, including cell syncytia. The cytopathic effect can be readily distinguished from that of other types of viruses by criteria listed below; ii) Production of a toxin capable of suppressing viral growth; iii) Production of lipid-like material; iv) Growth in cells from a wide range of species; v) Characteristic electron micrograph appearance, including the presence of viral particles in the nucleus, cytoplasm and cytoplasmic vacuoles; vi) Presence of inclusion bodies seen in association with the intracytoplasmic vacuoles; vii) Evidence on electron microscopy of viral particles showing signs of degradation; viii) Lack of staining of viral infected cells using typing antisera specific for cytomegalovirus, herpes simplex virus, human herpesvirus-6, varicella zoster virus and Epstein-Barr virus; ix) Positive staining of viral infected cells using Lendrum stain; x) Positive staining with sera from most normal individuals and from most, but not all, patients diagnosed as having

stealth virus infection; xi) Amplification using the polymerase chain reactiOn (PCR) of a region within some stealth viral isolates using single HTLV tax gene reactive primers. The amplified region is not the HTLV tax gene as shown by differences in molecular weight; xii) Amplification of a region of the viral genome with primers designed to amplify viruses other than stealth viruses. The distinction between the stealth virus and the virus against which the primers were designed is based on differences in the molecular size and the sequence of the PCR products generated by the PCR; xiii) Lack of hybridization with nucleic acids probes reactive with HTLV, cytomegalovirus, herpes simplex virus, human herpesvirus-6, varicella zoster virus and Epstein-Barr viruses using stringent hybridization condition; xiv) Ability to infect and to produce disease in both humans and animals, including but not limited to neurological disease; xv) A lack of an inflammatory response in viral infected tissues. These characteristics, which are discussed more fully, infra, are indicative of a stealth virus, and so that name is adopted for the purposes of this invention. However, the invention is not intended to be limited in any way by the choice of the term stealth virus for the virus described herein. Indeed, a superior term may be suggested once additional characterization of the viruses has been achieved using methods described in this application.

5.1 Isolation and In Vitro Culture of Stealth Viruses

Stealth viruses may be isolated by tissue culture from a blood sample obtained from a subject with symptoms associated with the chronic fatigue syndrome or with various atypical neurological psychiatric, rheumatological, glandular or liver diseases. Viruses may also be isolated from blood of animals infected with a stealth virus. In addition to blood, virus may be recovered from other biological samples including sur-

gical and fine needle aspiration tissue biopsies, post mortem organ biopsies, throat swabs and saliva, urine, cerebrospinal fluid (CSF), other body fluids, blood and blood products intended for transfusion or for in vitro uses, vaccines, foods and from the environment.

In a preferred embodiment, stealth virus is isolated from heparinized blood using a mixture of buffy coat granulocytes and ficoll-hypaque separated lymphocytes. In a particular embodiment described in detail in Section 8.2., infra, lymphocytes and granulocytes are obtained following a Ficoll-Hypaque density centrifugation of blood. Methods known in the art for viral isolation and propagation can be used. In a specific embodiment, isolation of stealth virus from blood and CSF is carried out as described as follows.

A permissive cell line is inoculated with a sample being tested for the presence of a stealth virus. Using appropriate conditions, stealth viruses infect and propagate in many mammalian cells in vitro, especially cultures of fibroblasts and other cell lines of mesodermal and neuroectodermal origin, e.g., primary kidney cell cultures, glial cells, myoblasts, lymphoid cells, etc. The distinctive CPE is, however, readily observed in primary fibroblast cultures. At present, therefore, these are the preferred cell lines to isolate the virus from clinical and animal samples. Suitable cells include human MRC-5 lung fibroblasts, primary human foreskin fibroblasts MRHF and rhesus monkey kidney RMK cells. These well known cell lines are available commercially (e.g., from Whittaker Bioproducts, Walkersville, MD). Preferably, all three cell lines are inoculated with a sample isolated from a patient or an animal. This helps ensures that a particular viral isolate will not be lost due to incompatibility with a particular cell line. Similarly, it should be noted that not all cell lines are permissive for growth and/or for the expression of a cytopathic effect (CPE). Conversely,

other cell lines may be more efficient in promoting the growth of stealth viruses than the fibroblast cell lines currently used. The tissue culture cell lines are maintained in the laboratory using methods well known in the art.

Stealth virus isolates may be cultured by inoculating and culturing permissive cells in vitro using any methods known in the art for virus cultures. In one embodiment, culture tubes containing MRC-5, MRHF and RMK cells are each inoculated with a cellular mixture of the buffy coat granulocytes and ficoll-hypaque separated lymphocytes derived from approximately the equivalent of one milliliter (ml) of heparinized blood. Typically, 5 mls of blood collected into a "green top" heparinized tube, are layered onto 3 mls of a ficoll-hypaque lymphocyte separation medium. Following 20 minutes centrifugation at 1,500 rpm, the lymphocyte, which collect at the plasma : ficoll hypaque interface, and the "buffy coat", present on the top of the erythrocyte layer, are collected into approximately 1 ml. An effort is made to minimize the number of erythrocytes in collecting all of the visible buffy coat. The cell mixture is washed once in 1 ml of 199 medium containing 7% FCS and resuspended into 1 ml. Aliquots of 0.2 ml of the cells are added to cell culture tubes containing 1 ml of 199 medium plus FCS. The tubes are placed in an incubator at 37°C for 45-60 minutes. The tubes are then rinsed to remove macroscopically visible erythrocytes and other non-adherent cells. Rinsing (washing) consists of emptying the fluid content of the culture tube by decanting or by aspiration; adding 2 mls of medium or phosphate buffer saline; rocking the tube for several seconds to suspend erythrocytes; and decanting the tube again. This important step is performed 2-3 times or until there are no macroscopically visible erythrocytes. Two mls of medium with 7% FCS, are added and the tubes returned to a 37°C incubator. Cultures are maintained in the incubator at

37°C, with refeeding (replacement of old medium with new medium) at 24, 48 and 72 hours. The tubes are examined microscopically after the 24 hour refeeding and, if residual erythrocytes are present, the tubes are rinsed in a manner similar to that performed at the 45-60 min. step. The tubes are refed three times each week by replacing the old medium with 2 mls of fresh medium. This procedure is designed to reduce the accumulation of a toxic component in the culture medium which tends to suppress viral growth. The tubes are examined three times per week for evidence of cytopathic effect (CPE). CPE is generally recognizable between 2-3 weeks after culturing.

The major criterion for recording a positive CPE is the appearance of rounded, slightly enlarged, refractile cells throughout the culture. In about one-quarter of affected cultures, the CPE progresses to very prominent collections of tightly packed, enlarged, foamy-cell appearing cells, with clearly defined cell syncytia and evidence of considerable cell destruction. The time to initial development of the characteristic CPE has become progressively shorter as culture methods have improved. With the current technique of daily or every second day refeeding of the cultures with medium 199 containing 7% FCS, the initial CPE is generally detectable between 10-20 days and shows progression over the next several days.

As stated above, the typical initial cytopathic effect (CPE), is the appearance of scattered, rounded refractile cells which are easily distinguished from the elongated fibroblasts. The affected cells, however, are clearly of fibroblast origin and stages between the spindle shape of the normal fibroblasts and the rounded appearance of affected cells can be seen. The nucleus is not noticeably enlarged and the cytoplasm remains readily visible. Several inclusions, consistent with vacuoles, can been seen within the cytoplasm. As their

numbers increase, affected cells form several tightly adherent clumps with indistinct cell boundaries. The affected cells continue to proliferate and scatter away from the cell clumps. This minimal level of CPE has to be transferable to secondary cultures for the culture to be recorded as a true positive (+).

A +/- (plus/minus) or equivocal effect refers to a minimal change in the culture in which the rounded cells are either not enlarged or non-refractile (possibly dead). Less than 4% of cultures from CFS patients have been recorded as equivocal, either because the CPE has been restricted to only a small region of the culture, or because it has failed to recur on secondary passage. These cultures have been excluded from analysis.

A ++ (two plus) strong positive response is recorded when vacuoles are clearly identified within enlarged, rounded, refractile cells and/or when multiple foci of cell clumping and/or destruction are apparent which are ringed by cells described as having a positive effect. A +++ (three plus) very strong positive effect refers to extensive ++ cellular changes throughout much of the culture and/or the appearance of large refractile vacuolated, foamy syncytial cell formation. A benefit of using multiple indicator cell lines, is that the CPE appearances in the different cell lines sometime complement each other. For example, cell syncytia are usually best observed in monkey kidney cells. The smaller rounded refractile cells are usually best observed in the human fibroblasts. In over 80% of positive cultures, the CPE is clearly observable in at least 2 of the 3 cell lines. As with any viral induced CPE, it is important to confirm transmission to fresh cultures. In recipient cultures, the stealth virus induced CPE generally develops more rapidly than in the primary culture with at least the same level of intensity.

Although the appearances of the CPE share some features in common with those associated with cytomegalo-

virus (CMV) and herpes simplex virus (HSV) infection, stealth virus cultures can be readily distinguished from cultures harboring these viruses. The CPE from HSV is much more rapid, occurring in a matter of days. The destruction is greater with large masses of dead cells. The CPE from CMV tends to initially develop in smaller, looser clusters and evolves more slowly than observed with stealth viruses. The intracytoplasmic vacuolization and syncytia formation are far less prominent with CMV than with stealth viruses. The stealth virus infected cells give more the appearance of "foamy cells" than does CMV. Indeed, with some stealth viral cultures, there appears to be a marked accumulation of lipid-like material attaching itself to the wall of the culture tube. Human CMV does not infect monkey derived cells. Both CMV and HSV infected cultures will stain specifically using typing antisera specific for these viruses, whereas stealth virus infected cells do not stain with these reagents. In my hands, human herpesvirus-6 does not induce any CPE in either human or monkey fibroblasts.

It should be noted that a lack of regular refeeding of the cultures can result in a tendency for the CPE to abort and not to progress. This effect is not seen with CMV infected cultures. In unfed stealth virus cultures and even with regular refeeding, one can observe a toxic effect on many of the remaining cells. Culture cells appear to lose a degree of vitality and become duller in appearance compared to control cultures. The fibroblasts can assume somewhat of a pavement appearance, instead of the elongated shape. Some of these changes can be mimicked using 5 nM of the polyether marine toxin okadaic acid (Cohen P, Holmes CF, Tsukitani Y. Okadaic acid: A new probe for the study of cellular regulation. Trends Biochem. Sci. 15: 98-102, 1990). CMV positive cultures do not demonstrate the toxic activity such as that observed with stealth viruses. Moreover, the

detection of CPE from CMV is readily seen in cultures containing only minimal essential medium and 2% FCS. This is the routine medium used in most virology laboratories and can be contrasted with the more enriched medium 199 and 7% FCS which I use to culture stealth viruses. The more enriched medium and the higher concentration of FCS, help to neutralize the toxic, stealth virus growth inhibiting effects, which would otherwise occur in the cultures. Photomicrographs demonstrating the typical CPE and signs of toxicity are shown in the Figures.

There is a degree of variability between the isolates from different patients. The intensity of the CPE for the three commonly used cell types can vary. The tendency to form cell syncytia and the size of the syncytia varies. It can be helpful to stain culture derived cells using either hematoxylin and eosin or special stains, including Lendrum, periodic acid schiff (PAS), oil red O, and trichrome. Infected cells display a foamy cell appearance with evidence of intracellular vacuolization and inclusions. Furthermore, one can prepare cell pellets for examination by electron microscopy. Cell syncytia and intracytoplasmic vacuolization can be readily screened for using electron microscopy. Intranuclear and intracytoplasmic viral particles may be visualized in cells from cultures with well established CPE.

Although presumptive of stealth virus infection, the CPE appearance may require additional confirmation for a definitive conclusion of stealth virus infection. As noted above, the infected cells can be stained with hematoxylin and eosin or other special stains. At least some cultures from patients will stain in immunofluorescence assays with many normal human sera at a dilution of 1:100. Infected cells can also be examined by electron microscopy.

(e.g., Figures 7-9). In another embodiment, molecular probe based hybridization assays on nucleic acid from infected cultures can be used to confirm the presence of stealth virus and/or to exclude the possible mis-identity with other types of viruses e.g. CMV, HSV, adenoviruses, etc. In using this approach, however, the molecular heterogeneity between different stealth virus isolates (see infra) will need to be considered.

A similar culture procedure, with or without additional confirmatory analyses, would be used to isolate stealth viruses from human CSF, throat swabs, urine, surgically removed and fine needle aspirated tissue samples, blood products and vaccines.

Successful stealth viral cultures from cats have been established on human fibroblast cells, and a human derived stealth virus has grown on a cat fibroblast cell line. As experience is gained, it may be more appropriate to include species related fibroblasts in attempts to culture viruses from non-human species. Fish cells for example, will require media with a different tonicity than mammalian cell cultures.

5.2 Cloning and Sequencing of Recombinant Stealth Virus DNA

Stealth viruses can be detected and their nucleotide sequence determined using molecular probe techniques. Because these viruses do not normally evoke an in vivo inflammatory response, they can easily go undetected in infected individuals. Similarly, even with the culture techniques described supra, it is difficult to obtain high yields of virus for conventional biochemical assays. For these reasons, the detection and characterization of stealth viruses are particularly suited for sensitive molecular probe based assays, including the polymerase chain reaction (PCR). In one embodiment described in detail in Sections 7-9 (infra), blood and the subsequent stealth virus culture from the patient

D.W., from whom the initial isolate of a stealth virus was achieved, showed a positive PCR assay, whereas control normal individuals and uninfected cultures tested negative. The PCR primers used in the initial molecular characterization of a stealth virus are not necessarily specific for the stealth virus. Rather the primer reactivity with a stealth viral DNA can be the result of cross priming. This can be explained because of the relatively low stringency conditions used in the PCR and in the hybridization assays. When applied to virus infected cultures, this low stringency PCR approach can allow the amplification and subsequent cloning and sequencing of a region of the stealth virus genome. In a preferred embodiment, cloning of PCR products can be achieved as follows: The PCR products generated in stealth virus infected cultures are blunt-ended, phosphorylated and cloned into pBluescript phagemid (pBluescript SK II [+] from Stratagene, La Jolla, CA) as follows: After completing the PCR, 2 unit of Klenow enzyme and 1 μM of each dNTP are added to the reaction mixture. After 30 minutes incubation at 14°C, the DNA is extracted using 100 μL of phenol and of chloroform. The DNA is precipitated using ethanol in the presence of KOAc and glycogen, washed once in ethanol and dried. The 5' end is phosphorylated using T4 kinase and ATP. Following a 37°C incubation for 30 min., the kinase is inactivated by heating the mixture at 65°C for 10 min. The reaction products are run in 0.8% low melting point agarose and the band of interest excised. A T4 ligase reaction is performed using purified pBluescript vector, previously cut with EcoRV and treated with calf intestinal phosphatase. The ligation reaction is allowed to proceed during an overnight incubation at 14°C. The enzyme is inactivated by heat (65°C for 10 min.). Transformation into XL-1 competent cells is achieved by a 40 min. incubation on ice followed by a heat shock at 42°C for 90 sec. The bacteria are plated on LB agar

containing ampicillin, IPTG and X-gal. Colorless colonies are screened for an insert using the PCR product as probed and confirmed using the bacteria as template in the PCR. Sequencing of the insert can be performed using the dideoxy/deoxy nucleotide chain termination method of Sanger F. Milkin S. and Coulson AR, 1977, Proc. Natl. Acad. Sci. USA 74:5463-5467. Once the sequence has been determined, highly specific primer sets can be prepared which should give clearly positive PCR responses in blood samples from the patient but not in blood samples from normal individuals.

Thus in summary, to obtain sequence data on new stealth viral isolates, one can begin by testing various PCR primer sets using relatively low stringent conditions. Amplified products can be isolated and tested for specific reactivity with DNA derived from viral infected cultures, compared to uninfected cultures or cultures infected with non-stealth viruses. If the PCR product proves to react specifically with the stealth virus infected cultures, it may be used as a probe. Moreover, using generally accepted methods, including the method outlined above, the PCR product can be cloned and sequenced. Specific PCR assays can then be constructed. Furthermore, the cloned DNA can be used to develop molecular probes for in situ hybridization. Once cloned DNA is available, one can proceed to the cloning and sequencing of the entire genome of the viral isolate. A detailed example of this approach is provided in Section 9.

Reactivity with various primer sets provides a method to distinguish among different isolates of stealth viruses. For example, PCR performed using the SK43' and SK44" primers on a stealth virus culture obtained from a CFS patient L.B., required that both primers be used. A 600 bp product was cloned and sequenced. Analysis of the sequence showed the incorporation of the SK43' primer at one end and of the SK44"

primer at the other end. The intervening sequence showed no relationship to the sequence of other known viruses or to the amplified sequences obtained using PCR with these primers on the stealth virus culture from patient D.W. (see Example 13, infra).

On the other hand, PCR reactive pattern seen on the stealth virus culture from patient D.W., was similar to that found on the stealth virus culture from the patient B.H.

Since the general nature of the CPE is a common characteristic of stealth viruses, it is predicted that there will be a common genetic region. Individual isolates appear to have unique genetic regions. Sequencing of such regions, will allow for the development of isolate specific PCR assays. These assays will be extremely useful in tracing the epidemiological spread of infection between individuals and between humans and animals. PCR against a common region will enable detection of multiple isolates.

The sequences within the cloned plasmids can be used to construct PCR primers that will read out through the 5' or 3' ends of the cloned sequence. When used in various combinations, these primer sets will yield additional PCR products that will bridge the regions of the stealth viral DNA between the regions presently sequenced. These additional products can be cloned and sequenced. Using this approach, additional DNA sequence data will become available on the isolated stealth virus from patient D.W.

In another approach, plasmids containing cloned regions of the stealth virus from a patient can be used to select clones derived from a genomic library obtained from the infected cultures. Genomic libraries can be created in Lambda vectors and the resulting clones hybridized with radiolabeled cloned inserts obtained from the pBluescript vectors so far isolated. The

selected clones can be sequenced and additional PCR primer sets established.

Using these approaches, the entire sequence of the stealth virus from patient D.W. can be derived. Furthermore, the molecular techniques, beginning with low stringency PCR using various primer sets, including the SK43' and SK44", and the CMV and EBV reactive primer sets, can be applied to other viral isolates, to yield comprehensive sequence data on other isolates.

The cloned plasmids can also be used to generate transcripts for use in in situ hybridization assays on infected cells both in vitro and in vivo.

5.3 Expression of Recombinant Stealth Virus Proteins

In addition to expression in virus infected fibroblast cultures, the production of stealth virus proteins can be achieved by recombinant DNA technology. For example, appropriate stealth virus nucleotide coding sequences which have been cloned as described supra can be expressed in appropriate host cells. As shown in the Example, supra, the polymerase chain reaction (PCR) technique can be utilized to amplify a segment of stealth virus DNA for subsequent cloning and expression of stealth virus DNAs (e.g., see U.S. patent numbers 4,683,202; 4,683,195; 4,889,818; Gyllensten et al., 1988, Proc. Nat'l. Acad. Sci. U.S.A. 85: 7652-7656; Ochman et al., 1988, Genetics 120: 621-623; Triglia et al., 1988, Nucl. Acids. Res. 16: 8156; Frohman et al., 1988, Proc. Nat'l. Acad. Sci. U.S.A. 805: 8998-9002; Loh et al., 1989, Science 243: 217-220). As described above, PCR derived products can further be used as a probes to screen genomic and cDNA libraries prepared from virally-infected tissues for stealth virus-specific DNA. These DNA probes can further be used to isolate the entire family of stealth virus protein genes from these cDNA libraries using methods which are well known to those skilled in the art. See,

for example, the techniques described in Maniatis et al., 1982, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y., Chapter 7.

Methods which are well known to those skilled in the art can be used to construct expression vectors containing the stealth virus genomic or fragments thereof, or nucleic acid sequences encoding stealth virus proteins or fragments thereof and appropriate transcriptional/translational control signals. These methods include in vitro recombinant DNA techniques, synthetic and in vivo recombinant/genetic recombination. See, for example, the techniques described in Maniatis et al., 1982, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y., Chapter 12. In a specific embodiment, described in an Example, infra, a PCR product may be cloned in a plasmid and cloned in a bacterial or eukaryotic vector.

A variety of host-expression vector systems may be utilized to express stealth virus proteins or fragments thereof. These include but are not limited to micro-organisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing a coding sequence for a stealth virus protein or fragment thereof; yeast transformed with recombinant yeast expression vectors containing a coding sequence for a stealth virus protein or fragment thereof; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing a coding sequence for a stealth virus protein or fragment thereof; or animal cell systems infected with recombinant virus expression vectors (e.g., adenovirus, vaccinia virus) containing a coding sequence for a stealth virus protein or fragment thereof.

The expression elements of these vectors vary in their strength and specificities. Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including consti-

tutive and inducible promoters, may be used in the expression vector. For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used; when cloning in insect cell systems, promoters such as the baculovirus polyhedrin promoter may be used; when cloning in mammalian cell systems, promoters such as the adenovirus late promoter or the vaccinia virus 7.5K promoter may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the inserted coding sequence for a stealth virus protein or fragment thereof.

In yeast, a number of vectors containing constitutive or inducible promoters may be used. For example, see Current Protocols in Molecular Biology, Vol. 2, 1988, Ausubel et al. (Eds.), Greene Publish. Assoc. & Wiley Interscience Ch. 13; Grant et al., 1987, "Expression and Secretion Vectors for Yeast," in Methods in Enzymology, Wu & Grossman (Eds.), Acad. Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C. Ch.3; Bitter, 1987, "Heterologous Gene Expression in Yeast," in Methods in Enzymology, Berger & Kimmel (Eds.), Acad. Press, N.Y., Vol. 152, pp. 673-684; and The Molecular Biology of the Yeast Saccharomyces, 1982, Strathern et al. (Eds.), Cold Spring Harbor Press, Vols. I and II. For complementation assays in yeast, DNAs for stealth virus proteins or fragments thereof may be cloned into yeast episomal plasmids (YEp) which replicate autonomously in yeast due to the presence of the yeast 2k circle. The stealth virus protein or fragment thereof sequence may be cloned so it is expressed under the control of either a constitutive yeast promoter such as ADH or LEU2 or an inducible promoter such as GAL ("Cloning in Yeast," Chpt. 3, R. Rothstein, in DNA Cloning Vol. 11, A Practical Approach, Glover (Ed.), 1986, IRL Press, Wash., D.C.). Constructs may contain the 5' and 3' non-translated regions of a cognate stealth virus protein mRNA or those

corresponding to a heterologous, e.g. yeast, gene. YEp plasmids transform at high efficiency and the plasmids are extremely stable. Alternatively, vectors may be used which promote integration of foreign DNA sequences into the yeast chromosome.

A particularly good expression system which could be used to express stealth virus proteins or fragments thereof is an insect system. In one such system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. AcNPV grows in Spodoptera frugiperda cells. A nucleic acid sequence comprising a coding sequence for a stealth virus protein or fragment thereof may be cloned into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of the polyhedrin gene results in production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect Spodoptera frugiperda cells in which the inserted gene is expressed (see, e.g., Smith et al., 1983, J. Biol. 46:586; Smith, U.S. Patent No. 4,216,051).

In an embodiment where an adenovirus is used as an expression vector, the coding sequence for a stealth virus protein or fragment thereof may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vivo or in vitro recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the stealth virus protein or fragment thereof in infected hosts (e.g., See Logan & Shenk, 1984, Proc. Nat'l. Acad. Sci. U.S.A. 81: 3655-3659). Alternatively, the vaccinia 7.5K promoter may be used (e.g., see Mackett et al., 1982, Proc. Nat'l. Acad. Sci.

U.S.A. 79: 7415-7419; Mackett et al., 1984, J. Virol. 49: 857-864; Panicali et al., 1982, Proc. Nat'l. Acad. Sci. U.S.A. 79:4927-4931).

Specific initiation signals may also be required for efficient translation of the inserted coding sequences for a stealth virus protein or fragment thereof. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire stealth virus protein genome, including its own initiation codon and adjacent sequences, are inserted into the appropriate expression vectors, no additional translational control signals may be needed. However, in cases where only a portion of the stealth virus protein coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame for the coding sequence of the stealth virus protein or fragment thereof, to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bitter et al., 1987, Methods in Enzymol, 153: 516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression driven by certain promoters can be elevated in the presence of certain inducers (e.g., zinc and cadmium ions for metallothionein promoters). Therefore, expression of the genetically engineered stealth virus protein or fragment thereof may be controlled. This is important where the protein product of the cloned foreign gene is lethal to host cells. Furthermore, modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have

characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed.

The host cells that contain the coding sequence for a stealth virus protein or fragment thereof may be identified by at least four general approaches: (a) DNA-DNA hybridization; (b) the presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by expression of stealth virus mRNA transcripts in host cells; and (d) detection of stealth virus gene products as measured by immunoassays or by biological activity.

In the first approach, the presence of the stealth virus protein or fragment thereof coding sequence inserted in a cloning or expression vector can be detected by DNA-DNA hybridization using probes comprising nucleotide sequences that are homologous to the stealth virus protein coding sequence or particular portions thereof substantially as described recently (Goeddert et al., 1988, Proc. Nat'l. Acad. Sci. U.S.A. 85:4051-4055). One such probe is a PCR product.

In the second approach, the recombinant expression vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the stealth virus coding sequence is inserted within a marker gene sequence of the vector, recombinants containing the stealth virus coding sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the stealth virus coding sequence under the control of the same or different promoter used to control the expression of the stealth virus coding sequence. Expres-

sion of the marker in response to induction or selection indicates expression of the stealth virus coding sequence.

In the third approach, transcriptional activity for the nucleic acid containing a stealth virus coding region can be assessed by hybridization assays. For example, RNA can be isolated and analyzed by Northern blot using a probe homologous to the stealth virus coding sequence or particular portions thereof substantially as described (Goeddert et al., 1988, Proc. Nat'l. Acad. Sci. U.S.A., 85: 4051-4055). Alternatively, total nucleic acids of the host cell may be extracted and assayed for hybridization to such probes.

In the fourth approach, the expression of the stealth virus protein or fragment thereof can be assessed immunologically, for example by Western blots, immunoassays such as radioimmunoprecipitation, enzyme-linked immunoassays and the like. In one embodiment, normal or patient sera may be used to detect expression product.

Once a recombinant that expresses a protein or fragment thereof is identified, the gene product should be analyzed. This can be achieved by assays based on the physical, immunological or functional properties of the product.

It will be recognized that the same techniques used to identify host cells that contain stealth virus protein coding sequences can be used to detect the presence of stealth virus in a sample. Furthermore, cloned stealth virus sequences provide a library of molecular probes for detecting stealth virus. In one embodiment, sample may be prepared, blotted on nitrocellulose and probed directly by hybridization assay with a stealth virus probe of the invention. As used herein, a "hybridization assay" refers to detection of hybridization of a probe to a hybridizable portion of a nucleic acid. A "hybridizable portion of a nucleic acid" is intended to be a nucleic acid sequence of at least about 8 nucleotides.

In another embodiment, rather than direct hybridization probing of DNA derived from the patient, a region of the stealth viral DNA (or RNA) can be amplified using the PCR reaction (PCR, and kits therefore provided by Perkin Elmer-Cetus). The choice of primer sequences will be determined on the basis of sequence data, e.g., from the PCR amplified material from isolates X and Y and other isolates obtained by the technologies described herein. Alternatively, the HTLV-I and II reactive primers can be used and the resulting products assayed with a stealth virus-specific probe. In a particular embodiment, the PCR products are probed with PCR products derived from virus X or Y as shown in examples, infra. Because of indications of heterogeneity among some of the isolates, several detecting probes can be used. In another embodiment, a consensus sequence based on common sequences from a large number of virus isolates can be prepared. Typical procedures for running PCR reactions are described in Section 6.1., infra; the procedure can be varied according to knowledge common in the art.

It is understood that molecular probe assays, including PCR, can be performed on tissue samples such as but not limited to CSF, urine, throat swabs and formalin-fixed, paraffin-embedded tissues, to mention but a few.

5.4 Antibodies and Immunoassays

The present invention additionally provides immunoassays to detect the presence of stealth viruses in order to diagnose and monitor disease.

In one embodiment, normal sera or sera from a patient may be used to detect stealth virus antigen. As shown in an Example infra (Section 7.2.5.) patient sera or normal sera can be used in an immunofluorescence assay to detect viral antigens in cells. Preferably, specificity of the reaction can be demonstrated by pre-adsorbing the antibody with supernatant from a virus culture, thus blocking spe-

cific binding. Non-adsorbed sera will decorate virally infected cells, while absorbed sera will not.

Other immunoassay techniques are also available according to the present invention. These techniques include but are not limited to competitive and non-competitive assays using techniques such as ELISA (Enzyme-Linked ImmunoSorbent Assay), "sandwich" immunoassays, radioimmunoassay, immunoradiometric assay, in situ immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, gel agglutination assays, hemagglutination assays, complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, to name but a few. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

In a specific embodiment, based on genetic sequence data and identification of open reading frames, synthetic peptides corresponding to the deduced primary amino acid sequence of stealth virus proteins can be produced (see Section 5.2., supra). Peptides or proteins can also be produced by expression of viral genes. In one instance, these genes are expressed by stealth virus-infected permissive cells, such as fibroblasts. In another embodiment, a recombinant method of production using a host cell transfected with a viral gene may be used to express the gene. These peptides and proteins can be used to detect antibody in patient sera specific for that antigen. Alternatively, the peptides and proteins can be used as immunogens to prepare anti-stealth virus antibody.

Various procedures known in the art may be used for the production of antibodies to stealth virus antigens.

Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and an Fab expression library. For the production of antibodies, various host animals, including but not limited to rabbits, mice, rats, etc., may be immunized by injection with a stealth virus or stealth virus antigen. In one embodiment, a stealth virus antigen may be conjugated to an immunogenic carrier. In another embodiment, a stealth virus epitope, e.g., a hapten, is conjugated to a carrier. As used herein, an "epitope" is a fragment of an antigen capable of specific immunoactivity, e.g., antibody binding. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

Monoclonal antibodies to stealth virus antigens may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein, (1975, Nature 256: 495-497), the more recent human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72) and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention, monoclonal antibodies specific to stealth virus antigens may be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Nat'l. Acad. Sci., U.S.A. 80:2026-2030), or by transforming human B cells with EBV virus

in vitro (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Nat'l. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule specific for stealth virus together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce stealth virus-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, Science 246: 1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for stealth virus.

Antibody fragments which contain sites specific for stealth virus may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')2 fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')2 fragments.

5.5 Analysis of Cell Mediated Immunity Against Stealth Viruses

The availability of stealth viruses, stealth virus infected cells, purified proteins of stealth viruses obtained from either infected cultures or by recombinant DNA technology, will allow the assessment of the cellular immune status of infected individuals against these antigens. This issue is very relevant given the evidence for a lack of an in vivo inflammatory response against stealth

viral infection. Cell mediated immunity can be assessed by culturing patients' or animal lymphocytes in the presence of stealth virus associated proteins. The response of the lymphocytes can be assessed by determining whether proliferation is enhanced as measured by radioactive thymidine incorporation or by enhanced conversion of the colorless tetrazolium substrate MTT. In addition, lymphocytes can be assessed for the production of various cytokines by assaying culture supernatants or by staining cells with immunological or molecular probes specific for the known cytokines. The lack of a positive response to stealth virus associated antigens could be reflected in the production of a cytokine with immunosuppressive activities. For example, Transforming Growth Factor-beta, appears to down regulate immune responses. The presence of an immunosuppressive cytokine can be assessed both by testing specifically for the cytokine and by testing whether stealth viral-proteins can suppress the positive immune response against an otherwise immunogenic antigen or mitogen. In a similar manner, the toxin found in association with stealth viral cultures (infra can be tested for immunosuppressive properties.

5.6 Vaccine Development

Once viral antigens are identified by their ability to react with antibody and/or to elicit an immune response is determined, the skilled artisan can proceed with a vaccine development program similar to those being pursued with other viruses. Animal models using the stealth viral isolates as challenge will assist in this development and in its application to humans. The isolated viruses, as well as synthetic peptides and recombinant DNA derived protein, can be used to monitor the development of humoral and cell-mediated immune responses in naturally infected and intentionally immunized individuals.

Preferably, a stealth virus vaccine will elicit a helper-T cell response, which in turn results in a

stronger antibody and cellular immune response (see, generally, Ada, 1989, "Vaccines," in Fundamental Immunology 2nd Edition, Paul (ed.), Raven Press, Ltd., New York, pp. 985-1032, incorporated herein by reference in its entirety).

Immunopotency of the stealth virus product can be determined by monitoring the immune response of test animals following immunization with the purified protein, synthetic peptide or protein, or attenuated stealth virus. In cases where the stealth virus protein is expressed by an infectious recombinant virus, the recombinant virus itself can be used to immunize test animals. Test animals can include but are not limited to mice, rats, cats, dogs, rabbits, primates, and eventually human subjects. Methods of introduction of the immunogen can include oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal or any other standard routes of immunizations. The immune response of the test subjects can be analyzed by one or more of the following four approaches: (a) the reactivity of the resultant immune serum to authentic viral antigens, as assayed by known techniques, e.g., enzyme linked immunoabsorbant assay (ELISA), immunoblots, radioimmunoprecipitations, etc. (See Section 5.3., supra); (b) the ability of the immune serum to neutralize viral infectivity in vitro; (c) stimulation of cell mediated immune response, including the production of cytokines; and (d) protection from stealth virus infection.

Many methods can be used to administer the vaccine formulations described herein to an animal or a human. These include, but are not limited to: oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, and intranasal routes. Since stealth virus transmission is likely to occur via the respiratory system, the secretory IgA antibodies produced by the mucosal associated lymphoid tissue can play a major role in protection against stealth virus infection by preventing the initial

interaction of the pathogens with the mucosal surface, or by neutralizing the important epitopes of the pathogens that are involved in infection/or spreading of the disease. Stimulation of mucosal immune responses, including production of secretory IgA antibodies, can be of major importance in conferring protection against transmission via lower and upper respiratory tract. When a live recombinant virus vaccine formulation is used, it may be administered via the natural route of infection of the parent wild-type virus which was used to make the recombinant virus in the vaccine formulation.

The proteins and polypeptides of the present invention that are related to neutralizing epitope(s) of the stealth virus are useful immunogens in a subunit vaccine to protect against stealth virus infection. Subunit vaccines comprise solely the relevant immunogenic material necessary to immunize a host. Vaccines prepared from genetically engineered immunogens of stealth virus proteins or fragments thereof, which are capable of eliciting a protective immune response, are particularly advantageous because there is no risk of infection to the recipients.

Thus, the stealth virus proteins and polypeptides can be purified from recombinant cells that express the neutralizing epitopes. Such recombinant cells include but are not limited to any of the previously described bacteria or yeast transformants, cultured insect cells infected with recombinant stealth virus protein(s) in baculoviruses or cultured mammalian cells that express stealth virus protein epitopes.

The stealth virus proteins or polypeptides are adjusted to an appropriate concentration and can be formulated with any suitable vaccine and adjuvant. The polypeptides and proteins may generally be formulated at concentrations in the range of 0.1 mg to 100 mg per kg/host. Physiologically acceptable media may be used as carriers. These include, but are not limited to: sterile

water, saline, phosphate buffered saline and the like. Suitable adjuvants include, but are not limited to: surface active substances, e.g., hexadecylamine, octadecylamine, actadecyl amino acid esters, lysolecithin, dimethyl-dioctadecylammonium bromide, N,N-dioctadecyl-N'-N'bis(2-hydroxyethylpropane diamine), methoxyhexadecyglycerol, and pluronic polyols; polyamines, e.g., pyran, dextransulfate, poly IC, polyacrylic acid, carbopol; peptides, e.g., muramyl dipeptide, dimethylglycine, tuftsin; oil emulsions; and mineral gels, e.g., aluminum hydroxide, aluminum phosphate, etc. The immunogen may also be incorporated into liposomes or conjugated to polysaccharides and/or other polymers for use in a vaccine formulation.

In yet another embodiment of this mode of the invention, the related protein or polypeptide is a hapten, i.e., a molecule which is antigenic but that cannot independently elicit an immune response. A hapten comprises an epitope, as defined in Section 5.3., supra. In such case, the hapten may be covalently bound to a carrier or immunogenic molecule; for example, a large protein such as protein serum albumin will confer immunogenicity to the hapten coupled to it. The hapten-carrier may be formulated for use as a subunit vaccine.

In a specific embodiment, the polypeptides and proteins of the present invention may be used when linked to a soluble macromolecular carrier. Preferably, the carrier and the polypeptides and proteins of the present invention are in excess of five thousand daltons after linking. More preferably, the carrier is in excess of five kilodaltons. Preferably, the carrier is a polyamino acid, either natural or synthetic, which is immunogenic in animals, including humans. The manner of linking is conventional. Many such linking techniques are disclosed in U.S. Patent No. 4,629,783 which is incorporated herein by reference. Many cross-linking agents that can be used are disclosed in 1986-87 Handbook And General Catalog, Pierce

Chemical Company, (Rockford, Illinois) pages 311 to 340, which pages are incorporated herein by reference.

Another embodiment of the present invention provides either a live recombinant viral vaccine or an inactivated recombinant viral vaccine, which is used to protect against disease symptoms of stealth virus. To this end, recombinant viruses are prepared that express stealth virus protein related epitopes. Where the recombinant virus is infectious to the host to be immunized but does not cause disease, a live vaccine is preferred because multiplication in the host leads to a prolonged stimulus, therefore, conferring substantially long-lasting immunity. The infectious recombinant virus when introduced into a host can express the stealth virus related protein or polypeptide fragments from its chimeric genes and, thereby, elicit an immune response against stealth virus antigens. In cases where such an immune response is protective against subsequent stealth virus infection, the live recombinant virus, itself, can be used in a preventative vaccine against stealth virus infection. Production of such recombinant virus may involve both in vitro (e.g., tissue culture cells) and in vivo (e.g., natural host animal) systems. For instance, conventional methods for preparation and formulation of smallpox vaccine may be adapted for the formulation of live recombinant virus vaccine expressing stealth virus proteins or polypeptides. Multivalent live virus vaccines can be prepared from a single or a few infectious recombinant viruses that express epitopes of organisms that cause disease in addition to the epitopes of stealth virus. For example, a vaccinia virus can be engineered to contain coding sequences for other epitopes in addition to those of stealth virus. Such a recombinant virus itself can be used as the immunogen in a multivalent vaccine. Alternatively, a mixture of vaccinia or other viruses, each expressing a different gene encoding for an epitope of

stealth virus and an epitope of another disease causing organism can be formulated in a multivalent vaccine.

In yet another embodiment, stealth virus, particles or proteins isolated from tissue culture may be used to prepare a vaccine. These particles should be attenuated to prevent infectivity, e.g., by heating. Where stealth virus-associated toxin is found in the culture, this toxin should be purified away from the vaccine components. Purification of viral components for vaccine use may be accomplished by ultracentrifugation, solvent extraction, or other means known in the art.

In pursuing this goal, it is recognized that while the establishment of immunity prior to exposure to stealth virus infection may be beneficial in preventing disease transmission, the occurrence of a cellular immune response in an already infected individual or animal could have deleterious effects. The various assays described above could be employed to detect such deleterious immune responses and allow for the development and the monitoring of means to reestablish the desired lack of cell mediated immunity against the resident virus.

5.7 Anti-Viral Agents

The viral isolates can be tested for susceptibility to existing anti-viral agents, such as interferon (IFN), acylovir, ganciclovir, azidothymidine (AZT), dideoxyinosine (DDI), ribovirin, etc. Such assays can be carried out by assessing the ability of a potential anti-stealth viral agent to protect cells in culture against the cytopathic effects of stealth virus. In one embodiment, an assay can be carried out using the culture techniques described in Section 5.1., supra. Furthermore, defining the mechanisms by which the viruses become activated and kill cells in vitro will lead to new approaches to interfering with viral replication and pathogenicity. In an Example infra, αIFN, is shown to prevent stealth virus infection in vitro. In another Example, cerulenin, an

inhibitor of lipid synthesis is shown to inhibit viral growth in vitro.

Compounds shown to be effective in vitro can be tested in vivo. In a preferred embodiment, an animal infected with the same stealth virus as known to affect a human, would be treated with the experimental drug and monitored for signs of clinical improvement. Efficacy in an animal model would support the experimental use of that agent in the human patient containing that virus. The animal could either be naturally infected as a result of contact with the patient or experimentally infected using the viral isolate obtained from the patient.

5.8 Origin of Infection in Individual Patients

In another embodiment, the present invention provides a method for screening sources of infection or transmission of stealth virus comprising assaying in a sample from the possible source of infection or transmission for the presence of a stealth virus. Possible sources of infection that can be screened include, but are not limited to, blood or blood products for transfusion, organs for transplantation, vaccines, particularly vaccines derived from cell culture (e.g., monkey kidney cells), farm animals, domestic pets, and food, especially but not limited to unpasteurized milk and uncooked or undercooked meat and seafood. In one embodiment, the presence of stealth virus may be detected by culturing stealth virus from a sample suspected of harboring the virus. In another embodiment, stealth virus may be detected by detecting binding of antibody or nucleic acid probes specific for stealth virus or by detecting stealth virus-associated toxic activity.

An important possible source of infection is from viral vaccines produced in primary cell cultures, especially of primate origin. Studies on stocks of vaccines may implicate the vaccine as the probable source of infection. Similarly, specific studies on possible blood transfusion derived infection would be indicated in those individuals

in whom such a suggestion is supported by epidemiological data.

The finding of tongue and salivary gland infection, and the isolation of stealth virus from throat swabs suggests that air borne transmission of infection may be common. This is consistent with the frequent occurrence of symptoms attributed to pharangeal involvement in CFS patients. School teachers are considered a risk group for the development of CFS possibly due to exposure to children. Medical, dental and veterinary personnel may also need to be considered at risk. The virus can be cultured from blood and, therefore, could also be transmitted by transfusion. Of concern, is whether stealth viruses may have contaminated some of the earlier live viral vaccines produced in primary monkey kidneys, e.g., polio and rubella. Unpublished data had indicated that polio vaccines may contain DNA other than that accountable by the genome of normal monkey cells, while rubella vaccines were known to have extraneous DNA, presumably derived from bacteriophages. Other potential sources of stealth viruses are farm animals and domestic pets. Interestingly, it has been noted that pets of patients with CFS may develop unexplained illnesses often involving the central nervous system. Furthermore, stealth viruses have been cultured from pets of patients with CFS using the culture method described in Section 5.1.

5.9 Stealth Virus-Associated Toxic Activity: Implications for Diagnosis and Therapy

The present invention further provides a stealth virus-associated toxin and its associated toxic activity. As described in the Examples infra, this toxic activity halts the viral CPE at the first stage. Normal fibroblasts treated with supernatant containing toxin become less permissive for virus growth. This toxic activity may, at least indirectly relate to the electron microscopic appearance of partially degraded viral particles in

infected cell cultures (described in Example 8 infra). The toxic activity also appears to adversely affect fibroblasts themselves. In particular, the cells lose vitality. At the extreme, the cells die. These effects can be detected by directly observing the indicator cells, e.g., fibroblasts. Alternatively, vitality assays such as tritiated-thymidine incorporation or the MTT assay (see Niks and Otto, 1990, J. Immun. Methods 130:140-151) can be used.

An interesting morphological feature of stealth virally infected fibroblasts is the presence of inclusions in addition to viral particles. These inclusions appear to stain with both a carbohydrate stain (periodic acid schiff) and with a lipid stain (oil red O). They are particularly evident by electron microscopy. Synthesis of the inclusions appears to accompany virus production in the cells and may contribute to viral assembly, e.g. as a source of tegument protein. The potential lipid component is particularly intriguing because, especially in late stage CPE, lipid-like debris is evident throughout the culture. It is known that polyether toxins are lipid soluble and that lipid containing vesicles are commonly found in dinoflagellates, a known source of polyether toxins. (Interestingly, it has been suggested that the lipid production provides buoyancy to the dinoflagellates. Thus, the profusion of intracytoplasmic inclusions and vacuoles and the apparent production of lipid-like substances in conjunction with viral infection appears to be related to the production of the toxin. The inclusions and/or vacuoles may serve to sequester toxin in order to buffer its toxic activity so as to moderate its toxic effects, or may serve to store newly made toxic activity in the cell.

There is some preliminary evidence that the toxin in viral infected cultures may be related to lipid soluble polyether type compounds. A search for such compounds was prompted by the appearance on electron microscopy of the

prominent vacuoles and inclusions in virally infected cells, and of lipid like materials in culture supernatants. While biological assays exist, a recently developed immunoassay procedure is available to detect toxic polyethers such as the ciguatera toxin found in fish. This approach was also prompted by the analogy between the symptoms of CFS and the residual effects seen in some individuals as a result of ciguatera poisoning. The immunoassay for ciguatera-type toxin gave a positive response with boiled supernatant from X virus infected cultures. The ciguatera toxins and other toxic polyethers such as okadaic acid can be tested for antistealth viral effects in vitro. Moreover, culture supernatants can be tested in various biological and chemical assays for polyether compounds.

The present culture systems provide a useful source of stealth virus-associated toxin. In one embodiment, the toxin is found in culture supernatant. In another embodiment, toxin is found in supernatant of lysed cells. In a further embodiment, the toxin is purified. Purification of toxin from culture supernatant or cell lysate may be by chromatography or solvent extraction, or by other means known in the art. In one embodiment, affinity chromatography with antibody specific for polyether toxins may be used.

If the stealth virus-associated toxin is similar in size to the ciguatera toxin-type polyethers, it will be of about 1,000 MW. Thus size exclusion chromatography on a support such as Sephadex 25 or 50 will separate the toxin from larger macromolecules (e.g., greater than 5,000-10,000 MW). Alternatively, reverse-phase HPLC can be used to purify the toxin. It may be possible to purify toxin by silica gel chromatography. Whatever chromatographic method is chosen, fractions of eluent nay be tested for toxic activity to determine where the toxin elutes. In another embodiment, capillary electrophoresis is used to separate the toxin from culture supernatant. The detec-

tion of toxin can be enhanced by prior derivatization of the components of the culture supernatants with a chemical moiety known to bind polyethers such as ciguatera, okadaic acid, palytoxin, brevitoxin, etc.

Alternatively, solvent extraction techniques may be used, in the expectation that the toxin is lipid soluble. In one embodiment, toxin may be extracted in 10% and 50% methanolchloroform.

In yet another embodiment, toxin can be prepared synthetically.

Recognition of an immunological relationship of stealth virus-associated toxin and the ciguatera-type polyether toxins suggests commonality of function. Given the similarity of symptoms of food poisoning resulting from consumption of ciguatera toxin with symptoms associated with stealth virus infection, such as severe neurologic manifestations in addition to gastrointestinal symptoms (Mebs, 1980, M.M.W. 122:1413-14), it is possible that some of the symptoms associated with stealth virus infection may directly result from toxic activity. Some of the marine polyether toxins are known to bind to ion and excitatory amino acid receptor molecules on the surface of neuronal cells. It is, therefore, possible to assay for the presence of stealth virus culture derived toxins using a competitive receptor binding assay in the presence of labeled known polyether toxin.

Furthermore, lingering cases of seafood poisoning may indicate transmission and infection with a virus, possibly from the seafood itself. It is noteworthy in this connection that stealth viruses have a wide range of host compatibility thus transmission of virus from seafood to an animal host is feasible.

Stealth virus-associated toxin may be a cellular anti-viral defense product, or may be an unusual viral product. If a viral product, it may be possible to delete the gene or genes encoding the toxin or the enzymes involved in synthesis of toxin from the virus. Such a

virus should not produce the cellular toxicity and viral growth suppressive activity of the wild-type stealth virus. This virus would be an ideal vaccine candidate, since toxicity that may be responsible for symptoms in stealth virus-associated disease, would be absent. In effect, this would be an attenuated virus.

Alternatively, if the toxic activity is a cellular defense response to viral infection, it may be possible to produce a permissive cell line that lacks the ability to produce toxin. Such cell lines can be produced by random mutagenesis, e.g., by treatment with mutagenizing agents or radiation, and selected on the basis of a lack of toxic activity in supernatant after viral infection. This cell line would be particularly useful for culturing stealth virus isolates, since no toxic activity would attenuate the cultures.

The present invention further provides antibody specific for stealth virus-associated toxin. In one embodiment, antibody reactive with ciguatera toxin may be used. In another embodiment, antibody to toxin activity can be prepared as described for stealth virus antigens in Section 5.3, supra. Preferably, the toxin is conjugated to an immunogenic carrier molecule to enhance the immune response. Suitable carrier molecules include serum albumin, keyhole limpet hemocyanin, and other macromolecular carriers, such as those described in Section 5.3 and 5.4., supra.

A useful assay to select for a monoclonal antibody specific for toxin is whether it can neutralize the toxic activity in an in vitro assay. In such an assay, the toxicity of stealth virus-associated toxin is tested in the presence and absence of antibody. In the presence of a neutralizing antibody, no toxic activity is observed, while in its absence, toxic activity is observed. Such an assay may be performed on a culture of fibroblast cells under culture conditions described in Section 5.1., supra.

Thus in addition to methods of neutralizing toxin comprising frequently refeeding of viral cultures and using a higher than normally used concentration of fetal calf serum, the present invention provides agents that neutralize toxin. In one embodiment, an antibody that can neutralize toxic activity, in vitro is such an agent. In another embodiment, chemical compounds known to inhibit the toxic effect of the toxin can be added to the culture. For instance, low doses of retinoic acid can inhibit some of the toxicity of okadaic acid on human fibroblasts. The addition of retinoic acid was found to enhance the growth of stealth virus.

In a particular embodiment, the present invention provides methods for detecting stealth virus-associated toxin in a sample. In yet a further embodiment, the level (i.e., amount or concentration) of toxin is measured. In one embodiment, a cell culture assay may be used to detect the presence of toxin. In another embodiment, antibody to stealth virus-associated toxin may be used to detect toxin in a sample. When the sample is from a patient, stealth virus or other viral infection can be detected. The degree of infection, progress of therapy, and level of toxicity can be measured. When the toxin is present in a food or vaccine, detection of the toxin can prevent inadvertent poisoning from consumption of the food or administration of the vaccine.

In another embodiment, the level of toxin in a sample from a patient or in an naturally infected or experimentally inoculated animal may be used to monitor disease progression. In one aspect, the level of toxin may be measured prior to and after treatment for virus infection or treatment to reduce toxin levels, a decrease in toxin level after treatment indicating therapeutic efficacy. In another embodiment, the toxin level in a patient or animal can be compared to the level in the patient or animal at an earlier time to monitor disease progression, the higher the level of toxin, the more advanced the disease. Yet

further, the level of toxin in a patient or animal can be compared to the level of toxin in normal controls or in other patients or animals in order to monitor, or diagnose, stealth virus infection.

Antibody to toxin can also be used to diagnose, monitor and treat food poisoning associated with polyether-type toxins. Furthermore, by monitoring the level of toxin after an episode of food poisoning, chronic toxicity suggesting persistent viral infection can be detected, even though a virus may not be detected.

In another embodiment, an agent that neutralizes toxin may be used to block the toxic activity. In a particular embodiment, the agent is an antibody. Blocking (neutralizing) antibody may be used in vitro, to inhibit toxicity, or it may be used therapeutically in vivo, to alleviate symptoms related to the toxicity. In one embodiment, this antibody can be used therapeutically to lower toxin levels in a patient in need of such treatment. In another embodiment, the agent can be a chemical able to reverse toxic activity.

5.10 Diagnosis and Therapy

The stealth viruses, culture methods, stealth virus molecular probes, stealth virus antigens, stealth virus associated toxin, antiviral agents, and antibodies of the invention can be valuable in the diagnosis and therapy of disease associated with stealth virus infections, as described in detail below.

5.10.1 Diagnosis

The present invention provides methods for diagnosing stealth virus infection associated with disease by detection of the presence of stealth virus in humans and in animals. Both in vitro and in vivo assays can be used, and include, but are not limited to, the culture and molecular probe based assays described in Sections 5.1, 5.2 and 5.3., supra. In addition, imaging tech-

niques can be used, in which an antibody of the invention or derivative or fragment thereof is bound to a label. The labeled antibody can then be administered in vivo to determine the location of stealth virus. In one embodiment, a magnetic resonance imaging agent may be used. In another embodiment, a radioactive imaging agent may be used.

In particular embodiments of the invention, the presence of stealth virus may be diagnosed by detecting the immunospecific binding of an antibody, or derivative or fragment thereof, reactive with an epitope of a stealth virus in a patient sample. In another embodiment, hybridization of a nucleic acid probe is detected, as described in Section 5.2., supra.

Assays specific for stealth virus-associated toxin may be used diagnostically, to determine whether toxin is present in a sample. The presence of toxin (by detection of it or its associated toxic activity) is indicative of viral infection. In another embodiment, the presence of toxin indicates food poisoning. The level of toxin may be monitored to indicate progression of disease and presumptive evidence for actual viral infection rather than ingestion simply of the toxin. The level of toxin can similarly be monitored to determine the efficacy of a treatment as described in Section 5.7, supra. In another embodiment, in vitro culture assays or immunoassays can be used to detect toxin for diagnosis or monitoring of disease, or to detect infection in animals, or contamination of food or vaccine preparations. In a preferred embodiment, a diagnostic kit comprising, in a suitable container, a stealth virus-associated toxin-specific antibody is provided.

The sample from a subject (i.e., a patient) may consist of any body fluid, including but not limited to peripheral blood, plasma, cerebrospinal fluid, urine, throat swabs, surgically excised and fine needle aspirated tissue samples, other body fluids. Preferably the sample is

blood, CFS, or affected tissue, i.e., brain biopsy. Binding of an antibody or a molecular probe may be accomplished and/or detected in vitro or in vivo. In vitro binding may be performed using histologic specimens or subfractions of tissue or fluid. In vivo binding may be achieved by administering the molecular hybridization (nucleic acid) probe or antibody (or fragment or derivative thereof) by any means known in the art (including but not limited to intravenous, intraperitoneal, intranasal, and intraarterial, to name but a few) such that specific binding may be detected; for example, by attaching a radioactive label or a magnetic resonance imaging agent to the molecular probe or diagnostic antibody, fragment, or derivative or by attaching such a label to its specific binding partner.

It should be understood that the diagnostic methods of the invention are best used in the context of other diagnostic parameters in order to obtain a comprehensive patient diagnosis. For example, a clinical diagnosis of the chronic fatigue syndrome (CFS) is preferably made based on the methods of the invention in the context of other clinical features of CFS such as long lasting fatigue and cognitive dysfunction. Clinical features of psychiatric, neurological, rheumatological, auto-immune, liver, salivary gland and other diseases may accompany stealth virus infections in different patients. The term multi-system stealth virus infection (MSVI), would appear to be an appropriate term to encompass the wide diversity of clinical manifestations in patients and animals with stealth virus infections.

As with any diagnostic criteria, the parameters disclosed in the present invention may not be sole determinants, or pathognomonic, of a particular disorder.

According to the invention, stealth virus infection may be diagnosed and monitored by detecting the presence of nucleic acid sequences homologous to a stealth virus gene in mRNA or DNA from a patient sample. Several proce-

dures could be used to correlate stealth virus genes or gene expression with disease. These involve (1) producing and analyzing cDNA or genomic DNA libraries obtained from a patient suffering from the disease to determine the presence of stealth virus genes; (2) analyzing disease samples by Southern blot to determine whether specific genetic polymorphisms (restriction fragment length polymorphisms, RFLPs) or oligoclonal stealth virus sequences exist; (3) analyzing disease samples by the polymerase chain reaction (PCR) amplification, and analysis of PCR products using electrophoresis and/or hybridization procedures. The PCR procedure represents a more efficient procedure in the time required for analysis and in the number of patients that can be analyzed to detect a disease correlation. A fourth procedure using in situ hybridization of cells may also be extremely useful.

5.10.2 Therapy

Anti-viral agents of the invention, such as are discussed in Sections 5.5. and 5.7., supra, can be provided therapeutically, according to the present invention. One of ordinary skill in the art would be able to determine a therapeutic dose of an antiviral agent based on in vitro assays and standard dosage testing. For many of the agents described in Section 5.5., effective dosages are known. If the stealth virus-associated toxin is used as an anti-viral agent, it is administered so that the toxin levels in vitro do not exceed the level tolerated by individuals with toxin present in serum, as can be determined by the methods described in Sections 5.7. and 5.8.1., supra. These parameters are readily determined using the assays provided by the invention.

In another embodiment, a neutralizing agent for the toxin is administered therapeutically. In one embodiment, antibody reactive with toxin, and capable of neutralizing toxin in vitro, is provided. Neutralization of toxin may

also be useful in the treatment of ciguatera-type toxic poisoning.

The therapeutic agents of the invention may also contain appropriate pharmaceutically acceptable carriers, diluents and adjuvants. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium carbonate, magnesium stearate, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. These compositions can take the form of solutions, suspensions, tablets, pills, capsules, powders, sustained-release formulations and the like. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the active compound together with a suitable amount of carrier so as to provide the form for proper administration to the patient. While intravenous injection is a very effective form of administration, other modes can be employed, such as by injection, or by oral, nasal or parenteral administration.

5.10.3 Kits

Also provided by the present invention are kits for in vitro culture of stealth virus and diagnosis of stealth virus-associated disease. A kit for in vitro culture of a stealth virus isolate comprises, in a suitable container, an neutralizing agent of stealth virus-associated toxin. The neutralizing agent may be provided in lyophil-

ized form and reconstituted prior to use, or it may be provided in solution. In one embodiment, the neutralizing agent of toxin is an antibody specific for toxin.

A kit for detecting the presence of stealth virus in a sample is also provided. Such a kit comprises, in a suitable container, a stealth virus-specific probe. In one embodiment, the probe is an antibody specific for a stealth virus antigen. In another embodiment, the probe is a nucleic acid (molecular probe) capable of hybridizing to a stealth virus nucleic acid sequences. In yet another embodiment, the probe is an antibody specific for toxin.

The invention will be further clarified by the following Examples, which are intended to be purely exemplary of the invention.

6. Example: Evidence, Based on PCR Assays, for a Novel Virus Infection in Patients with the Chronic Fatigue Syndrome

The discovery of human herpesvirus-6 prompted the suggestion that it may be causally related to the chronic fatigue syndrome. To decided to explore this possibility using the polymerase chain reaction. Blood from CFS patients and controls were tested using a PCR primer set specific for HHV-6. No differences could be detected between patients and controls. I next decided to reduce the stringency of the PCR so that the HHV-6 reactive primers might begin to detect a virus related to, but different from HHV-6. In a similar approach, I evaluated various primer pairs to determine if conditions could be established in which a primer set would amplify all of the known human herpesviruses. This was possible using a primer set reactive with the gene coding the gp64 major late antigen of human cytomegalovirus. In other words, this primer set and a detecting probe was able to give a positive PCR response, as detected in a dot blot hybridization assay using a ^{32}P-labeled detecting oligonucleotide, with all of the known human herpesviruses (Herpes simplex I and

II, varicella zoster virus, cytomegalovirus, Epstein-Barr virus, and HHV-6. This primer set was used to test blood from CFS patients and from normal controls.

6.1 Materials and Methods

The PCR primers chosen to amplify cross-reactive herpesviral genes were based on sequences contained in the gene coding the gp64 late antigen of human cytomegalovirus (CMV). The sequences of these primers were CGTTTGGGTTGCGC AGCGGG (primer A) and CCGCAACCTCGTGCCCATGG (primer B). Products from the herpes virus primers were probed with a 40-bp oligonucleotide specific for CMV late antigen. Preliminary studies showed that under the conditions used, these primers could give a positive PCR assay with CMV, HSV-1 and HSV-2, varicella zoster, Epstein-Barr virus and human herpesvirus-6. In addition to these primers, I obtained primers that would be specific for each of the known human herpesviruses.

PCR was performed on crude DNA extracts from heparinized whole blood. Fifty μl of blood are lysed using a Triton X buffer and the white cells washed twice with phosphate buffered saline (PBS). The proteins are partially digested by incubating for 1 hour at 56°C with proteinase K (100 μg in 50 μl PCR buffer). Following digestion, the template DNA samples are placed in boiling water for 10 min. The PCR is performed in 100 μl containing 10 μl of the DNA template, 1 μM of each primer, 2.5 units Taq polymerase, 200 μM of each dNTP and 2 mM Mg++. Forty cycles with annealing temperature 42°C (30 sec); extension temperature 720 (30 sec); and denaturation temperature 94°C (30 sec) are used with rapid ramping. The PCR products are examined by either dot blot (using 50 μl) or Southern blot analysis (using 15 μl). Membranes are UV cross-linked and baked in an oven at 80°C for 30 min. Hybridization is generally performed for 3 hours using a detecting oligonucleotide probe which had been 32P labeled at the 5' end using a T4 kinase reaction. Mem-

brane washing consists of sequential incubations with 2X SSC twice for 5 min periods at room temperature, followed by IX SSC for 30 min at 50°C and a 4th wash with 0.1X SSC for 30 min at 55°C. All wash solutions contain 0.2% SDS. The washed membrane is monitored to check the negative and positive controls. If necessary, an additional wash is performed using 0.1 SSC at 60°C. The membranes are exposed to photographic film using 2 screens at -70°C for 12 to 18 hours.

6.2 Results

PCR assays were performed on over 100 patients referred with a clinical diagnosis of chronic fatigue syndrome. Consistent, but weak responses, were seen in approximately one-third of the CFS patients tested. An occasional patient gave a unmistakable positive response of greater than 2/4 plus intensity. In over 50 normal controls, not even a weak positive responses were observed. This was due to the fact that the PCR conditions, especially the final washing of the membrane, were specifically monitored so as not to give false positive responses with blood samples from normal controls. Although other investigators in the field were suggesting human herpesvirus-6 as a possible etiologic agent in CFS patients, negative results, using a PCR primer set based on specific sequences present in HHV-6, were obtained in all but a single patient. Similarly, the PCR patients tested negative using primer sets shown to be specific for cytomegalovirus and for Epstein-Barr virus.

6.3 Conclusion

Although difficult to interpret, these preliminary data provided encouragement to continue to search for a novel type of virus using broadly reactive PCR primers. I, therefore, also decided to extend this approach to the use of PCR primer sets designed to react with human retroviruses.

7. Example: Evidence, Based on PCR Assays, for a Novel Virus Infection in Patients with Atypical Neurological Illnesses

Although a byproduct of the study on the possible cause of the chronic fatigue syndrome, the following patient examples did indicate that a virus, detectable by PCR, may be unaccompanied by the usual inflammatory manifestations of an infectious disease. These studies employed the broadly reactive herpesviral PCR primers and primer sets based on the combined sequences of HTLV-I and HTLV-II. This primer set was chosen in an attempt to amplify sequences of any potential retroviral related viruses.

7.1 Materials and Methods

The PCR assays were performed as described supra. The retroviral primers were constructed, with slight modifications, from those described by other investigators to detect the various genes of HTLV-I and HTLV-II viruses (Ehrlich et al., 1990, "Detection of human T-cell lymphoma/leukemia viruses," in PCR Protocols: A Guide to Methods and Applications, Innis et al. (eds.), Academic Press, pp. 325-336). Primers against the transactivating tax gene of HTLV was eventually chosen because it performed well in the PCR assays and because of evidence that various retroviruses may possess shared transactivating activity (Rethwilm et al., 1991, Genetics 88:941-945). One primer was designed to react with the region of HTLV I from nucleotide 7248-7267 and the region of HTLV II from nucleotide 7358-7377. The sequence of this primer was 5-CGGATACCCCGTCTACGTGT-3. These sequences were used in the previously published HTLV primer SK43' for HTLV-I, with the exception that I substitution cytidine for adenosine in position 10). The second primer was identical to the SK 44" primer designed to amplify HTLV-II and covering the sequence 7406-7386 of HTLV-II. The sequence of this primer was 5-GAGCTGACAACGCGTCCATCG-3. Sequence analysis

showed that SK43' and SK44" would be expected to react (with one and two mismatches respectively) with HTLV-II for SK43', in the region covered by nucleotide 7358-7377 of HTLV-II; and with HTLV-I for SK44" in the region covered by nucleotides 7516-7496 of HTLV-I. The ^{32}P-labeled detecting probe was identical to SK45 and corresponded to sequences 7447-7468 of HTLV-I. The detecting probe was 85% homologous to sequences present in HTLV-II.

7.2. Results

Patient DA. This 37 year old school teacher was criticized for having misspelled a word in a note sent to parents of one of her pupils. Her condition showed a marked deterioration eight months later with onset of near complete expressive dysphasia and dyspraxia. Her CSF showed no abnormality, yet periventricular lesions were noted on MRI and CAT scan. A stereotactic biopsy from an affected area was performed at University of Southern California Medical Center. The tissue showed gliosis and demyelination but no inflammation. As requested by the neurosurgeon, the tissue was examined using PCR for JC virus. No evidence for this virus was found. In a PCR assay using the common herpesvirus primers, however, a weak but definite positive response was noted. On further electron microscopic examination, intracytoplasmic viral particles were seen on electron microscopy. The appearance of these particles and lack of inflammatory response provided support for the contention that the positive PCR assays, did in fact, reflect the presence of virus. The type of virus could not be morphologically identified but appeared most consistent with a herpesvirus.

Patient PM. This is a 19 year old male. Three years previously, he developed an acute illness suggestive of viral encephalitis. The institution of therapy was delayed, however, because of lack of any changes in his cerebrospinal fluid (CSF). After three days in the hospital, his neurological condition markedly deteriorated

and a clinical diagnosis was Herpes simplex encephalitis. Acyclovir therapy (the standard treatment for Herpes simplex infection) was started but with poor response. He currently exhibits major sequelae including a near complete failure to comprehend verbal language or to express coherent thoughts. Ten years earlier, his younger brother had suffered from encephalitis, causing coma but from which apparent full recovery had been made. A sample of this patient's CSF was obtained to try to resolve whether his illness had, in fact, been due to Herpes simplex virus. Although, his CSF gave a negative PCR using a HSV specific primer set, it did gave a strong response with both the broadly reactive herpesvirus and retrovirus PCR primer sets. A positive PCR response was also detectable in a blood sample obtained from this patient.

Patient BU. This patient was delivered at only 23-5/7 weeks gestation. At day 75 of life, the baby had a severe clinical deterioration with apnea, bradycardia and seizures. He also developed pneumonia. Neurologically, the baby showed severe clonus and jitteriness. Hypertonia of the upper and lower extremities with tight heel cords and increased extensor tone of the neck and shoulders. His electroencephalogram showed evidence of seizure activity. He showed a gradual improvement with no overt neurological signs during the month prior to discharge except for mild cerebral atrophy detected by CT scan. Lumbar punctures at the onset of the illness and during the course of the illness showed no evidence of inflammation by chemistries or by cell counts. On the other hand, PCR assays with both the common herpesviral and the HTLV tax reactive primers gave strongly positive responses on each of two occasions.

7.3 Conclusion

These studies clearly established that the PCR assay could be positive in the absence of other signs of infection. The data strongly suggested that the virus (or

viruses) responsible for the positive PCR were different from known viruses and, although difficult to detect, could cause severe neurological illness. In proceeding with these studies, many additional examples were found in which patients with atypical neurological diseases who otherwise showed no inflammatory evidence of an infection, were nevertheless, clearly positive on PCR assay testing. The patients include a six-year old child who developed difficulties with school work, especially with reading. She was reluctantly diagnosed as having lupus cerebritis based on a slightly elevated ANA titer. Other patients have had clinical diagnoses which have included: dysimmune myeloradiculopathy, post viral encephalopathy, lupus cerebritis, polyneuritis and polymyositis, prosac-associated hepatitis, atypical multiple sclerosis, childhood attention deficit, and unexplained mental deterioration. The patient with the myeloradiculopathy, a 76-year old male, is interesting since his illness began within 6 months of open heart surgery, which would have required blood transfusions. Positive PCR assays were also continuing to be observed in CFS patients. In one analysis of 110 CFS patients, the responses were gauged from a weak positive through a + (one-plus) to a ++++ (four-plus) positive range. Using the HTLV primer set, the distribution of results was as follows: negative, 57; weak positive, 16; one-plus positive, 21; two plus positive or more, 16. A normal control DNA was included in all assays and yielded negative results. While a weak positive was considered non-informative, a one plus or greater response was considered abnormal. In spite of the PCR data, there were no other indications of viral infection in either the patients with the atypical neurological illnesses, nor in the PCR positive CFS patients. The apparent difficulty in detecting the putative virus responsible for the positive PCR assays, suggested to me the term "stealth viruses". This term will be used in the subsequent examples.

8. Example: Evidence of Stealth Virus Infection in a Patient with CFS Based on Virus Culture and Molecular Probe Based Assays

8.1 Case Study

The 43 year old patient (initials D.W.) from whom the virus was isolated was in her usual state of good health prior to an acute onset illness in August 1990. This illness was characterized by intense headaches, generalized myalgia and fever, developing 1 week after a sore throat. She was hospitalized with an admitting diagnosis of possible encephalitis/meningitis. The CSF examination, however, was unremarkable. She was subsequently diagnosed as having CFS based on continuing severe fatigue necessitating elimination of evening and weekend social activities; a marked reduction in her capacity for work; impaired cognitive functions, including short term memory loss and a major difficulty in naming items (dysnomia); headaches; and non-restorative sleep. She has been on disability for CFS since June of 1991.

Evidence for viral infection in the patient: The patient's blood was initially tested for possible viral infection shortly after the onset of her illness. Positive PCR assays were repeatedly obtained using a primer set reactive with the tax gene and common herpesvirus primer sets described supra. An example of the strongly positive PCR assay on the patient's blood using the SK43' and SK44" primer set and SK45' detecting probe is shown in Figure ?.

8.2 Material and Methods

Following these observations, heparinized blood from the patient was cultured on human foreskin fibroblasts (MRHF, Whittaker Bioproducts, Walkersville, MD). The inoculum consisted of ficoll hypaque separated mononuclear cells admixed with the granulocytes from the buffy coat at the top of the erythrocyte layer. The cultures were washed at 1 hour and again at 24 and 48 hours to remove

contaminating erythrocytes. The typing sera for HSV, CMV, adenoviruses and enteroviruses were obtained from Baxter Labs. Sacramento CA. The broadly reactive anti-HHV-6 monoclonal antibodies BO145 (against p41) and BO151 (against gp102) were obtained from Universal Biotechnology Inc. Rockville Md. The reactivity of these antibodies are described by Ablashi DV, Balachandran N and Josephs SF, et al; Genomic polymorphism, growth properties and immunologic variations in herpesvirus-6 isolates. Virology 184: 545-52, 1991; and by Schirmer EC, Wyatt LS, Yamanishi K, et al; Differentiation between two distinct classes of viruses now classified as human herpesvirus-6. 1991 Proc. Nat'l Acad Sci. 88: 5922-5926. The sera and monoclonal were used as recommended by the suppliers. The testing for anti CMV and HIV antibodies was performed at an outside Reference Laboratory. Anti-HTLV (Abbott, ELISA) and anti HHV-6 (Universal Technology Inc., immunofluorescence) testing was performed using undiluted as well as diluted antisera. Normal and patient's serum samples were diluted 1/10-1/100 and placed on acetone or alcohol-fixed cells from in vitro cultures. After 30 min incubated with serum, the slides were washed and a FITC-labeled rabbit-anti-human antibody added. Decorated cells were visualized under ultraviolet light. Viral infected or uninfected tissue culture cells were mounted for transmission electron microscopy (EM) according to standard procedures. Cultured cells were pelleted. The pellet was fixed with glutaraldehyde-osmium tetroxide, sectioned and placed on an EM grid. Electron micrographs were obtained by the Los Angeles County/University of Southern California Medical Center Pathology Department, Electron Microscopy Service and from the Doheney Eye Institute.

8.3 Results

A cytopathic effect (CPE) - characterized by the formation of dispersed refractile cells, was observed after 8 weeks of culture. The CPE was readily transferable to

MRHF cells, human lung diploid fibroblasts (MRC-5 cells), primary monkey kidney cultures (RMK) and murine (3T3), feline (H927) and hamster (CHO) fibroblast cell lines. The virus also grew in various long term cell lines of glial, muscle and lymphoid origin. The distinctive CPE is, however, most readily observed in primary fibroblast cultures. In these cells, the CPE progresses to extensive cell destruction and syncytial (foamy) cell formation (Figure 2). Electron microscopic examination of an infected culture showed numerous enveloped viruses approximately 180 - 200 nm in diameter. The viral particles were seen lining cytoplasmic vacuoles and were also present elsewhere within the cytoplasm (Figure 3). Prominent dense inclusions were present in association with the cytoplasmic vacuoles containing viral particles. Non-enveloped viral particles were seen in the nucleus. Extracellular virus, often showing signs of either incomplete development or partial degradation, was also seen.

The viral infected cells did not stain by direct immunofluorescence with commercial typing antisera for Herpes simplex virus (HSV), cytomegalovirus (CMV), HHV-6, human adenovirus or human enteroviruses. Positive staining, in an indirect immunofluorescence assay, was observed on infected cells using serum from the patient as well as sera from normal individuals, to a dilution of approximately 1:100. The patient's sera gave negative reactions in ELISA assays for CMV, HIV and HTLV and in immunofluorescence assays for HHV-6.

To exclude the possibility of laboratory contamination or other possible causes of a false positive culture, additional blood samples from the patient has been cultured on MRC-5, MRHF and other fibroblast cell lines. On ten of twelve separate occasions during the past 18 months, the blood cultures have yielded the same foamy cell CPE, transmissible to secondary and tertiary cultures. With daily refeeding of the cultures, the CPE from fresh inocula of the patient's blood is observable at

approximately 2 weeks. On passage, CPE can be seen within 2-3 days. Each of six positive cultures examined by electron microscopy have shown the same characteristic virus. The virus has also been cultured from CSF collected from the patient during a 1991 hospital admission. The CSF was otherwise unremarkable with normal protein (51 mg/dl), glucose (49 mg/dl) and only a single white cell per cu mm.

8.4 Conclusion

The repeated viral culture results confirmed that patient D.W. had a persistent virus infection. Isolation of the virus from otherwise normal CSF indicated that the virus was probably neurotropic and unable to evoke an inflammatory response. The foamy cell appearance of the CPE and the HTLV tax gene PCR reactivity of the patient's blood initially suggested that the virus may belong to a sub-family of retroviruses termed spumaviruses. The electron micrographic appearance, however, was more suggestive of a herpesvirus. Spumaviruses can be considered as recombinant viruses containing non-retroviral genes inserted between the envelope coding gene and the 3' long terminal repeat genes. Transgenic mouse studies indicate that the "bel" genes of a HSRV, a spumavirus isolated from a human, can cause neuropathological effects. I considered the possibility that the stealth virus may be a recombinant virus containing both retroviral and herpesviral sequences.

9. Example: Cloning and Sequencing of DNA in Stealth Virus Isolated from CFS Patient D.W.

9.1 Materials and Methods

PCR is performed on tissue culture cells as follows: cells from a single culture test tube are scraped into PBS, washed once and digested with 100 μg proteinase K. The subsequent procedures are identical to those used for proteinase K digested whole blood. Labeling of PCR products was performed using the random primer method

described by Feinberg AP and Vogelstein B; A technique for radiolabeling DNA restriction endonuclease fragment to high specific activity. Anal Biochem. 137: 266-269, 1984; using reagents from US Biochemical Cleveland, OH, to a specific activity of approximately 10^9 dpm/ug.) Coning of PCR products is achieved as follows: The PCR products are blunt-ended, phosphorylated and cloned as follows: Following completion of the PCR, 2 units of Klenow enzyme and 1 μM of each dNTP are added to the reaction mixture. After 30 minutes incubation at 14°C, the DNA is extracted using 100 μL of phenol and of chloroform. The DNA is precipitated using ethanol in the presence of KOAc and glycogen, washed once in ethanol and dried. The 5' end is phosphorylated using T4 kinase and ATP. Following a 37°C incubation for 30 min., the kinase is inactivated by heating the mixture at 65°C for 10 min. The reaction products are run in 0.8% low melting point agarose and the band of interest excised. A T4 ligase reaction is performed using purified pBluescript vector, previously cut with EcoRV and treated with calf intestinal phosphatase. The ligation reaction is allowed to proceed during an overnight incubation at 14°C. The enzyme is inactivated by heat (65°C for 10 min.). Transformation into XL-1 competent cells is achieved by a 40 min. incubation on ice followed by a heat shock at 42°C for 90 sec. The bacteria are plated on LB agar containing ampicillin, IPTG and X-gal. Colorless colonies are screened for an insert using the PCR product as probed and confirmed using the bacteria as template in the PCR. Sequencing of PCR products is performed according to the dideoxy/deoxy nucleotide termination method of Sanger F. Milklen S and Coulson AR. 1977. Proc Natl. Acad. Sci. USA 74; 5463-5467.

9.2 Results

PCR assays, using the HTLV tax gene primers SK43' and SK44", were performed on virus infected MRC-5 and MRHF cultures derived from CFS patient D.W. The HTLV primers

consistently yielded an unexpectedly large band when examined by ethidium bromide stained agarose electrophoresis. The band had an apparent size of 1.5 kbp. The 1.5 kbp band was clearly distinct from several smaller products generated in the same PCR and from the 158 bp product obtained using the tax primers on HTLV-I infected cultures (Figure 4). The individual HTLV tax primers SK43' and SK44" were tested in PCR assays on viral infected cultures. As has been noted in other PCR assays using single primers (Wang WP, Myers RL Chiu IM. Single primer-mediated polymerase chain reaction: application in cloning of two different 5'-untranslated sequences of acidic fibroblast growth factor mRNA. DNA Cell Biol. 10:771-7,1991.), the SK44" primer used as a single primer was as effective in generating the 1.5 kbp band as was the combination of the two primers. Similarly, the SK43' primer set used alone could generate a discrete PCR product from the stealth virus infected culture from patient D.W. (see infra).

The 1.5 kbp PCR product(s) generated from the virus infected culture was excised from the agarose gel, labeled with alpha-^{32}P dCTP and used as a probe. It hybridized with extracts from infected cultures from the patient and with extracts from a positive culture from a patient (B.H.) who is described infra. It did not hybridize with material extracted from uninfected MRC-5 cells or cells infected with CMV, HSV, HHV-6, HTLV-I or HTLV-II. As expected, PCR generated products using the tax primers on the infected cultures hybridized strongly to the labeled probe. Results from a hybridization assay are shown in Figure 5.

Cloning and sequencing of individual recombinant plasmids showed that there were two distinct PCR products generated in virus infected cultures using the SK44" tax gene reactive primer. One product cloned into plasmid number 15-5-2 contains 1484 bases while the other product, cloned into plasmid number 15-5-4, contains 1539 bases. The sequences of the plasmid inserts are shown in Tables 1 and

2. Both inserts are flanked by the EcoRV cloning site (GAT/ATC) and the SK44" primer used in the PCR. Computer assisted analysis (FastA Program available from Genetic Computer Group, Wisconsin) showed no apparent homology between the sequence in plasmid 15-5-2 with any viral or non-viral sequence contained in the entire GenBank data base (updated as of 12/91). Analysis of the sequence of plasmid 15-5-4, however, showed highly significant, partial, homology with the AD169 strain of human cytomegalovirus (GenBank Accession number: X17403). FastA analysis revealed a 58% identity over a 1,201 bp overlap. The overlapping regions extended from nucleotide 140 to 1,311 of the insert and nucleotide 44,705 to 45,891 of the CMV genome. This region of the CMV genome is contained within the transcripts of both the UL33 and UL34 genes and is part of the protein coding sequence of the UL34 gene which extends from nucleotide 44,500 to 46,011 (Chee MS, Bankier AT, Beck AT, et al. Analysis of the protein coding content of the sequence of human cytomegalovirus strain AD169. 1990 Curr. Topics Micro. Immunol. 154: 126-169. Welch AR, McGregor LM, Gibson W. Cytomegalovirus homo-logs of cellular G protein-coupled receptor genes are transcribed. 1991 J. Virol 65: 3915- 3918). No significant sequence homologies were identified for the sequence beyond the region of overlap with the UL34 coding gene. In particular, the flanking regions adjacent to where the primer had been incorporated bore no significant relationship to the sequence of HTLV.

The sequences of the two plasmids were used to design sets of virus specific primers and detecting probe for use in the PCR. The regions used are indicated in Tables 1 and 2. These primers gave no detectable products when the PCR was performed on blood samples from normal individuals or on uninfected cultures or cultures infected with CMV, HSV, HTLV-I or HTLV-II. Strongly positive PCR responses, shown by a well defined band of the expected size on agarose electrophoresis and by Southern blot hybridization

with labeled probe, occurred when the PCR was performed on viral cultures derived from the patient D.W. even over a 3 log dilution. All six independently derived cultures from patient D.W. gave strong positive PCR. Moreover, frozen blood samples collected from patient D.W. over an 18 month period tested positive. Figure 6 shows the banding pattern of the PCR products obtained using seven stored blood samples. These data establish that the virus was derived from patient D.W.

The SK43' primer used by itself was also able to generate a PCR product of 660 bp. The sequence of this product, which shows no significant homology with known viruses is shown in Table 3.

It is possible to screen multiple primer sets for such cross-reactivity. As a further example, a positive PCR can be obtained from the stealth viral culture of patient D.W. using a primer that corresponds to a region of the Epstein-Barr virus. This product has been isolated, cloned and sequenced. Its sequence is shown in Table 4.

9.3 Conclusion

The available sequence data confirm that I have isolated a novel virus with at least some homology with a herpesvirus. To date, I have not identified known retroviral sequences in the virus. Using the available virus-specific plasmids, I am currently proceeding to isolate additional regions of the viral genome for sequencing. The example shows the use of PCR primers, which fortuitously bound to the viral template DNA, to derive clones and sequence data from stealth virus infected cultures. The same approach can be taken in using paraffin-embedded tissue sections or other non-viable samples containing stealth viral genomes.

Table 1. Sequence of PCR amplified product obtained from virally infected cells and cloned into plasmid 15-5-2.

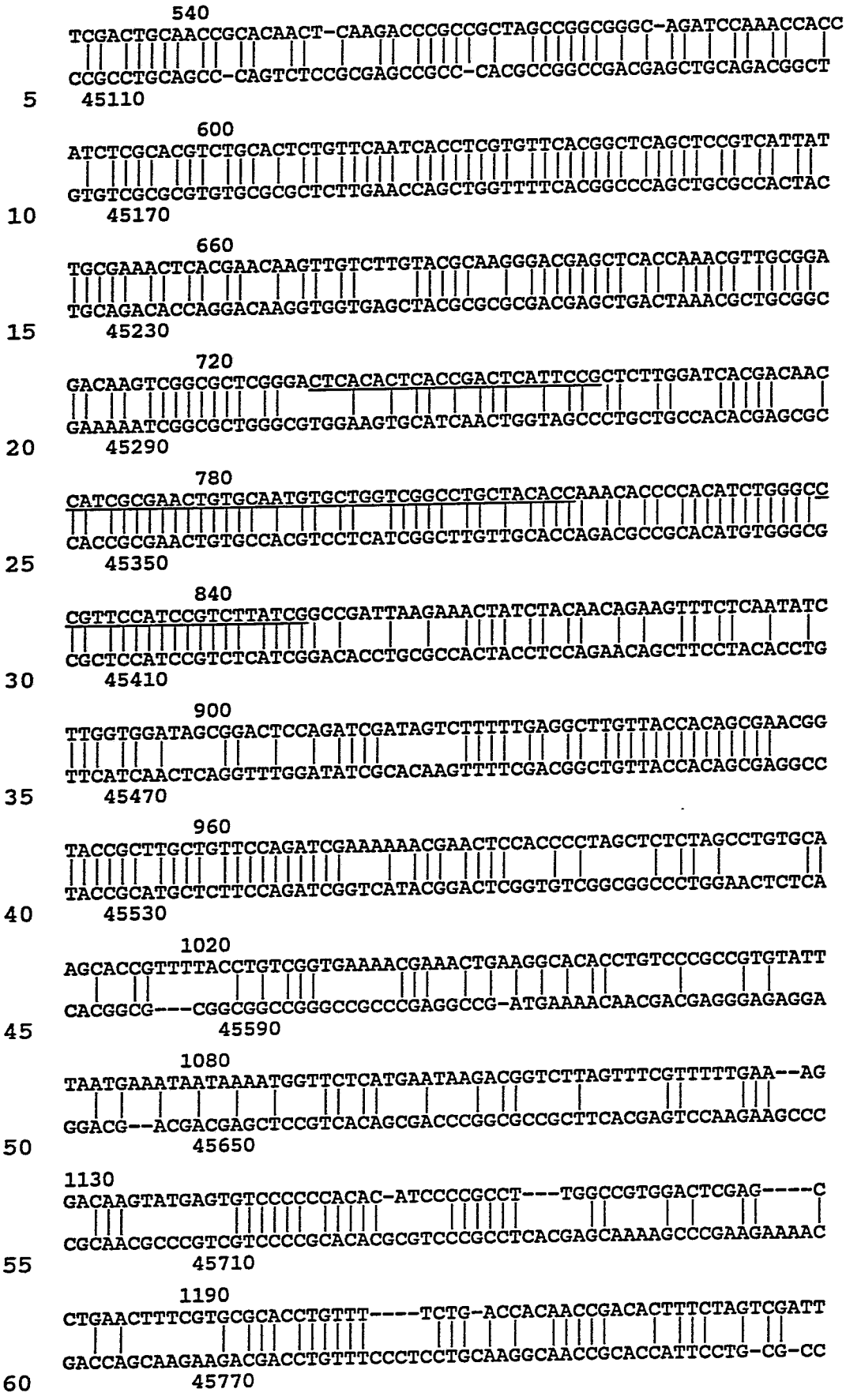
5' GATGAGCTGACAACGCGTCCATCGGAACATGACAGCACGGAAAC
TACCCCTATCTTGTCCGCCTCGCACAAACCCAGCACGAACACACCCCCGG
CTGTCTCACCTAAGCCAACCATCTCCAACGGCACCAAAAAGCCCATTGTT
CCACCAAAACCTAAACCGAAGCCAAAGCCGACGATGCTCCAGTTCCCCGC
ACCCAAAAGCGCCAGACCACGCCCAAGAACACTCAAAGTCCCAAAGTGTT
TACTTTTAACGAGCGTGACATAAGAAAGCACAAAGAAGAGATGGGCGCGG
AGGCCACGAAACCTAGAATCATCCATCACACAGAAGACAGAACCACCGTT
GACAGCGTCCTAACGCCGCTACTGCCACCTCCACCGCCAGCTCCCCGACG
GTATCAACATCGCGGGTGCCAGTGATGATTCCCTGGGACAACACGCAATC
TCCCGCGAAGATGAGTCCCTGGAAAGACACTTGCGAATCCCTGCCCACGG
AGCTGGACCCTTGGGAGTTTAGGCCCCGCCGTGGTTGTAGTAAAAAGAAC
TTGGACTGTCAATCAAACTGCAGATTGTATAGCTATTTAAACTTTATTTC
TGTATATATGTGTAAATAATAAATTTATTCCTCGTATCACACATCTGCAT
CCTGGTCATTCACATCTAGTATTCGCAGCGCAATTCGGGGCCCGGGAGGG
TGCATCATGGCGTCCGGGGCTATCTCTTCCATGATTAAGATAATCTCATC
TCCCAACGGAGGCCTGTTTTCCTGGTCCTCAGCCTGTATTTCCATAGCGA
TCTCTACCACCTCAGCCACCTCGGGAGGAAACTGCAACGGCTGGATCTGC
AGTTGAAGCTGCTGTCGTTGCAGATAGTTCTGAAACAGTCTCCGCCGAGC
CCGAGGAGCATACATGCCTAGAGGGCGATGGGCAGGTTGGTTTAAACGAT
AAAACGAAGCCCGGATAGAAGGCATGAGACCGCGGACGGCCTCTGATAAG
AGGGGATCGGTGCTGCCTCCCATAGTGCCAGTAAGATTCTGGGAGAGTAG
ACATTCCTAAATACTAGCCTGGATCTGACGTCAACACTATGATTCACGCC
CAATTCCACCCACAAAGCCCGTTAGAATACCAGACAACGTCCCCGTTAGT
GATGCCACCCACACAAGATATTTAATGATAACAGAGTTTCAGACCGCCTT
TGTTGAATCGATTCCAATACCGGCATCATGAGGAATCTACAGCTGATCGC
AACTTTGCTAGTTATCGGTTTGGTGGCAGTTCATGCCATCCCCAGGTTGG
AATATGTAACCATATATATCGCGCCCTAGTTATAACCATATTTGACCTGA
AATACTAATGATTCTTTCCTCTCACATGTGCAGGAGAACCACAGATGATA
ATAAAAAGACAACAAACTCAGACTTAAGCAACCAGACCGACGGTGGCGCT
GGCTTCGGCAGCACATACGATGGACGCGTTGTCAGCTCATC-3'

The sequence is shown as read from the T3 sequencing primer of the plasmid. SK44" primer sequences are indicated by bold type. The underlined segments show

the positions of the primers and the detecting probe which were synthesized to enable plasmid-specific PCR amplification. A second plasmid (15-5-1) gave essentially identical results, as did sequencing of a cloned Pst I digest of the PCR products.

<u>Table 2. Sequence of PCR amplified product obtained from virally infected cells and cloned into plasmid 15-5-4 and comparison with the sequence of CMV</u>.

```
                  10
5'-GATGAGCTGACAACGCGTCCATCGGCCAGATACATAAGTTTACACTCACTGGTACTTGTC
5'-GTGGCGCCCCGGCTTCATTATAACGCCACGTCGGAGCCCCTGCGCGCCACAACGCCGTCC
   44570

             70
ACTCACTTGATCACTTTGCTGCGGACGTGACGGCCAATCGTTTCGGGCAGGAGTGGCCAA
GGCGCAACTTCTGTCTCGGCACGGTACGATAAAAACAACGTCCCCCGTCGACGTTGTTTT
44630

            130
ACGGCATTATAACGAAACGCCGACCGGGGCCACACGCCAC-TTGGAAACGCCGCCGTTAG
CTCCGAGCGGTGATCGTTCCCGTCCCTCTCCTCCCTCCGCGGCCCCCACGGCGGCGGCCT
44690

             190
TCCTTTTTTCAACGGTACGATATCGG--CAATCCCCAT-GAC--TATGAACATAATTATA
GCTCGCACGGACCTATACTATTACCGCCCGACCGCCGTCGTCGTCATGAACTTCATCATC
44750

     240
ACCACCCGCGAATTCTCCAATGACGAATCCGTCACAGAGAG-CACGGAACCGCAAGACAA
ACCACCCGAGACTTCTTCAACGACGATTCAGTC-CTGCGAGCCGCCGAGATGCGTGACAA
44810

      300
CGTTGCCAATAACCTTTCTAAAGCTTACCGAGGTACGATTCGCGCGGAAGGTAAGAAGAA
CGTGGCAGGCTCGATTTCCAAAGCTTACCGAGGTACGATTCGCGCGGAAGGTAAGAAGAA
 44870

      360
ACTGCTTATTCGCAACCTG--CCTGCC-ACTTTCGGCTGCACTCGCCGCAACAGTAATTT
GCTGCTGCTGAAGCACTTGCCCGTGCCGCCCGGCGGCTGCTCGCGCCGCAACAGCAACCT
 44930

          420
ATTTATATTTTATAACGACCGGGACTATCGAAAGTTCCATCAAGGCATCATACAGTTAAA
CTTCGTTTTCTGCACCGAGCGCGACTACCGCAAGTTCCACCAGGGCATCGCACAGCTCAA
 44990

          480
ACGAACTCGCACGCGAGTTGATTCATCACAGATCGTGAACGTTACAAAAAATATAAAGTC
GCGCGCGCCGGCCGAACTGGACCGCCACGAGATCCAGCAAGTCACGGCCAGTATCCGCTG
 45050
```


540
TCGACTGCAACCGCACAACT-CAAGACCCGCCGCTAGCCGGCGGGC-AGATCCAAACCACC
CCGCCTGCAGCC-CAGTCTCCGCGAGCCGCC-CACGCCGGCCGACGAGCTGCAGACGGCT
45110
600
ATCTCGCACGTCTGCACTCTGTTCAATCACCTCGTGTTCACGGCTCAGCTCCGTCATTAT
GTGTCGCGCGTGTGCGCGCTCTTGAACCAGCTGGTTTTCACGGCCCAGCTGCGCCACTAC
45170
660
TGCGAAACTCACGAACAAGTTGTCTTGTACGCAAGGGACGAGCTCACCAAACGTTGCGGA
TGCAGACACCAGGACAAGGTGGTGAGCTACGCGCGCGACGAGCTGACTAAACGCTGCGGC
45230
720
GACAAGTCGGCGCTCGGGACTCACACTCACCGACTCATTCCGCTCTTGGATCACGACAAC
GAAAAATCGGCGCTGGGCGTGGAAGTGCATCAACTGGTAGCCCTGCTGCCACACGAGCGC
45290
780
CATCGCGAACTGTGCAATGTGCTGGTCGGCCTGCTACACCAAACACCCCACATCTGGGCC
CACCGCGAACTGTGCCACGTCCTCATCGGCTTGTTGCACCAGACGCCGCACATGTGGGCG
45350
840
CGTTCCATCCGTCTTATCGGCCGATTAAGAAACTATCTACAACAGAAGTTTCTCAATATC
CGCTCCATCCGTCTCATCGGACACCTGCGCCACTACCTCCAGAACAGCTTCCTACACCTG
45410
900
TTGGTGGATAGCGGACTCCAGATCGATAGTCTTTTTGAGGCTTGTTACCACAGCGAACGG
TTCATCAACTCAGGTTTGGATATCGCACAAGTTTTCGACGGCTGTTACCACAGCGAGGCC
45470
960
TACCGCTTGCTGTTCCAGATCGAAAAAACGAACTCCACCCCTAGCTCTCTAGCCTGTGCA
TACCGCATGCTCTTCCAGATCGGTCATACGGACTCGGTGTCGGCGGCCCTGGAACTCTCA
45530
1020
AGCACCGTTTTACCTGTCGGTGAAAACGAAACTGAAGGCACACCTGTCCCGCCGTGTATT
CACGGCG---CGGCGGCCGGGCCGCCCGAGGCCG-ATGAAAACAACGACGAGGGAGAGGA
45590
1080
TAATGAAATAATAAAATGGTTCTCATGAATAAGACGGTCTTAGTTTCGTTTTTGAA--AG
GGACG--ACGACGAGCTCCGTCACAGCGACCCGGCGCCGCTTCACGAGTCCAAGAAGCCC
45650
1130
GACAAGTATGAGTGTCCCCCCACAC-ATCCCCGCCT---TGGCCGTGGACTCGAG-----C
CGCAACGCCCGTCGTCCCCGCACACGCGTCCCGCCTCACGAGCAAAAGCCCGAAGAAAAC
45710
1190
CTGAACTTTCGTGCGCACCTGTTT----TCTG-ACCACAACCGACACTTTCTAGTCGATT
GACCAGCAAGAAGACGACCTGTTTCCCTCCTGCAAGGCAACCGCACCATTCCTG-CG-CC
45770

```
      1240
TAGTAACCCAAAGCTGCAGCGGCTATGTGGGACTG--TGTAACGCCGGAATTCCCATCCC
CAGAACCCTCCGTCTCCAACGACGACG-GCAACGGCGGCGAACGCTGCGACACGC-TAGC
 45820
      1300
CACCTATGTACTGGAAACGCTAATTGACTTTCAGGTTCCAACCACATACACGAAAATTAA
GACCGCCCTGC-GGCATCGCGCCGACGAAGAAGACGGACCTCTAGCCAGCCAGACCGCTG
      45880
       1360
GCCCATTGCCGTAAAAGTGCTAAAAATCTGTATTCTGGCTAACTACCTAAAAAACAGTAA

TGCGGGTCGCCGCGACCCCCTCACCTTCAGTCACCCCAGCCCTTACCCCCGTCACGTCCC
       45940
       1420
AGAATTATGGATTGATTTCAAAGCTAACCTAGACGAGATTAATTCTGGTGCAAATAAGCA

CCATAACCCCCGTTGTGTATTTAACGTCACTGGAGGACAATAAAGCGTTGATTTCTCAACT
       46000
       1480
AAGACTGTACAGAGGCTTTTACAAACTATGTCGTGATAAAAACTCGATGCACGCGTTGTC

TCCGCTCTGGTTTTGGTTTCGTTTTCAAAGGGAGCCCCATCATGGCCCAACGATCGCGAG
       46060
AGCTCATC-3

CCCCATCC-3
```

The upper row in each pair of sequence is that of plasmid 15-4-4 read from the T7 primer. The lower row in each pair is that of human CMV (Genbank accession number X17403). The nucleotide numbers are shown. The SK44" primer sequences incorporated into the plasmid are indicated by bold type. The underlined segments show the positions of the primers and the detecting probe which were synthesized to enable plasmid-specific PCR amplification. A second plasmid (15-6-1) gave essentially identical results, as did sequencing of a cloned Xho I digest of the PCR products. Note the sequences identified in this viral isolate are present in some but not in the majority of other cultured stealth viral isolates.

Table 3. Sequence of PCR amplified product obtained using SK43' primer on viral culture from patient D.W.

5-GGAATTCGATCGGATACCCCGTCTACGTGTATTAATATTACATAAAATAGGCTT
TTTTTTAAAAAAAAGAAAAGACATTTTTCACTAATGGTGTCATATCATTATAAT
AAACCTTGTTTTCATCAGGAAGGTATAAAAACAAATTCATATGCACTAAATAAT
ATAGATTCAAAACAAATAAGGCAAAAATCAATGGCAACAGAATAAGCATATATA
TAAACATGGTGAAAAATTACATATAAACACCAAGAATGTGGAAGATTTAGCTGT
GATTAGCAAATTTTGCCTAATGGATATATATGTATAAACTTGTCCCAATATCTA
CAGAGTACTCATTCCTATCAAACACAAATAAAACAGTTCTTAAAAATTCAGTAC
ATATTGTGTCAATTTTAAAAATAAGCTTCAAAGTTTTGATACTATAATTTAGAA
ACTATCTCGAGGGAAATAATATAAATAGTTTAAATAAAAGTGAGGTGAAACTAA
TGTATATTTAGATGAAGCAGTATAGTTTTAAATTTACATATTATAAAAGAAGAA
TATTAATGAACTAAACATACATCCTAAGAAGTTAGAAATAGAATAGCAAAATAA
ACTCAAAGAAAGCATAAAAAAAGAAACTGGTGGAACGGGAAACACACGTAGACG
GGGTATCCGATCAAGCTT-3

10. Example: Evidence Based on Viral Culture of Stealth Virus Infection in Blood of Additional CFS Patients

A large number of patients diagnosed with CFS have been tested for stealth virus infection using the culture method described in detail in this Section.

Table 4. Sequence of PCR amplified product derived from stealth virus from patient D.W.

The product was amplified using a single EBV reactive primer, cloned into pBluescript and sequenced as described above. The plasmid is designated number 7.

5'-
TATCGATAAGCTTGATTTCGCGTTGCTAGGCCACCACTAATGCATGATTTTTCTTT
CAAATATACCAACACATAAAATACGATAGTAGCCACACAGCAACAAATAATGAAAT
CATGTACCGAAGAGGTTCAGGTCCAGTTAAAAATAGAAAAGTATGAATAAAGTGCC
TCCATCCCTTAGGGAATTCGATTTCGCGTTGCTAGGCCACCGCTTTGTTTTTTGCA
ATCTCCTACGGTAAAAGTAATACAAGGGAATGGAGAGCCGCCGCTCGATACGCACT
AGCACTGCAATTGGAAATTCGATCCAAAGAAGAACCGTGGACGCCACTTGAACCTC
GCATATTTCAGCGCGTGTATTTGGAACACGACACGACTTGCGAACTCAACAATGAT

CAACTACATGTCAGCGGAACTGTGATTGGAAATTTTACAAATACAGCTTGGATGCA
TGTTAGTCTGAGTTATCCTAAGTTCAAGGAAATGTTCGTCATGTCTACCAACCCAG
ACATCACAGTGAA-3'

10.1 Materials and Methods

The following procedure was used to isolate stealth viruses from blood. Blood was collected into an anticoagulant (heparin, EDTA or citrate). Approximately 5 ml of blood was layered onto 3 ml of Ficoll-Hypaque in a centrifuge tube. The tube was centrifuged for 20 minutes at 800 x g. Lymphocytes collect at the interface of the plasma and the Ficoll-Hypaque, while granulocytes are present within the upper region of the erythrocyte pellet, which passes through the Ficoll-Hypaque. Both the lymphocytes and the granulocytes (mixed with some erythrocytes) were collected in a volume not exceeding 1 ml, with effort to minimize the number of erythrocytes. The collected cells are washed once with tissue culture medium (Medium 199, Whittaker Bioproducts supplemented with 7% FCS), and resuspended into 1 ml of medium. The cells (0.2 ml aliquots) were then added to test tubes containing monolayers of human MRC-5 lung fibroblast, MRHF primary human foreskin fibroblasts and RMKC rhesus monkey kidney cells. The cultures are available from Whittaker BioProducts, Maryland. Prior to inoculation, the 2 ml of media in the tubes (which contain 2% FCS) is replaced with 1 ml of medium with 7% FCS. The inoculated tubes were returned to a 37°C incubator for 40-60 min (less time if erythrocytes are abundant). The tubes were then rinsed with phosphate buffer saline to remove macroscopic evidence of remaining erythrocytes. Two mls of medium containing 7% FCS were added. At 24 hours the tubes were again rinsed to remove remaining erythrocytes, as assessed by microscopic examination under low power magnification, and refed. Refeeding was also performed at 48 and at 72 hours. Thereafter, refeeding is performed at two day intervals, with the

exception that routine refeeding of cultures was not normally performed on Sundays. The tubes were examined for CPE as described in Section 5, supra, at two day intervals. Passage of infection was achieved by scrapping of the adherent cells into suspension and vortexing the tube. Volumes of 0.1 - 0.2 were used with refeeding at 24 and at 48 hours. Control blood samples were obtained from a variety of sources including medical students, mothers of children visiting a pediatrician, University clerical and laboratory personnel and friends of CFS patients.

10.2 Results

Samples were considered positive for stealth virus if positive CPE was observed in at least one of the three cultures, that could be transmitted to a secondary culture. It is worth noting, however, that some cultures that showed evidence of an imminent CPE failed. This was particularly true early in the study, before the culture techniques were refined to account for the toxicity seen in infrequently fed cultures (see Section 13., infra). The overall results of this study, as well as the more recent results obtained during 1992, are shown in Table 4.

Table 4. Stealth Virus Detection in CFS and Normal Patients

Total number of culture performed on patients provisionally identified as having CFS	1,342
Total number of cultures scored as positive for stealth virus infection	547
Overall percent positive cultures	41%
Total number of cultures performed on normal control individuals	235*
Total number of cultures scored as positive	0
Overall percent positive cultures	0%

* 35 performed during 1992

Number of cultures performed during 1992 on patients provisionally identified as having CFS	194
Number of cultures scored as positive for stealth virus infection	131
Percent positive cultures	68%
Number of cultures performed in 1992 on patients in whom the inventor was consulted and in whom there was well defined evidence for severe CFS	34
Number of these patients showing a positive culture for stealth virus	28
Percent positive culture of a CFS illness	82%

10.4 Conclusion

Not all clinically diagnosed CFS patients have tested positive using the fibroblast culture system. During the last 6 months, approximately 70% of CFS diagnosed patients have given positive cultures. This may indicate a limitation in the present culture system or the existence of different processes involved in the pathogenesis of this complex syndrome.

11. Example: Evidence Based on Viral Culture of Stealth Virus Infection in CSF of Additional CFS Patients

11.1 Materials and Methods

Stealth virus was cultured from cerebrospinal fluid (CSF). Approximately 0.5 ml aliquots of CSF were each used to inoculate MRC-5, MRHF and RMK cells. The cultures were rinsed once after an hour. The cultures were subsequently processed in a manner similar to that for whole blood.

11.2 Results

Positive stealth virus cultures have been obtained from the cerebrospinal fluid of in 8 of 25 CSF samples cultured. Included in the 8 positive cultures were 4 individuals in whom the lumbar puncture was performed on the same day as the culture.

11.3 Conclusion

The result confirm that stealth virus can be isolated from CSF. Consideration needs to be given to the use of a viral transport medium for those specimens that cannot be cultured on the day of collection.

12. Example: Evidence Based on Viral Culture of Stealth Virus Infection in Patients with Atypical Neurological, Psychiatric and Rheumatological Diseases

I have applied the same culture technique to test for viral infection in patients who have presented diagnostic

difficulties to their attending neurologists, psychiatrists and rheumatologists.

12.1 Materials and Methods

The same culture methods were used as described for CFS patients.

12.2 Results

The following section briefly describes several patients presenting with atypical symptoms in whom repeated positive viral cultures have been obtained. The clinical histories have been abstracted from the patients' medical records and from discussions with the patients and/or their attending physicians.

Patient B.H. This 23 year old woman was admitted to a general hospital from a community mental hospital. She had been diagnosed four years previously as having a manic-depressive illness and had received lithium therapy. In early 1991, the patient was admitted to the community hospital after becoming acutely delusional, with visual, oratory and tactile hallucinations. She continued to exhibit speech perseveration, grandiose delusion and thought disorganization. Seventeen days later, she became comatosed with seizures and was admitted to a teaching hospital as a suspected drug overdose (for which no evidence was subsequently found). Her EEG showed marked disorganization. The patient underwent a transient cardiac arrest following which her EEG was considered indicative of irreparable anoxic damage. A virus capable of inducing foamy syncytial cells was cultured from the patient's CSF obtained soon after her admission to the hospital and subsequently also from blood cultures. Interestingly, the patient's CSF at the time of the positive viral culture was otherwise unremarkable (protein 23 mg/dl, glucose 80 mg/dl, 2 WBC per high power field). By electron microscopy, the viral infected cells showed the

characteristic vacuolated appearance with abundant viral particles within the cytoplasmic vacuoles (Figure 1).

Patient J.T. This is a 51 year old women admitted under restraint to a County psychiatric hospital because of aggressive behavior with her neighbors. She had been diagnosed two years previously as chronic paranoid schizophrenia. On admission she was described as having auditory and visual hallucinations. She also complained of vague somatic pains involving her head and back. On interview, her major symptoms over the last two years had been tiredness, palpitations on effort and social withdrawal. She attributed her social difficulties to her uncertainty as to what she was saying to others. During her present admission, both haloperidol and tricyclic medication were provided but were discontinued because of toxicity. She improved clinically on lithium therapy but remained virus culture positive. Eight of 12 additional psychiatric in-patients with atypical affective disorders considered either manic depressive or schizophrenia, have tested positive by viral culture.

Patient T.R. This 31 year old female patient has had intermittent episodes of ptosis of the right eyelid beginning 8 years previously. Typically, each episode would last 1-2 weeks with gradual improvement. Paresthesia and a sense of altered pain sensations were also noted. Her CSF showed an elevation in immunoglobulin synthesis (5.7 mg/day compared to a normal value of less than 3.3 mg/day) with 2 oligoclonal bands. A diagnosis of multiple sclerosis was made at that time. The patient also began to experience a chronic pain syndrome. She has undergone ten abdominal operations for pelvic pain attributed initially to ovarian cysts and later to abdominal adhesions. She has had recurring headaches and an episode diagnosed as pseudotumor cerebri (3) (opening CSF pressure of 350 mm). Over the last several years, she has developed severe myalgia, pain, swelling and redness of the joints of her finger and toes. The patient was referred by her neurol-

ogist to a rheumatologist. Although, tests for antinuclear antibodies and rheumatoid factor were negative, a skin biopsy showed a positive "lupus band" test and a diagnosis of systemic lupus erythematosus was made. She continues to experience cluster headaches (4), arthralgia and has electromyographic evidence for proximal muscle myopathy. The patient's blood has repeatedly tested positive for virus induced CPE with marked syncytial cell formation. Her CSF is also positive for CPE. While the protein and glucose levels were normal, there were 17 lymphocytes and a single erythrocyte per cu mm. The CSF opening pressure was elevated (320 mm). Four of 10 additional patients with a clinical diagnosis of SLE have tested positive by culture. One of these patients T.M. is described infra.

Patient G.P. This 50 year old man presented 6 years previously with bilateral testicular and groin pain with bilateral parotid enlargement. A biopsy of his right testis was unremarkable but the operation was complicated by bacterial infection, necessitating unilateral orchiectomy. Testicular, and to a lesser extent parotid gland pain, has persisted and has incapacitated the patient. His therapy has included various attempts at pain management including phenol injections into the remaining testis, narcotics and psychological support. The patient has experienced frequent overwhelming episodes of fatigue, sleeplessness, impaired concentration, difficulty in assimilating complex information and in decision making. His blood culture, as well as a throat culture, have been repeatedly positive for CPE and the presence of the characteristic virus confirmed by electron microscopy. A fine needle aspirate of an enlarged submandibular gland showed oncolytic ductal change and slightly swollen acini. There was no evidence of inflammation. By electron microscopy, however, disrupted foamy cellular debris with viral-like particles were seen (Figure 2). The patient's wife has

increasing depression, headaches and fatigue. On testing, her blood is also virus culture positive.

12.3 Conclusion

An important conclusion from this study is that it has help confirm a link between stealth virus infection in patients with CFS and some patients presenting with more severe neurologic, psychiatric and/or rheumatologic disease. Stealth virus infection may, therefore, be associated with a wide range of clinical illnesses affecting the central nervous system (CNS) and peripheral tissues. The various neurological and psychiatric manifestations of infection may represent the relative involvement of different regions of the CNS and may also be influenced, in part, by the cultural background and self expectation of the affected individual. Highly educated individuals are more prone to comment on cognitive dysfunction and more guarded in expressing irrational thoughts. The clinical case definition of CFS is somewhat ambiguous in that relatively mild neuropsychiatric symptoms can support the diagnosis, yet more severe symptoms of psychiatric illness are considered exclusionary criteria in ruling out the diagnosis (Kendell RE Chronic fatigue, viruses and depression. Lancet 337: 160,1991. Hickie I, Lloyd A, Wakefield D, et al. The Psychiatric status of patients with the chronic fatigue syndrome. Brit J Psychiatry 156: 534-540, 1990.).

In addition to major psychiatric diagnoses (schizophrenia and manic depressive illness), viral infection has been seen in association with severe encephalopathies. As in the CFS patients in whom virus has been isolated from CSF, there was no or only minimal evidence of an accompanying inflammatory reaction.

The isolation of virus from a throat culture of one of the patients (G.P.) and positive viral culture obtained from his wife suggest a possible means of horizontal transmission of infection. In fact, several examples exist in

which both husband and wife were found to be stealth virus positive. In one couple, the wife had clearly defined CFS, whereas her husband (J.L.) showed no cognitive dysfunction but had elevated liver transaminase enzyme. His liver biopsy showed steatosis (fatty change) with Mallory bodies (normally an indication of alcoholic hepatitis). This individual did not drink. Nor were there signs of inflammation. Stealth virus was cultured both from this patient's blood and from his liver biopsy.

13. Example Evidence of Molecular Heterogeneity Among Different Isolates of Stealth Viruses

Partial sequencing of the stealth virus from patient D.W. has been completed and virus specific primers made. The question was raised whether these viral sequences were detectable in other stealth viral isolates. The viral cultures isolated from patients B.H., J.T., T.R., G.P. and two CFS patients (N.R. and L.B.) were analyzed using PCR assays. The primers included the HTLV tax gene primers (SK43' and SK44" and the specific primers based on the sequences in plasmids 15-5-2 and 15-5-4 containing PCR products amplified from cultures from patient D.W.

13.1 Materials and Methods

PCR assays and the cloning and the sequencing of PCR products was as described supra.

13.2 Results

The SK43' and SK44" primers generated PCR products from the various stealth viral cultures. When examined on agarose gels, however, only the culture from patient B.H. gave a banding pattern similar to that seen with the viral culture from patient D.W. This finding was consistent with the previously observed cross-hybridization seen with labeled PCR products between these two cultures. PCR products could also be generated on the culture from patient B.H., using the primers based on the sequences of the

cloned plasmids obtained from the virus infecting patient D.W. In spite of this molecular similarity, at least in the regions so far examined, the fine details of the CPE associated with the viruses from patients D.W. and B.H. show clear differences. For example, the size of the cell syncytia is larger in cultures from patient D.W.

A PCR product of about 600 bp which was generated using the SK43' and SK44" primer set on the culture of the patient L.B. was cloned and sequenced (Clone 18). The PCR product contained both the SK43' and the SK44" primer sequences. This, is in contrast to the situation with the cloned PCR products generated from the cultures from patient D.W., which contain either the SK43' or the SK44" primer, but not both. The sequence of the product derived from the L.B. culture is shown in Table __. It shows no relationship to known viruses or to the previously sequenced PCR products from patient D.W. In spite of this, the electron microscopic appearance is quite similar to that of the virus from both patients D.W. and B.H.

Table 5. Sequence of PCR Product Generated Using the SK43' and SK44" Primers Stealth Virus Culture From Patient L.B.

AAGCTTGATCGGATACCCCGTCTACGTGTAACACCTGGAAAGTTAATGTT
CAGTGAAGCGCCCCAATGTCGCTGAATCCACCCAGCTCCTCACCTGCAAG
TTGGCCAACATGATGTGTCAAGTTGGGGACATGAATGCTTGTCCCACCTG
CCCTGGGAGAAAAGATCATAGAAGTGAAATGACCTTGTAAACAGCAAAGT
CCTGTGCAAATATAATGGTCCTTGTTGAGTCTTTTCCACATTCATAATCG
ATGTTTGTCTGACGCTGACCCCTGCTCCAGAACCACCCCCCCCACTCCCC
GGTCTGCTGTCGGGGAGCGCCAGGACACACTTGGCTCTTGGGCAGTTTTA
AGTAGGTTTAACGTTCTCACACTGATAGAAGTGGTGTACTTTAAAGATGA
ATTAAAATGAATACTTTATTAGTAACTCAGCTGTGCTTACTGCTAGATTC
CTTAAAATAATGCCCCTGCCTTTCCCACAATGACAGGGCTTGAATTTCTT
TTTTTGCGAAGTGTGGTGGTGAGTCACAATCATTTCCGATGGACGCGTTG
TCAGCTCATCGAATTCC

13.3 Conclusion

These findings establish that various stealth viruses may differ in their genetic composition. In spite of this difference, the viruses show common characteristics when cultured in fibroblasts. A homologous genetic region responsible for the common biological property, such as the induction of foamy cells in vitro, is anticipated to be found with additional sequencing. An advantage of sequence heterogeneity among isolates is that it can be used to trace disease transmission.

14. Example: Evidence for Stealth Virus Culture from Animals

Domestic pets, including dogs and cats, of patients with the chronic fatigue syndrome (CFS) appear to have a higher than expected incidence of neurological disease. A patient T.M. who had been clinically diagnosed as having systemic lupus erythematosus (based on an anti nuclear antibody titer of 1:1,280) also has many of the cognitive signs typical of CFS patients. As an example in some cognitive tests she would score in the 99 percentile, while in another she scored in the 9th percentile. A stealth virus had been grown from the blood of this patient on several occasions. Recently, the patient noted that her cat showed an acute onset of marked lethargy and had several episodes of confusion and seemingly irrational behavior. For example, the cat was distracted by a noise as it was about to begin eating its food. The cat became perplexed and had to be reminded of the presence of the food. Once this registered, the cat rapidly went back to eating. On another occasion, the cat showed fear when approaching its food. Rather than playing, the cat now prefers to sit about the house. Blood from the cat was, therefore, cultured for stealth virus.

14.1 Materials and Methods

The methods used were essentially similar to those used for human blood. The same indicator cell lines were used. In other cultures from cats and dogs, the volumes have had to be reduced.

14.2 Results

Within 3 weeks, the blood from the cat gave a positive stealth virus CPE on both MRC-5 and MRHF cells. The CPE was successfully transferred to secondary cultures. Molecular cloning and sequencing of this virus and of the original isolate from patient T.M. are in progress. Plans are underway to attempt experimental therapy in the cat using cerulenin (infra).

14.3 Conclusion

This study provides direct evidence for potential human - domestic pet transfer of infection under natural circumstances. In a related study, Dr. Tom Glass of the University of Oklahoma had performed autopsies on two cats of CFS patients to see if there was evidence of a foamy cell inducing viral infection. Pathological changes, by light microscopy were seen in the brain and in several other organs in the cats. Blood from one of the cats was transferred to a healthy cat which subsequently became sick. In a collaborative study, I determined that: i) Viral particles similar to those of stealth viruses could be seen by electron microscopy in the brain. ii) The affected brain tissue not only showed evidence for gliosis, but that some of the glial cells showed positive staining using an antibody reactive with interleukin-6. iii) Amplified products could be generated using formalin-fixed, paraffin embedded tissues from the brain of the cats. These products will be isolated and sequenced. iv) The donor of the first cat was positive for stealth virus infection by culture. The cat studies provide an excellent model to study the pathogenesis of stealth virus

infection in humans. Along with similar studies in dogs, these findings highlight the concern for animal - human transmission as well as the potential of disease transmission within the food chain.

15. Example: Evidence for Experimental Transmission of Human Derived Stealth Virus to Animals

The stealth virus cultured from patient D.W. on which considerable DNA sequence information was available, was provided to Dr. R. Glass of the University of Oklahoma for inoculation into two cats. The inoculum consisted of the equivalent of the content of a single test tube of MRC-5 cells showing a 2?4 plus CPE. Control cats received either boiled virus culture supernatant or the content of an uninfected culture. The cats were observed for signs of illness. Cats receiving the viable virus shows significant changes in temperament becoming very difficult to handle. They shy away from light and from human contact. The animals were noted to frequently rub their bodies against their cages and experienced considerable fur loss. The illness became most intense two weeks after inoculation and gradually receded. Blood obtained at 3 weeks was cultured and the stealth virus recovered.

15.1 Conclusion

This study confirms the potential transmission of stealth virus between humans and animals. It also provides a well controlled setting to perform experimental therapeutic studies. Using all necessary precautions, additional studies are planned to determine potential transmission of the stealth virus to farm animals, fishes, microalgae, bacteria, fungi and various human parasites.

16. Example. Virus is Destroyed by Boiling but is Resistant to Freezing and to Drying

The supernatants and cell extracts from virus infected cultures can readily transmit the infection to

secondary cultures. Depending upon the concentration of virus used, the CPE in the secondary culture can be first observed 2-4 days following transfer. If the inoculum is boiled for 10 minutes, it loses its capacity to transmit infection. As discussed infra, there is a transient toxic effect demonstrable in the recipient culture but infectious virus cannot be recovered from the secondary culture. In contrast to this result, if the culture supernatant from an infected culture is frozen and stored at either -10°C or -80°C, upon thawing it is still infectious for secondary cultures. If the fluid in an infected culture is allowed to evaporate to dryness and the dried tubes stored for several days at room temperature, infectious virus can still be reconstituted by the addition of fresh medium and passage to secondary cultures. This experiment indicates that infection may be transmitted from environmental sources contaminated with virus even if the material proceeds to dryness.

17. Example Toxic Effects of Stealth Virus Culture Medium and Patient Sera

Culture supernatants and patient sera were tested for toxin. The supernatant and patient sera were boiled and added to cultures of normal fibroblasts. After three-days exposure to the toxin the fibroblasts were observed to show many of the same toxic characteristics that had previously associated with cultures showing signs of active infection. In particular, many of the fibroblasts appear to lose their normal appearance of vitality, and in some cases became swollen and died. The normal "streaming" appearance of the fibroblast cultures diminished substantially, and some of the cells acquired a dullness and showed evidence of retraction. No "foamy cell" associated CPE was observed with these cells, indicating that no viral activity was present. This was confirmed by attempting to passage the infection to additional cultures.

There was no evidence that the boiled supernatant was able to transmit infection.

The effect of the boiled supernatant on the growth of subsequently added non-boiled supernatant was investigated. Prior exposure of normal fibroblast cultures to boiled supernatants significantly delayed and in many cases completely suppressed the outgrowth of the virus associated CPE. In other words, fibroblasts exposed to the toxin did not support virus growth.

The effect of using sera from stealth virus positive patients on the in vitro growth of stealth virus was also investigated. Several sera from chronic fatigue syndrome patients had a toxic effect on normal fibroblasts and appeared to be slightly inhibitory to stealth virus growth compared to normal sera. A similar effect was seen in testing cerebrospinal fluid from a CFS patient.

These findings led to efforts to determine the importance of frequent refeedings of cultures to allow for viral growth. Parallel cultures showing early CPE changes were refed at varying intervals extending from daily to weekly. The CPE tended to regress in cultures refed at intervals greater than 2 days and could be completely reversed in cultures not refed for 5-7 days. Similarly virus growth was inhibited in cultures simply refed with "spent" medium from other infected but not uninfected cultures. The susceptibility to these virus growth suppressive effects was most pronounced on early passage isolates. With frequent refeeding and attention to optimal concentrations of fetal calf serum, the time required to develop CPE in cultures from patient D.W. has steadily lessened.

The toxic changes seen in normal fibroblasts exposed to boiled supernatants was reminiscent of some of the morphological changes seen in fibroblast cultures exposed to the marine polyether toxin okadaic acid. Indeed, the addition of 5 nM [nano Moles) of okadaic acid was able to significantly delay viral growth. Conversely, the addi-

tion of retinoic acid, a compound which neutralizes some of the toxic effects of retinoic acid on human fibroblasts showed a tendency to enhance stealth virus growth.

18. Example. Changing Culture Conditions Decreased Culture to CPE Time

A practical application of the detection of a virus suppressive activity in culture supernatants was the improvement seen in the stealth virus culture assay. Specific changes in culture method, including early passage of cells, increasing the level of FCS, and frequent replacement of culture medium, resulted in a decreased culture to CPE time. This improvement is reflected in the progressively shorter time required for CPE in repeat stealth viral cultures from patient D.W. Table 6.

Table 6. Improvement in Culture Time with Improved Culture Methods

Date of Culture* (days)	Culture to CPE Time
0	61
55	48
62	31
75	14
97	15
152	9
176	8

* Date of culture is the number of days from initiation of the first in vitro culture isolated from patent DW. Each culture was initiated by inoculation of MRHF, MRC-5 and RMK cells with sample from the patient.

The results in Table 6 indicate that progressive improvement in culture to CPE time resulted from increasing the frequency of refeeding cells (replacing culture medium) and increasing the concentration of FCS above 2%. Pres-

ently, a FCS concentration of 5%-10% (7% in standard cultures) has been found to be adequate, as has refeeding every second day, as opposed to daily refeeding.

19. Example. Detection of a Product in Stealth Virus Infected Cultures Using an Immunoassay for Ciguatera Toxin

A sample of boiled supernatant was provided to Dr. Doug Park, Department of Nutrition and Food Science, University of Arizona, for testing. In particular, the supernatant was tested for the presence of polyether compounds antigenically related to okadaic acid and to ciguatera toxins of marine origin. Ciguatera toxin is associated with particularly virulent seafood poisoning in which some patients progress to a chronic illness with clinical features somewhat similar to those associated with the chronic fatigue syndrome e.g., fatigue and impaired short term memory. Using broadly reactive immunoassay capable of detecting ciguatera polyether toxin found in seafood (see Juranovic and Park, 1991, supra), a four plus positive reaction was reported for the supernatant of viral infected culture compared to a negative finding with the supernatant of uninfected culture. Subsequent to this, sera from several patients with CFS was sent to Dr. Parks. One of the sera (from patient N.R.) also gave a strong positive (four-plus) response similar to that seen in the culture supernatant. In these assays, the positive control consisted of 0.5 μg okadaic acid which gave a five-plus near maximum response. It was of interest that the stealth virus culture from patient N.R., had previously been noted as showing evidence for production of lipid-like material.

These findings led to the suggestion that chronic disease following apparent ciguatera poisoning may be the result not of the toxin itself but of infection with a toxin producing microorganism, possible a stealth virus. Efforts were made to infect dinoflagellates (Gambierdiscus

toxicus and Prorocentrum concavum from Bigalow Laboratory for Ocean Science, Maine), with a stealth virus. The preliminary experiment gave an indication of infection but could not be confirmed in a subsequent attempt, possibly because the dinoflagellates had died.

19.1 Conclusion

A stealth virus-associated toxin is found in tissue culture fluid of in vitro cultures and in the serum of some patients with chronic fatigue syndrome. This toxin may itself cause symptoms of CFS or of other illnesses associated with stealth virus infection. The immunological cross-reactivity of the stealth virus associated toxin with the polyether marine toxins such as ciguatera toxin and okadaic acid, together with the knowledge that these seafood toxins can cause neurological symptoms is particularly intriguing. It suggests a possible functional relationship between stealth virus associated toxin and ciguatera or other marine toxins. It also suggests that chronic illness resulting from ciguatera associated poisoning may reflect not simply the ingestion of the toxin but rather actual infection with a stealth virus capable of continued production of the toxin in the affected subject. Ciguatera and other marine polyether toxins such as palytoxins, brevitoxin, were previously thought to originate as part of the normal metabolism of various planktons, especially some of the dinoflagellate microalgae species. Through the process of feeding, these toxins were thought to pass up to food chain through various fishes. Ingestion of the fish containing such a toxin was considered the cause of illness. One conclusion from the studies described above is that a virus, rather than the toxin itself may be involved in the transmission of the toxin within this food chain. The issue of animal to human transmission of infection is also highlighted by the previously

described finding of stealth virus infection in domestic animals.

20. Example Anti-Viral Activity of Various Biological and Chemical Compounds

The successful in vitro culturing of stealth viruses enables the testing of various biological and chemical agents for their effect on viral growth. The principal goal of this endeavor is the development of a safe and effective therapy for use in stealth virus infected humans.

20.1 Materials and Methods

A standardized inoculum of tissue culture derived virus was aliquoted into multiple samples and stored frozen. The tissue culture infectious dose of the aliquots was determined by making serial 10 fold dilutions and transferring a set amount (100 μL) to MRC-5 cells. The inoculated cells were examined daily and the time at which CPE was first noted was recorded. Thereafter, the rate of progression of CPE was recorded daily using a + (one plus) to ++++ (four plus) scale. The experiment was repeated except that into test samples, various biological and chemical compounds were added. The addition could either precede, be given at the same time or be given after the virus inoculum. The effect on the subsequent development of CPE was recorded. In cultures in which the CPE either did not develop, or developed more slowly than in controls, the cultures were tested for the presence of infectious virus by passage of cells and/or supernatant into fresh cultures of MRC-5 cells. Normal MRC-5 cells were also used to test for any inherent toxicity of the biological or chemical compound under study. Specificity can be established using multiple isolates of stealth viruses and various other types of viruses which can be propagated in MRC-5 cells.

20.2 Results

Examples of compounds which were found to completely suppress growth of the stealth virus derived from patient P.W. include the following: i) Boiled culture supernatants from stealth virus positive cultures. ii) okadaic acid. iii) Alpha interferon (10^{-3} units per ml. iv) Cerulenin (an inhibitor of fatty acid synthesis) at 20 nM. Partial inhibition was seen with 30 mM lithium carbonate but not with 10 mM. No inhibition was seen with AZT or with Brefeldin at 2 μg per ml. or with non-toxic doses of AZT. Many additional compounds are awaiting testing in this system.

20.3 Conclusions

Preliminary data, especially the use of cerulenin, are encouraging. New therapies will be suggested as additional insight is gained into the pathogenesis of stealth virus infections becomes defined. Based on in vitro findings, the biological or chemical compound can be administered to animals either naturally or experimentally infected with stealth viruses. The effect of therapy can be monitored using clinical signs and culture and molecular probe based assays.

21. Deposit of Microorganisms

The stealth virus isolated from patient D.W. (virus-X infected MRC-5 cells) was deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedures on 9-17-91, and were assigned accession no. VR-2343.

The present invention is not to be limited in scope by the microorganisms deposited or the specific embodiments described herein since such embodiments are intended as but single illustrations of one aspect of

the invention and any microorganisms which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

It is also to be understood that all base pair sizes given for nucleotides are approximate and are used for the purpose of description.

Various references are cited herein, the disclosures of which are incorporated by reference herein in their entirety.

Claims

1. A method of diagnosing a stealth virus-associated disease in a human or animal subject suspected of having the disease, comprising detecting in a sample from the subject the presence of a stealth virus.

2. The method of claim 1 in which the presence of the stealth virus is detected by (i) inoculating a permissive cell line with a sample from the subject suspected of containing stealth virus; and (ii) detecting in vitro a cytopathic effect in the permissive cell line.

3. The method of claim 2, in which the cell line is maintained in culture medium, and further comprising frequently replacing the culture medium.

4. The method of claim 1 in which the presence of a stealth virus is detected by a method comprising isolating DNA from the sample, exposing the DNA to a nucleic acid hybridizable to a stealth virus nucleic acid under conditions such that hybridization can occur, and detecting any resulting hybridization.

5. The method of claim 1 in which the presence of a stealth virus is detected by a method comprising exposing protein in the sample to an antibody specific for a stealth virus antigen under conditions such that immunospecific binding can occur; and detecting any resulting immunospecific binding.

6. The method of claim 1 in which the presence of a stealth virus is detected by detecting the presence of a stealth virus-associated toxin.

7. The method of claim 1 in which the disease is chronic fatigue syndrome.

8. The method of claim 1 in which the disease is an atypical neurological disease.

9. The method of claim 1 in which the disease is an atypical psychiatric disease.

10. The method of claim 1 in which the disease is an atypical rheumatological disease.

11. The method of claim 1 in which the disease is an atypical auto-immune like disease.

12. The method of claim 1 in which the disease is an atypical disease involving liver, testis, ovary, salivary glands, lymph nodes, intestine or any other organ shown to be susceptible to infection with stealth viruses in humans and in animals.

13. A method of culturing a stealth virus associated with an atypical neurological disease comprising inoculating permissive cells with a sample containing the stealth virus and culturing the cells in vitro.

14. The method of claim 13 further comprising neutralizing stealth virus-associated toxic activity.

15. The method of claim 14 in which the neutralizing comprises frequently replacing culture medium.

16. The method of claim 14 in which the neutralizing comprises adding a neutralizing agent to the culture.

17. The method of claim 16 in which the neutralizing agent is selected from the group consisting of an amount of fetal calf serum yielding greater than 5% fetal calf serum in the culture medium, an antibody specific for the

toxin and a chemical agent capable of reversing the virus growth suppressive effect of the toxin.

18. A method of diagnosing chronic fatigue syndrome in a subject suspected of having chronic fatigue syndrome and/or an illness shown to be associated with the possible presence of a stealth virus infection, comprising detecting in a sample from the subject the presence of a stealth virus.

19. An isolated stealth virus corresponding to a virus whose presence in a subject is positively correlated with the presence of chronic fatigue syndrome in the subject.

20. The virus of claim 19 in which the disease is selected from the group consisting of multiple sclerosis, unexplained encephalopathy, acute encephalopathy, psychiatric disorder, Alzheimer's disease, fibromyalgia, myositis, muscle neuritis, chronic pain syndrome, salivary gland enlargement, autoimmune disease, and unexplained hepatitis.

21. The virus of claim 15 in which the disease is a chronic fatigue like syndrome occurring in a domestic cat.

22. An isolated virus as contained in the MRC-5 cell line deposited with the ATCC and assigned accession no. VR 2343.

23. An isolated stealth virus infected MRC-5 cell having all of the identifying characteristics of the MRC-5 cell line deposited with the ATCC and assigned accession no. VR 2343.

24. A purified stealth virus-associated toxin.

25. The toxin of claim 24 which is characterized by: (i) weak activity in a ciguatera toxin-specific immunoassay; (ii) the ability to induce loss of vitality of fibroblasts in vitro; and (iii) the ability to suppress stealth virus cytopathic effect in vitro.

26. A pharmaceutical composition comprising stealth virus-associated toxin in an amount sufficient to suppress stealth virus infection but subtoxic for the patient, and a pharmaceutically acceptable carrier.

27. A method of treating a human or animal subject suffering from a stealth viral infection comprising administering to the subject a therapeutically effective amount of an anti-stealth viral agent.

28. The method according to claim 27 in which the anti-stealth viral agent is selected from the group consisting of stealth virus-associated toxin, ciguatera toxin, α-interferon, lithium and cerulenin.

29. The method according to claim 27 in which the anti-stealth viral agent is selected from compounds shown to possess anti-stealth virus activity in vitro or to be active against other viruses known to affect human or animal subjects.

30. A method of treating a human or animal subject suffering from chronic fatigue syndrome or other stealth virus associated diseases, comprising administering to the subject a therapeutically effective anti-stealth viral agent.

31. A method of monitoring a stealth virus associated disease in an human or animal subject comprising detecting the level of stealth virus and/or stealth virus-associated toxin in a sample from a subject.

32. A kit for in vitro culture of stealth virus comprising an agent of that neutralizes stealth virus-associated toxic activity.

33. The kit of claim 32 in which the neutralizing agent is an antibody to the stealth virus-associated toxin.

34. A kit for detecting the presence of stealth virus in a sample comprising in a container a nucleic acid probe capable of hybridizing to a stealth virus nucleic acid.

35. A kit for detecting the presence of a stealth virus in a sample comprising in a container an antibody capable of immunospecifically binding a stealth virus antigen.

36. A purified antibody specific for an antigenic portion of a stealth virus associated with human or animal disease.

37. The antibody of claim 31 which is a human antibody.

38. A purified antibody specific for an antigenic portion of a stealth virus associated with a human or animal disease.

39. A nucleic acid probe comprising a purified first nucleic acid capable of hybridizing to a second nucleic acid of a stealth virus associated with a human or animal disease in which the first nucleic acid does not hybridize to nucleic acids from HTLV-I, HTLV-II, CMV, HSV, HHV-6, or adenovirus under the most stringent hybridization conditions which allow the probe to hybridize with the stealth virus nucleic acid.

40. The nucleic acid probe of claim 34 which is DNA.

41. The DNA probe of claim 35, in which the probe comprises a cloned stealth virus nucleic acid fragment or a portion thereof.

42. An isolated nucleic acid sequence encoding at least a hybridizable portion of a stealth virus nucleic acid sequence.

43. The stealth virus nucleic acid sequence of claim 42 as contained in a stealth virus infected MRC-5 cell deposited with the ATCC, and assigned accession no. VR 2343.

44. A vaccine comprising an immunogenic amount of a stealth virus antigen or fragment thereof containing an epitope, in a suitable vaccine carrier.

45. The vaccine of claim 44 comprising an isolated attenuated stealth virus.

46. The vaccine of claim 45 in which the stealth virus is an attenuated derivative of the virus as contained in MRC-5 cells deposited with the ATCC, and assigned accession no. VR 2343.

47. A vaccine comprising a vector capable of expressing an immunogenic amount of a stealth virus antigen, in a suitable vaccine carrier.

48. A method for screening a suspected human or animal source of infection or transmission of stealth virus comprising assaying in a sample from the suspected source of infection or transmission for the presence of a stealth virus.

49. A method for screening a food or other environmental substance or object suspected as being a possible source of stealth viral infection or transmission, comprising assaying in a sample from the suspected source of infection or transmission for the presence of a stealth virus.

PCR AMPLIFICATION ON CSF OF ADULT
WITH ENCEPHALITIS-LIKE ILLNESS

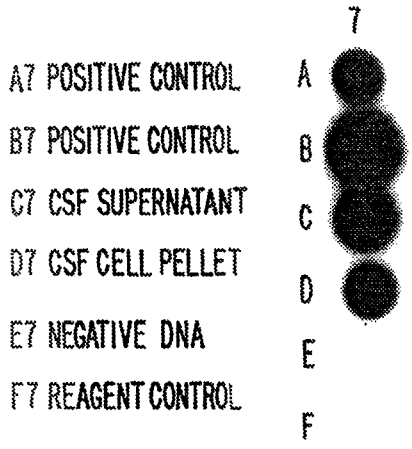


FIG. 1.

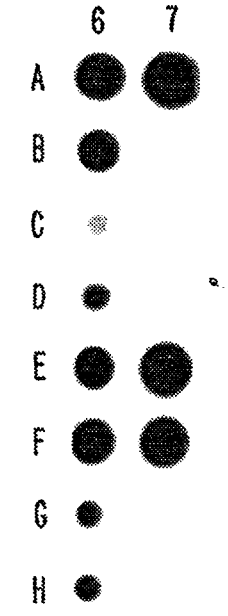
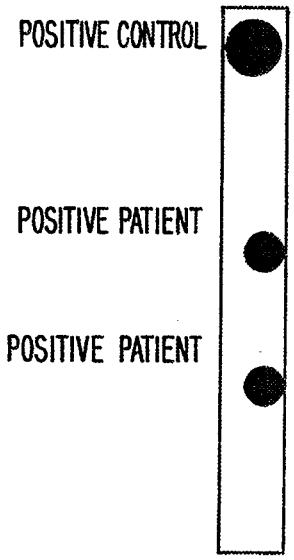


FIG. 2.

FIG. 3.



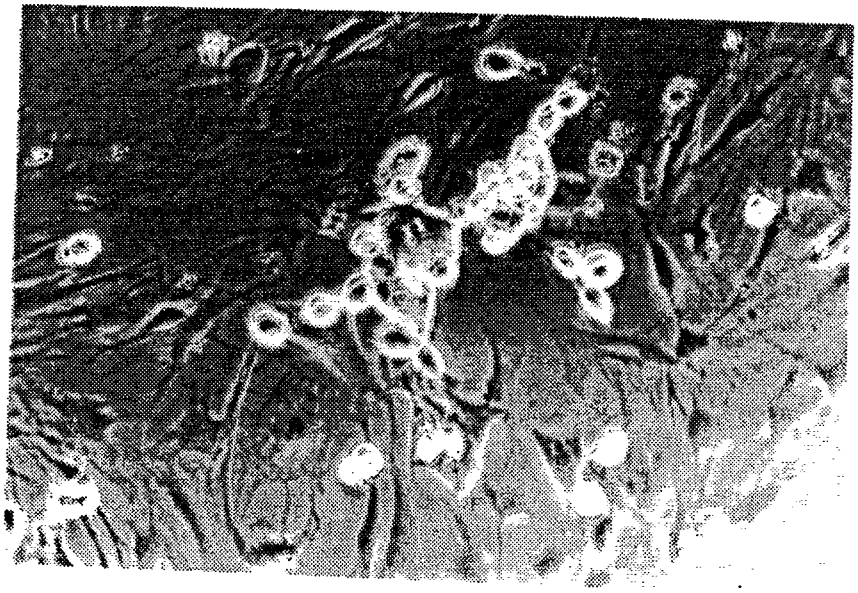


FIG. 5.

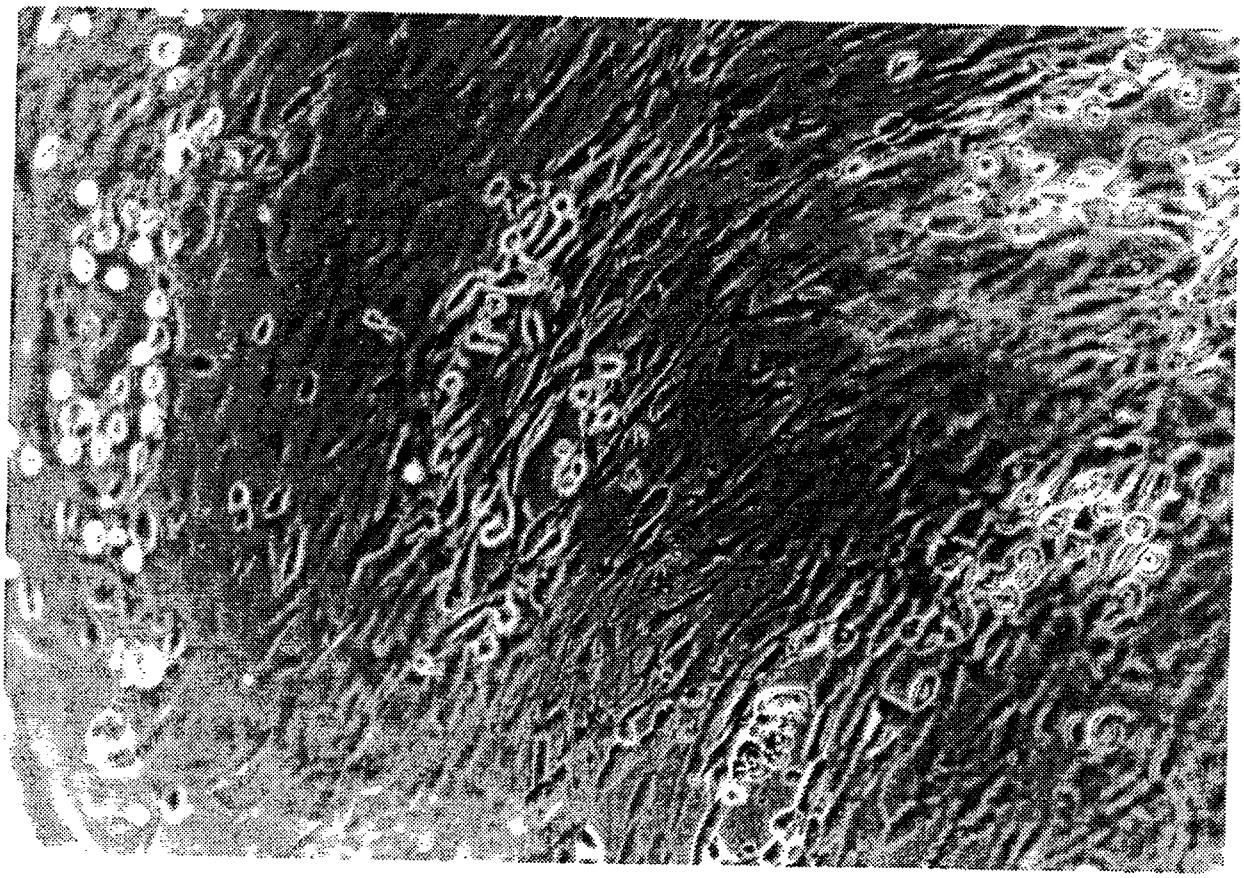


FIG. 4.

FIG. 5a.

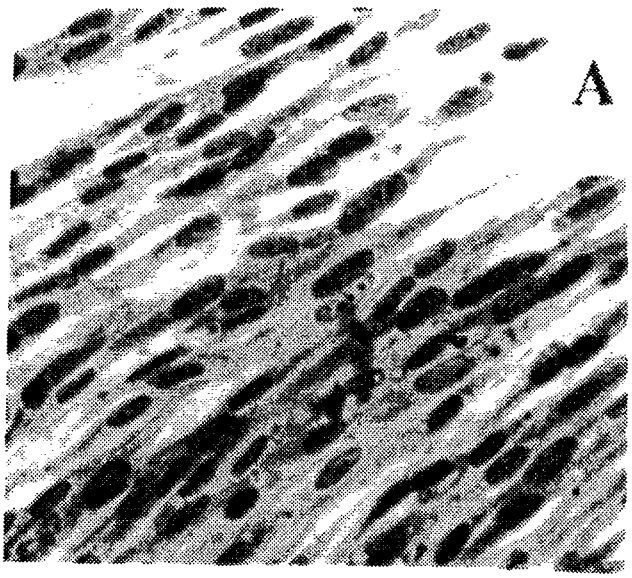


FIG. 5b.

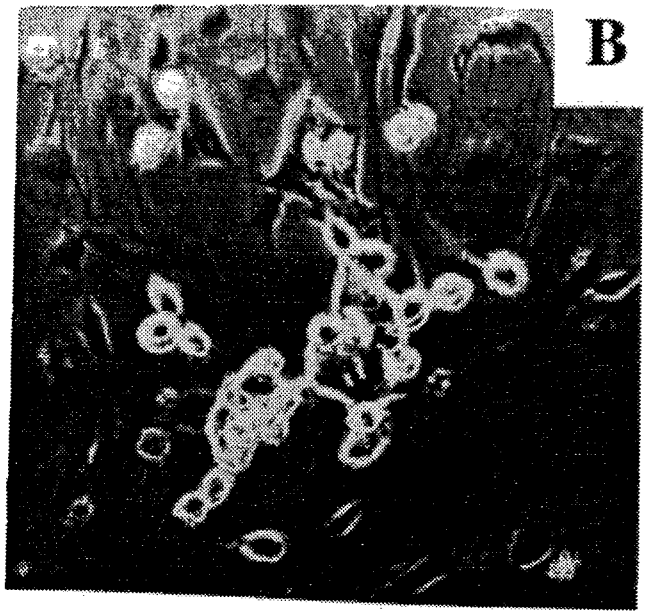
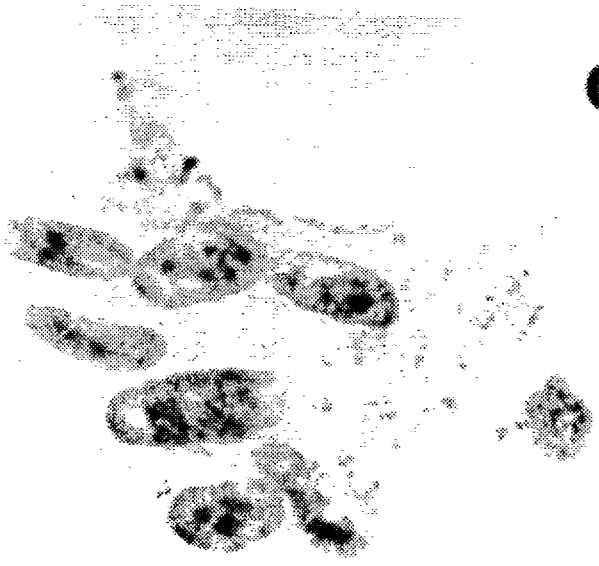


FIG. 5c.



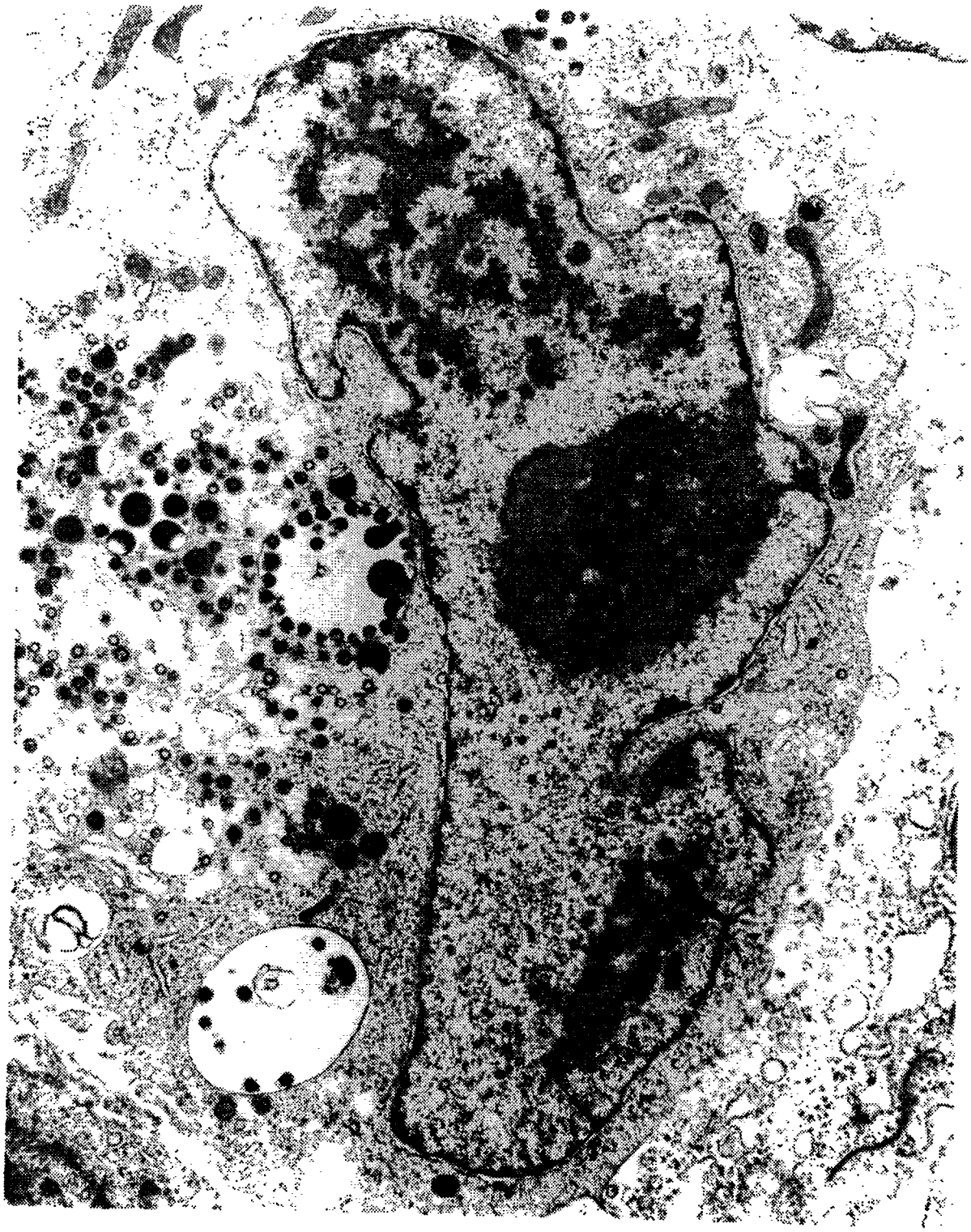


FIG. 6.

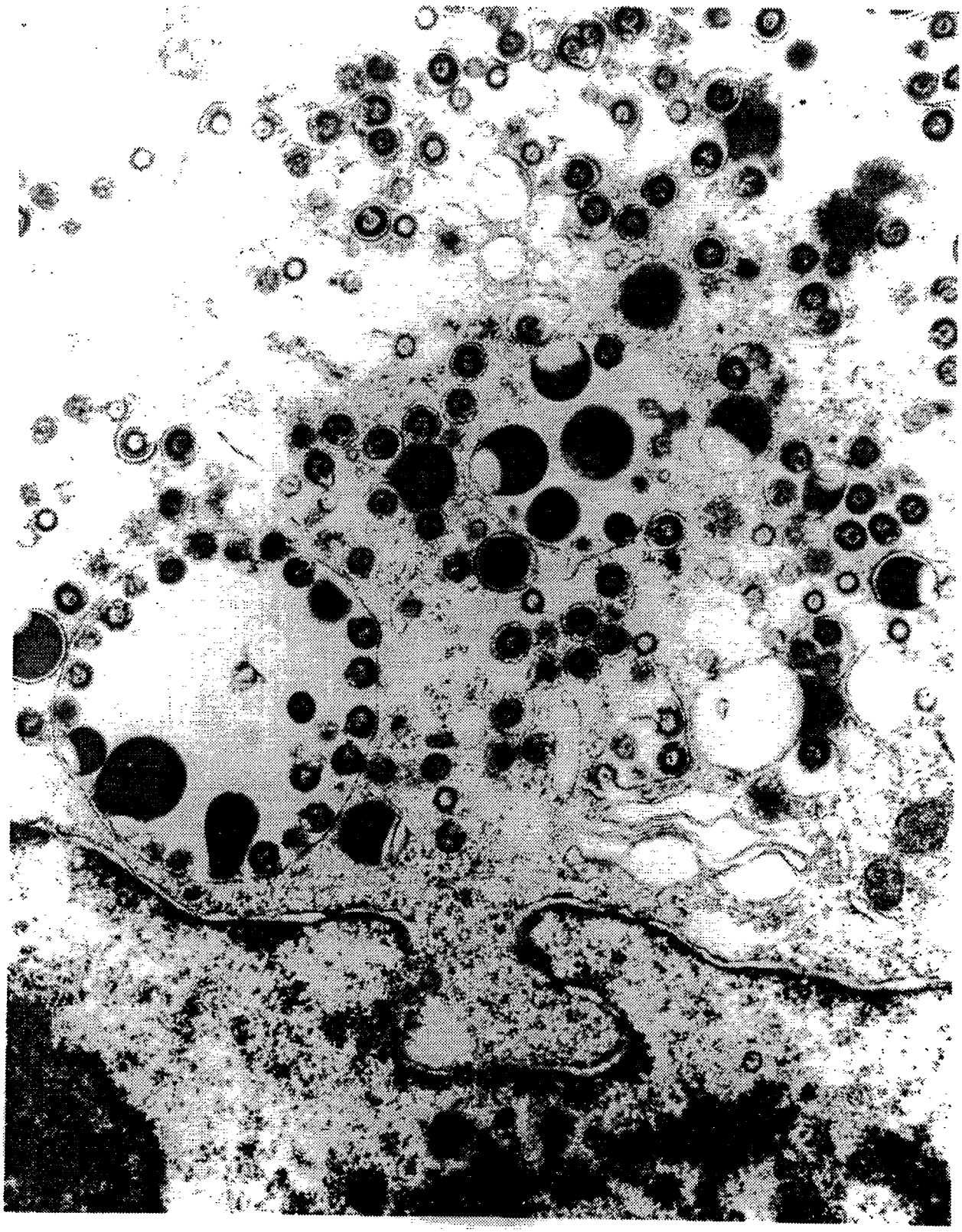


FIG. 7.

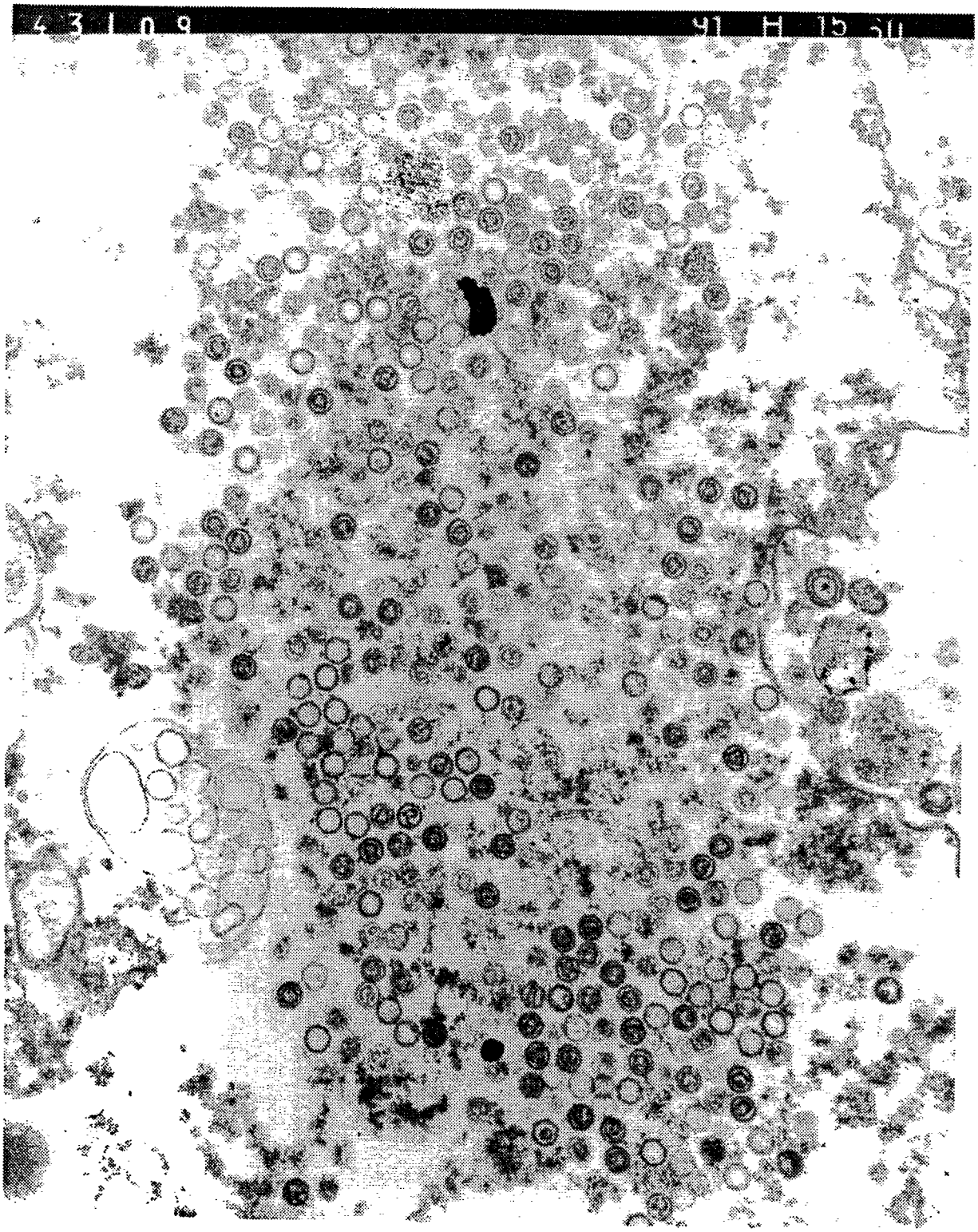


FIG. 7a.

PCR GENERATED PROBE USING X-VIRUS AS TEMPLATE

LANE 1 X-VIRUS PCR
LANE 2 HTLV PCR
LANES 3,4 SIZE MARKERS

BEFORE EXCISION OF BANDS A

AFTER EXCISION OF BANDS B

FIG. 8.

REACTIVITY OF LABELED X-VIRUS PCR PRODUCT

			1	2
A1	X VIRUS CULTURE	A		
B1	Y VIRUS CULTURE	B		
C1	CMV CULTURE	C		
		D		
E2	X VIRUS POST PCR	E		
F2	Y VIRUS POST PCR	F		
		G		
H2	UNINFECTED CELLS	H		

17 HR EXPOSURE, 1 SCREEN, EXP. DH-5

FIG. 9.

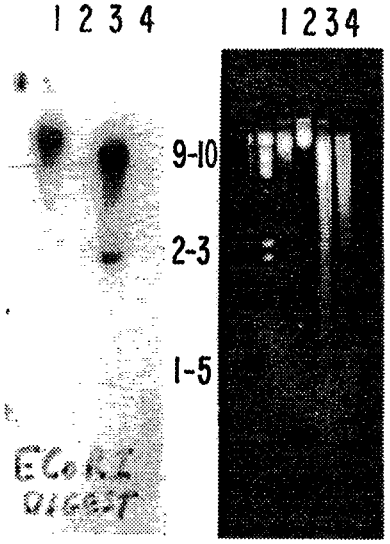


FIG. 10.

GR49① PCR ON VIRAL CULTURE

1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIG. 11.

PCR ON BLOOD

1 2 3 4 5 6 7 8 9

FIG. 12.

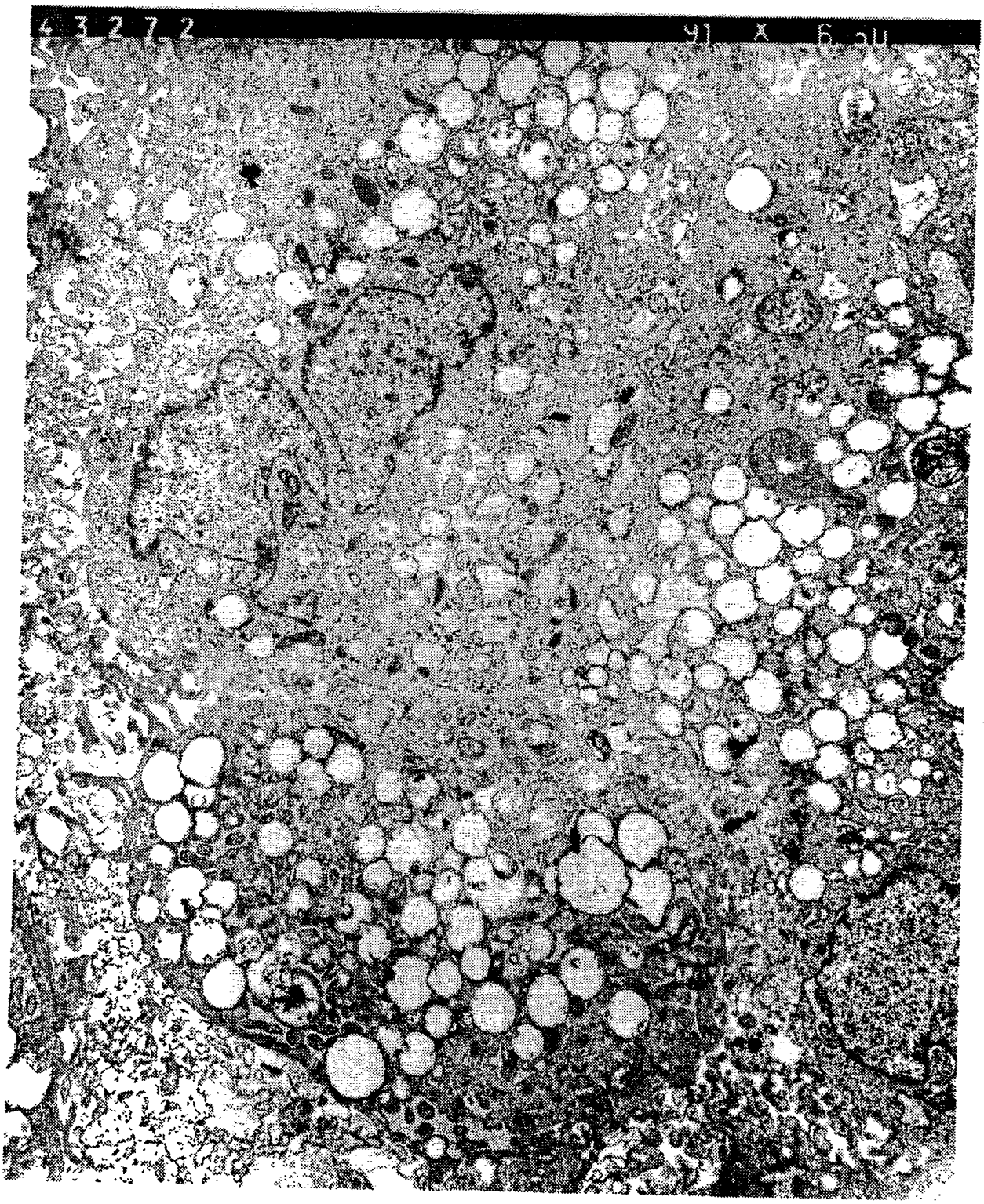


FIG. 13.

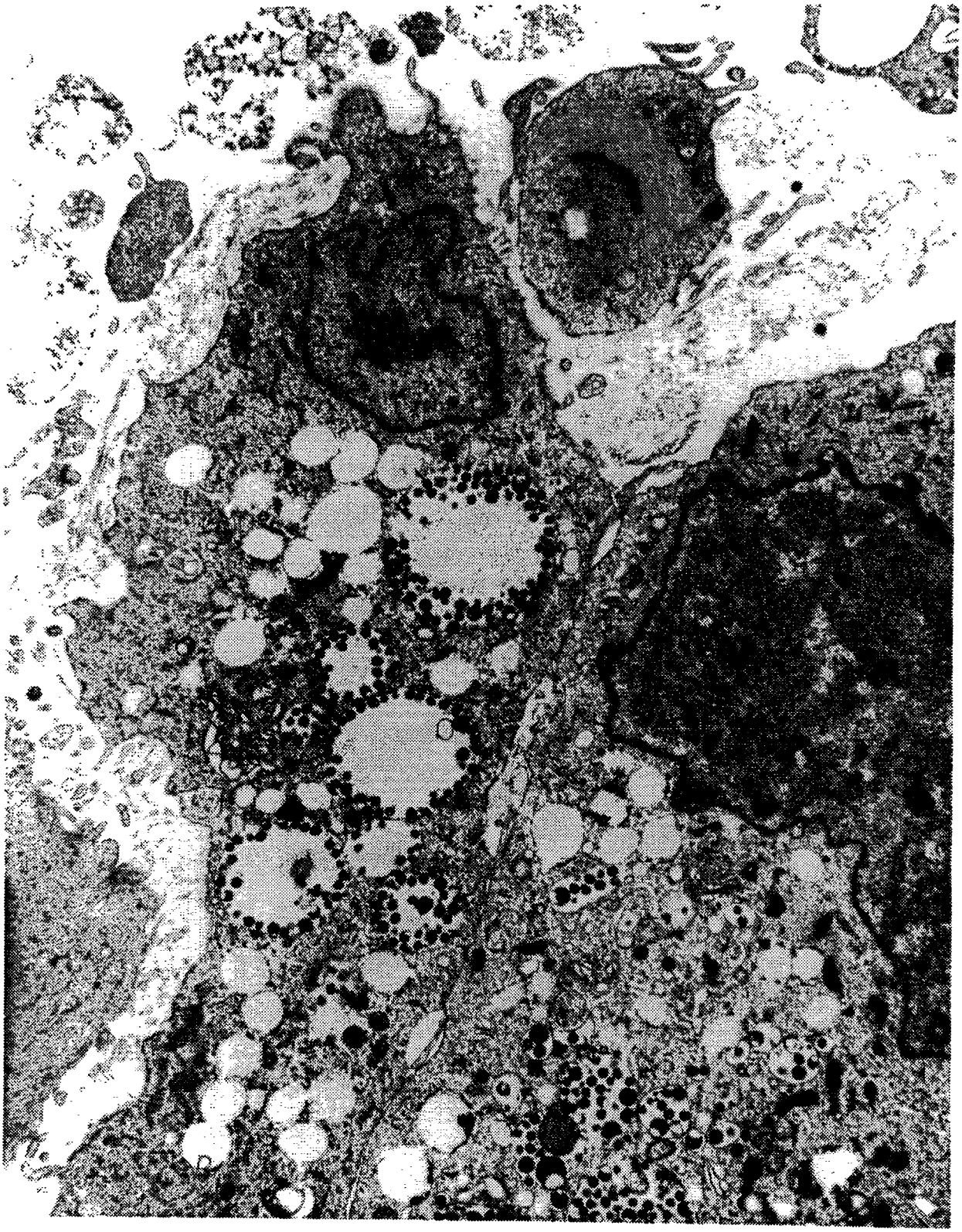


FIG. 14.

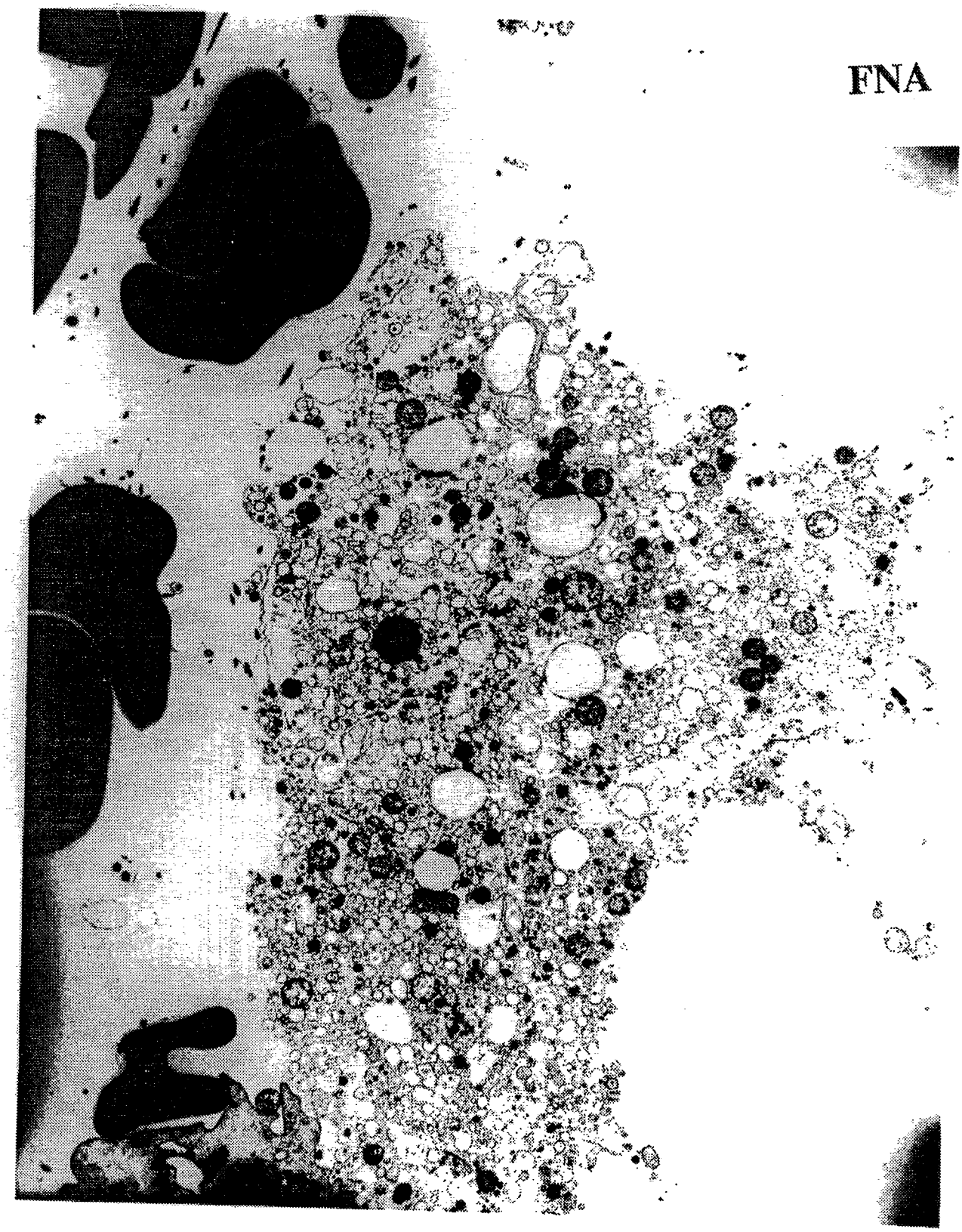


FIG. 15.

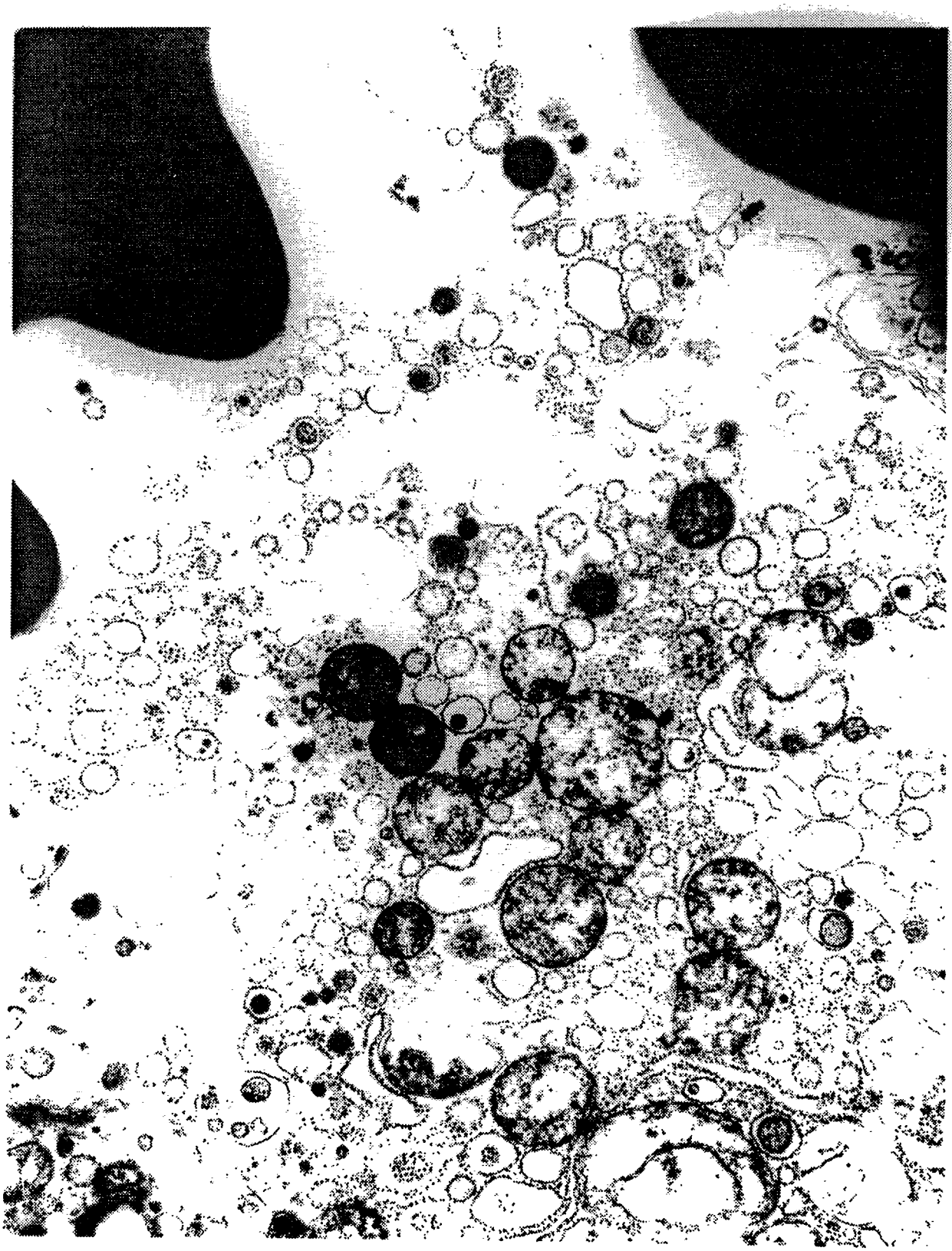


FIG. 16.

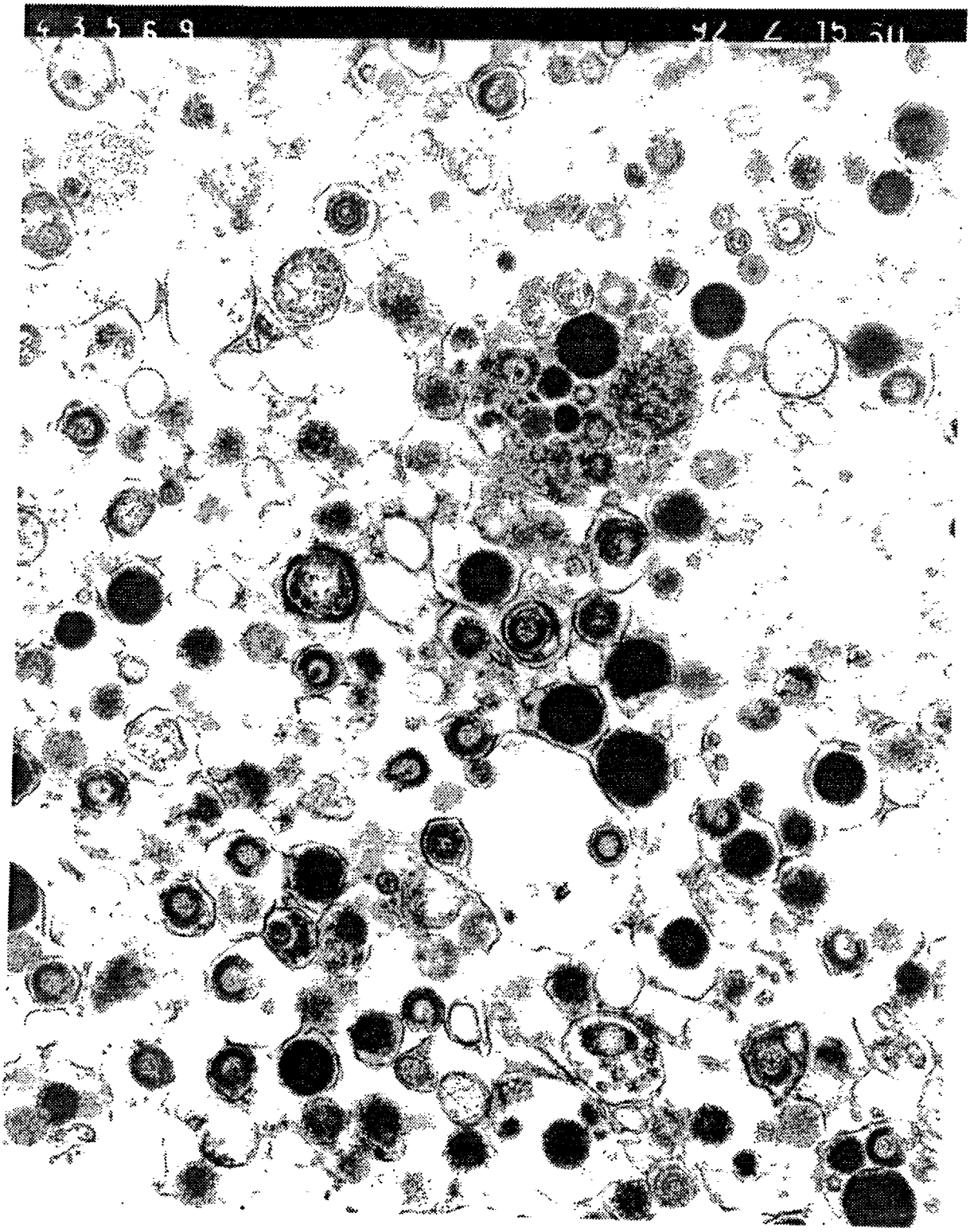


FIG. 17.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/04314

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :C12N 7/02, 7/00, 7/06; C07H 21/04; C07K 15/28
US CL :435/239, 235.1, 238; 530/388.3; 536/27
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. : 435/239, 235.1, 238; 530/388.3; 536/27

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, Biosis, World Patents Index.
Search terms: chronic fatigue syndrome, CFS, virus.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The Lancet, issued 4 August 1979, Werner et al., "Isolation of foamy virus from patients with De Quervain thyroiditis", pages 258-259. See entire article.	1-49
Y	Nucleic Acids Research, Vol. 18, No. 4, issued 1990, Rethwilm et al., "Infectious DNA of the Human spumaretrovirus", pages 733-738. See entire article.	1-49
A	Scandavian Journal of Immunology, Vol. 33, issued March 1991, "A comprehensive immunologica analysis in chronic fatigue syndrome", pages 319-327. See entire article.	1-49
Y	International Journal of Cancer, Vol. 42, issued 1988, Ablashi et al., "Utilization of human hematopoietic cell lines for the propagation and characterization of HBLV (human herpesvirus 6)", pages 787-791. See especially page 790.	1-49

[X] 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 19 August 1992	Date of mailing of the international search report [illegible] 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer PHILIP W. CARTER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/04314

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences USA, Vol. 85, issued December 1988, Frohman et al., "Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer", pages 8998-9002. See entire article.	4, 39-43
Y	Chemical Abstracts, Vol. 114, issued 1991, DeFreitas et al., "Retroviral sequences related to human T-lymphotropic virus type II in patients with chronic fatigue immune dysfunction syndrome", Abstract No. 205331c, Proc. Natl. Acas. Sci. USA 88(7), 2922-2926 (1991). See entire abstract.	1-49
A,P	Science, Vol. 254, issued December 1991, Palca, "On the track of an elusive disease", pages 1726-1728. See entire article.	1-49
Y	Science, Vol. 249, issued 14 September 1990, Palca, "Does a retrovirus explain fatigue syndrome puzzle?", pages 1240-1241. See entire article.	1-49
Y	Journal of Clinical Microbiology, Vol. 27, No. 5, Ng et al., "Reliable confirmation of antibodies to human immunodeficiency virus type 1 (HIV-1) with an enzyme-linked immunoassay using recombinant antigens derived from the HIV-1 gag, pol, and env genes", pages 977-982. See entire article.	5