



UNIVERSITÀ DEGLI STUDI DI FIRENZE

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**ENDOGENOUS RETROVIRUSES AS CONFOUNDING FACTORS IN THE
PATHOGENESIS OF AIDS**

Tesi di Laurea di:

Chiara Matteuzzi

Relatore: *Prof. Stefania Pacini*

Correlatore: *Prof. Marco Ruggiero*

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FOREWORD

The starting point of this thesis is the recent statement of Professor Luc Montagnier in which he declares: “We can be exposed to HIV many times without being chronically infected. Our immune system will get rid of the virus within a few weeks”. This statement of the Nobel Laureate indicating that immunodeficiency is the cause of chronic HIV infection and not vice versa is fully consistent with previous statements of his; for example, in 1990 he declared, on an American television program, that HIV was not the only cause of the syndrome AIDS (he then postulated the action of other infectious co-factors) and that HIV’s role might only be secondary in the destruction of the immune system of infected people. This statement, confirmed and amplified by experimental data presented in Vienna at the XVIII International Conference on AIDS (July 2010), reverses the long-assumed cause-effect relationship between HIV and AIDS: it is the immunodeficiency that causes chronic HIV infection and not vice versa, as believed thus far. Furthermore, these statements of Professor Luc Montagnier received experimental confirmation by the finding that stimulation of the immune system eradicates HIV infection in humans (J Med Virol. 2009 Jan;81(1):16-26).

In the first part of this thesis I analyzed current hypotheses on AIDS aetiology and pathogenesis with particular reference to Human Endogenous Retroviruses (HERVs) as confounding factors.

In the second part of this thesis, I describe the experiments performed to elucidate molecular details of stimulation of the immune system leading to eradication of HIV infection.

PART I

CHAPTER 1

HUMAN ENDOGENOUS RETROVIRUSES AND AIDS RESEARCH

Diseases caused by animal retroviruses have been recognized since 19th century in veterinary field. Most livestock and companion animals have own retroviruses. To disclose the receptors for these retroviruses will be useful for understanding retroviral pathogenesis, developments of anti-retroviral drugs and vectors for human and animal gene therapies. Of retroviruses in veterinary field, receptors for the following viruses have been identified; equine infectious anemia virus, feline immunodeficiency virus, feline leukemia virus subgroups A, B, C, and T, Jaagsiekte sheep retrovirus, enzootic nasal tumor virus, avian leukosis virus subgroups A, B, C, D, E, and J, reticuloendotheliosis virus, RD-114 virus (a feline endogenous retrovirus), and porcine endogenous retrovirus subgroup A. Primate lentiviruses require two molecules (CD4 and chemokine receptors such as CXCR4) as receptors. Likewise, feline immunodeficiency virus also requires two molecules, i.e., CD134 (an activation marker of CD4 T cells) and CXCR4 in infection. Gammaretroviruses utilize multi-spanning transmembrane proteins, most of which are transporters of amino acids, vitamins and inorganic ions. Betaretroviruses and alpharetroviruses utilize transmembrane and/or GPI-anchored proteins as receptors.

Thus it is acknowledged that retroviruses are an important group of pathogens that cause a variety of diseases in humans and animals. Four human retroviruses are currently known, including human immunodeficiency virus type 1, which causes AIDS, and human T-lymphotropic virus type

1, which causes cancer and inflammatory disease. For many years, there have been sporadic reports of additional human retroviral infections, particularly in cancer and other chronic diseases.

Unfortunately, many of these putative viruses remain unproven and controversial, and some retrovirologists have dismissed them as merely "human rumor viruses." Work in this field was last reviewed in depth in 1984, and since then, the molecular techniques available for identifying and characterizing retroviruses have improved enormously in sensitivity. The advent of PCR in particular has dramatically enhanced our ability to detect novel viral sequences in human tissues. However, DNA amplification techniques have also increased the potential for false-positive detection due to contamination. In addition, the presence of many families of human endogenous retroviruses (HERVs) within our DNA can obstruct attempts to identify and validate novel human retroviruses.

As far as HERVs and the HIV/AIDS hypothesis are concerned, a recent paper of Prof. De Harven, intergrally quoted below, elucidates their role as confounding factors in the pathogenesis of AIDS. In fact, not much if anything was known about HERVs at the beginning of the AIDS era. By now, a great deal has been found out, and some of it is directly relevant to various conundrums and controversies about HIV. A recognition of the existence and characteristics of HERVs offers the possibility of resolving differing views, as to whether HIV exists or whether it exists but is harmless. In fact, this paper explains why is it so hard to isolate and purify human immunodeficiency virus (HIV). Why has no one been able to see, by electron microscopy, a single HIV particle in the blood of AIDS patients, even those who have a "high viral load". Why does HIV seem to mutate with startling rapidity. All this considering the fact that AIDS researchers have not been able to come up with answers to these questions.

As a matter of fact, HERVs might provide explanations that have been overlooked for 20 years, writes Professor Etienne de Harven, M.D., in the fall 2010 issue of the Journal of American

Physicians and Surgeons,. HERVs are present in all of us, and fragments of their DNA may be confused with HIV in the polymerase chain reaction (PCR) tests used to estimate viral load.

The beautiful photographs of HIV published in both lay and scientific journals are embellished with special effects from computerized image reconstruction. Since they come from cell cultures, which are likely to be contaminated, the particles may be “elegant artifacts” rather than the exogenous virus—a virus of external origin—believed to cause AIDS, de Harven states.

About 8 percent of the human genome consists of sequences incorporated from retroviruses. When cells break down, DNA fragments are released into the circulation—including these viral sequences. Patients with clinical AIDS carry a large spectrum of infectious diseases, so a high level of circulating DNA is expected.

While some sceptics may challenge the role of HIV in AIDS, or even its existence, they are obligated to explain the observations of clinicians and researchers. HERVs are, at a minimum, a confounding variable that needs to be investigated, de Harven notes.

Puzzles involving the interpretation of diagnostic tests for HIV, the epidemiology and transmission patterns of AIDS, and strategies for prevention and treatment cannot be solved without broadening AIDs research beyond the narrow confines accepted by the “Orthodoxers,” de Harven believes. Alternate hypotheses need to be objectively assessed, and conclusions must be based on scientific evidence rather than consensus.

CHAPTER 2

THE PAPER OF PROFESSOR DR HARVEN, PUBLISHED IN THE JOURNAL OF THE ASSOCIATION OF AMERICAN PHYSICIANS AND SURGEONS

www.aapsonline.org

THE HIV CONSENSUS

The hypothesis that the acquired immunodeficiency syndrome (AIDS) is caused by an exogenous retrovirus, the human immunodeficiency virus (HIV), initially proposed in the early 1980s (Science 1984;224:500-503; Ann intern Med 1985;103:689-693; Science 1983;220:859-862), has exclusively dominated AIDS research for the past 25 years, although many investigators have repeatedly stressed the lack of scientifically acceptable verification of this hypothesis. Alerted to the numerous shortcomings of the official retroviral hypothesis by eminent retrovirologist Peter Duesberg (Cancer Res 1987;47:1199-1220; Regnery 1996), a group of AIDS “Rethinkers,” founded by molecular biologist Charles Thomas in 1991, called for the “Scientific Reappraisal of the HIV/AIDS Hypothesis” in 1996.

This group (www.rethinkingaids.com) released a mission statement co-signed by thousands of scientists and concerned citizens, including Nobel laureates Walter Gilbert and Kary Mullis. Other well-respected scientists, notably Sonnabend, Stewart, Lang, Papadopoulos, Rasnick, and Geshekter (Nature 1984;210:103; Lancet 1989;336:1325; Regnery 1996; Springer Verlag 1997; Med Hypothesis 1988;25:151-162; Reappraising AIDS 1996 Aug; Reappraising AIDS 1997 Sept/Oct;5) and distinguished scientific writers such as Celia Farber, John Lauritsen, Neville Hodgkinson, Joan

Shenton, Christine Maggiore, Renaud Russeil, Djamel Tah, Jean-Claude Rousez, and Janine Roberts (Spin 1989 Nov; New York Native 1987 Oct 19; Sunday Times (London) 1993 Sept 5; London-New York:IB Tauris 1998; American Foundation for AIDS Alternatives 2000; Chêne-Bourg/Genève:Editions Vivez Soleil 1996; Continuum 1997 winter; Belgium:Marco Pietteur 2005; UK:Impact Investigative MediaProductions 2009) have also described the multiple failings of the HIV hypothesis. Between 1992 and 2000, another group based in London, UK, made highly significant contributions to scientific/public education by publishing magazine, under the leadership of Huw Christie (Continuum 1997 winter). A medical team directed by Eleni Papadopulos in Perth, Australia, has also presented information questioning the validity of the HIV hypothesis (Bio/Technology 1993;11:696-707; Emerg Med 1993;5:5-147; Curr Med Res Opin 1998;14:185-186). In May 2000, the controversy concerning HIV and the antiretroviral (ARV) drugs used to treat it became the topic of international inquiry when President Thabo Mbeki of South Africa, convened a debate between 35 academic scientists, “Orthodoxers” as well as “Rethinkers” together (A synthesis report of the deliberations by the panel of experts invited by the President of the Republic of South Africa, the Honourable Mr. Thabo Mbeki, March 2001). A similar debate took place in 2003 at the European Parliament in Brussels, Belgium, when Paul Lannoye, a Belgian member of parliament, organized a public debate on “AIDS inAfrica.” (Embourg, Belgium:Marco Pietteur 2004).

Reports by AIDS Rethinkers are readily accessible on numerous websites, the early and most significant ones being www.virusmyth.com, www.rethinkingaids.com, www.theperthgroup.com, www.sidasante.com, and www.altheal.org.

In spite of innumerable scientific and public conferences and publications by AIDS Rethinkers, many in the medical community either ignore, or bluntly reject the existence of any HIV controversy (Science 2008;321:530-532; AIDS Behav 2010;14:237-247; Nature 2009;459:168), or

claim that AIDS “denialism” undermines AIDS prevention (Int J STD AIDS 2008;19:649-655). As a result, the monumental budgets allocated throughout the world to combat AIDS have been, and still are totally and exclusively restricted to HIV research. This can neither be explained nor justified by the lack of alternative hypotheses of AIDS causation, since nonviral factors (chemical, pharmacological, nutritional, and behavioral) associated with the clinical symptoms attributed to AIDS have been well documented and reviewed by others (J Biosci 2003;28:383-412). The retroviral hypothesis linking HIV to AIDS received a precipitous acceptance, not on the basis of scientifically verifiable data, but based on a so-called “consensus”—a consensus enthusiastically supported by the pharmaceutical industry. This review will focus primarily on the scientific facts (or artifacts) that impact the credibility of AIDS research.

FACTORS THAT GAVE APPARENT CREDIBILITY TO THE HIV HYPOTHESIS

In the extensive HIV/AIDS literature, one finds that the claimed “evidence” that AIDS is caused by HIV-1 or HIV-2 is presumably “clear-cut, exhaustive and unambiguous,” (Nature 2000;406:15-16) and comprises four groups of data: (1) identification of retroviral molecular markers, (2) observation of retroviral particles by transmission EM, (3) claimed efficacy of antiretroviral (ARV) drugs, and (4) epidemiological data.

1. Identification of Retroviral Molecular Markers

In a long list of presumed HIV molecular markers, the most emblematic one is the enzyme reverse transcriptase (RT) (Nature 1970;226:1209-1211; Nature 1970;226:1211-1213). Importantly, however, the activity of this enzyme has been readily demonstrated in practically all living cells of the biological universe (Cr Acad Sci (Paris) 1972;274:2801-2804; Sci Am 1987;257:48-54), making it imperative to verify the purification of viral samples before making any claim for a specific link between RT and retroviruses. Sample contamination by cell debris can, by itself, explain the

presence of RT activity. This is of considerable importance because attempts to isolate and purify HIV by sucrose gradient ultracentrifugation of supernatant from supposedly HIV-infected cell cultures have provided samples heavily contaminated with microvesicular cell debris, readily demonstrated by EM (Virology 1997;230:134-144; Virology 1997;230:125-133).

Anti-HIV antibodies are regarded as another class of molecular markers, used in so-called “HIV tests,” such as the enzyme-linked immunosorbent assay (ELISA)(Abbott Laboratories Diagnostic Division 66-8805/R5 1997 Jan;5; Sunday Time (London) 1993 Aug 1). The lack of specificity of this test, however, was clearly documented by C. Johnson (Continuum 1996;4(3):4) who reported, as early as 1996, that almost 70 medical conditions having nothing to do with AIDS or HIV may result in a positive antibody test. These conditions include tuberculosis, malaria, leprosy, hepatitis, blood transfusions, influenza vaccination, multiple pregnancies, and others. Such a lack of specificity came as no surprise to those who were aware that the method used to prepare “HIV” antibodies was based on a circular argument, as discussed early on by Neville Hodgkinson. Moreover, the method initially used in ELISA tests included a 400-fold plasma dilution. Without such high dilution everybody turned out to be “HIV positive,” as originally demonstrated by Roberto Giraldo in 1998 (The Business 2006 May 21).

Protein antigens of claimed retroviral origin represent a group of HIV markers used in another “HIV test,” the western blot test (WB). The WB test is used to confirm the ELISA test, and is based on the identification by electrophoresis on polyacrylamide gels of 10 presumably HIV proteins, such as p120, p41, p32, p24/25, and others. However, prior successful isolation and purification of HIV would be required to verify that all of these proteins actually originate from HIV particles, a purification that has never been achieved, as recognized by Luc Montagnier himself (Continuum 1999;5(5):9-11).

The considerable difficulty in isolating and purifying HIV was recognized, as early as 1993, by Eleni Papadopulos et al., who correctly concluded that without successful HIV purification, the retroviral nature of the “HIV marker proteins” was most uncertain. Papadopulos emphasized that these proteins are most likely cellular, originating from the abundance of cell debris in poorly “purified” HIV samples. The uncertainty and shortcomings of WB testing were already reported in 1991(Continuum 1997;5(2):30-39). Soon afterwards, Papadopulos et al. raised the question: “Is a positive western blot proof of HIV infection?” (Bio/Technology 1993;11:696-707). That WB tests are not reliable is evidenced by the variability of the protein criteria required for a “positive” test in different countries. The test is not even approved for diagnostic purposes in Great Britain. The considerable difficulties experienced in attempts to purify “HIV” have never been resolved (Lancet 1991;337:286-287). Recently, Henry Bauer has reviewed evidence that supports the conclusion that “HIV tests are not HIV tests.”(European Parliament, Brussels 2003 Dec 8) “HIV tests” only indicate the presence of antibodies supposedly directed against HIV. They do not indicate the presence of the virus itself.

The question then arises of whether the so-called “viral load”(J Am Phys Surg 2010;15:5-9) tests are more reliable, as they are based on polymerase chain reaction (PCR) technologies for recognizing and quantifying HIV. This appears highly questionable; Nobel laureate Kary Mullis himself, the discoverer of PCR, has indicated that his method is not expected to provide a reliable result in HIV diagnosis.

A second reason to question “viral load” data is that “viral load” implies the existence of viremia, i.e. the presence of virus particles in the peripheral blood, although no one has ever observed, by EM, one single retroviral particle in the blood of HIV/AIDS patients, even in those patients tagged as presenting with a “high viral load.”(Interim Report of the AIDS Advisory Panel, Pretoria, South Africa, May 2000) Moreover, the PCR methods used for “viral load” determination bypass the

problems of isolation of retroviral particles. The question therefore arises: what is actually measured in “viral load” determinations? To date, no satisfactory answer has been provided.

Still, various amounts of claimed retroviral nucleotide sequences are routinely identified and quantified in a patient’s plasma. They are interpreted as originating from HIV, and used in the clinical assessment and therapy of AIDS patients. When Luc Montagnier was asked, “What is actually measured in viral load assessments?” during the discussion of a major HIV/AIDS debate in the European Parliament in 2003, his answer was less than clear and convincing (Embourg, Belgium:Marco Pietteur 2004). The contradiction remains that genomic retroviral sequences are routinely recognized by PCR, and interpreted as originating from HIV particles, while nobody has actually visualized them by EM. More critical attention should be given to the true nature of these retroviral sequences, the origin of which is at present unclear.

II. Observation of Retroviral Particles by Transmission Electron Microscopy

All the images of particles supposedly representing HIV and published in scientific as well as in lay publications derive from EM studies of cell cultures. They never show HIV particles coming directly from an AIDS patient (AIDS 1991;5:617-637). The pictures are always embellished by computerized image reconstruction, with attractive colors and refined three-dimensional effects. The endless, worldwide publication in the media of these elegant artifacts has done much to persuade scientists and lay people alike to accept the existence of HIV as a key part of the orthodox consensus.

Cell cultures have been the major tool that permitted the development of modern virology. Unfortunately, these cultures are frequently contaminated by microorganisms such as viruses and/or mycoplasma, readily identifiable by EM. These contaminants, well known and documented for a long time (London-New York:Academic Press 1973), frequently made the interpretation of experimental data rather laborious, because to demonstrate the cytopathic effects of a given virus on

cultured cells, it would have been much preferable to experiment with “clean” (i.e. virus-free) cells. Unfortunately, such cells are hard to obtain! Actually, it was difficult to study the Friend leukemia virus (FLV) in cell cultures, using murine cells, because EM readily demonstrated that most available murine cell lines were chronically carrying retroviruses!

The 1983 study from Institut Pasteur in Paris (Science 1983;220:868-871) is illustrated by an EM(their Figure 2) showing unquestionable budding retroviruses on the surface of human cord blood lymphocytes. The interpretation of this figure by Luc Montagnier and his team, that these retroviruses originated from a pre-AIDS patient, was based on the fact that the cord blood lymphocytes were exposed to the cell-free supernatant of “infected” co-cultures. But the authors did not provide any evidence for “infection” in their co-cultures, nor for the presence of retrovirus particles in the supernatant of these cultures. Therefore, another explanation for the origin of the observed retroviruses on the surface of these cultured cord blood lymphocytes must be sought.

III. The Claimed Efficacy of Antiretroviral (ARV) Drugs

Drugs such as azidothymidine (AZT), aDNAchain “terminator,” as well as non-nucleoside analog RT inhibitors (such as nevirapine) and protease inhibitors (such as ritonavir), are currently used in various combinations such as “highly active retroviral therapy” (HAART), and repeatedly claimed to be “life saving.” Manufacturers of these drugs, however, strongly emphasize their toxicity. Lethal effects of AZT became dramatically evident when mortality of seropositive hemophiliacs suddenly increased sharply in 1987, precisely at the time high dosages of AZT started to be prescribed (Rethinking AIDS 1996 Feb 4;1-4; Nature 1995;377:79-82). Hopes that AZT might have preventive value were shattered by the Concorde study, when mortality of AZT recipients was found 25% higher than that of the untreated control group of symptom-free HIV-positive individuals (Lancet

1994;343:871-881). These important studies have been reviewed by Duesberg (Regnery 1996), by Hodgkinson (London, England:Fourth Estate Limited 1996), and others.

Equally perplexing is that deaths of ARV-treated patients very frequently result from acute liver failure (14th International AIDS Conference, Barcelona, Spain 2002 Jul), conflicting with the fact that HIV is not known for liver toxicity, while ARV drugs are.

If the effects of ARV drugs could still be regarded as proving that HIV is the cause of AIDS, one would at least expect some patients to be cured by these drugs. However, not a single case of “cure” has ever been reported. Instead, the clinical evidence points to the high toxicity of ARV drugs and their immunodepressive effects which actually mimic AIDS itself (Regnery 1996).

Patients with severe AIDS have frequently been reported to be transiently, but remarkably, improved by ARV drugs. Such “Lazarus” type observations have been interpreted as evidence for an antiretroviral effect on HIV, supporting the existence/role of HIV. However, as most of these patients frequently suffer from pneumonia with *Pneumocystis Carinii*, mycosis with *Candida Albicans*, or both, and because protease inhibitors, introduced in antiretroviral therapy in 1996, have marked anticandidal (J Infect Dis 1999;180:448-453) and antipneumocystis (J Infect Dis 2000;181:1629-1634) effects, this interpretation is questionable at best. When anti-proteases help block such opportunistic infections, this has no direct relevance to HIV, and certainly does not “automatically support the “HIV model.”(Rethinking AIDS 000;8(9):1- 2)

IV.Epidemiologic Data

Maneuvering for major federal budget allocations, AIDS public health policies have been relying on media amplification of fear (Spin 1992 June Fawnskin, Calif:Personhood Press 2004). Catastrophic prediction of heterosexual transmission of the disease, prophecies of a worldwide pandemic, and

reliance on CDC and WHO statistical reports were all linked to the assumption that AIDS was a contagious disease, possibly transmitted in the general population by sexual intercourse.

Renowned epidemiologist Gordon T. Stewart did much, however, to dispel these erroneous predictions. In a letter to *The Lancet* (*Lancet* 1993;341:898), he stated “the UK Government is beginning to retreat from its pessimistic certainty about pandemics of heterosexual transmitted AIDS” and exposed to scrutiny “the claim that AIDS has already spread by heterosexual transmission to the general populations.” (*Genetica* 1995:95:173-193) Stewart’s conclusions correlate well with the complete absence of HIV among female sex workers not using IV drugs.

This “prostitute paradox” (i.e. no increased risk for AIDS among female sexworkers) was reviewed from worldwide studies by Root-Bernstein in 1993 (*Rethinking AIDS* 1993 Mar), and re-emphasized more recently by Etienne de Harven and Jean-Claude Roussez (*Trafford Publishing* 2008). The lack of evidence for heterosexual transmission of AIDS was clearly presented by Padian et al., who could not observe one single case of seroconversion in a follow-up study of 175 HIV-serodiscordant couples over a period of six years (*Am J Epidemiol* 1997:146:350-357). That heterosexuals are not at risk for AIDS was stressed by Christian Fiala in his 1997 book (*Lieben wir gefährlich? Do We Love Dangerously?*)(Vienna, Austria:Deuticke Verlag 1997). Safe-sex practices (e.g. condoms) remain essential, however, for the prevention of diseases proven to be sexually transmitted, such as syphilis and gonorrhea.

Certain African countries, such as Uganda and Tanzania, had been regarded as epicenters of an AIDS “pandemic.” The lack of evidence supporting this, initially recognized by Philippe Krynen (*Sunday Times* (London) 1993 Oct 3), was clearly documented by Charles Geshekter (*Ethics & Politics* 2007;9:330-370) and by science writers Celia Farber (*Spin* 1993 Mar) and Neville Hodgkinson (London, England:Fourth Estate Limited 1996). Twenty years later, national census figures have shown spectacular demographic increases in several sub-Saharan countries, clearly

demonstrating that their populations had not been devastated, as officially predicted, by a deadly AIDS pandemic of historic proportions(Presentation at RA2009, Oakland, Calif., November 2009). The most authoritative conclusions presented in 2008 by experienced epidemiologist James Chin, former Chief of the Unit of the Global Programme on AIDS of the World Health Organization (WHO) in Geneva, in his book *The Collision of epidemiology with Political Correctness* (Oxford,UK:Radcliffe Publishing 2007) brought to a close any possible debate on heterosexual AIDS transmission. Chin stated that AIDS was, and still is restricted to a small population of homosexuals and intravenous drug users, and that the heterosexual population is not at risk. Chin's conclusions have raised serious questions on the reliability of WHO statistics.

AIDS epidemiological data have been further confused by several consecutive changes in the official definition of the syndrome, and have failed to support the current HIV=AIDS dogma. The hypothesis of an exogenous retrovirus "HIV" causing AIDS appears unsupportable by the scientific evidence concerning molecular markers, EM findings, ARV drugs, and epidemiology. However, two intriguing findings deserve further attention: the identification of genomic retroviral sequences in AIDS patients' blood ("viral load") and the EM demonstration of retroviruses in cord blood lymphocytes (Science 1983;220:868-871). Simply concluding that "HIV does not exist" is not sufficient unless alternative, satisfactory explanations for these two observations are found.

"VIRAL LOADS" AND RETROVIRAL SEQUENCES

"Human endogenous retroviruses (HERVs) represent footprints of previous retroviral infection and have been termed 'fossil viruses.' They are transmitted vertically through the germline and are thus inherited by successive generations in a Mendelian manner," stated Nelson et al. in a review entitled "Demystified... Human Endogenous Retroviruses."(Cancer Res 1990;50(suppl):5636-5642; J Gen Virol 1992;73:2463-2466) The molecular basis of HERVs was recognized 20 years ago (Proc Natl Acad Sci USA 1996;93:5177-5184). They appear defective, and rarely produce virus particles. As

molecular footprints, they are “in all of us,” as recognized by Lower et al. in 1996, and represent approximately 8% of the human genome, actually consisting of nucleotide sequences analogous to the retroviral genome (Reprod Sci 2009;16:1023-1033). Expression of HERVs, i.e. particle formation, seems to be a rare event, although it has been observed in placenta (Curr Top Pathol 1979;66:175-189) and in tumor cell lines (J Virol 1987;61:2059-2062). HERV retroviral sequences have also been detected by PCR in the peripheral blood mononuclear cells (PBMC) of healthy individuals (Cancer Res 1990;50(suppl):5636-5642). The possible role of HERVs in human pathology (autoimmune diseases and cancers) has received considerable attention (Clin Microbiol Rev 1996;9:72-99), as has the classification of their numerous families (Nucleic Acid Res 2004;32 Database issue D50:1-7).

Since 1996, real-time PCR has been used to claim quantification of a postulated HIV viremia, termed “viral load,” in AIDS cases. These methods have been based on the study of patients’ plasma (Clin Microbiol Newsletter 1997;19:1-5) samples: initially, samples originated from nuclei of peripheral blood mononuclear cells, and later from low-speed centrifugation pellets of plasma. The various methods applied to the PCR measurement of the so-called “viral load” have one point in common: they all bypass direct isolation of retroviral particles demonstrable by EM. These methods are not expected to isolate, nor concentrate any retrovirus. Moreover, as clearly stated during the South African 2000 conference (A synthesis report of the deliberations by the panel of experts invited by the President of the Republic of South Africa, the Honourable Mr. Thabo Mbeki, March 2001), not one single particle of retrovirus has ever been seen, by EM, in the blood plasma of any AIDS patient, even in those patients identified as presenting with a high so-called “viral load.” That statement, widely publicized, has never been refuted nor challenged (Available at: www.altheal.org/isolation/prize.htm. Accessed Jul 28, 2010). Human plasma carries various amounts of circulating DNA.

Suspected for a long time, this was first demonstrated by modern technologies in 1999, by P. Anker et al. (*Cancer Metastasis Rev* 1999;18:65-73), in the blood of cancer patients. The significance of circulating nucleic acids, as possible molecular markers in the study of cancer, was extensively reviewed in a New York Academy of Sciences conference in 2006 (*Ann NY Aca Sci* 2006;1075:1–353). The origin of free circulating DNA is complex, and seems to depend primarily on cell apoptosis. “If the engulfment of apoptotic bodies is impaired or cell death is increased enough to produce substantial amounts of circulating DNA, inflammation would definitely be a problem and autoimmunity would occur frequently in cancer and other conditions involving increased circulating DNA.” (*Clin Chem* 2007;53:2215-2218).

Apoptosis and a large spectrum of infectious diseases are constant components of all clinical AIDS cases. Circulating DNA is expected, therefore, in the plasma of all symptomatic AIDS patients. Amounts can vary, as a function of more or less rapid removal of DNA by clearance mechanisms. Apoptotic bodies and/or fragments of PBMC nuclei are certainly expected in low-speed centrifugation plasma pellets, such as those used in PCR “viral load” measurements, and most likely increase the amount of recognizable DNA. Human DNA always contains approximately 8% of retroviral nucleotide sequences. It’s no surprise, therefore, that RT-PCR study of plasma pellets shows, and amplifies, retroviral nucleotide sequences. Unfortunately, such findings are frequently misinterpreted as originating from hypothetical exogenous “HIV,” although, as stated above, not one single retroviral particle has ever been found by EM in plasma samples. Quantifying a presumed “viral load” has, therefore, probably nothing to do with an exogenous “HIV.” It simply reflects variable amounts of circulating DNA.

Retroviral sequences in plasma pellets being easily explained by the presence of variable amounts of circulating DNA, one should not, however, expect that these nucleotide sequences would be identical in all cases. Quite to the contrary, since “nucleotide sequences that diverged from co-

linearity with the typical retroviral genome (LTRgag-pol-env-LTR) considerably increase the number of HERV families,”(Nucleic Acid Res 2004;32 Database issueD50:1-7) the large number of HERV families resulting apparently from frequent recombinational deletions (J Virol 2007;81:9437-9442). Expected variations in the observed nucleotide sequences have, unfortunately, often been misinterpreted as an indication for a high rate of HIV mutations! It seems much more likely, however, that the numerous variations in the observed retroviral nucleotide sequences in circulating DNA reflect the large number of HERV families they originate from, and have nothing to do with presumed “mutations” of a hypothetical HIV.

Reference to HERVs and/or to circulating DNA can hardly be found in the extensive literature on “viral load” measurements, interference of HERVs, and of circulating DNA being consistently ignored by the HIV/AIDS orthodoxy.

Conclusively, RT-PCR identification, and presumed quantification of so-called “HIV viral load,” can easily be explained by the variable amounts of HERV-derived retroviral nucleotide sequences present in the circulating DNA of AIDS patients.

RETROVIRUSES ON THE SURFACE OF CORD BLOOD LYMPHOCYTES

In their 1983 Science paper (Science 1983;220:868-871), Barré-Sinoussi et al. failed to demonstrate, by EM, any retrovirus in their co-cultures. Still, the supernatant of these co-cultures has been used to “infect” human cord blood lymphocytes. This theory requires one to subscribe to infection via a virus that is not visible by EM. If the authors had included EM evidence for retroviruses in their co-cultures and their supernatant, their interpretation would have been more convincing. Unfortunately, such data were not provided.

Nevertheless, their Figure 2 unquestionably demonstrates “budding” (J Biphys Biochem Cytol 1960;7:747-752) retroviruses on the surface of cultured human cord blood lymphocytes. Its origin needs to be better clarified.

Cord blood lymphocytes are placenta-derived cells. The human placenta is well known for its high content of HERVs, with EM recognizable retrovirus particles. Cord blood lymphocytes are, therefore, likely to carry similar HERVs (Reprod Sci 2009;16:1023-1033). The 1983 paper (Science 1983;220:868-871) demonstrated that HERV particle (Curr Top Pathol 1979;66:175-189) expression had been successfully activated in cultured cord blood lymphocytes, under culture conditions that included 2g/ml of Polybrene. It does not demonstrate, however, that the EM-observed retroviruses originated from the studied pre-AIDS patient. Along-overdue control experiment would be to study, by EM, cultured cord blood lymphocytes under conditions that would reproduce exactly those used at the Pasteur Institute in 1983. Dourmashkin presented some data addressing this issue in 1992 (J Med Virol 1993;39:229-232), although his presentation did not satisfactorily resolve the problem, since his cord blood lymphocytes were not cultured under conditions identical to those used at Pasteur in 1983.

The EM observation of typical retroviral particles in the 1983 Pasteur paper can alternatively be explained by the presence of placenta-derived, Polybrene-activated HERVs. However, this EM observation does not support the existence of an AIDS-related, exogenous retrovirus.

Obviously, confounding by HERVs cannot be ignored in the objective analysis of clinical as well as basic HIV/AIDS research.

DISCUSSION

All AIDS Rethinkers are united in the fundamental opinion that HIV is not the cause of AIDS (Nature 2000;407:286). However, they diverge on the important question of the very existence of the Human Immunodeficiency Virus (HIV).

Some of them (Regnery 1996; Reappraising AIDS 1996 Aug) maintain that HIV is a “harmless passenger virus,” while others (Bio/Technology 1993;11:696-707;Curr Med Res Opin 1998;14:185-

186) claim that HIV “does not exist” at all. Since neither of these two positions explains the pertinent observations, an alternative interpretation, compatible with all the available scientific evidence, is needed.

Claiming that HIV is a harmless passenger virus raises at least two critical problems. First, if HIV is “harmless” it cannot be linked to immune deficiency (a very severe pathological condition), as implied in its name. Therefore, the name of the virus should at least be changed in order to fit with a claimed “harmless” character. Secondly, in the general classification of animal virology, very large numbers of viruses are nonpathogenic, as was well illustrated in the 1960s in a special conference, at the New York Academy of Sciences, under the title “Viruses in Search of Diseases.” Obviously, all non pathogenic (i.e. “harmless”) viruses are clearly visible under the EM. Pathogenic and non pathogenic viruses look identical under the EM. In AIDS research, retroviral particles were observed by EM only in complex cell culture systems (AIDS 1991;5:617-637), never directly in the plasma, nor in the tissues of any AIDS patient.

Claiming simply “HIV does not exist” is not satisfactory either, because it fails to explain the two sets of data discussed in this review, namely the presence of retroviral genomic sequences in the plasma of AIDS patients, and the EM evidence for retrovirus particles in the “historical” 1983 Pasteur paper (Science 1983;220:868-871).

Others have previously emphasized that HERVs cannot be ignored and that they actually represent “confounding factors for human retrovirus discovery.”(Haematologica 1998;84:385-389) Their role having been confirmed and amplified, this review shows that HERVs, in addition, offer a rational, alternative interpretation for the two above-mentioned problems.

The existence of endogenous human retroviruses has been known for some time, but their interference in HIV/AIDS research has yet to be widely appreciated. Of course, HIV should not be considered an HERV, since the hypothetical HIV is supposed to be an exogenous, infectious

microorganism, while HERVs are fundamentally endogenous, non-infectious, vertically transmitted, defective viruses. Still, HERVs have been a “confounding” factor in HIV/AIDS research (Haematologica 1998;84:385-389), and have caused confusion in interpreting the concept of “viral load.” Moreover, HERVs put HIV researchers on the wrong track, creating the illusion of continuous HIV mutations—mutations that improperly served to explain the extreme difficulty in preparing anti-HIV vaccines. However, difficulties in developing anti-HIV vaccines might not be explained by a constantly mutating HIV, but rather by a lack of exogenous HIV.

As emphasized years ago by Papadopoulos (Bio/Technology 1993;11:696-707), Lanka (April/May 1995), and others (Microbiol Mol Biol Rev 2008;72:157-196), there is no scientifically verifiable evidence to confirm the existence of a hypothetical exogenous HIV. However, stating simply that “HIV does not exist” is an incomplete statement that fails to explain the complexity of HIV/AIDS research. To that statement, one should always add that HERVs have heavily interfered with HIV/AIDS research in a way that cannot be ignored. Adequate understanding of HERVs as confounding factors opens the way to a better, more objective analysis of AIDS research.

Finally, the question as to whether HIV exists, or of whether researchers have been studying a harmless passenger virus, is a question that should be subject to open debate and careful consideration of scientific evidence or lack thereof. Alternative explanations for findings should be decided by the scientific evidence, not by consensus. The advancement of our understanding of AIDS demands nothing less.

CHAPTER 3

ANCIENT RETROVIRUSES AND THE EVOLUTION OF GENOMES IN THE PATHOGENESIS OF AIDS: A NOVEL PERSPECTIVE

The following considerations come from the unpublished work of Dr. Christl Meyer who kindly shared with us her work. These considerations, albeit from a different perspective in comparison to those presented above, offers the possibility of resolving differing views as to whether HIV exists or whether it exists but is harmless.

From the work of Dr. Meyer

As previously shown in a recent paper entitled “Reconciliation between Pure Scientists and AIDS Dissidents: Could an ancient retrovirus, RNA-interference and stress be the answer to the divergent opinions ?“ there is evidence, that HIV is an ancient retrovirus acting as a gene that can be influenced by environmental stress and small RNAs.

The Human Genome Project (HGP) gave us the information about less protein coding genes than we were aware before. Instead we are now eagerly discovering the Human Epigenome (HEP), looking for the methylated genes (Curr.Issues Mol. Biol. 2002;b4:111-128; PLOS Biol 2004;2(12):e405) and as to Jenuwein and Allis (Science Vol. CCXCIII, No. 5532, 2001 Aug 10;1074:1080) histones of the DNA because of the big importance of gene expression in the different cells and tissues of our bodies (Gene Expr. 1996;5(4-5):245-53).

The Human Microbiome Project (HMIP) tries to specify the genes of the microbes living on and in our bodies (Washington University in St. Louis,School of Medicine). We have 10 fold more

microbes than we have cells in one person and the genes of our symbioses, which contribute to our digestion, vitamin supplementation and gene activation are of high interest for research. The co-evolution of bacteria and their viruses gives new information about the acceleration of evolution (Nature; Dep. Of Biol. Sciences, Stanford University CA) and lateral (horizontal) gene transfer from microbes to their hosts (J.Virol. 2000;74:7079). The Human Protein Project (HPP) and the Human Metabolom Project (HMP) will give additional information on the complexity of life and evolution.

I. The Diversity of Humans

“The genetic structure of the indigenous hunter-gatherer peoples of southern Africa, the oldest known lineage of modern human, is important for understanding human diversity “(Nature 2010 Feb 18;463:43-947). These hunter-gatherers, known as Khoisan, San, or Bushmen, are genetically divergent from other humans. In term of nucleotide substitutions, the Bushmen seem to be, on average more different from each other than, for example, a European or an Asian. There is also a discontinuity between local hunter-gatherers and central Europe’s first farmers (Science 326,137:140). By analyzing ancient DNA of neolithic hunter-gatherer and contemporary Scandinavians, Malstrom et al. revealed a lack of continuity (Curr.Biol.2009;19:785-62). Humans underwent an adaption process which was influenced by geography (PLoS Gen. 2009;5:1000500). This resulted in differences including the immune system. People of African descent show reduced neutrophil count due to a regulatory variant (Gen. 2009;5:1000360). There is also an extensive genetic diversity in the HLA class II region of Africans from Gambia and Malawi. This diversity is twice as extensive as found in northern Europeans (Institute of Molecular Medicine. Univ. of Oxford, John Radcliffe Hospital, Un. Kingd).

In consequence we find differences in humoral responses between Ethiopian and Swedish persons

who are claimed to be “infected” by HIV (Clinical And Diagnostic Laboratory Immunology 1997 Sept;627-629:071-412X/97\$04.00+0). Therefore it is necessary to investigate the nature and biological background of the immune system which is responsible for cell activation, receptor and antibody generation and the communication between all partners involved in the immune response.

II. The MHC/HLA System and its Origin

Most interesting is the study from the Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 ISA, UK published in Nature/Vol 425/23 October 2003 “The DNA sequence and analysis of human chromosome 6” (Nature Vol. CDXXV, 2003 Oct. 23. The Wellcome Trust Sanger Institute, Genome Campus, Hinxton, Cambridge CB10 ISA, UK; J. of Vir. 1996 Dec;9064:9068).

This chromosome constitutes about 6% of the human genome and harbours 1,557 genes and 633 pseudo-genes. Within the essential immune loci of the major histocompatibility complex, HLA-B was found to be the most polymorphic gene. Among these are genes directly implicated in diseases like cancer and autoimmunity. Having a look at the supplementary tables, which give full lists of HLA allele-associated HIV polymorphisms in Protease, Reverse Transcriptase, VPR and Nef, they show that more than 240 gene variants of Nef are encoded by HLA. There are also tremendous variants for the other genes. The main effect of Nef is to block transport of MHC-I molecules to the cell surface, leading to accumulation in intracellular organelles (Virology, Vol. CCLXXXII, No.2, 2001 Apr 10;267-277).

This study and the report from Brumme et al. “Evidence of Differential HLA Class I Mediated Viral Evolution in Functional and Accessory/Regulatory Genes of HIV-1” (PLoS Pathog 2007;3(7): e94) confirms the nature of HIV – showing that it is of endogenous origin – part of our genome and constituted by the high variability of the genes, which elucidates the variability of the HIV-variants and the immune escape.

As Hedrick already stated in 1994 there is a high heterozygosity, reaching over 60% at some amino acid sites with primary function. In some populations there is an observed deficiency in homozygotes. There seems to be a balancing selection in the MHC region related to the function for protection against microbes. Probably there is a selection at the MHC involved in non-random mating and maternal-fetal interactions in pregnancy (Naturalist, Vol. CXLIII, No. 6, 1994 Jun;945-964).

The Mexican cohort (Retrovirology, 2009 Aug 10;6 :72) and the ANRS Genome Wide Association Study (PLoS ONE 2008;3(12): e3907) conclude that HLA/MHC controls HIV-reservoir and replication.

Gyllenstein et al. declared an allelic diversity that is generated by intraexon sequence exchange at the DRB1 locus of primates (Proc. Of the Nat. Ac. Of Sci. US 1991;88(9):3686-90 ; Proc. Of the Nat. Ac. Of Sci. US 1991;88(9):3686-90). In correspondence Doxiadis et al. state a phylogenetic evidence that supports the notion of the generation of new HLA- DRB genes as a dynamic and steadily ongoing process. This is due to the presence of indels (insertions/deletions), mainly mapping to intron. The research compared a large number of full-length sequences of rhesus macaques, chimpanzees and humans. As no evidence was found for convergent evolution, the combination of these observations indicates that ancient peptide binding motifs are frequently reshuffled among duplicated members of the HLA-DRB multigene family (Mol Immunol. 2008 May;45(10):2743-51. Biomed. Prim. Res. Centre, Dep.of Comp. Gen. And Refinement, The Netherl.). Oosterhout stated in his review on population genomics and epidemiology the concern of transposons in the MHC of the vertebrate immune system.

Transposons constitute a large proportion of the vertebrate genome, and on average more than 40% of the mammalian genome consists of these parasitic elements. TEs have a non-random distribution throughout the genome, and they show an increased density in the MHC in a wide range of vertebrates (Heredity 2009;103:190-191.Univ. of Hull, UK). Genetic drift may play an important

role in the population genetics.

John, Moore, James and Mallal from the Centre of Clinical Immunity and Biomedical Statistics, Royal Perth Hospital and Murdoch University, Western Australia published a paper “Characteristic non-synonymous mutation in HIV-reverse transcriptase sequence encoding an HLA-B7 restricted CTL epitope is associated with increased viral load”. Characteristic mutations in HIV RT are evident at a population level. For-example, the presence of HLA-B7 in the Western Australian HIV Cohort Study is strongly associated with non-synonymous mutation at position 135 of HIV RT which is an anchor residue of a HLA B51 restricted CTL epitope. This mutation allows escape from the host CTL response. These results are important because of the occurrence on drug induced mutations (Centre for Clinical Immunology and Biomedical Statistics, Royal Perth Hospital and Murdoch University, Western Australia, Program Abstr 8th Conf Retrovir Oppor Infect Conf Retrovir Oppor Infect 8th 2001 Chic III. 2001 Feb;4-8:93 (abstract no. 174)).

Among the many host cell-derived proteins found in HIV-1, HLA-II appears to be selectively incorporated onto virions (J. of Virology 2003 Dec;12699-12709). The genetic diversity of the envelope glycoprotein from HIV-1 isolates from 8 countries in Africa is studied by Louwagie et al. The data confirm the existence of several genetic subtypes and broaden the genetic variability observed for envelope subtypes. The geographic spread of different subtypes was shown to be substantial (J. of Virol. Vol. LXIX, No.1, 1995 Jan;263-271).

“Rapid Evolution of Major Histocompatibility Complex Class I Genes in Primates Generates New Disease Alleles in Humans via Hitchhiking Diversity” The diversity created by single nucleotide variations (SNV) was not evenly distributed. It was rather concentrated within the gene-clusters HLA-A and HLA-B/C. These polymorphisms seem to be species specific. They might have been selected in adaption to the constantly evolving microbial antigenic repertoire (Genetics, Vol. CLXXIII, 2006 July;1555-1570). The Mexican cohort (Retrovirology, 2009 Aug 10;6 :72) and the ANRS Genome Wide Association 01 Study (PLoS ONE 2008;3(12): e3907) find that HLA/MHC

controls HIV-reservoir and replication.

Clerici and Shearer (Immunology Today, Vol. XV, No.12, 1994) present a model “The Th1-Th2 hypothesis of HIV infection: new insights” where they claim activation-induced, cytokine modulated, programmed cell death as a major factor in the pathogenesis of HIV infection in AIDS. Immunoregulatory cytokines are also produced by non T-cells, including monocytes/macrophages, natural killer cells and Bcells. So the authors prefer the terms type 1 and type 2 responses. They suggest an endogenous imbalance in the immunoregulatory cytokine network. HIV resistance in female sex-workers in Northern Thailand seems to be influenced by synergistic impact of HIVspecific cytotoxic T lymphocytes, HLA-A11, and chemokine –related factors (AIDS Research and Human Retroviruses 2001 May;17(8):719-734).

Transcriptional analysis for host factors required by HIV-1 was performed by RNA interference. More than 250 HIV-dependency factors were identified. These proteins participate in cellular functions. Transcriptional analysis revealed that these genes were enriched for high expression in immune cells (How. Hughes Med. Inst. Science 2008 Feb 15;319(5865):921-6). In addition HIV incorporates HLA-DR which is a cell-surface protein in big quantities. Other proteins that have been found are HLA class I and various cell adhesion proteins as well as proteins from inside the cell like cyclophilin A, actin and ubiquitin (The Molecules of HIV).

Thus HIV is not a virus.

Conclusion: HIV is an evolutionary adapted and partly active variable and heritable gene construct of our immune (MHC/HLA)-system.

III.Sperm Proteins, Pregnancy and Protection of Health

The influence of human semen on immunity is of importance for estimating the impact on reactions concerning sexuality and child development. Jeremias et al. claim that human semen is both an inducer of an anti-inflammatory TH2 immune response and an inhibitor of TH1 cell mediated

immunity. The induction of interleukin 10 and 70 kDa heat shock protein gene transcription and IFN-I was examined (Molec. Hum. Reproduc., Vol 4, No.11;1084:1998). Virus-encoded (ORF) homologs of cellular interleukin-10 range in sizes for cellular Il-10 proteins (J. of Virol. 2009 Oct;9618:29). The result in both cases is a shift from T1 to T2 immune response which protects cells from immune attack an absolute necessary prevention for the fetus which could be otherwise attacked by maternal response to paternal antigens in the womb. There is a cross-reactivity of sperm and T-lymphocyte antigens that results in higher titers of antibodies in couples with antisperm immunity as compared with “normal” couples (Am J Reprod Immunol. 1981;1(3):113-8). A number of studies have suggested that an immune response to human leukocyte antigen HLA alloantigens may contribute to protection against HIV infection [34]. Infectivity for HIV through heterosexual transmission is low, and sexual transmitted diseases (STDs) may be the most important cofactor for transmission (Am J Epidemiol. 1997 Aug 15;146(4):350-7).

Heterosexual and homosexual monogamous partners practising unprotected sex develop CD4+ and CD8+T cell proliferative responses to the partners’ unmatched cells and a minority may be tolerated. These together with other research results suggest that allogeneic immunity may play a significant role in HIV (PLoS ONE, Vol. IV, No.11, 2009 Nov;e7938).

HIV-1 gp120 is an immunoglobulin superantigen which can bind to pre-immune serum Ig. The level of pre-immune anti-gp120 IgG is a polymorphic population trait, and low levels are a potentially specific and significant factor in homosexual transmission of HIV infection (J Clin Invest. 1996 Oct 15; 98(8):1794-1801).

HIV-1 binds and enters normal sperm and can transfer HIV-1 like particles to normal human oocytes (J Cell Biol. 1994 Nov;127(4):903-14). Already in 1991 at the AIDS conference in June Calarco and Whitmer showed that HIV-1 expression can occur during early mammalian development (International Conference on AIDS 1991 jun 16-21;7:62 (abstract no. TH.A.26)).

Background maternal (rather than paternal) allergy confers stronger allergy risk for the offspring.

Maternal responses to fetal antigens were related to fetal immune responses and subsequent allergy. The number of previous pregnancies was associated with stronger maternal responses to fetal alloantigens (Clinical and exp. Allergy Source, Vol. XXXV, No.4, 2005;417:425 [9 page(s) (article)] (25ref.)).

Conclusion: HIV is a natural product in sperms which has its origin in the HLA and protects the fetus from maternal rejection of paternal antigens by shifting T1 to T2. Heterosexual transmission of HIV is only suggested with additional pathogens in STDs. Homosexual transmission is due to rejection of alloantigens. Allogeneic immunity protects from infection but can be related to allergies also in the offspring.

IV.Auto, Alloreaactions and Diseases

Concerning to the afore mentioned HIV is a regulatory and even life promoting element of the immune system which has evolved in millions of years as a symbiotic partner that interacts in health and disease. Multiple interactions in cell communication are proofed concerning HIV specifically in GALT (gut associated lymphoid tissue) which makes sense for protecting the body from strange invaders. Mehandru et. al. from the Mount Sinai School of Medicine, and the Aaron Diamond AIDS Research Center emphasize that the gastrointestinal tract - associated lymphoid tissue constitutes the largest immune compartment in the body. More than 60% of the bodies total lymphocytes is estimated to be T-cell associated with the small intestinal epithelium [45].

Dissemination of virus to GALT is mediated by an integrin and Gp 120 leading to the formation of virological synapses, which facilitate efficient cell-to-cell spreading of HIV-1 (Nature Immunology 2008;9:301-309).

Retroviral assembly is driven by Gag release which is promoted by clathrin adaptor complex AP-1 to intracellular sites of active budding – the machinery that forms intraluminal vesicles of the multivesicular body MVB. Protein sorting is critical for diverse cellular functions, like receptor

down-regulation, degradation of membrane proteins and lysosome like organelles, which includes attachment of ubiquitin to cargo proteins (Mol. Biol. Of the Cell, Vol. XVIII, 2007 Aug;3193:3203). Teis et al. state that a certain complex (ESCRT) is required for cargo sequestration and vesicle formation during MVB sorting (The EMBO Journal 2010 March 03;29:871-883). In addition direct cell-cell communication mediated by plasma membrane-spanning gap junction (GJ) channels is vital to all aspects of cellular life. Cells internalize GJ in response to various stimuli. In this process clathrin, dynamin (GTPase) and other proteins are involved in internalizing double-membrane vesicles into cells (FEBS Lett. 2008 Aug 20;582(19):2887-92).

Exosomes correspond to the multivesicular body and are released upon exocytic fusion with the plasma membrane. They function in intercellular communication during the immune response. They might be involved in tissue developmental processes and seem to be of ancient origin (2002 May;3(5):321-330). Stephen J. Gould has created the “Trojan exosome hypothesis” in which he and his colleagues propose that retroviruses exploit a cell-encoded pathway of intercellular vesicle traffic, exosome exchange and last but not least that alloimmunity is a central component of antiretroviral immunity (PNAS, Vol. C, No.19, 2003 Sept 16).

Antibodies against HLA neutralize HIV-1 in vitro. This was proved by alloimmune sera from polytransfused patients (AIDS Res. and Human Retrov. 1999 Apr;15(6):533-543). An Article from Frank P. Ryan published in the Journal of The Royal Society of Medicine from 2004 talks about “mutualistic symbiosis” and HERVs in our genomes that have lost the ability to survive independently, but their removal from our genome would also make us extinct” (J of the Royal Society of Medicine, Vol. XCVII, 2004 Dec).

The American Society of Microbiology regards the subject of viral contribution to host evolution as so important that it has commissioned Luis Villareal to write a book to educate the next generation of scientists (Viruses and the evolution of life) (Washington DC: Am Soc. For Microb).

The envelope glycoprotein of HIV-1 gp120 has been identified as a member of the Immunglobulin

superantigens (Ig-SAg) which bind selective to an unusually high proportion of endogenous nonimmune Ig, that are members of the VH3 Ig gene family (The J. Immunol. Vol.CLV, No.1, 1995;5151:5159).

The importance for diseases might be that the up-regulation of expression of endogenous retroviral superantigens has substantial implications for understanding the pathology of virus infections i.e. Epstein-Barr virus (Trends in Microbiology, Vol.XXIII, No.2, 2002 Feb;57:58). SAGs seem to be involved in allergy and autoimmune diseases (Indian J Med Microbiol 2004;22:204-211). Thus they might be used as a therapeutic agent in the treatment of cancer. The light chain subunits of antibodies cloned from patients with systemic lupus erythematosus bind and hydrolyze gp120 sAg (Online 2007 Jan 12). Already in 1990 research on other lupus patients stated about one third of them produced antibodies to the p24 gag protein of HIV-1 as demonstrated in Western blotting (J Clin. Invest. 1990;85(6):1866-1871). In a study concerning HLA class I and II antigens in South African Blacks with Grave's disease there was a significant increase in the frequency of HLA-DR3 in patients compared to control subjects, and a relationship in the DRI locus (Indian J Med Microbiol 2004;22:204-211). Grave's disease is due to autoimmunity. A study from multiple sclerosis patients provides "direct proof" that HTLV-I, which is similar to HIV, is involved in MS disease process (The Associated Press 1989 Jan). Approximately 25% of severe haemophilia A (HA) patients develop antibodies to factor VIII protein, which is due to impact of polymorphisms of the MHC complex class II and other factors like interleukin-10 (J Thromb Haemost. 2009 Dec;7(12):2006-2015). As antibodies to blood products were defined as HIV contaminated in previous studies (The Lancet, Vol. CCCXXI, No.8331, 1983 Apr 30;956:958-30) the reported data from 2009 may now be interpreted as gene expression of distinct SNPs.

There is also a relation of HIV-1 acquisition to hormonal contraception and to herpes simplex virus type 2 among Kenyan women (AIDS 2007 Aug 20;21(13):1771-7). The genetic predisposition to type 1 -diabetes is associated with genes of the HLA system, specifically with HLA-DR and -DQ

(Arq Bras Endocrinol Metabol. 2006 Jun;50(3):436-44). Research concerning transgenic mice resulted in activation of gene expression in HIV by Mycobacterium tuberculosis and suppression after antimycobacterial chemotherapy (The J of Infec. Diseases 2007;195:246-254). This proves the bystander function of HIV in tuberculosis. As a result HIV is not the cause of the disease but tuberculosis is the disease and HIV is part of the communication system and part of an active immune system. Gene regulation is subject to hormone control specifically to corticosteroids in retroviral systems and to pregnancy in women (AIDS Research and Human Retroviruses 1990 Apr;6(4):553-560). This might indicate stress and placental involvement of testing HIV positive. The HIV-1 VPR-protein might be protective against cancer by inducing apoptosis in tumour cells (Cancer Cell International 2009;9:20). Cell surface MHC class I-like proteins are up-regulated upon cell stress, including viral and bacterial infection and tumour transformation and are recognized by NKG2D a C-type lectin- activating receptor (J. Exp. Med. The Rockefeller Univ. Press, Vol.CIC, No.7, Apr.5, 2004;1005:1010). Stress and depressive symptoms are associated with decrease of protective NK and CD8+ T lymphocytes in HIV-infected men (Men Arch Gen Psychiatry. 1997; 54(3):279-285). Individuals from Central Africa have a higher level of immune system activation compared with non-African populations that might be due to multiple and frequent exposures to viral, bacterial and parasitic antigens (Viral Immunology 1992 Winter;5(4):243-8).

By comparing medical treatment for “HIV-infection” resistance to zidovudine was significantly higher in individuals with disease progression than in those from the control group (Journal of Acquired Immune Deficiency Syndromes 1993). The variable region (V3) of the gp 120 surface envelope glycoprotein of HIV-1 is a highly variable disulfide-bonded structure which triggers cell infection and escape from antiviral drugs, specifically entry inhibitors and is a target for neutralizing antibodies (AIDS Res Hum Retroviruses 2005 Feb;21(2):171-89).

After effective highly active antiretroviral therapy (HAART) people with HIV might experience an “immune restoration syndrome” which is established by lymphocyte recovery period and might be

manifested by infectious agents such as cytomegalovirus or mycobacterium avium intracellulare or a sudden onset of sarcoidal granulomatous reactions.

An uncontrolled Th1 response as a result of cytokine alterations via Il-2 is the causative mechanism (Online publication: 9 October 2002) as the authors of this study claim. In HIV Il-1 is increased which shifts the reactions to Th2 response. Reuse, Calao Kabeya et al. from Belgium propose a synergistic activation of HIV-1 expression by deacetylase inhibitors and prostratin as implications for treatment of latent infections. This might reduce the size of latent HIV-1 reservoirs in HAART-treated patients (PLoS One 2009 Jun 30;4(6):e6093). A study from 2008 found an inactivation of HIV-1 by modification of nucleocapsid zinc fingers (Elsevier Inc. online 2008 March 4).

Conclusion: HIV is a symbiotic agent in GALT and other tissues, thus protective against microbes due to nutrition. Exosomes are produced and function in cell communication processes. Gp 120 is active as a superantigen that increases Th2 related antibody production in infections as a “booster” and might be due to allergy and autoimmunity.

Stress is involved in gene expression. HIV is protective to cancer. Medications and HAART might have different (negative) impacts on the balance of the Th1 / Th2-system.

V.Evolution Never Ends

Human endogenous retroviruses (HERVs) have been estimated to be part of the genome and are replication incompetent. HERV-W encodes a highly fusogenic membrane glycoprotein within functional retrovirus genes which has been proposed to play a role in normal placental development not only in humans but also in simian and pig cells. The HERV-W entered the genome of primates approximately 25 million years ago. They are replication defective because of mutations within functional retrovirus genes. The existence of individual open reading frames corresponding to gag, pol and env have been shown to encode proteins in some cases. HERVs could be potentially

assembled into infectious virions through transcomplementation with virion proteins encoded by different HERVs (J Virol. 2001 Apr.;3488:3489).

This research from 2000, published in the Journal of Virology 2001, by Dong Sung An et al. from UCLA AIDS Institute, Los Angeles, California was done before the Human Genome Project was published and the tremendous occurrence of HERVs were detected in the Human genome specifically in the MHC/HLA. The authors also claim that a functional envelope glycoprotein would confer the ability to be transmitted vertically and / or horizontally. Kumar et al from All India Institute of Medical Sciences, New Delhi, India revealed a two fold higher expression of CXCR4 mRNA in early as compared to term human placenta. Chemokines and their receptors may play a crucial role in angiogenesis and proliferation in cell function. The receptor expression may be developmentally regulated and its role in the early stages of pregnancy is implicated when embryogenesis and organogenesis takes place (Elsevier Ltd).

They suggest that CXCR4 may not have a direct role in HIV infection, as only 1-2% of the placental transmission of HIV takes place in the early placenta. Allogeneic stimulation in early pregnancy improves pre- and postnatal ontogenesis by activation of the female immune system and enhancement of rise of plasma progesterone. The dissimilarity of mother and foetus induces stress resistance in the progeny of BALB/cLac mice (Journal of Reproduction and Development, Vol. XLVI, No. 6, 2000). This evolutionary development acts also as a mechanism for creating phenotypic diversity (Retrovir. 2006 Dec 21;3(Suppl 1):P15). In seropositive children a well known cross-reactivity between HLA-DR and gp 120 is marked (Int Conf AIDS 1993 Jun 6-11;9:257 (abstr. No. PO-A-31-0737); The Molecules of HIV and Cellular Proteins in HIV Virions).

Conclusion: These research results indicate an evolutionary effect of HERVs and cell receptors and other proteins of the immune system and an allogeneic stimulation in pregnancy which is protective for life.

CHAPTER 4

HIV TESTS AND HIV ISOLATION: FURTHER CONSIDERATIONS

The following considerations were collected from Dr. Meyer at the Conference on AIDS held in Vienna in July 2010. Here we integrally report her work.

Over recent months debate has been taking place regarding the wisdom of taking up the issue of HIV isolation as an argument in our fight against mainstream AIDS science. According to some dissidents this question should not be raised because:

1. it will provide HIV/AIDS protagonists with additional ammunition with which to discredit us;
2. it makes little difference if people are being killed in the name of a non-existent or a merely harmless virus;
3. it is an "existentialist" discussion.

From the very beginning the Perth group questioned the evidence which may well prove the most significant "inconvenient" fact to fly in the face of all HIV/AIDS protagonists. If the data do not prove beyond reasonable doubt the existence of HIV then, in terms of a putative exogenous retrovirus, there can be no "observations...to provide strong support for the official theory".

In 1996, when Peter Duesberg wrote a paper claiming the Continuum prize, he directly challenged the Perth group. At present there are four reasons why it is necessary to question the isolation and thus the existence of HIV:

1. Since 1996 it has become clear that, as far as the existence of HIV is concerned, the Group for

Reappraising AIDS is divided. Some of the best known HIV experts are aware of this fact and it is now too late to pretend otherwise.

2. There is no proof that such an entity exists. To claim the opposite is to deny the scientific evidence. Certainly conduct an anti-HIV debate avoiding this issue as exemplified by many in our midst. However, in arguing against the HIV hypothesis of AIDS in this manner one has to be content with half truths.
3. "HIV" is the main obstacle, indeed, the only obstacle, in deconstructing the HIV theory of AIDS.
4. Demonstrating that HIV has not been isolated is not an "existentialist" debate. In fact we consider this to be the strongest argument we can muster.

If we accept there is no proof for the existence of HIV then undoubtedly "the construction AIDS, also called HIV disease, collapses immediately and all so called "HIV tests" are automatically unmasked as being useless". If, on the other hand, we accept the existence of HIV, the debate could be endless, no matter how courageously one fights and what sacrifices one makes.

Why HIV isolation is necessary?

Use of this word signals the reader that the experimenter is claiming that the data presented proves that a virus exists. What all scientists must consider is whether the data presented as "isolation" do indeed justify the claims.

A virus is an obligatory intracellular, replicating particle of particular physical and chemical properties. Thus the first absolutely necessary, but not sufficient step in proving the existence of a retrovirus is to isolate retrovirus-like particles. There are many reasons for this including the following:

1. To prove that the retrovirus-like particles are infectious, that is, the particles are a virus.

Finding a retrovirus either in vitro or in vivo is not proof that it originated from outside, that is, the virus is infectious, exogenous. To prove that retrovirus-like particles observed in a culture are virus particles one must isolate (purify) the particles, characterise their proteins and RNA and introduce them in a secondary culture, preferably containing cells of a different type than the primary culture. If any particles are released in the secondary culture, isolate them and prove that their proteins and RNA are exactly the same as those of the particles isolated from the primary culture.

2. To determine their biological effects.

For this one must use pure particles otherwise it is impossible to determine whether the effects are due to the virus particle or to contaminants. As Peter Duesberg has pointed out in "Koch's second postulate: The microbe must be isolated from the host and grown in pure culture"

3. To characterise the viral proteins.

The only way to prove that a protein is a viral protein is to obtain it from that object, or when the object is very small, as is the case of viruses, from material which contains nothing else but virus particles.

4. To characterise the viral genome.

As for viral proteins the only way to prove that a stretch of RNA is viral, it is to obtain it from material which contains nothing else but virus particles.

5. To use it as a gold standard.

Just because a virus or viral protein reacts with an antibody present in a patient's sera, this does not prove that the antibody is directed against the virus or its proteins. That is, the reaction is specific.

The only way to perform hybridisation and PCR studies is to use the viral RNA or its cDNA or small fragments of it, as probes and primers. However, as with antibodies which react with viral

proteins, a positive result, especially a positive PCR result, does not guarantee that what is detected is viral RNA. To determine the specificity of the PCR the virus must be used as a gold standard. All retrovirologists agree that one of the principal defining physical characteristics of retroviruses is their density. In sucrose density gradients they band at the density of 1.16g/ml. Using the method of sucrose density banding in 1983 Francoise Barre-Sinoussi, Luc Montagnier and their colleagues claimed to have isolated a retrovirus, that is, to have obtained material which contained nothing else but "purified labelled virus" which now is known as HIV. Similar claims were reported by Robert Gallo's group in 1984. It goes without saying that if the material was pure HIV, then all the proteins present in such material must be HIV proteins. Instead, only the proteins which were found to more often react with sera from AIDS patients and those at risk were said to be HIV proteins, and the antibodies which reacted with them the specific HIV antibodies. Since then the reaction of these proteins with antibodies is considered proof for HIV infection. Again, if their material was pure HIV then all the nucleic acids present in their material must be the HIV genome. Instead, only some fragments of RNA rich in adenine were arbitrarily chosen and were said to constitute the HIV genome. Since then, these fragments have been used as probes and primers for hybridisation and PCR studies, including the determination of "viral load".

The biggest problem in accepting Montagnier's and Gallo's groups claims is the fact that neither published even one electron microscope picture of the "pure" HIV to prove that the material contained nothing else but isolated, retrovirus-like particles, "purified labelled virus". In 1997 Montagnier was asked by the French Journalist Djamel Tahy why such pictures were not published. Incredibly Montagnier replied because in what his group called "purified" HIV there were no particles with the "morphology typical of retroviruses". When he was asked if the Gallo group purified HIV, Montagnier replied: "I don't know if he really purified. I don't believe so". If this is the case then the 1983 Montagnier findings and the 1984 Gallo's finding, prove beyond all

reasonable doubt that they did not have any retrovirus much less a unique retrovirus, and that the proteins and the RNAs which were present in their "purified" material could not have been of retroviral origin.

In the same year, 1997, some of the best known HIV experts accepted that no evidence existed which proved HIV isolation and thus a "virus to be used for biochemical and serological analyses or as an immunogen".

IS A POSITIVE WESTERN BLOT PROOF OF HIV INFECTION?

It is currently accepted that a positive Western blot (WB) HIV antibody test is synonymous with HIV infection and the attendant risk of developing and dying from AIDS. In this communication we present a critical evaluation of the presently available data on HIV isolation and antibody testing.

The available evidence indicates that:

1. The antibody tests are not standardised;
2. The antibody tests are not reproducible;
3. The WB proteins (bands) which are considered to be coded by the HIV genome and to be specific to HIV may not be coded by the HIV genome and may in fact represent normal cellular proteins;
4. Even if the proteins are specific to HIV, because no gold standard has been used and may not even exist to determine specificity, a positive WB may represent nothing more than cross-reactivity with the many non-HIV antibodies present in AIDS patients and those at risk, and thus be unrelated to the presence of HIV. We conclude that the use of the HIV antibody tests as a diagnostic and epidemiological tool for HIV infection needs to be reappraised.

To date, the only routinely used methods for demonstrating the presence of HIV in vivo are the ELISA and WB antibody tests. In the ELISA, the "HIV proteins" are present as a mixture. For the

WB, the HIV proteins are dissociated and placed on a polyacrylamide gel slab. After electrophoresis, which separates the proteins by molecular weight and charge, the proteins are transferred to a nitrocellulose membrane by electroblotting. In performing the antibody test, in both ELISA and WB, the patient's serum is added to the antigen preparation. It is assumed that if HIV antibodies are present, they will react with the HIV proteins which, after washing, are visualised by an enzyme anti-human-immunoglobulin chromogen reaction. In the ELISA the reaction is read optically. For the WB, individual proteins are recognised and interpreted visually as coloured bands, each of which is designated with a small "p" (for protein), followed by a number, (which is the molecular weight in kilodaltons), for example p41 (Fig. 1). The WB is believed to be highly sensitive and specific, and a positive result is regarded as synonymous with HIV infection. A positive HIV status has such profound and far reaching implications that no one should be required to bear this burden without solid guarantees of the verity of the test and its interpretation. In this paper, the evolution of the antibody tests, the basis of their specificity, and the validity of their interpretation are evaluated.

Acceptance of an antibody test for HIV as being scientifically valid and reliable requires the following:

1. A source of HIV specific antigens;
2. Standardisation;
3. Determination of the test's reproducibility.

The proteins considered to represent HIV antigens are obtained from mitogenically stimulated cultures in which tissues from AIDS patients are co-cultured with cells derived from non-AIDS patients--usually established leukaemic cell lines. Following the detection of the enzyme reverse transcriptase (RT) in the cultures, the supernatant, and more often the cell lysates, are spun in

density gradients. Material which bands at 1.16 gm/ml is considered to represent "pure HIV" and consequently the proteins found at that density are considered to be HIV antigens.

The immunogenic HIV proteins are thought to be coded by three genes, namely gag, pol and env.

The gag gene codes a precursor p53/55, which is then cleaved to p24/25 and p17/18. The pol gene codes for p31/32, and the env gene codes the precursor protein p160 which is cleaved to p120 and p41/p45 (Nature 1985 Jan;313:277-284).

The p120 protein. The generally accepted view is that p120 and p41 are cleavage products of p160, which is found only in infected cells and not in the virus. However:

1. p120 is a component only of the knobs (spikes) on the surface of HIV particles;
2. The knobs are found only in the budding (immature) particles; and not in cell free (mature) particles;
3. immature particles are "very rarely observed" (J. Virol. Meth 1987;16:125-137).

The p41 protein. p41 is one of the proteins detected by both Gallo's and Montagnier's groups in the first HIV isolates. However:

1. Montagnier and his colleagues observed that AIDS sera reacted with a p41 protein both in HIV and HTLV-I infected as well as non-infected cells, and concluded that the p41 band "may be due to contamination of the virus by cellular actin which was present in immunoprecipitates of all the cell extracts" (Science 1983;220:868-871).
2. Actin is an ubiquitous protein which is found in all cells as well as bacteria and several viruses.
3. Platelets from healthy individuals also contain a p41/45 protein which reacts with sera from homosexual men with AIDS and immune thrombocytopenic purpura (ITP) and which "represents non-specific binding of IgG to actin in the platelet preparation" (NEJM 1985;313:1375-1380).

The p32 protein. In 1987 Henderson isolated the p30-32 and p34-36 of "HIV purified by double banding" in sucrose density gradients.

The p24/25 protein. Detection of p24 is currently believed to be synonymous with HIV isolation and viraemia. However:

1. Apart from a joint publication with Montagnier where they claim that the HIV p24 is unique, Gallo and his colleagues have repeatedly stated that the p24s of HTLV-I and HIV immunologically cross-react (Nature 1985;317:395-403);
2. Genesca et al. (Lancet 1989;2:1023-1025) conducted WB assays in 100 ELISA negative samples of healthy blood donors; 20 were found to have HIV bands which did not fulfil the then (1989) criteria used by the blood banks for a positive WB. These were considered as indeterminate WB, (WBI), with p24 being the predominant band, (70% of cases). They concluded that WBI patterns "are exceedingly common in randomly selected donors and recipients and such patterns do not correlate with the presence of HIV-1 or the transmission of HIV-1", "most such reactions represent false-positive results";
3. Antibodies to p24 have been detected in 1 out of 150 healthy individuals, 13% of randomly selected otherwise healthy patients with generalised warts, 24% of patients with cutaneous T-cell lymphoma and prodrome and 41% of patients with multiple sclerosis (NEJM 1988;318:448-449);
4. In another study, "In half of the cases in which a subject had a positive p24 test, the subject later had a negative test without taking any medications that would be expected to affect p24 antigen levels...the test is clinically erratic and should be interpreted very cautiously" .

The p17/18 protein

1. In addition to the p24 band, the p17/18 band is the most often detected band in WB of healthy blood donors (Abstracts VII International Conference on AIDS Vol. I, Florence 1991);
2. Sera from AIDS patients bind to a p18 protein in mitogenically stimulated HIV infected T-cells, but not to non-infected, unstimulated lymphocytes. However, when the lymphocytes are mitogenically stimulated, but non-infected, the AIDS sera bind to a p18 protein in these non-infected lymphocytes (Nature 1987;327:710-713);
3. AIDS patients and those at risk have high levels of antibodies to the ubiquitous protein myosin (Ann. Inst. Pasteur/Immunol. 1987;138:223-233), which has two subunits of molecular weights 18,000 and 25,000.
4. In view of all the above evidence it is difficult to defend the view that the bands p41 (and thus p160 and p120), p32, p24 or p18 represent specific HIV proteins.

I. Standardisation of HIV Antibody Tests

An antibody test becomes meaningful only when it is standardised, that is, when a given test result has the same meaning in all patients, in all laboratories, in all countries. From the first antigen-antibody reactions performed by Montagnier's 4 and Gallo's (Science 1984;224:497-500) groups (Fig.2, Fig.3) it was found that: (a) not all of the "HIV proteins" react with all sera from AIDS patients or even sera from the same patients obtained at different times; (b) sera from AIDS patients may react with proteins other than those considered to be HIV antigens. Because of these variable reactions, an essential requirement was to establish criteria as to what constitutes a positive WB.

Untill 1987 "there were as many WB procedures as there were laboratories doing the assay"(AIDS Clinical Review, 1991, 1-15). Since then, all major laboratories have changed their criteria for WB interpretation but in the United States there are still no nationally agreed criteria, even among the

major laboratories:

1. In 1987 the Food and Drug Administration (FDA) licensed a WB kit manufactured by DuPont. It specifies "extremely stringent" criteria for a positive result namely "specific bands representing three different gene products: p24 (gag), p31 (pol), and an env band, either gp41, gp120 or gp 160"(AIDS Clinical Review, 1991, 1-15);
2. The American Red Cross defines a positive result as presence of antibodies to at least one gene product from each of the gag, pol and env genes, without specifying which bands;
3. The Association of State and Territorial Public Health Laboratory Directors/Department of Defence/CDC consider a WB positive if two out of p24, gp41 and gp120/160 are reactive;
4. The Consortium for Retrovirus Serology Standardization (CRSS) defines a positive WB as the presence of antibodies to at least p24 or p31/32, and gp41 or gp120/160(JAMA 1988;260:674-679).

For all laboratories, a negative result requires the absence of any and all bands including bands which do not represent "HIV proteins".

When the FDA criteria are used to interpret the WB, only a minimal number (less than 50%) of AIDS patients have a positive WB, that is, are infected with HIV. If the criteria of the CRSS are used, the percentage of AIDS patients testing positive increases to 79%. Henderson and his colleagues have shown that p31/32 is a non-HIV protein.

Despite the above evidence, even at present, the p160, p120 and the p41 bands are considered to represent distinct viral envelope glycoproteins. In fact, the current WHO guidelines consider a serum positive for HIV-1 antibodies if "two envelope glycoprotein bands (with or without) other viral specific bands are present on the strip"(Australian Prescriber 1992;15:11-13).

II.Reproducibility

The Transfusion Safety Study (TSS) Group in the USA submitted approximately 100 patient samples weekly for WB testing to three reference laboratories over three separate periods of several months. With the 100 patient samples, they submitted aliquots from four quality control (QC) plasmas, two positive and two negative. HIV positivity or negativity "was based on the collective experience with each plasma using:

1. licensed EIA systems of five manufacturers,
2. an immunofluorescence assay,
3. IB in four reference laboratories, and
4. a radioimmunoprecipitation assay in an additional laboratory". (EIA=ELISA;IB=WB).

The samples were then sent to reference laboratories which were aware of quality control testing, but "the labels and codes did not permit identification of the QC specimens as such or linkage to previous QC specimens". QC1#(+) was submitted 40 times to laboratory A, 5 times to laboratory B and 45 times to laboratory C.

A reported the following band patterns: p24, p32 and gp41/120, 7 times; p24, gp41/120, 28 times; p24 only, 5 times.

B reported: p24, p32, gp41/120, 4 times; p32, gp41/120, on one occasion.

C reported: p24, p32, gp41/120, 26 times; p24, gp41/120, 10 times; p24, p32, twice; p24 only, 5 times; "others", once; no bands, once.

In considering the results detailed above, one must bear in mind that they occurred in Reference Laboratories, that is, first class laboratories which constitute only a small number of the total number of laboratories which perform WB testing in the USA.

III. Specificity of HIV Antibody Test

The task of authenticating a new diagnostic test in clinical medicine requires an alternative independent method of establishing the presence of the condition for which the test is to be employed. This method, often referred to as the gold standard, is a crucial sine qua non, and represents the tenet upon which rests the scientific proof of validity. The only possible gold standard for the HIV antibody tests is the Human Immunodeficiency Virus itself. Obviously, the clinical syndrome and the decrease in T4 cells cannot be considered a gold standard.

Colonel Donald Burke and his colleagues from the Walter Reed Army Institute in the USA are credited as having most thoroughly researched the problem of defining HIV antibody specificity in a large population and his data is widely believed to represent the current state of the art (NEJM 1988;319:1010-1012). Burke et al. (NEJM 1988;319:961-964) tested a highly selected healthy subpopulation of 135,187 individuals chosen for a very low prevalence of HIV infection--1/10th that of a much larger pool of applicants (1.2 million). All applicants were screened with an initial ELISA. All reactive ELISA tests were repeated in duplicate. Then an initial WB was performed and, if diagnostic or reactive, a second WB was performed on another fresh blood specimen. Beginning in May 1987, the method of preparing blot strips was modified so that antibodies to gp120 and gp160 could be detected reproducibly. A positive WB was diagnosed if and only if the first and second serum samples were diagnostic on WB. All of the diagnostic WB samples were then assayed with four other antibody tests. A WB was considered "true positive if all four assays on all available serum samples from an applicant were reactive and diagnostic", but was considered "false positive if all four assays on all available serum samples from an applicant were non-reactive, non-diagnostic or both". From the 135,187 applicants, there were 16 positive tests. In one of these, the serum was unavailable for further testing and one applicant declined to provide a second

sample. Serum from 27 of the 29 samples from the 15 applicants found positive were tested by the four other antibody tests. Fourteen samples were found positive by all four assays and all four were negative for one applicant. From this Burke and his associates calculated the false positive rate as 1 in 135,187 or 0.0007%. They calculated the overall prevalence of 1.48 per 1000 in the entire pool as equivalent to 200 per 135,187. Assuming that the false positive rate is the same for the whole population they estimated that since there will be 200 true positive tests per 135,187 persons of which only one will be a false positive then the "predictive value of a positive diagnosis in the program is 99.5%, and a specificity of 99.9%" (NEJM 1988;319:961-964; NEJM 1988;319:1010-1012). Much of Burke's and his colleagues' reasoning is open to criticism:

1. There is no gold standard for defining HIV infection.
2. They define:
 - The true positive tests as samples which repeatedly test positive in four similar tests.
 - The false positive tests as samples which repeatedly test negative in four similar tests.
 - The false positive rate as the number of false positive results divided by the number of samples tested.

These definitions bear no resemblance whatsoever to those described in standard texts (Ann. Int. Med. 1981;94 (Part 2):559-563). The correct definitions are:

- A true positive is a positive test occurring in an individual who is HIV infected as defined by an independent gold standard;
 - A false positive is a positive test which occurs in an individual who, by application of the gold standard, does not have HIV infection, (but is not necessarily healthy);
 - The false positive rate in the number of false positive tests as a fraction of total number of individuals who, by application of the gold standard, are not infected with HIV.
3. The Burke et al premises are quite the opposite to those of Gallo et al where all positive test

results in healthy individuals are regarded as false positive.

4. Burke's extrapolation to the entire 1.2 million applicants is invalid. In the rest of the population the false positive rate may have been much higher for example as a result of higher concentrations of globulins in general or of autoantibodies in particular.
5. It is impossible to define specificity, sensitivity and predictive value with the algorithm used by Burke and his associates.

Burke et al, like Gallo et al, determined specificity without reference to sick individuals. The definition of specificity requires that the test is evaluated in persons who do not have the disease which is under scrutiny, including sick individuals who have other diseases where antibodies, some of which may interact with HIV antigens, may be produced for other reasons. The specificity of the HIV antibody tests must be determined by testing individuals who are immunosuppressed and/or who have symptoms and clinical signs similar to AIDS, but who are not considered to have AIDS or HIV infection. This point is well illustrated by the serological tests for syphilis. Persons with BFPS were also found "to have a high frequency of other serological abnormalities including anti-nuclear factors, autoantibodies, and alterations of gamma globulin". It is of significance that a high proportion (14%) of AIDS patients were also found to have false positive syphilis serology (AIDS 1990;4:467-471).

That a positive WB may not represent proof of HIV infection but is only a non-specific marker for AIDS, is suggested by the following data: In drug addicts there is a strong association between high serum globulin levels and a positive HIV antibody test and this was the "only variable which remained significant in a logistic regression model" (Alcoholism Clin Exp Res 1988;12:687-690). In children, using WB as a gold standard, hyperglobulinaemia identified HIV infected children with a specificity of 97% (Lancet 1991;337:253-260). Sixty three sera obtained from 23 patients before and immediately after immunoglobulin infusion were tested for HIV antibodies using WB. Of the

63 sera, 52 (83%) were found positive.

IV.Hiv Isolation

It goes without saying that virus isolation can be used as a gold standard only if it provides conclusive genetic, virological and molecular evidence for the existence of a unique virus. For retroviruses, as a first step towards this goal one must:

1. Find particles with morphological characteristics similar to other retroviruses;
2. Demonstrate that these particles have a unique set of structural components including RNA and proteins which belong only to these particles and to no other entity.

Peyton Rous (J. Exp. Med. 1911;13:397-411) is credited with the discovery and isolation of the first retrovirus. In 1911 he was able to repeatedly induce tumours in a particular breed of chickens by means of tumour derived, cell free filtrates. Rous contemplated that either a "minute parasitic organism" or "a chemical stimulant" might form the basis of his observations; nevertheless, the tumour inducing filtrates became known as "filterable viruses" or oncoviruses.

In the 1950s, in animal cultures and in fresh tissue, especially tumour tissue, particles later attributed to retroviruses were readily detectable with electron-microscopy (EM). In 1970, the enzyme reverse transcriptase (RT) which transcribes RNA into DNA, was discovered in oncoviruses. Because of this, in the 1970's, oncoviruses became known as retroviruses. In the preceding decade, density gradient centrifugation was introduced to separate and isolate sub-cellular particles including viruses. Because some cellular constituents were found to have the same buoyant density as viruses, when viruses were isolated from cell cultures, the best results could be obtained with supernatant fluids which: (a) had high viral concentration; (b) had low cellular contaminants.

Nonetheless, as many eminent retrovirologists point out, contamination of the viral preparation with virus-like particles which contain RT, but could be nothing more than "cellular fragments", microsomes from disrupted cells, "membraneous vesicles which may enclose other cellular constituents including nucleic acids", especially when "inadvertent lysis of cells" was induced, could not be avoided (Adv. Vir. Res. 1972;17:129-186; Cold Spring Harbor Laboratory. 1982; Comprehensive Virology Vol. IV, 1975, 275-331). Because of this, to prove that the material which banded at 1.16 gm/ml contained nothing else but particles with "No apparent differences in physical appearances", and that the particles were indeed retroviruses, every retrovirus preparation was further analysed using the following assays:

1. Physical EM for virus count, morphology and purity;
2. Biochemical RT activity, viral and cellular RNA, total protein, gel analyses of viral and host proteins and nucleic acids;
3. Biological infectivity in vivo and in vitro (Spectra No. 4, 1973, 225-235; Spectra No. 4, 1973, 237-243).

Unlike animal virus cultures where the particle concentration is very high, in the AIDS cultures/co-cultures the particle concentration is low, so low that both Gallo's and Montagnier's group had difficulty in detecting them. Unlike most animal retroviruses, HIV is considered to be a cytopathic virus. If this is so, then cell culture supernatants will contain many cellular constituents. If, as has been recently proposed, "a single unique mechanism", HIV induced apoptosis, can account for T4 cell death (Today 1991;12:102-105), then the supernatant must also contain apoptotic bodies, that is, membrane bound cellular fragments which, (like many retroviruses), bud from the cell surface. The presently available evidence indicates that only about 20% of the proteins which band at 1.16 gm/ml are "HIV proteins", the rest are cellular, including beta-2 microglobulin and

HLA-DR proteins (4.4%) (J. Virol. 1987;61:629-632; Hum. Immunol. 1987;18:39-52).

It must be pointed out that the terms in the AIDS literature "HIV", "HIV isolation", "pure particles", "virus particles", "virions" and "infectious particles" have a variety of meanings and include all of the following, but most often without proof of the presence of a particle: (a) "RNA wrapped in protein" (Lancet 1992;339:805-806); (b) material from the cell culture supernatants which passes through cell tight filters but through which organisms such as Mycoplasmas may pass (Res. Virol. 1990;141:5-16); (c) the pellet obtained by simple ultracentrifugation of the culture supernatant (J. Virol. 1990;64:1745-1755); (d) recently, very often, detection in AIDS cultures of p24.(J. Med. Virol. 1988;26:23-32; Lancet 1992;340:863-867).

However, isolation is defined as separating the virus from everything else and not detection of some phenomena attributed to the virus (RT, antibody/antigen reactions [WB]); or similar to it, (particles). Phenomena can only be used for viral detection--even then, if and only if, the phenomena have been identified as being specific for the virus, by using the isolated virus as a gold standard. Although this has not been done, the presently available indirect evidence (that is, evidence that has been obtained without a gold standard) from both general retrovirology and AIDS research, indicates that RT, RVP and the antigen/antibody reactions are not specific for HIV, (or even retroviruses).

V. Particle Detection

Retroviruses are enveloped infectious particles about 100-120nm in diameter with a core comprising a protein shell and a ribonucleoprotein complex. Retroviruses are classified into three Subfamilies--Spumavirinae, Lentivirinae and Oncovirinae. Retroviruses belonging to the latter Subfamily are divided into Type-A, B, C and D particles.

The particles detected in AIDS cultures/co-cultures are considered by all AIDS researchers as being HIV. However:

1. There is no agreement as to which Genus or even Subfamily of retroviruses they belong. Sometimes agreement is not found even within the same group.
2. Gelderblom et al put forward an HIV model (Fig. 5) which has a well defined morphology and composition, including surface knobs made of p120, a protein considered to play a crucial role in cytopathogenesis and to be indispensable for HIV infectivity (Virology 1987;56:171-176). The model has been accepted and is well known. However, the same group using EM and immune electron-microscopy has shown that: (a) knobs are found only in immature (budding) particles. Immature particles are "very rarely observed", and are seen only "on metabolically impaired cells" (J. Virol. Meth. 1987;16:125-137; Z. Naturforsch. 1987;42C:1328-1334); (b) mature particles are "hardly, if at all, labelled" by AIDS and ARC sera. Immature particles are "highly labelled", but so is the rest of the cell from which they are budding, which "might be due to the fact that natural immune sera are indeed polyspecific" (J. Virol. Meth. 1987;16:125-137; Lancet 1985;2:1016-1017) (c) like sera, antibodies to p120 react preferentially with immature particles (Virology 1987;56:171-176).
3. The T-cell and monocyte "HIV infected cultures" contain in addition to particles with the morphologies attributed to HIV, many other "viral particles" unlike any of the "HIV particles" (J. Exp. Med. 1988;167:1428-1441; Micron Microsc. 1988;19:41-60; S. Afr. Med. J. 1986;69:793-794; J. Gen. Virol. 1988;69:2455-2469). Budding and mature type-C particles appear in metabolically impaired but non-HIV infected lymphoma cells (Blood 1976;47:447-459). "Retroviral particles" antigenically related to HIV have been found in cultures of salivary gland extracts from patients with Sjogren's syndrome (Science 1990;250:1127-1129).

VI. Comments on "Isolation"

One can conclude then that neither the antigen/antibody reaction, nor the particles nor RT can be

considered specific for retroviruses. Even if they were, their finding cannot be considered as synonymous with the detection of an externally acquired retrovirus, as is claimed to be the case for HIV. Such findings may represent the expression of endogenous retrovirus (vide infra) or other exogenous retrovirus.

Lately, "several laboratories reported retroviral activity [RT, particles] in cells of patients who appear not to be infected by HIV", an activity said to be "from endogenous retrovirus" (Culliton, B.J. 1992). The cell line most often used in AIDS research is the leukaemic cell line H9. It is known that H9 is a clone of HUT78, which was derived from a patient with adult T-cell leukaemia. Since the causative agent of this leukaemia is accepted to be HTLV-I, another exogenous retrovirus, the H9 cultures should have both RT and retroviral particles even in the absence of HIV. Because about 25% of AIDS patients have antibodies to HTLV-I, about 25% of cultures should have in addition to particles and RT, a positive WB to HTLV-I. However, since the proteins from HIV and HTLV-I share the same molecular weights, the HTLV-I WB bands will appear to be positive for HIV. As far back as 1988, researchers at the CDC in the USA realised that no correlation exists between "HIV isolation" and a positive antibody test (which they call documented infection), and more importantly, between "HIV isolation" in vitro and its presence in vivo--"correlation between these two methods is limited; they are inconsistent, in that virus cannot be detected in every person with a documented infection. Furthermore, the culture technique determines the ability of infected cells to produce virus in vitro but does not necessarily indicate the status of virus expression in vivo" (Lancet 1988;2:596-599).

VII. Genomic Investigations

In the decades following Rous' experiments, Rous as well as other researchers performed similar investigations with several animal species. However, although neoplasia could be induced by injection of filtrates from tumour tissues, (infectious retroviruses, exogenous retroviruses), no

epidemiological evidence existed to suggest an infectious origin of cancer. In 1939 Andrews "speculated on the possible activation of latent viral infectious particles in cancerous tissues", and in 1948 Darlington postulated "that such viruses [endogenous viruses] could arise from cellular genetic elements which he named proviruses" (Cold Spring Harbor Laboratory. 1982). In the 1950s and 1960s the following experimental evidence was considered proof of the proviral hypothesis:(a) healthy animals in which no complete virus could be detected had viral antigens similar to those of exogenous virus; (b) DNA genomes or partial genomes of the infectious retroviruses were found to be integrated into the genomes of normal non-virus producing cells; (c) "Final proof came with the isolation of infectious viruses from uninfected cells".

At present it is generally accepted that "one of the most striking features that distinguishes retroviruses from all other animal viruses is the presence, in the chromosomes of normal uninfected cells, of genomes closely related to, or identical with, those of infectious viruses" (Cold Spring Harbor Laboratory. 1982).

Depending on conditions, the provirus genome remains unexpressed or part or all of it may be expressed. The latter may or may not lead to the assembly of viral particles (endogenous retrovirus((Cold Spring Harbor Laboratory. 1982).In other words, the finding of a viral genome (DNA) or even of RNA, antigens and antibodies to them, is not proof of the presence of infectious particles.

Although most of the above findings are from animal experiments, at present, evidence exists that "The human genome carries DNA sequences related to endogenous retroviral genomes that are subdivided into families according to sequence homology. Some are present in only a few copies, whereas others are present in hundreds to thousands of copies" (Cytogenet. Cell Genet.

1991;57:18-22). Animal data also shows that new retroviruses may arise by (a) phenotypic mixing; (b) genetic recombination and deletion. When a cell contains two proviruses, progeny may be found that possess the genome of one but the structural proteins of either or both viruses present.

Conversely, the RNA may be viral but at least some of the proteins may be cellular. In other instances, the particles do not have a genome at all, or one or more genes are missing (genetically defective viruses). The retroviral replicative cycle "involves three distinct steps: reverse transcription, DNA polymerization, and the synthesis of RNA from a DNA template (transcription). Any errors made by the polymerase enzyme during the first and the third steps are not subjected to proof reading, the result being pronounced sequence variability" (Nature 1990;347:18).

To date, the data on the HIV genome has not altered the prediction and shows that many problems may exist with the use of the genomic studies in efforts to prove infection of AIDS patients with a unique exogenous retrovirus, HIV. Some of these problems can be summarised as follows:

1. No two HIV genomes are the same. (a) No two identical HIV have been isolated even from the same person. b) from the same person at a given time more than one HIV can be isolated (J. Med. Virol. 1987;23:51-66; J. Clin. Microbiol. 1987;25:1411-1415); (c) many, if not all of the proviruses detected in vivo and in vitro are defective (AIDS 1989;3:S13-S18); (d) In one and the same patient, the genomic data in monocytes differs from that in T-lymphocytes (AIDS Res. Hum. Retroviruses 1992;8:261-268); (e) the genetic data obtained in vitro does not correlate with the data obtained in vivo--"To culture is to disturb" (Cell 1989;58:901-910); (f) The type of virus isolated is determined by the cell types used for HIV isolation (J. Clin. Microbiol. 1987;25:1411-1415; AIDS Res. Hum. Retroviruses 1987;3:401-408).
2. There is no correlation between "isolation" of HIV and detection of the HIV genome.
3. HIV sequences cannot be found in all AIDS patients.
4. To improve detection, the polymerase chain reaction (PCR) method was introduced. However, "a striking feature of the results obtained so far" with this method, as with the standard hybridisation technique, "is the scarcity or apparent absence of viral DNA in a proportion of patients" (J. Virol. 1990;64:864-872) and, when viral RNA or DNA is found, the "signal" is very low. It must be pointed out that a positive PCR, even if found in all patients as is claimed in

some publications (J. Virol. 1990;64:864-872), cannot be regarded as signifying the presence of the whole HIV genome. With the PCR "only small regions may be amplified, a gene at best" (AIDS 1989;3:S13-S18) that is, one does not detect the whole viral genome, and, since most HIV "isolates" to date are defective, detection of part of or a whole gene, or even several genes, cannot be considered synonymous with the whole HIV genome. Furthermore, the PCR is not standardised and to date, there has been only one study in which the reproducibility, sensitivity and specificity of PCR were examined. In this study, the gold standard used was serological status. The PCR was found not to be reproducible and "false-positive and false negative results were observed in all laboratories (concordance with serology ranged from 40% to 100%).

In 1984 when Gallo and his associates conducted their first hybridisation studies, they found that when the results were positive, the hybridisation bands were "faint", "low signal". The "low signal" was interpreted as proof that HIV infected individuals contain provirus in small numbers of peripheral blood mononuclear cells and at low copy numbers. However, according to Gallo and his associates, "theoretically this low signal intensity could also be explained by presence of a virus distantly homologous to HTLV-III in these cells" (Science 1984;226:1165-1171).

As far back as 1989 researchers at the Pasteur Institute concluded that "the task of defining HIV infection in molecular terms will be difficult" (Cell 1989;58:901-910). They confirmed their conclusion in a recent study where they "described the enormous heterogeneity found in vivo within HIV-1 populations" and the possibility "that an HIV carrier may harbour easily in excess of 1010 proviruses, most of which will be genetically unique". They conclude: "It is therefore possible that the sheer size of variants within an infected individual will allow HIV to explore totally new genetic possibilities". The appearance of "radically different genetic" retroviral structures may be the result of "rearrangement, duplication, deletion or hypermutation. The transduction of host cell DNA represents possibly the most startling genetic trait of retroviruses".

CONCLUSIONS OF PART I

The relationship between HIV infection and AIDS: correlation but not causation

1. Many different agents impair immune system function and may cause AIDS independently of signs HIV infection.
2. Most available evidence does not support a causative role for HIV in AIDS.
3. The very existence of HIV as an independent pathogenic virus is questioned.
4. The putative pathogenic mechanism of HIV is unknown.
5. As Prof. Montagnier stated, is the immunodeficiency that causes chronic HIV infection and not vice versa.
6. The well documented association between HIV-seropositivity and AIDS could simply be indicative of pre-existing immunosuppression. *i.e.*, different agents cause immunosuppression and immunosuppression (in addition to many other confounding factors such as HERVs) leads to HIV-seropositivity.
7. Signs of HIV infection could then just be “symptoms” of immunodeficiency, just like left shoulder and arm pain are symptoms of myocardial infarction.

PART II

THE EXPERIMENTAL APPROACH

The rationale: if the association between HIV-seropositivity and AIDS is simply indicative of pre-existing immunosuppression *i.e.* signs of HIV infection are “symptoms” and not cause of immunodeficiency, *then* stimulation of the immune system would eradicate HIV infection. This would prove that immunodeficiency is the cause of HIV infection and not vice versa as thus far believed.

Our experimental work is based on a seminal paper published by the group of Prof. Yamamoto in 2009 that we quote integrally because of its importance in HIV/AIDS research.

CHAPTER 1

THE PAPER OF PROF. YAMAMOTO ON THE ERADICATION OF HIV INFECTION

INTRODUCTION

Human immunodeficiency virus (HIV) can lead to acquired immunodeficiency syndrome (AIDS) that is characterized by opportunistic infections and by “opportunistic neoplasms” (e.g., Kaposi’s sarcoma) as an evidence of immunosuppression (Nature (London) 1984;312:763–767). Impaired phagocytosis and bacteriocidal process of the phagocytes of AIDS patients have been reported from a number of laboratories (Clin Res 1984;32:491–499.; J InfectDis 1985;152:409–412.; 1986; Eur J Clin Invest 1986;16:262–270; Clin Exp Immunol 1990;82:10–15; Exp Immunol 1992;88:23–28). Macrophages are the major phagocytic and antigen presenting cells. Since macrophage activation for phagocytosis and antigen-presentation to B and T lymphocytes is the first indispensable step in development of both humoral and cellular immunity (AIDS Res Human Retrovirus 1995;11:1373–1378; Immunol Cell Biol 1998;76:237–244), lack of macrophage activation leads to immunosuppression. In fact, immunosuppression in AIDS (AIDS Res Human Retrovirus 1995;11:1373–1378; AIDS Res Human Retroviruses 2006;22:262–271) and advanced cancer (Cancer Res 1996;56:2827–2931; Cancer Res 1997;57:295–299) patients is caused by lack of macrophage activation.

Microbial infection induces inflammation that results in macrophage activation as for innate defense. The inflammation-derived macrophage activation is the process for generation of the principal macrophage activating factor (Cancer Res 1987;47:2008-2013; Proc Soc Exp Biol Med

1990;193:118-124; Proc Natl Acad Sci USA 1991;88:539-543). Administration of an inflamed tissue lipid metabolite, lysophosphatidylcholine (lyso-Pc) or other lysophospholipids, to mice rapidly activates macrophages for greatly enhanced phagocytic and superoxide generating capacities (Cancer Res 1987;47:2008-2013; Clin Exp Immunol 1990;79:307-313; Proc Soc Exp Biol Med 1990;193:118-124; Inflammation 1994;18:311-322). Inflammation-derived macrophage activation process requires serum Gc protein (known as vitamin D3-binding protein) (Proc Natl Acad Sci USA 1991;88:539-543; J Immunol 1991;147:273-280; Mol Immunol 1996;33:1157-1164; U.S. Patent Number: 5,177,002.1993) and participation of B and T lymphocytes (Proc Soc Exp Biol Med 1990;193:118-124; Proc Natl Acad Sci USA 1991;88:539-543; Mol Immunol 1996;33:1157-1164). Gc protein carries one trisaccharide composed of N-acetylgalactosamine with dibranched galactose and sialic acid termini at 420 threonine residue (Proc Natl Acad Sci USA 1991;88:539-543; U.S. Patent Number: 5,177,002.1993; Mol Immunol 1996;33:1157-1164; J Immunol 1993;151:2794-2802). Stepwise hydrolysis of Gc protein with the inducible membranous β -galactosidase (Bgli) of inflammation-primed (or lyso-Pc-treated) B lymphocytes and the Neu-1 sialidase of T lymphocytes yields the macrophage activating factor (MAF) (Proc Natl Acad Sci USA 1991;88:539-543; U.S. Patent Number: 5,177,002.1993; Academic Press Ltd. London 1998a;2494-2499; Immunol Lett 1994;43:143-148), the protein with N-acetylgalactosamine as the remaining sugar (Fig. 1a). Thus, Gc protein is the precursor for the principal MAF (Proc Natl Acad Sci USA 1991;88:539-543; Mol Immunol 1996;33:1157-1164; Academic Press Ltd. London 1998a;2494-2499).

When peripheral blood mononuclear cells (PBMCs) containing monocytes/macrophages (macrophages for short) and lymphocytes of HIV-infected patients were treated with lyso-Pc (1 mg/ml) and cultured in a serumfree medium containing purified Gc protein (1 ng/ml) or healthy human serum (0.1%) as a source of Gc protein, the lyso-Pc-primed lymphocytes of all patients were

fully capable of converting Gc protein to MAF, resulting in activation of their macrophages (AIDS Res Human Retrovirus 1995;11:1373–1378). However, cultivation of lyso-Pc-treated patient PBMCs in medium containing patient own serum (0.1%) resulted in lack or reduced levels of macrophage activation (AIDS Res Human Retrovirus 1995;11:1373–1378; U.S. Patent Number: 5,620,846.1997; U.S. Patent number:5,985,545.1999), as a consequence of lost or decreased MAF precursor activity of the patient serum Gc protein (AIDS Res Human Retrovirus 1995;11:1373–1378; U.S. Patent Number: 5,620,846.1997; U.S. Patent Number:5,985,545.1999). Loss of MAF precursor activity is due to deglycosylation of serum Gc protein by α -N-acetylgalactosaminidase (termed Nagalase) secreted from HIV-infected cells (Fig. 1b) (AIDS Res Human Retrovirus 1995;11:1373–1378; U.S. Patent Number: 5,620,846.1997; U.S. Patent Number: 5,712,104.1998b; AIDS Res Human Retroviruses 2006;22:262–271). Since Nagalase is an intrinsic component of the envelope gp120 (U.S. Patent Number:5,985,545.1999; AIDS Res Human Retroviruses 2006;22:262–271), serum Nagalase activity is the sum of enzyme activities carried by both HIV virions and unassembled envelope proteins (gp160 and gp120) released from HIV-infected cells. Because of Nagalase being an HIV viral component, serum Nagalase is already complexed with patient own immunoglobulin G (anti-HIV IgG) (U.S. Patent Number:5,985,545.1999; AIDS Res Human Retroviruses 2006;22:262–271).

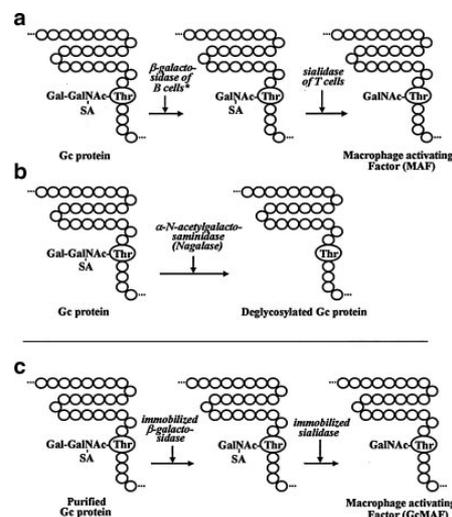


Fig. 1. Schematic illustration of formation of macrophage activating factor (a), deglycosylation of Gc protein by α -N-

acetylgalactosaminidase (Nagalase) (b) and enzymatic preparation of GcMAF (c). *, inflammation-primed B cells: B cells can be treated with an inflamed membranous lipid metabolite, for example, lysophosphatidylcholine (lyso-Pc).

The IgG-bound Nagalase retains enzyme activity that can initiate fusion for HIV-infection process and also deglycosylate Gc protein (AIDS Res Human Retroviruses 2006;22:262–271). The deglycosylated Gc protein cannot be converted to MAF and as a consequence macrophages cannot be activated, leading to the development of immunosuppression (AIDS Res Human Retrovirus 1995;11:1373–1378; U.S. Patent Number: 5,985,545.1999; AIDS Res Human Retroviruses 2006;22:262–271). As disease advances serum Nagalase activity increases while the MAF precursor activity decreases (AIDS Res Human Retrovirus 1995;11:1373–1378; Yamamoto, 2006). Lack of macrophage activation and severe immunosuppression explain why AIDS patients die from overwhelming infection (e.g., pneumonia) (Am J Obst Gyn 1992;167:152–157; Am J Med 1992;93:387–390; AIDS Res Human Retrovirus 1995;11:1373–1378; AIDS Res Human Retroviruses 2006;22:262–271).

However, exogenously given MAF can bypass the deglycosylated Gc protein and directly act on macrophages for activation. Stepwise treatment of purified Gc protein with immobilized b-galactosidase and sialidase generates probably the most potent MAF (termed GcMAF) (U.S. Patent Number: 5,177,002.1993, 1996, Academic Press Ltd. London 1998a;2494-2499; J Immunol 1993;151:2794-2802; Immunol Lett 1994;43:143-148) (Fig. 1c) ever discovered, which produces no side effects in humans (Mol Immunol 1996;33:1157-1164, Academic Press Ltd. London 1998a;2494-2499; Immunology. Italy:Medmond, Bologna 2004a;201-204). The optimal human dose of GcMAF, to achieve phagocytic capacity by 30-fold increased ingestion index and 15-fold increased superoxide generating capacity of peripheral blood monocytes/macrophages, was found to be \approx 100 ng/human (Int J Cancer 2008a;122:461–467). GcMAF administration greatly enhanced

phagocytic and superoxide generating capacities of systemic macrophages in 3.5 hr (Immunol Cell Biol 1998;76:237-244). GcMAF also has a potent mitogenic capacity to act on the progenitor cells, resulting in a 40-fold increase in systemic macrophage cell counts in 4 days (Immunol Cell Biol 1998;76:237-244). Such highly activated systemic macrophages are chemotactically recruited to inflamed lesions, as manifested by a 180-fold increase in local macrophage counts (Immunol Cell Biol 1998;76:237-244). The activated macrophages develop a large amount of Fc-receptors (Proc Natl Acad Sci USA 1991;88:539-543; Mol Immunol 1996;33:1157-1164) as well as an enormous variation of receptors (J Immunother 2005;28:642; Int J Cancer 2008a;122:461–467) that recognize a variety of antibody-bound and unbound viral and bacterial antigens and cells with surface abnormality such as cancer cells (J Immunother 2005;28:642; Int J Cancer 2008a;122:461–467). Mouse macrophages, by in vitro treatment with 10–50 pg/ml GcMAF for 3 hr, are maximally activated for phagocytosis (Mol Immunol 1996;33:1157-1164) and superoxide generation (Immunol Lett 1994;43:143-148; Academic Press Ltd. London 1998a;2494-2499). Four administrations of 100 pg GcMAF to Ehrlich ascites tumor bearing mice eradicate the tumor (Cancer Res 1997;57:2187-2192; Proc Soc Exp Biol Med 1999;220:20-26). When human macrophages are treated in vitro with 100 pg/ml for 3 hr, they are highly tumoricidal and kill approximately 50% of both prostate cell line LNCaP and breast cancer cell lines MCF-7 and MDA-MB-321 in 4 hr (Immunology. Italy:Medmond, Bologna 2004a;201-204; Int J Cancer 2008a;122:461–467). Weekly administrations of 100 ng GcMAF to metastatic adenocarcinoma (breast and prostate cancer) patients (n=432) and metastatic colorectal cancer patients eradicate tumors in 16–25 weeks and 32–50 weeks, respectively (Immunology. Italy:Medmond, Bologna 2004a;201-204; J Immunother 2005;28:642; Int J Cancer 2008a;122:461–467; Translational Oncol 2008b;1:65–72; Cancer Immunol Immunother 2008c;57:1007–1016). With the same therapeutic procedure (i.e., administration of 100 ng GcMAF/week) in a preliminary study of HIV-infected patients, both cell-free virions and HIV-infected cells were eradicated in 10–18 weeks (Immunology. Italy:Medmond,

Bologna, 2004b;197:200; Proc 13th Int Cong Immunol. Italy:Medimond, Bolog 1997;38:38).

MATERIALS AND METHODS

I. Chemicals, Reagents and Cells

Phosphate buffered saline (PBS) contained 1 mM sodium phosphate and 0.15 M NaCl. Macrophage-SSM serum free medium was purchased from GIBCO BRL Life Science Technology (Grand Island, NY). When peripheral blood monocytes adhere to vessel substratum, they behave like macrophages which show increased synthesis of hydrolases. For manipulation in vitro and cultivation of peripheral blood mononuclear cells (PBMCs) containing monocytes/macrophages (macrophages for short) and lymphocytes (B and T cells), 0.1% egg albumin supplemented medium RPMI 1640 (EA medium) (Mol Immunol 1996;33:1157-1164) was used. Gc1F protein (the major isoform of Gc protein) was isolated from Gc1F homozygous donor serum. Lysophosphatidylcholine (lyso-Pc) and p-Nitrophenyl N-acetyl-a-D-galactosaminide were purchased from Sigma Chemical Co. (St. Louis, MO). Mitomycin C, a provirus inducing agent (Arch Virol 1977;54:333-343), was obtained from ICN pharmaceutical, Inc. (Costa Mesa, CA). HIV-infected PBMCs were treated with 5 mg mitomycin/ml for 30 min, washed and plated on a plaque-forming indicator monolayer for detection of infectious centers (AIDS Res Human Retroviruses 2006;22:262–271), or cultured in Macrophage-SSM medium supplemented with 5% FCS for 72 hr for induction of the provirus (AIDS Res Human Retroviruses 2006;22:262–271). Protein G-Sepharose to be used for precipitation of IgG-immunocomplex (AIDS Res Human Retroviruses 2006;22:262–271) was obtained from Pharmacia Biotech, Inc. (Piscataway, NJ).

II. Procedure for Preparation of GcMAF

Procedure for preparation of GcMAF was described previously (U.S. Patent Number: 5,177,002.1993; Immunol Lett 1994;43:143-148; Int J Cancer 2008a;122:461–467; Cancer

Immunol Immunother 2008c;57:1007–1016). Briefly, Gc1F protein was purified using 25-hydroxyvitamin D3-affinity chromatography (Anal Biochem 1986;157:262–269). Stepwise incubation of the purified Gcprotein with immobilized β galactosidase and sialidase yielded probably the most potent macrophage activating factor (GcMAF) ever discovered (J Immunol 1993;151:2794–2802; Immunol Lett 1994;43:143–148; Mol Immunol 1996;33:1157–1164) (Fig. 1c). GcMAF was filtered through a low protein-binding filter, Millex-HV (Millipore Corp., Bedford, MA) for sterilization.

The molecular structure of GcMAF is identical to that of the native human MAF. Numerous administrations (more than 10 times for 3–6 months period) of GcMAF (100–500 ng/human) to 12 humans showed no sign of side effects (U.S. Patent Number: 5,177,002.1993, 1996; Int J Cancer 2008a;122:461–467; Translational Oncol 2008b;1:65–72; Cancer Immunol Immunother 2008c;57:1007–1016). Quality control of the preparation of GcMAF was performed for activity, sterility and safety tests.

III. GcMAF Therapy of HIV-Infected Patients

Participants. A cohort of 15 asymptomatic HIV-1- infected patients was included in this study.

These patients must carry HIV-1 provirus in their PBMCs as demonstrated by increased Nagalase production and infectious center (plaques) formation after mitomycin treatment of PBMCs (AIDS Res Human Retroviruses 2006;22:262–271). They received GcMAF therapy exclusively and excluding combination with erythropoiesis induction. Thus, anemic HIVinfected patients were not eligible in the program. The study was approved by The Institutional Research and Ethic Committees of Nagasaki Immunotherapy Group (Nagasaki, Japan) and The Institutional Review Board of Hyogo Immunotherapy group (Hyogo, Japan). The participants gave written informed consent before entering the study.

GcMAF administration. Because the half-life of the activated macrophages is approximately 6 days

(Cancer Res 1988;48:6044-6049; Proc 13th Int Cong Immunol. Italy:Medimond, Bolog 1997;38:38; Int J Cancer 2008a;122:461–467), 100 ng GcMAF was administered intramuscularly once a week.

IV.Procedures Used for Prognostic Analysis

Serum and blood samples were weekly or biweekly collected immediately prior to each GcMAF administration and used for prognostic analyses. Precursor activity of serum Gc protein, serum Nagalase activity, p24 antigen, HIV-1 RNA copies, CD4 β and CD8 β Tcells counts, viral load in plasma and proviral load in PBMCs were always determined during GcMAF therapy.

The MAF precursor activity of serum Gc protein and serum Nagalase activity were determined to show recovery rate of immuno-potency during the early stage of GcMAF therapy. These prognostic analyses were compared with amounts of p24 antigen and HIV-1 RNA and changes in CD4 β and CD8 β T-cell counts. Since serum Nagalase activity is the total sum of Nagalase activities carried by HIV virions and unassembled envelope proteins in the patient blood stream and is detectable until complete eradication of HIV infection (U.S: Patent Number: 5,985,545.1999; AIDS Res Human Retroviruses 2006;22:262–271), assessment of curative response to GcMAF therapy was performed by determining serum Nagalase activity as a prognostic index during the entire kinetic course of GcMAF therapy.

V.CD4 and CD8 Cell Counts

The blood samples were processed immediately within 2 hr of collection, for determining the absolute counts of CD4 β cells and CD8 β cells by two color immunophenotyping on the single platform fluorescence activated cell sorting (FACS) count system (Becton Dickinson Pvt. Ltd, Mountain View, CA), using fluorochrome labeled monoclonal antibodies to CD4 β and CD8 β T-cells, following manufacturer's instructions.

VI. Immunoassay of HIV-1 p24 Antigen

HIV p24 ELISA (Lentivirus Quantitation Kit, Cell Biolabs, Inc., San Diego, CA) was used.

An anti-HIV p24 monoclonal coating antibody is adsorbed onto a microtiter plate. P24 antigen present in the sample or standard binds to the antibodies adsorbed on the plate, a biotin-conjugated goat anti-p24 antibody is added and binds to p24 antigen captured by the first antibody. Following incubation and wash steps, Streptavidin-HRP is added and binds to the biotin conjugated anti-p24. Following unbound Streptavidin- HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells. A colored product is formed in proportion to the amount of p24 antigen present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from recombinant HIV-p24 protein and sample p24 concentration is then determined.

VII. Detection of HIV-1 RNA in Serum by RT-PCR

HIV-1 RNA in serum was measured by a RT-PCR kit (Amplicator HIV-1 Monitor, Roche Diagnostic Systems, Basel, Switzerland) as described by Hashida et al. (2000). The cutoff value of this assay is 400 copies/ml.

VIII. Determination of MAF Precursor Activity of Serum Gc Protein of HIV-Infected Patients

Assay procedure for determination of MAF precursor activity of patient serum Gc protein has been described previously (AIDS Res Human Retrovirus 1995;11:1373-1378, Cancer Res 1996;56:2827-2931; Cancer Res 1997;57:295-299; Proc Soc Exp Biol Med 1999;220:20-26; AIDS Res Human Retroviruses 2006;22:262-271). Briefly, healthy control PBMCs were treated with lyso-Pc (1 mg/ml) for 30 min and cultured in EA medium containing 0.1% serum of HIV-infected patients or healthy controls as a source of Gc protein. After 3 hr cultivation the extent of macrophage activation

was assayed for superoxide generating capacity (Cancer Res 1996;56:2827-2931; U.S: Patent Number: 5,620,846.1997). The data were expressed as nmol of superoxide produced per minute per 10⁶ cells (macrophages). These values represent the MAF precursor activity of patient serum Gc protein (Cancer Res 1996;56:2827-2931; U.S: Patent Number: 5,620,846.1997). Lost or reduced MAF precursor activity of patient serum Gc protein are exhibited as a decrease in superoxide generation as compared with control healthy human Gc protein. Thus, this procedure measures the ability of individual patient to activate macrophages as for immune potential.

Cultivation of the mixture of lyso-Pc-treated lymphocytes and macrophages in EA medium without addition of serum results in the production of 0.5–0.8 nmol superoxide/min/10⁶ macrophages (Inflammation 1994;18:311-322). Thus, if patient serum (0.1%) generates <0.8 nmol superoxide/min/10⁶ macrophages, the precursor activity of the patient serum Gc protein is considered to be lost.

IX. Detection of Nagalase in HIV-Infected Patient Sera

Patient sera (300 ml) were precipitated with 70% saturated ammonium sulfate. The precipitates were dissolved in 50 mM sodium citrate buffer (pH 6.0) and dialyzed against the same buffer at 4°C for 2 hr. The dialysates were made up to 1 ml in volume and assayed for Nagalase activity (AIDS Res Human Retrovirus 1995;11:1373-1378; Cancer Res 1996;56:2827–2931; Cancer Res 1997;57:295–299; 2008a). Substrate solution (250 ml) contained 5 mmol of p-nitrophenyl N-acetyl- α -D-galactosaminide in 50 mM citrate buffer (pH 6.0). The reaction was initiated by addition of 250 ml of the dialyzed samples, kept at 37°C for 60 min and terminated by adding 200 ml of 10% TCA. After centrifugation of the reaction mixture, 300 ml of 0.5 M Na₂CO₃ solution was added to the supernatant. The amount of released p-nitrophenol was determined spectrophotometrically at 420 nm and expressed as nmol/min/mg

protein (AIDS Res Human Retrovirus 1995;11:1373-1378, Cancer Res 1996;56:2827-2931; Cancer Res 1997;57:295–299;Int J Cancer 2008a;122:461–467). Protein concentrations were estimated by the Bradford (Anal Biochem 1976;72:248–254) method.

Healthy control sera exhibit very low levels (0.35–0.68 nmol/min/mg) of the enzyme activity. This is the enzyme activity of α -galactosidase that can also catabolize the chromogenic substrate (i.e., p-nitrophenyl N-acetyl- α -D-galactosaminide) for Nagalase (AIDS Res Human Retrovirus 1995;11:1373-1378, Cancer Res 1996;56:2827-2931; Cancer Res 1997;57:295–299). A reduction in serum Nagalase activity to 0.68 nmol/min/mg or less in patient during GcMAF therapy serves as demonstration that HIVinfection has been eradicated.

X.Determination of the Viral Load in Plasma and the Proviral Load in PBMCs of the HIV-Infected.

Patients Assay methods for plaques for viral load in plasma and plaques as infectious centers for proviral load in PBMCs were employed. Monolayers of MT-4 cells were prepared for plaque assay (J Immunol Methods 1986;92:177–181; J Gen Virol 1989;70:3327–3333). Six well tissue culture plates were treated with 50 mg/ml of poly-L-lysine for 1 hr at room temperature. The wells were then washed three times with distilled water and left to dry in air. MT-4 cells were washed in serum free EA medium twice and placed at a final cell concentration of 2×10^6 /well. Cells were then allowed to adsorb to the plates for 30 min at room temperature. Unbound cells were subsequently removed by aspiration.

For determination of the viral load in patient plasma as the amount of cell-free virions, patient plasma (at 100 μ l) were added to the cells. After adsorption of virus for 1 hr at room temperature, the cells were overlaid with 1.5 ml of 0.6% molten agarose (Sea Plaque agarose, FMC Bioproducts, Rockland, ME) containing nutrient medium (RPMI-1640 medium supplemented with 10% FCS, 100 IU of penicillin and 100 mg of streptomycin per ml). After the agar overlay solidified, the

plates were incubated in a CO₂ incubator at 37°C. After 3 days of incubation, the cells were overlaid with another 1.5 ml of agarose medium. After 5 or 6 days of incubation, some plaques were visible.

The HIV virions in plasma of some patients do not produce distinct plaques. The plaques were then fixed and stained with peroxidase-labeled antibodies (J Clin Microbiol 1987;25:1305–1307). Three milliliters of 95% methanol was poured onto each dish, and the cells were fixed overnight at room temperature. Methanol was changed three to four times during fixation. After fixation, 3 ml of formamide was added to each dish to melt the agarose. The dishes were washed with running water and 1 ml of 1:1,000-diluted human antiserum against HIV was added to each dish. After incubation for 1 hr at 37°C, the dishes were washed three times with PBS, and 1 ml of biotinylated anti-human immunoglobulin G (1:500 dilution in PBS; Amersham International plc, Buckinghamshire, England) was added and incubated for 1 hr at 37°C. The dishes were washed three times with PBS, and 1 ml of streptavidin-biotinylated horseradish peroxidase complex (1:1,000 dilution in PBS; Amersham) was added and incubated for 1 hr at 37°C. After dishes were washed with PBS three times, brownish stained spots were visualized by the addition of 1 ml of a staining mixture containing 0.5 mg of 3,3'-diaminobenzidine tetrahydrochloride per ml and 0.02% H₂O₂ in PBS. When the stain had reached a suitable intensity, the dishes were washed with running water and dried. The numbers of the immunostained plaques were counted.

For determination of the numbers of HIV-infected PBMCs as the proviral load in the PBMCs, 0.1 ml of mitomycin-treated patient's PBMCs was added to 1.5 ml of 0.6% molten agarose (Sea Plaque agarose, FMC Bioproducts, Rockland, ME) containing nutrient medium (RPMI-1640 medium supplemented with 10% FCS, 100 IU of penicillin and 100 mg of streptomycin per ml). After the agar overlay solidified, the plates were incubated in a CO₂ incubator at 37°C. After 3 days of incubation, the cells were overlaid with another 1.5 ml of agarose medium. Neutral red stain was added in the second agarose overlay at a concentration of 0.00032%, incubated in the dark for 20 hr

and the plaques for cell-free virions and plaques for infectious centers were counted using an inverted microscope (Yamamoto, 2006). Since an infectious center is produced by infection of MT-4 cells with a large number of HIV virions released from mitomycin-treated cells, peroxidase-labeled immunostaining is generally not required for detection of infectious centers.

The total assessment of viral load in the HIV-infected patients is accomplished by the plaques for cell-free HIV virions and the infectious centers for determination of HIV-infected patient PBMCs. Since the macrophages activated by GcMAF phagocytize cell-free HIV virions, eradication of the proviral carrying PBMCs follows that of cell-free virions. If no infectious centers are detected after GcMAF therapy, HIV-infection is eradicated.

RESULTS

I.MAF Precursor Activity of Serum Gc Protein and Serum Nagalase Activity of HIV-Infected Patients

Because the fate and staging of the HIV-infected patients correlate with the level of immunosuppression and viral load (AIDS Res Human Retrovirus 1995;11:1373-1378; U.S. Patent Number: 5,985,545.1999; AIDS Res Human Retroviruses 2006;22:262–271), the MAF precursor activity of Gc protein and serum Nagalase activity for each of 15 patients were determined before entering GcMAF therapy. As shown in Table I, patients having lowerMAFprecursor activity had higher serum Nagalase activity. This inverse correlation between the MAF precursor activity and serum Nagalase activity is consistently observed with a number (>230) of HIV-infected patients screened (AIDS Res Human Retrovirus 1995;11:1373-1378; U.S. Patent Number: 5,620,846.1997; U.S. Patent Number:5,985,545.1999; AIDS Res Human Retroviruses 2006;22:262–271). In contrast, quantitative correlation between the MAF precursor activity (or serum Nagalase activity) and CD4pvalue of the patients has not been feasible (AIDS Res Human Retrovirus 1995;11:1373-1378; U.S. Patent Number: 5,620,846.1997; U.S. Patent Number:5,985,545.1999). The majority of

this HIV patient group carries low CD4 β cell counts ranging from 156 to 336 cells/ml with a few exception (Table I). However, only a small proportion of cells expressing CD4 β cells is infected (Science 1989;245:305–308; AIDS Res Hum Retroviruses 1993;9:287–289). Thus, CD4 β cell counts do not reflect the frequency of HIV-infection (AIDS Res Human Retrovirus 1995;11:1373-1378) and could not be used for quantitative kinetic study of GcMAF therapy.

TABLE I. Precursor Activity of Serum Gc Protein and Nagalase Activity Detected in the Blood Stream and CD4 β Cell Counts of 15 HIV-Infected Patients

Patient No.	Precursor activity(a)	Nagalase specific		
	superoxide produced (nmol/min/10 ⁶ cells)	activity (nmol/min/mg)	Pre-GcMAF therapy CD4 β cells/ml	Post-GcMAF therapy CD4 β cells/ml
1	0.85	5.38	236	828
2	1.53	4.59	326	725
3	1.94	3.72	312	626
4	1.72	4.08	475	818
5	1.10	5.02	156	784
6	0.88	5.42	224	997
7	1.62	4.56	282	807
8	2.73	4.19	422	652
9	2.80	4.98	253	908
10	2.05	3.77	326	561
11	2.58	3.18	336	846
12	0.72	5.58	214	794
13	2.76	3.06	526	883
14	2.78	4.04	312	787
15	1.63	4.88	264	942

Control(b)	6.25	0.23(c)	824
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(a) Precursor activity was measured by superoxide generating capacity of healthy human macrophages when treated with 0.1% of patient's serum as a source of Gc protein.

(b) Healthy control (average of 5).

(c) This uninfected human enzyme is known to be α -galactosidase (AIDS Res Human Retrovirus 1995;11:1373-1378; Cancer Res 1996;56:2827-2931). This enzyme is unable to deglycosylate Gc protein though it is able to hydrolyze p-nitrophenyl N-acetyl- α -D galactosaminide (AIDS Res Human Retrovirus 1995;11:1373-1378; Cancer Res 1996;56:2827-2931). Because α -N-acetylgalactosaminidase and α -galactosidase are evolutionary conserved, carry 46.9% amino acid sequence homology and share a common chromogenic substrate for their catabolic capacities (AIDS Res Human Retrovirus 1995;11:1373-1378; Cancer Res 1996;56:2827-2931; Cancer Res 1997;57:295-299).

II. Demonstration of an Excess Antibodies Beyond Anti-HIV Antibodies Already Complexed With Serum Nagalase in HIV-Infected Patient Blood Stream.

Because of Nagalase being a viral component, Nagalase in the patient blood stream was complexed with patient's own immunoglobulin G (i.e., polyclonal anti- HIV IgG) (U.S: Patent Number: 5,985,545.1999; AIDS Res Human Retroviruses 2006;22:262-271). To test whether each patient has an excess amount of anti-HIV IgG antibodies beyond antibodies already complexed with serum Nagalase, 1 ml of a patient serum was mixed with a culture lysate (1 ml) of another patient HIV-infected PBMCs which have been treated with MitomycinC (MC: a provirus inducer (Arch Virol 1977;54:333-343)) and cultured for 37°C for 72 hr (AIDS Res Human Retroviruses 2006;22:262-271). After 1 hr incubation of the mixture at 37°C, protein G-Sepharose (40 mg) was added and centrifuged to isolate IgG-Nagalase immunocomplexes. The supernatant was assayed for Nagalase activity. Precipitated protein G-Sepharose bound IgG-Nagalase immunocomplexes were washed with 50 mM citrate phosphate buffer (pH 6.3), and the enzyme was eluted from the protein G-Sepharose with citrate phosphate buffered saline containing 0.15 M NaCl (pH 6.5) and assayed for Nagalase activity (AIDS Res Human Retroviruses 2006;22:262-271). As shown in Table II, the

total amount of Nagalase activity was precipitated with protein G-Sepharose. The result indicates that sera of all four HIV-infected patients had ample amounts of anti-HIV IgG antibodies to form immunocomplexes with the exogenously given Nagalase which has been mitomycin- induced from HIV-infected PBMCs of another patient. This reveals that HIV-infected patients have an ample amount of anti-HIV IgG antibodies capable of complexing with Nagalase as soon as secreted from HIVinfected cells.

III.MAF Precursor Activity of Gc Protein and Serum Nagalase Activity as Prognostic Parameters During GcMAF Therapy of HIV-Infected Patients.

MAF precursor activity and serum nagalase activity during GcMAF therapy. When systemic macrophages are activated by administration of 100 ng GcMAF, macrophages develop a large amount of Fcreceptors as well as an enormous variation of receptors (Proc Natl Acad Sci USA 1991;88:539-543; U.S. Patent Number: 6,410,269.2002; Int J Cancer 2008a;122:461–467; Translational Oncol 2008b;1:65–72;Cancer Immunol Immunother 2008c;57:1007–1016). Since serum Nagalase is immunocomplexed with the patient’s own immunoglobulin G (anti-HIV IgG), we anticipated that the macrophages activated by GcMAF rapidly phagocytize IgGbound Nagalase via Fc-receptor mediation. Thus, the two prognostic indices,MAFprecursor activity of serum Gc protein and serum Nagalase activity of the first five patients (Table I) were analyzed during the first 6 weeks of GcMAF therapy.

Because the half-life of activated macrophages is approximately 6 days (Cancer Res 1988;48:6044-6049; Mol Immunol 1996;33:1157-1164; Proc 13th Int Cong Immunol. Italy:Medimond, Bolog 1997;38:38na; Int J Cancer 2008a;122:461–467), 100 ng ofGcMAF were administered weekly and patient serum samples were collected weekly at the end of a week but immediately prior to each GcMAF administration. As GcMAF therapy progressed, the MAF precursor activity of all five

patients increased while their Nagalase activity decreased inversely as shown in Table III. Because serum Nagalase activity is the sum of Nagalase activities carried by HIV virions and unassembled envelope proteins, as serum Nagalase activity decreased the amount of HIV virions in the blood stream decreased while the MAF precursor activity as the immuno-potency increased.

TABLE II. HIV-Infected Patients Carry Ample Amounts of Anti-HIV-1 IgG Capable of Complexing With Nagalase Activity Secreted From HIV-Infected Patient PBMCs

Patient No.	Serum	MC-induced HIV-PBMCs(a)	Nagalase (mol/min/mg)		
			Control(b)	Protein-G: precipitate(c)	Supernatant(d)
S1	+	-	5.38	5.06	0.22
	-	+	3.18		
	+	+	8.52	8.22	0.43
S2	+	-	4.59	4.35	0.38
	-	+	2.29		
	+	+	6.78	6.62	0.36
S3	+	-	3.72	3.16	0.33
	-	+	2.86		
	+	+	6.12	6.08	0.59
S4	+	-	5.42	5.26	0.32
	-	+	3.56		
	+	+	8.84	8.59	0.73

(a) Culture medium of Mitomycin C-induced HIV-infected PBMCs of another patient.

(b) Sixfold diluted patient serum (300 ml), Mitomycin-induced PBMCs culture medium and their mixture were assayed for Nagalase activity (AIDS Res Human Retroviruses 2006;22:262–271).

(c) Nagalase-antibody complex was eluted from protein G-Sepharose with 0.1 M citrate and assayed for Nagalase activity.

(d) Supernatant after protein G-Sepharose precipitation was assayed for the enzyme.

Effect of GcMAF therapy on quantity of HIV-1 RNA and p24 antigens and cell counts of CD4⁺ and CD8⁺T-cells. Nagalase is the major viral constituent required for infectious process (AIDS Res Human Retroviruses 2006;22:262–271). Loss or decrease of serum Nagalase activity during GcMAF therapy was always compared with quantitative change of HIV-1 RNA and p24 antigen and cell counts of CD4⁺ and CD8⁺T-cells. Table III shows that as GcMAF therapy progressed both RNA copy number and p24 antigen rapidly decreased and often reached to undetectable levels at the 6th week of GcMAF therapy. Furthermore, as Nagalase activity decreased during GcMAF therapy, CD4⁺ cell counts increased while CD8⁺ cell counts decreased.

Eradication of nagalase carriers. Since patient anti-HIV antibodies do not inhibit Nagalase activity (AIDS Res Human Retroviruses 2006;22:262–271), the antibodies of IgG-Nagalase immunocomplexes are bound to the structure of Nagalase carriers (i.e., HIV virions and envelope proteins) other than the active center of Nagalase. As shown in Figure 2a, weekly decrease of serum Nagalase activities of five patients suggests that IgG-bound Nagalase carriers in the patient blood stream were rapidly phagocytized by the activated macrophages via Fc receptor mediated. Although serum Nagalase carriers were replenished by spontaneous induction from HIV-infected cells, serum Nagalase activity decreased steeply during the first 6 weeks of GcMAF therapy (Table III and Fig. 2a). We continued to assay serum Nagalase activity as a measure for a decrease in the amount of Nagalase carriers. Since spontaneous induction for HIV-infected cells to release HIV virions is at a high frequency (AIDS Res Human Retroviruses 2006;22:262–271), continuous removal of Nagalase carriers by the activated macrophages may soon result in exhaustion of HIV-infected cells. Time course weekly prognostic analysis of GcMAF therapy was continued for additional 6–12 weeks. As shown in Figure 2a, the Nagalase activities of patients #2, 3, 4, and 5 reached to the control level in 10–13 weeks, indicating eradication of both Nagalase carriers and HIV-infected cells while the Nagalase activity of patient #1 reached to the control level in 18 weeks. When these

patient PBMCs were treated with mitomycin C and plated on MT-4 cell monolayers, no infectious centers were detectable (Table IV), indicating eradication of HIV-infection. From these results we conclude that GcMAF therapy of HIV patients cures HIV disease.

IV. Time Course Study of Serum Nagalase Activity of HIV-Infected Patients During GcMAF Therapy

Based on findings of the curative process of HIV infection with GcMAF therapy of five HIV-infected patients described in the preceding section, we conclude that eradication of both cell-free HIV virions and HIV-infected cells by GcMAF therapy can be monitored by serum Nagalase activity as a prognostic index and confirmed by no infectious center formation of mitomycin-treated PBMCs. This conclusion deserves substantiation with GcMAF therapy of more HIV-infected patients. Time course analyses of serum Nagalase activity of additional ten HIV-infected patients were performed to assess the efficacy of GcMAF therapy for HIV disease. Serum Nagalase activities of these patients were analyzed weekly for the first 2 weeks and followed by biweekly analysis up to 18 weeks. These patients had the initial Nagalase activities ranging from 3.06 to 5.58 nmol/min/mg (Table I).

TABLE III. Correlation Between Serum Nagalase Activity, Quantity of p24 Antigen and HIV-1 RNA and Cell Counts of CD4 β and CD8 β T-Cells of Five HIV-1 Infected Patients During GcMAF Therapy

Patient No.	Assayed weeks	Precursor	Nagalase	p24 (pg/ml)	RNA	CD4	CD8
		activity (nmol)	(nmol/min/mg)		(copies/ml)	(cells/ μ l)	(cells/ μ L)
1	0	0.85	5.39	343	432,000	236	1,174
	1	1.52	4.65				
	2	1.94	3.72	85	58,000	495	852
	3	2.32	3.23				
	4	2.61	2.75	22	7,200	648	598
	5	2.72	2.40				
	6	2.88	2.22	4.2	900	806	583
2	0	1.53	4.59	127	386,000	326	1,023
	1	1.85	3.77				
	2	2.10	3.18	34	48,000	504	689
	3	2.66	3.62				
	4	3.13	1.82	7.2	5,200	692	593
	5	3.54	1.05				
	6	3.96	0.78	<0.2	UD	716	599
3	0	1.94	3.72	68	165,000	312	897
	1	2.46	3.04				
	2	2.82	2.21	17	11,600	513	605
	3	3.65	1.60				
	4	3.72	1.16	<0.2	1,400	615	525
	5	3.86	1.04				
	6	4.04	0.80	<0.2	UD	632	593

4	0	1.72	4.08	92	238,000	475	1,010
	1	1.98	3.38				
	2	2.59	2.72	19	28,000	608	754
	3	2.96	2.08				
	4	3.22	1.63	4.8	2,300	802	585
	5	3.36	1.36				
	6	3.48	1.14	<0.2	UD	811	536
5	0	1.10	5.02	305	378,000	156	1,085
	1	1.72	4.24				
	2	1.98	3.54	69	52,000	525	834
	3	2.54	2.98				
	4	2.70	2.42	13	3,800	705	712
	5	2.96	2.11				
	6	3.26	1.78	<0.2	UD	788	558
Control		4.25	0.45(a)			872	524
	(average of 5)						

UD indicates undetectable copies less than the cutoff value of 400 copies/ml.

aHealthy subjects carry a-galactosidase that shares the same substrate with Nagalase (AIDS Res Human Retrovirus 1995;11:1373-1378).

As shown in Figure 2b, the serum Nagalase activity of all 10 patients decreased as GcMAF therapy progressed. Thus, the activated macrophages rapidly phagocytized IgG-bound Nagalase carriers. After less than 18 weekly administrations of GcMAF, their serum Nagalase activities decreased to the healthy control value (<0.60 nmol/min/ mg), suggesting that these patients were free of both HIV virions and HIV-infected cells. In fact, these patients plasma did not produce plaques (Table IV). When these patient PBMCs were treated with mitomycin C and plated on MT-4 cell monolayers (AIDS Res Human Retroviruses 2006;22:262–271), no infectious centers were

detectable, indicating eradication of HIV-infection as shown in Table IV. All 15 patients carried healthy CD4 β cell counts, 798_213 cells/ml (Table I). During 7 years observation after completion of GcMAF therapy, these patients showed no increase in their serum Nagalase activities, indicating no recurrence of the disease. HIV-1 RNA and p24 antigen were never detectable in these patient blood stream. Furthermore, the above healthy CD4 β cell counts of these patients were maintained for the entire 7-year period after GcMAF therapy.

DISCUSSION

HIV-1 infection appears to induce some antibodies in patients who nonetheless develop inadequate immunity for the disease (AIDS Res Human Retroviruses 2006;22:262–271). Consequently, a persistent infection is established. Although HIV-1 is one of the retroviruses, it has lytic capacity upon infection to susceptible host cells. This characteristic of HIV-1 allows plaque assay for quantitation of the virions. If cells did not lyse by HIV-1 infection, however, the cells become latently infected but the HIV provirus is rather unstable (AIDS Res Human Retroviruses 2006;22:262–271). Thus, the cells latently infected with HIV constantly release HIV virions and unassembled envelope proteins carrying Nagalase activity into the patient blood stream (AIDS Res Human Retroviruses 2006;22:262–271).

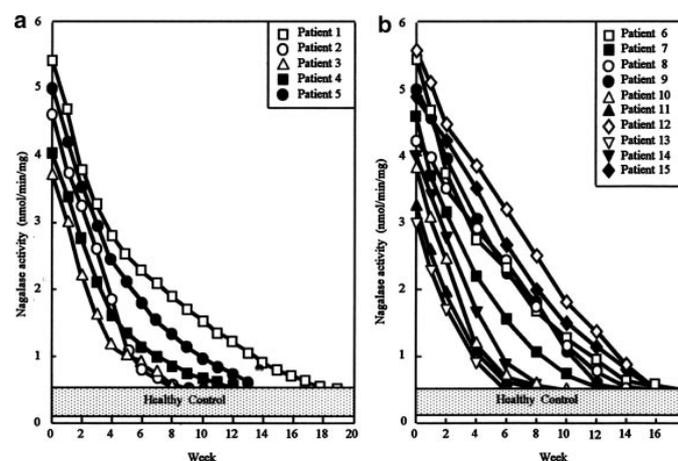


Fig. 2. Therapy of HIV-infected patients with GcMAF. Time course analysis of serum Nagalase activity as a prognostic index during GcMAF therapy of five HIV-infected patients (a) and additional ten patients (b).

The latency of HIV-1 provirus was also confirmed by increased Nagalase production and infectious center (plaque) formation after mitomycin treatment of patient PBMCs (Yamamoto, 2006). Because patient sera contain ample amounts of anti-HIV IgG, these spontaneously released Nagalase carriers were immediately immunocomplexed with anti-HIV antibodies. These antibodies are largely not neutralizing antibodies because of blood borne infectivity of HIV, although Wendler et al. (AIDS Res Human Retroviruses 1987;3:157–163) reported that some patients develop neutralizing antibodies. The IgGbound virions still retain Nagalase activity (AIDS Res Human Retroviruses 2006;22:262–271) that is required for infectivity of the HIV virions and also able to deglycosylate serum Gc protein (AIDS Res Human Retroviruses 2006;22:262–271). The deglycosylated Gc protein cannot be converted to MAF, leading to the development of immunosuppression (AIDS Res Human Retrovirus 1995;11:1373-1378; U.S. Patent Number: 5,620,846.1997; U.S. Patent Number:5,985,545.1999). Monocytes/macrophages activated by GcMAF administration immediately stop DNA replication and rapidly synthesize a large amount of Fc-receptors as well as an enormous variation of receptors (Proc Natl Acad Sci USA 1991;88:539-543; U.S. Patent Number: 6,410,269.2002; J Immunother 2005;28:642; Int J Cancer 2008a;122:461–467). The macrophages activated by GcMAF phagocytize multi-IgG-complexed larger object (i.e., virion) more quickly than IgG-complexed protein (i.e., envelope protein). This may explain why HIV-1 RNA copies decreased rapidly during GcMAF therapy as shown in Table III. Although these activated macrophages constantly intercept both IgG-bound and unbound HIV virions as soon as secreted from HIV-infected cells to prevent reinfection, they preferentially phagocytize IgG-bound virions rapidly via Fc-receptor mediation (Proc Natl Acad Sci USA 1991;88:539-543; U.S. Patent Number: 6,410,269.2002). Since spontaneous induction of HIV-infected cells to release HIV virions is at a high frequency (AIDS Res Human Retroviruses 2006;22:262–271), continuous removal of cell-free HIV virions by the activated macrophages soon results in exhaustion of HIV-infected cells. This explains why HIV-infected patients are rapidly cured by GcMAF therapy. Eradication of HIV-

infection was firmly confirmed by no infectious centers produced by mitomycin-treated patient PBMCs after GcMAF therapy (Table IV).

Envelope hemagglutinating protein HA-1 of influenza virus also carries Nagalase activity which is detectable at a level of 1.5–2.0 nmol/min/mg in patient sera immediately after influenza infection (Microbes Infect 2005;7:674–681). Upon infection of HIV the hosts develop flu-like symptoms with serum Nagalase activity similar to the influenza acute state.

TABLE IV. Viral Load in Plasma and Proviral Load in PBMCs During GcMAF Therapy of 15 Patients

	Before therapy	4th week	Post-therapy(a)
Median plasma viral load (plaques/ml)	6.7×10^2	1.6×10^1	0
Median proviral load (infectious centers/ml)	1.1×10^2	1.2×10^2	0

(a) Patient serum Nagalase activities reached to the healthy control level by 10–18 weeks

Like serum Nagalase activity of influenza virus-infected patients, serum Nagalase activity is the sum of enzyme activities carried by HIV virions and unassembled envelope proteins released from HIV-infected cells. Thus, serum Nagalase activity is readily detectable at all stages of HIV infection (AIDS Res Human Retrovirus 1995;11:1373-1378; AIDS Res Human Retroviruses 2006;22:262–271) whereas HIV-1 RNA and p24 protein are undetectable in the early stage of HIV-infection (Clinical Diagnostic Lab Immunol 2000;:872–881). Similarly after 6 weeks of GcMAF therapy the majority of patients, HIV-1 RNA and p24 antigen are undetectable (Table III). Because of the availability of the precision measurement of serum Nagalase, the cure rate measurements of HIV-infection during GcMAF therapy have been possible. Therefore, the significance of GcMAF therapy of HIV-infection has been greatly enhanced by the discovery of HIV-specific Nagalase that can accurately monitor the cure rate during GcMAF therapy of HIV-infected patients. In the present

study GcMAF therapy was given to nonanemic HIV-infected patients and found to be highly curative. Anemia is a common manifestation of HIV infection, occurring in approximately 30% of patients with asymptomatic infection and in as many as 75–80% of patients with AIDS (J Acquir Immune Defic Syndr 2001;26:28–35). A number of studies have consistently found anemia to be associated with reduced survival (Clin Infectious Dis 1999;29:44–49). Anemic HIV and AIDS patients have yet to be tested with GcMAF therapy.

CHAPTER 2

CONSIDERATIONS ON PROF. YAMAMOTO'S PAPER

The paper quoted above raises several interesting considerations.

HIV AND MACROPHAGE FUNCTION

Impaired phagocytosis and bactericidal process of the phagocytes of AIDS patients have been reported from a number of laboratories. Macrophages are the major phagocytic and antigen presenting cells. Since macrophage activation for phagocytosis and antigen-presentation to B and T lymphocytes is the first indispensable step in development of both humoral and cellular immunity, lack of macrophage activation contributes to immunosuppression independently of the number of Cd4+ lymphocytes. In fact, it is believed that immunosuppression in AIDS and advanced cancer patients is caused by lack of macrophage activation. According to the theories presented above, several agents (drugs, malnutrition, chronic infections other than HIV) can lead to deficient macrophage activation and thus to AIDS. The presence of signs of HIV infection, rather than being causative for AIDS, could then reflect an impaired macrophage function. Thus, it is conceivable that restoring macrophage function could eradicate signs of HIV infection.

Microbial infection induces inflammation that results in macrophage activation as for innate defence. The inflammation-derived macrophage activation is the process for generation of the principal macrophage activating factor (MAF). Administration of an inflamed tissue lipid metabolite, lysophosphatidylcholine (lyso-Pc) or other lysophospholipids, to mice rapidly activates macrophages for greatly enhanced phagocytic and superoxide generating capacities. Inflammation-

derived macrophage activation process requires serum Gc protein (also known as vitamin D3-binding protein). Gc protein is the precursor for the principal MAF, termed GcMAF. When peripheral blood mononuclear cells (PBMCs) containing monocytes/macrophages (macrophages for short) and lymphocytes of HIV-infected patients were treated with lyso-Pc and cultured in a serum-free medium containing purified Gc protein or healthy human serum (0.1%) as a source of Gc protein, the lyso-Pc-primed lymphocytes of all patients were fully capable of converting Gc protein to MAF, resulting in activation of their macrophages. However, cultivation of lyso-Pc-treated patient PBMCs in medium containing patient own serum (0.1%) resulted in lack or reduced levels of macrophage activation, as a consequence of lost or decreased MAF precursor activity of the patient serum Gc protein. It was demonstrated that loss of MAF precursor activity is due to deglycosylation of serum Gc protein by α -N-acetylgalactosaminidase (termed Nagalase). In fact, the MAF precursor activity of Gc protein is lost or reduced in a variety of chronic conditions, from cancer to those conditions leading to immunodeficiency, because serum Gc protein is deglycosylated by Nagalase, possibly secreted from cells under (oxidative?) stress. Deglycosylated Gc protein cannot be converted to MAF, resulting in no macrophage activation.

In brief, an increase in serum Nagalase activity is found in most common cancers and in patients with signs of HIV infection. Increased Nagalase activity leads to decreased levels of active GcMAF with consequent decreased macrophage stimulation and eventually immunodeficiency.

For example, advanced cancer patients have high serum Nagalase activities, resulting in no macrophage activation and severe immunosuppression that explain why cancer patients often die with overwhelming infection (e.g., pneumonia). The same holds true for AIDS patients.

Exogenously administered MAF can bypass the deglycosylated Gc protein and directly act on macrophages for activation with impressive results on cancer patients and on individuals harbouring

signs of HIV infection. GcMAF administration greatly enhances phagocytic and superoxide generating capacities of systemic macrophages in few hr. GcMAF also has a potent mitogenic capacity to act on the progenitor cells, resulting in a 40-fold increase in systemic macrophage cell counts in 4 days. When human macrophages are treated in vitro with 100 pg/ml for 3 hr, they are highly tumoricidal and kill approximately 50% of both prostate cell line LNCaP and breast cancer cell lines MCF-7 and MDA-MB-321 in 4 hr. Weekly administrations of 100 ng GcMAF to metastatic adenocarcinoma (breast and prostate cancer) patients and metastatic colorectal cancer patients eradicate tumors in 16–25 weeks and 32–50 weeks, respectively.

According to Dr. Yamamoto (J Med Virol 2009 Jan;81(1):16-26) , Nagalase is an intrinsic component of the envelope HIV protein gp120 and, in HIV-infected patients, serum Nagalase activity is the sum of enzyme activities carried by both HIV virions and unassembled envelope proteins (gp160 and gp120) released from HIV-infected cells. Because of Nagalase being an HIV viral component, serum Nagalase is already complexed with patient own immunoglobulin G (anti-HIV IgG). The IgG-bound Nagalase retains enzyme activity that can initiate fusion for HIV-infection process and also deglycosylate Gc protein. The deglycosylated Gc protein cannot be converted to MAF and as a consequence macrophages cannot be activated, leading to the development of immunosuppression.

This mechanism however is most likely independent of HIV infection since the same Authors described increased serum Nagalase activity in a variety of common human cancers totally independent of HIV infection, *i.e.* prostate cancer (Transl Oncol 2008 Jul;1(2):65-72), metastatic colon cancer (Cancer Immunol Immunother 2008 Jul;57(7):1007-16), metastatic breast cancer (J Cancer 2008 Jan 15;122(2):461-7), and even in Ehrlich ascites tumor-bearing mice (Proc Soc Exp Biol Med 1999 Jan;220(1):20-6). The observed correlation between increased Nagalase activity and

tumor burden was so stringent that the Authors propose to use Nagalase activity as a prognostic factor for prostate cancer in addition to prostate specific antigen (PSA) (Transl Oncol 2008 Jul;1(2):65-72). Thus, it appears that conditions totally independent of HIV infection increase serum Nagalase activity thus leading to deficient macrophage activation.

It is difficult to envisage a common denominator for conditions so different as prostate, colon, and breast cancer, Ehrlich tumor and HIV infection. In a very superficial way it could be postulated that all these conditions are associated with a significant, generic cellular “stress”. The increased serum Nagalase activity could then just reflect such a non-better-defined “stress”; nevertheless increased serum Nagalase activity can lead to immunodeficiency and a reversion of this condition by exogenous administration of GcMAF could be beneficial in such diverse conditions. In molecular terms, one candidate as the common denominator for this non-better-defined “stress” could be the transcription factor NF-kappaB, which is constitutively active in most tumors and is induced by carcinogens (such as cigarette smoke), tumor promoters, viral proteins (HIV-tat, HIV-nef, HIV-vpr, KHSV, EBV-LMP1, HTLV1-tax, HPV, HCV, and HBV), chemotherapeutic agents, and gamma-irradiation. This transcription factor regulates the expression of several pro-inflammatory gene products that mediate a critical role in suppression of apoptosis, proliferation, angiogenesis, invasion, and metastasis. Among these gene products are TNF and members of its superfamily, IL-1alpha, IL-1beta, IL-6, IL-8, IL-18, chemokines, MMP-9, VEGF, COX-2, and 5-LOX (Biochem Pharmacol. 2006 Nov 30;72(11):1605-21).

Bearing these considerations in mind we decided to study the molecular mechanisms underlying the effect of GcMAF on human PBMCs. Given the reported effects of GcMAF on angiogenesis and the relationships between angiogenesis and HIV infection (Angiogenesis 2002;5:141–151), we also studied the effects of GcMAF in the chick embryo chorioallantoic membrane assay.

CHAPTER 3

EXPERIMENTAL RESULTS OBTAINED WITH Gc-MAF

1. Effects of GcMAF on Human Monocyte Viability and Proliferation in Subjects Harboring Different VDR Genotypes

It is well assessed that GcMAF stimulates human monocytes (Molecular Immunology, Vol. XXXIII, No. 15, 1996; pp. 1151-1164); in preliminary experiments, however, we had noticed that the effects of GcMAF on human monocyte viability and proliferation showed significant inter-individual variation (not shown). Thus, we selected donors for VDR genotypes; to our knowledge, the association between VDR polymorphisms and GcMAF effects on monocytes has not been studied before. The most frequently studied single-nucleotide VDR polymorphisms are the restriction fragment length polymorphisms FokI (rs2228570) and BsmI (rs1544410), as defined by the endonucleases FokI and BsmI, respectively. The FokI restriction fragment length polymorphism, located in the coding region of the VDR gene, results in the production of a VDR protein that is three amino acids longer. Although no significant differences in ligand (vitamin D) affinity, DNA binding or transactivation activity is found between these two VDR forms when studied independently, in transient transfection assays with a vitamin D-responsive reporter gene, the shorter VDR variant (i.e. the one identified by the “F” allele) displays higher potency than the longer one (“f”) when stimulated with vitamin D (Pharmacogenetics Genomics 2005 May; 15(5):349-55). BsmI (together with ApaI and TaqI) polymorphism is located in a regulatory site at the 3' end of the VDR gene; these three polymorphic sites (BsmI, ApaI and TaqI) are in linkage disequilibrium and none of them affects the structure of the VDR. Thus, BsmI, unlike FokI, does not alter the amount, structure or function of the VDR protein produced. However, although not functional, it is

strongly linked with a poly(A) microsatellite repeat in the 3' untranslated region which may influence VDR messenger RNA stability; the allele ("b") is the one associated with the strongest response to vitamin D (J Nephrol 2008 Nov-Dec;21(6):843-9; Gene 2004 Sep 1;338(2):143-56)

When monocytes of subjects harbouring the homozygous "FF/bb" VDR genotype were challenged with GcMAF, we observed the maximal response in terms of cell proliferation and survival. The effect was dose-dependent and maximal stimulation was obtained using 0.1 ng/ml GcMAF.

Heterozygous subjects (i.e. "Ff/Bb") showed an intermediate response, whereas subjects harbouring the homozygous genotype "ff/BB" showed little or no response. It is worth noting that in subjects harbouring "FF/bb" genotypes, GcMAF sustained cell viability for about 98 h, whereas unstimulated cells were no longer viable after 48 h, as if, in those subjects, GcMAF had rescued monocytes from apoptosis (Cell Death Dis 1, e30).

The stimulatory effect of GcMAF was comparable to that achieved by the highest concentration of endotoxin (lipopolysaccharide; 1 µg/ml) taken as positive control (Cellular Immunology 1999;194,6–11); in this case, however, we did not observe any significant difference between genotypes, thus indicating that monocyte responsiveness to other stimuli was not associated with VDR genotypes. The effects of GcMAF were compared to those of a known ligand of VDR, commonly used in clinical treatment, i.e. paricalcitol, a non-hypercalcemic vitamin D analogue. It is well assessed that vitamin D and its analogues inhibit immune cell function and viability (Nature Med Vol XII/IV, 2006 Apr;389) and, as expected, we observed that the "F" and "b" allele of the DR gene were associated with the strongest inhibition of cell viability and proliferation. Thus, paricalcitol, showed the strongest inhibitory effect on subjects harbouring the homozygous "FF/bb" genotype. The highest concentration of paricalcitol (240 nM) showed some inhibitory effect on the heterozygous "Ff/Bb" genotype, whereas in the homozygous "ff/BB" genotype no significant effect was observed. These results indicate that "F" and "b" alleles are associated with the strongest

responses to both GcMAF and paricalcitol even though GcMAF stimulated monocyte proliferation whereas paricalcitol decreased monocyte viability.

II. Effects of GcMAF and Paricalcitol on cAMP Production in Human Monocytes

The signal transduction pathway of GcMAF received little attention, in particular as far as intracellular second messengers are concerned; thus we decided to study cAMP formation in human monocytes treated with GcMAF. GcMAF (100 pg/ml) significantly stimulated cAMP formation in a manner associated with VDR polymorphisms. Also in this case, thus paralleling the effects observed in studying cell proliferation, the “F” and “b” alleles were associated with the strongest response. Those healthy subjects harbouring the homozygous “FF/bb” genotype showed the most significant response in terms of GcMAF-stimulated cAMP formation; heterozygous subjects had an intermediate response, whereas the homozygous “ff/BB” genotype showed little or no response. Paricalcitol evoked a stimulatory response quite similar to that of GcMAF and intracellular cAMP formation was strongest in the homozygous “FF/bb” genotype and non significant in the homozygous “ff/BB” genotype. In the heterozygous “Ff/Bb” genotype, the highest paricalcitol concentration (240 nM) elicited a statistically significant response, thus paralleling the results observed studying macrophage viability.

III. Effects of GcMAF on Angiogenesis

It was previously demonstrated that GcMAF inhibited angiogenesis, a key feature in its anti-tumour properties (J Natl Cancer Inst 2002;94:1311–19; Angiogenesis 2005;8:349–360 Springer 2006). The effects of GcMAF on angiogenic growth factor-induced cell proliferation, chemotaxis, and tube formation were previously examined in vitro by using cultured endothelial cells (murine and porcine cells, human umbilical vein endothelial cells) and in vivo by using a mouse cornea micro-pocket assay. It was demonstrated that GcMAF had direct antiangiogenic effects on endothelial

cells independent of tissue origin. On the basis of these considerations, we decided to study the effects of GcMAF on an angiogenesis model quite different from those used before, i.e. the CAM assay. We tested its effects both in basal conditions and when administered together with powerful stimulators of angiogenesis (i.e. PGE2, 1 mg/ml and the human breast cancer cell line, MCF-7). Also in this case, the effects of GcMAF were compared to those of paricalcitol.

Neither GcMAF nor paricalcitol altered basal angiogenesis or chick embryo development. As expected, MCF-7 cells, directly implanted in CAM, strongly stimulated angiogenesis to an extent comparable to that of a known stimulator of angiogenesis, i.e. PGE2 (J. Environ. Pathol. Toxicol. Oncol. 2009;28:85-8). Both GcMAF (1 ng/ml) and paricalcitol (240 nM) significantly inhibited PGE2- and MCF-7-stimulated angiogenesis. It is worth noting that the GcMAF concentration required to achieve full inhibition of stimulated angiogenesis was 1 ng /ml, i.e. a concentration 10-fold higher than that required to stimulate monocytes in our experimental conditions.

DISCUSSION OF THE EXPERIMENTAL RESULTS

These results contribute elucidating the cellular and molecular mechanisms through which administration of GcMAF proved useful in cancer and other diseases (Journal of Medical Virology 2009;81:16–26; Transl Oncol 2008 Jul;1(2):65-72; Cancer Immunol Immunother 2008 Jul;57(7):1007-16.; Int J Cancer 2008 Jan 15;122(2):461-7; Cancer Res 1996 Jun 15;56(12):2827-31; Comparative Biochemistry and Physiology Part A 132 2002;1–8; Cancer Lett 2009 Oct 8;283(2):222-9; Epub 2009 Apr 25). Thus, we demonstrated that GcMAF has potent mitogenic activity on human monocytes in vitro and these data are consistent with the notion that stimulation of monocyte proliferation is the hallmark of immune system stimulation (Herbal Supplements and Athlete Immune Function. David S. Senchina,¹ Nisarg B. Shah,² Danielle M. Doty,¹ Cole R. Sanderson,³ Justus E. Hallam³). Our data are also consistent with the observation that intravenous

administration of GcMAF increased the systemic cell counts of the activated macrophages to >200-fold presumably because of interaction of GcMAF with myeloid progenitors in bone marrow. (Prof. Yamamoto, personal communication).

In addition, our results demonstrate for the first time that the response of human monocytes to GcMAF is associated with VDR gene polymorphisms. It is worth noting that the alleles “F” and “b” are also associated with the highest sensitivity to vitamin D and its analogues; an interconnection of vitamin D and GcMAF signalling pathways can thus be hypothesized even though the effects of the two compounds on monocyte viability and proliferation were opposite. In our opinion it is highly unlikely that GcMAF exerts its effects through direct interaction with the VDR and it is most likely instead that VDR polymorphisms influence the response to GcMAF in an indirect manner. As a matter of fact, VDR polymorphisms indirectly influence a variety of conditions, from cancer (Carcinogenesis Vol.XXX, No.7, 2009;1170–1180) to AIDS (Journal of Steroid Biochemistry & Molecular Biology 89–90 (2004) 199–207) and chronic kidney disease (Kidney Int 2009 Nov;76(9):931-3). Further studies will elucidate whether VDR polymorphisms are also associated with the known polymorphisms of the Gc protein (Anticancer Res 2004 Sep-Oct;24(5C):3361-6) or with polymorphisms of the gene coding for the GcMAF receptor as well as with its regulation. Whatever the case, these results can prove instrumental in identifying those subjects that could benefit the most from GcMAF treatment, in particular considering that such a treatment is currently being proposed in different settings (see for example: <http://www.gcmaf.eu/info/> or <http://immunemedicine.com/available-therapies/gcmaf>).

As far as the stimulatory effect of GcMAF on intracellular cAMP formation is concerned, to our knowledge this is the first time that such an effect is described. In fact, there is very limited information about GcMAF signalling; we found a preliminary report approved for public release

demonstrating that GcMAF blocked the phosphorylation of a band with the approximate molecular weight of 75 KDa in prostate cancer cell lines (W81XWH-04-1-0010. Treatment of Prostate Cancer with a DBP-MAF-Vitamin D Complex to Target Angiogenesis and Tumorigenesis. Michael W. Fannon, Ph.D. University of Kentucky Research Foundation Lexington, Kentucky 40506-0057. U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012. Approved for Public Release; Distribution Unlimited). However, we were unable to find any study concerning second messenger formation in GcMAF-stimulated cells. Also this effect of GcMAF was associated with VDR polymorphisms; those alleles associated with the strongest response in terms of monocyte proliferation were also associated with the strongest response in terms of cAMP formation. Comparison of the effects of GcMAF and paricalcitol, however, suggests that cAMP probably is only one of the components of the GcMAF signalling cascade. In fact, the two compounds had opposite effects on monocytes (with GcMAF stimulating and paricalcitol inhibiting), whereas both stimulated cAMP formation in a VDR-associated manner. Since VDR regulates a number of other genes in response to ligand binding, it is quite conceivable that, although vitamin D and GcMAF may share some component of the signalling cascade such as cAMP, other components (i.e. other second messengers and/or transducing proteins) are different so that the final molecular targets (and cell responses) are quite distinct. To this point, it is worth noting that, in a different experimental model, increasing cAMP levels activated protein kinase A and protein kinase A activation then prevented the phosphorylation and subsequent degradation of I κ B, which in turn prevented translocation of the NF- κ B p65 subunit to the nucleus to transactivate a number of genes (J Immunol 2008 Mar 15;180(6):3670-9). The ensuing scenario of GcMAF-induced cAMP production and NF- κ B inhibition, is indeed intriguing and could help explaining several of the reported effects of GcMAF in cancer and other diseases. In fact NF- κ B is constitutively active in most tumours and is induced by carcinogens, tumour promoters and viral proteins (HIV-tat, HIV-nef, HIV-vpr, KHSV, EBV-LMP1, HTLV1-tax, HPV, HCV, and

HBV) (Biochem Pharmacol 2006 Nov 30;72(11):1605-21). In addition, it is worth noting that in autoimmune conditions such as SLE and in immunodeficiencies, including AIDS, it has been demonstrated that the regulatory effect of the signalling pathway of cAMP and cAMP-dependent protein kinase A is abrogated. Thus it is postulated that modulation of the cAMP-protein kinase A pathway could represent a potential useful strategy for therapeutic interventions of dysfunctional T cells associated with SLE and HIV infection (Curr Drug Targets 2005 Sep;6(6):655-64). Therefore, the observed stimulation of cAMP formation might also help elucidating the mechanisms responsible for the reported effects of GcMAF in autoimmune diseases such as SLE (Clin Immunol Immunopathol 1997 Mar;82(3):290-8).

Finally, inhibition of angiogenesis by GcMAF on an assay completely different from those used in previous studies, reinforces the hypothesis that this effect of GcMAF is independent of the tissue of origin and of the stimulus used to induce the angiogenic response (Journal of the National Cancer Institute, Vol. XCIV, No. 17, 2002 Sept 4). GcMAF-induced increase of cAMP formation could account for this effect since it was demonstrated that elevated cAMP level inhibited angiogenesis in CAM assay (J Vasc Res 1994 Jul-Aug;31(4):195-204). GcMAF-induced inhibition of angiogenesis could then be crucial in determining its therapeutic effects in conditions where angiogenesis plays a key role in the progression of the disease, from cancer (Exp Cell Res 2010 May 1;316(8):1304-8) to HIV infection (Angiogenesis 2002;5: 141–151).

FINAL REMARKS

The following considerations were communicated to Prof. M. Ruggiero by Dr. P. Cheney from Asheville, USA. We quote them literally.

“What strikes me about these ideas which were articulated years ago by nobel laureate Professor Luc Montagnier is the likelihood that XMRV (the retrovirus associated with the Chronic Fatigue Syndrome, CFS) and Professor Ruggiero's and Professor Montagnier's view of HIV may be correct and that it is possible to obliterate XMRV and HIV with appropriate immunotherapy such as GcMAF. Yamamoto wrote a convincing paper on this possibility and Dr. Ruggiero confirmed Yamamoto. One also has to explain why perhaps most people exposed to XMRV and the macaque exposure to XMRV as well are then capable of wiping XMRV out of the blood though it can come back under certain conditions and remains activated in some people.”

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