

Has Gallo proven the role of HIV in AIDS?

ELENI PAPADOPULOS-ELEOPULOS,
VALENDAR F TURNER, JOHN M PAPADIMITRIOU

ABSTRACT

The evidence that Robert Gallo and his colleagues presented on 4th May 1984 regarding HTLV-III (HIV) isolation and the role of HIV in the pathogenesis of AIDS is critically analysed. It is concluded that the evidence does not constitute proof of the isolation of a retrovirus, that the virus is exogenous, or that the virus is causally related to AIDS.

Introduction

In 1982, Robert Gallo from the National Cancer Institute in the USA, put forward the hypothesis that the cause of AIDS is a retrovirus. One year later Essex and his colleagues¹ found that AIDS patients had antibodies to the Human T-cell Leukemia virus Type-1 (HTLV-I), a virus discovered by Gallo a few years earlier. At the same time, Gallo and his colleagues² reported the isolation of HTLV-I from AIDS patients and advocated a role for this retrovirus in the pathogenesis of AIDS.

This hypothesis however, was not without a few problems:

1. While HTLV-I was accepted to induce T4-cell proliferation and cause adult T-cell leukaemia,³ the "hallmark" of AIDS was T4-cell depletion, and the incidence of leukaemia in AIDS patients was no higher than in the general population;

2. The highest frequency of antibodies to this virus was found in Japan, yet no AIDS cases had been reported from that country;⁴

3. In the same month in which Gallo's and Essex's groups reported their data, Luc Montagnier and his colleagues from the Pasteur Institute described the isolation of a retrovirus, later known as Lymphadenopathy Associated Virus (LAV), from the lymph nodes of a homosexual patient with lymphadenopathy.⁵ Although this virus was similar to HTLV-I, one of its proteins, a protein with a molecular weight of 24,000 (p24), did not react with monoclonal antibodies to the HTLV-I p24 protein. Samples of this virus were, on several occasions, sent to Gallo's laboratory.

In May 1984, Gallo, Popovic and their colleagues published four papers in *Science* in which they claimed to have isolated from AIDS patients, another retrovirus similar to HTLV-I, which they called HTLV-III.^{6,7,8,9} On the 23rd of April 1984, before the *Science* papers were published, Gallo and Margaret Heckler, the then Health and Human Services Secretary called a press conference to announce that Gallo and his co-workers had found the cause of AIDS and had developed a sensitive test to show whether the "AIDS virus" is present in blood.

In 1985, the Pasteur Institute alleged that Gallo had misappropriated LAV in developing the blood test. The ensuing conflict, which reached the American courts, was eventually settled by a negotiated agreement signed in 1987 by Gallo, Montagnier, US President Reagan and French Premier Chirac. The agreement declared Gallo and Montagnier to be co-discoverers of the AIDS virus, presently known as the

Eleni Papadopulos-Eleopulos
Physicist
Department of Medical Physics
Royal Perth Hospital

Valendar F Turner
Staff Specialist
Department of Emergency Medicine
Royal Perth Hospital

John M Papadimitriou
Professor of Pathology
Department of Pathology
University of Western Australia

Correspondence to:
Eleni Papadopulos-Eleopulos
Department of Medical Physics
Royal Perth Hospital
Box X2213 G.P. Perth

The subject matter of this invited paper was presented to the Annual Scientific Meeting of the Australasian College for Emergency Medicine, Sydney, November 1992.

Human Immunodeficiency Virus (HIV). Nevertheless, the misappropriation conflict attracted the attention of John Crewdson, an investigative journalist, and US Senator John Dingell. In November 1989, Crewdson published a lengthy article in the *Chicago Tribune* newspaper, "With allegations that Robert C Gallo stole from French scientists the virus he discovered to be the cause of AIDS."¹⁰

This led to a National Institute of Health (NIH) internal "inquiry" into the allegation with "an outside committee of expert but disinterested parties [led by Yale biochemist Frederic Richards] to oversee the activity of the internal panel".¹¹

Following the inquiry, which was viewed as a fact-finding mission, the Richards committee insisted on a "formal investigation", "on suspect data in one of four seminal papers published by Gallo's lab in *Science* on 4 May 1984".¹² In this paper, the first of the series of four, with Mikulas Popovic the principal author, "there appears to be differences between what was described in the paper and what was done".¹⁰ A draft report of the formal investigation written by NIH Office of Scientific Integrity (OSI), was published in September 1991. In the draft report, Popovic is accused "of misconduct for misstatements and inaccuracies" that appeared in the paper, and that Gallo, as laboratory chief, "created and fostered conditions that give rise to falsified/fabricated data and falsified reports". However, Gallo's actions were not considered to "meet the formal definition of misconduct".¹³

The final draft report of the OSI, completed in January 1992, was immediately criticised by the Richards Panel as well as Senator Dingell. This led to a review of the OSI report by the Office of Research Integrity (ORI) which found Gallo guilty of scientific misconduct. Nonetheless, the scientific misconduct is said not to "negate the central findings of the [1984 *Science*] paper".^{13,14} In other words, despite the above findings, at present, it is still accepted, as Gallo and his colleagues concluded, "The results presented in our four papers provided clearcut evidence that the aetiology of AIDS and ARC was the new lymphotropic retrovirus, HTLV-III"¹⁵ [ARC = AIDS related complex]. Although

the findings of the Gallo investigation are of considerable importance, in what follows, with few exceptions, we will consider that there were no "differences between what was described in the paper and what was done". However, the data will be critically analysed with regard to the following:

1. Whether the experimental method described constitutes irrevocable evidence of viral isolation;
2. Whether the authors have presented evidence proving a causal role for HIV in AIDS.

To facilitate this analysis it may be useful to consider what is generally accepted as retroviral isolation.

Retroviral isolation

Peyton Rous¹⁶ is credited with the discovery and isolation of the first retrovirus. In 1911 he was able repeatedly to induce tumours in a particular breed of chickens by means of tumour derived, cell free filtrates. It is instructive to repeat Rous' own thoughts on his observation: "The first tendency will be to regard the self-perpetuating agent active in this sarcoma of the fowl as a minute parasitic organism. Analogy with several infectious diseases of man and the lower animals, caused by ultramicroscopic organisms, gives support to this view of the findings, and at present work is being directed to its experimental verification. But an agency of another sort is not out of the question. It is conceivable that a chemical stimulant, elaborated by the neoplastic cells, might cause the tumour in another host and bring about in consequence a further production of the same stimulant". The tumour inducing filtrates became known as "filterable viruses" or oncoviruses and, more recently, exogenous retroviruses or infectious retroviruses.¹⁷ 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.¹⁸ 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 had high viral concentration and low cellular contaminants. This was best satisfied by non-cytopathic viruses and by culture conditions which maintained maximum cellular viability. All retroviruses isolated prior to HIV satisfy the above conditions.¹⁹ Taking advantage of the above retroviral properties, by repeated suspensions and sedimentation in sucrose density gradients, one could obtain, at a density of 1.16 gm/ml, a relatively pure concentration of retroviral particles- that is, obtain retroviral particles separate from everything else, and thus isolate them.¹⁹ Nonetheless, as many eminent retrovirologists point out, contamination of the viral preparation with particles which contain RT, but could be nothing more than "cellular fragments", microsomes from disrupted cells, "membranous vesicles which may enclose other cellular constituents including nucleic acids", especially when "inadvertent lysis of cells" was induced, could not be avoided.^{17,18,19,20} 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: (a) physical- EM for virus count, morphology and purity; (b) biochemical- RT activity, viral and cellular RNA, total protein, gel analyses of viral and host proteins and nucleic acids; (c) biological- infectivity *in vivo* and *in vitro*.^{19,21}

In other words, the first step in the effort of isolation of a retrovirus is the demonstration that:

1. The particles seen in the cultures band at 1.16 gm/ml;
2. In the 1.16 gm/ml band there is little present but the particles;
3. "No apparent differences in physical appearances" between particles are seen.

Has Gallo proven the role of HIV in AIDS?

Isolation of HTLV-III (HIV).

In the first, seminal paper on HIV isolation, entitled "Detection Isolation and Continuous Production of Cytopathic Retroviruses (HTLV-III) from Patients with AIDS and Pre-AIDS",⁶ Popovic, Gallo and their colleagues first described a leukaemic T-cell line, HT. This cell line was exposed "to concentrated culture fluids harvested from short-term cultures of T-cells... obtained from patients with AIDS or pre-AIDS. The concentrated fluids were first shown to contain particle-associated RT". The finding in the HT cell line as well as in eight clones derived from it including H4, H9 and H17, of: (a) RT; (b) cell immunofluoresence with serum from a haemophilia patient with pre-AIDS, and "Rabbit antiserum to HTLV-III", was considered evidence for the existence in these cultures of a retrovirus which was named HTLV-III. "Both virus production and cell viability of the infected clone H4 (H4/HTLV-III) were monitored for several months. Although virus production [RT activity] fluctuated (Fig. 2a), culture fluids harvested and assayed at approximately 14 day intervals consistently showed particulate RT activity [RT activity in the material which banded at 1.16 gm/ml] which has been followed for over 5 months... Thus the data shows that this permanently growing T-cell population can continuously produce HTLV-III". Electron microscopy examination of the H4 clone culture showed "the presence of extracellular viral particles". Some of the findings of the Gallo investigation are relevant to the above experiments:

1. The HT cell line was not cultured with concentrated fluids originating from individual AIDS patient T-cell cultures as is implied in the paper but from fluids pooled, first from the individual cultures of three patients and ultimately from the individual cultures of 10 patients.²² The Gallo investigation found this procedure to be "of dubious scientific rigor". One scientist described it as "really crazy".¹¹
2. According to the OSI inquiry, "the statement in the published papers that the samples were "first" shown to be secreting RT, "is contradicted by the evidence of the notebooks that only one of the three [initial cultures] was tested".²² In evidence which

Popovic gave to the inquiry he said that he had pooled the supernatant fluids from the ten cultures because none "individually was producing high concentrations of reverse transcriptase" (the levels of RT are not given).

However:

1. It is important to note that RT is determined by estimation of the incorporation of [³H] labelled nucleotides into DNA and is reported as counts per minute (cpm);

2. It is acknowledged that background radioactivity, that is, radioactivity in the absence of infection, can be as high as 0.4×10^4 cpm.²³

The above findings give rise to additional questions: If the first HTLV-III was isolated from HT cell cultures with the pooled supernatants, then how was the "Rabbit antiserum to HTLV-III" obtained for the immunofluorescence studies? How was it possible to ensure the specificity of rabbit antisera to a virus before the virus has been isolated? Similarly, how was it possible, before viral isolation, to ascertain that patient serum used to test material from the cultures did indeed interact specifically with the same virus?

3. The OSI found the claim that "the culture" was continuously producing HTLV-III (RT activity), was incorrect since the culture was "reinoculated on at least two occasions" with more supernatant.^{11,22}

In the second paper,⁷ the authors describe their attempt to isolate HTLV-III from mitogenically stimulated T-cell cultures obtained from 115 patients with AIDS, pre-AIDS and clinically normal homosexual men. In Table I entitled "Detection and Isolation of HTLV-III from patients with AIDS and pre-AIDS", they state: "Samples exhibiting more than one of the following were considered positive: repeated detection of a Mg²⁺ dependent reverse transcriptase activity in supernatant fluids; virus observed by electron microscopy (EM) [retroviral particles in the cultures]; intracellular expression of virus-related antigens detected with antibodies from seropositive donors or with rabbit antiserum to HTLV-III; or transmission of particles". By transmission of particles was meant detection of reverse transcriptase or particles in cultures of

"human cord blood, bone marrow, or peripheral blood T lymphocytes", cultured with concentrated fluids from the cell cultures from tissues obtained from AIDS patients.

In further experiments:^{8,9}

1. Lysates of the H4/HTLV-III and H17/HTLV-III "infected" cell lines were tested with patient sera using the Western blot (WB) technique. (Appendix 1);

2. "The specificity of the reaction [for HTLV-III] was studied by comparing lysates of H4/HTLV-III and H17/HTLV-III with lysates of the same clones, H4 and H17, before viral infection. No antigen from uninfected clones reacted with the sera, with the exception of a protein of molecular weight 80,000 in H17 which bound antibodies from all the human samples tested". They concluded: "These results show clearly that the antigens detected after virus infection are either virus-coded proteins or cellular antigens specifically induced by the infection".

3. The reaction with patient sera of the H4/HTLV-III cells was then compared with the reaction of the material from the H4/HTLV-III culture fluids which in sucrose density gradients banded at 1.16 gm/ml. Of the proteins which banded at 1.16 gm/ml, two, p41 and p24, were found to react with some patient sera. They concluded: "p24 and p41 may therefore be considered viral structural proteins";

4. Finally, they used the ELISA (Appendix 2) technique to test for HTLV-III antibodies. Eighty eight percent (43/49) of patients with AIDS, and 79% (11/14) patients with pre-AIDS "but less than 1 percent of heterosexual subjects", had antibodies "reactive against antigens of HTLV-III".

"To understand the molecular nature of the antigens recognized by ELISA", the sera were analysed by Western Blot (WB). "...the antigen most prominently and commonly detected among all of the sera from AIDS patients had a molecular weight of 41,000 (p41)...Reactivity to p24 of the virus was generally very weak and was clear only in two cases".

From the above data it is obvious that by HTLV-III (HIV) isolation was meant detection of more than one of the following phenomena:

1. RT, either in the culture fluids, or in the material from these fluids or cellular lysates which in sucrose density gradients band at 1.16 gm/ml;

2. In culture fluids, but not in the material which bands at 1.16 gm/ml, particles with morphological characteristics of retroviruses (RVP);

3. Proteins, (p41, and in some cases, p24), which, in sucrose density gradients, band at 1.16 gm/ml, (but without proof that they are unique constituent parts of the particle), and react with patient sera.

However, isolation is defined as separating an object, (HIV), from everything else, and not the detection of some phenomena attributed to it (RT, WB), or similar to it, (RVP). Phenomena can only be used for retroviral detection, not isolation, and even then if, and only if, it is first shown that each is specific for the virus by use of the only valid gold standard, HIV itself, "HIV isolation". It is important to note that in the earlier (1983) report by Montagnier's group on HIV (LAV) isolation, the same experimental procedures and findings as those described by Gallo were reported. The only exception was that Montagnier's group did not "infect" an immortalised cell line, yet Gallo's group considered that Montagnier and his colleagues had not described "true isolation".⁶ In fact, in 1984, evidence existed that RT, antigen-antibody reactions (WB), and RVP, are non-specific for retroviruses. The indirect evidence, that is, evidence that has been obtained without a gold standard from recent AIDS research, has confirmed the above.

Reverse transcriptase

Although Gallo has described the enzyme reverse transcriptase as "unique to retroviruses", this is not the case, a fact stressed by its discoverers, (both Nobel laureates).¹⁸ Reverse transcription can be found in leukaemic T-cells,²⁴ (HT and its clones including H9, from which the first "HTLV-III (HIV) virus was isolated", is a leukaemic cell line), normal spermatozoa,²⁵ and, according to Harold Varmus, another Nobel laureate, more recently, in the uninfected cells of yeasts, insects and mammals.²⁶ As far back as 1973, Gallo himself was the first to show that RT can be

Has Gallo proven the role of HIV in AIDS?

found in "PHA stimulated (but not unstimulated) normal human blood lymphocytes".²⁴ Confirmation of this was reported at the 1991 Florence AIDS conference where evidence was presented that the drug AZT can inhibit the action of normal cellular RT,²⁷ and this was postulated as a mechanism for drug toxicity.

Retroviral particles

By definition, retroviral particles are enveloped infectious particles 100-120nm in diameter with a core comprising a protein shell and a ribonucleoprotein complex. RVP are further categorised according to the site of core assembly, that is, within the cytoplasm or at the cell membrane, and by certain other morphological features. Included in this taxonomy are the Subfamilies Oncoviruses which include Type C and Type D particles, as well as the Subfamily Lentiviruses.

Prior to the AIDS era, many retrovirologists showed that the finding of a particle with morphological features similar to retroviruses does not constitute sufficient proof that they are retroviruses or that they are infectious particles, even if they are found to band at 1.16 gm/ml.¹⁸ In 1976 Gallo himself pointed out that in human leukemic tissue "virus-like particles morphologically and biochemically resembling type-C virus but apparently lacking the ability to replicate, have been frequently observed".²⁸ Particles with the morphological characteristics of retroviruses were reported in milk, cultures of embryonic tissues and "in the majority, if not all, human placentas".^{29,30,31} However, they were considered to be "an intriguing and important problem that remains to be solved".³² Evidence from AIDS research shows that:

1. There is no agreement on the precise taxonomic classification of HIV. Initially, HIV was reported as an Oncoviral type-C particle,⁵ then a type-D particle,³³ and then as a member of a different Subfamily, a Lentivirus,³⁴

2. The T-cell and monocyte "HIV infected cultures" contain in addition to particles with morphologies attributed to HIV, many other "viral particles" unlike any of the "HIV particles".³⁵⁻³⁸ "Non-HIV-infected" HT (H9)

cells, the cell line from which the Gallo team isolated the first HIV (HTLV-III) and from which most of the published electron micrographs of "HIV particles" have originated, as well as other cells used for "HIV isolation", CEM, C8166, EBV transformed B-cells, and cord blood lymphocytes, express virus-like particles albeit they are somewhat different from the variety of particles accepted as HIV.³⁹ The above data raise questions not only in regard to the origin and role of the "non-HIV particles", but also to the "HIV (HTLV-III) particles". Furthermore, neither Gallo's team, nor anybody else before or since has published electron micrographs of the material derived from AIDS cultures/co-cultures which bands at 1.16 gm/ml. Thus it is impossible to know which, if any, of the particles band at that density;

3. Most importantly, it is generally accepted that particles reported in the lymph nodes of AIDS patients are HIV. However, in the only EM study⁴⁰, either *in vivo* or *in vitro*, in which suitable controls were used and in which extensive blind examination of controls and test material was performed, particles attributed to HIV were found in 90% (18/20) of patients with persistent generalised lymphadenopathy and in 87% (13/15) of patients with non-HIV lymphadenopathies, leading the authors to conclude: "The presence of such particles do not, by themselves indicate infection with HIV".

Antigen-antibody reactions

One can claim that a given protein is an antigen derived from an exogenous retrovirus if first it is shown that:

1. The protein is a structural component of a particle;
2. The particle is a retrovirus;
3. The protein is coded exclusively by a viral and not a cellular gene.

Once the above are demonstrated, the only way to prove that the antibodies found in AIDS patient sera are directed against the viral antigen is to use the antigen or the isolated virus as a gold standard. The mere finding that a protein from the AIDS cultures bands at 1.16 gm/ml and reacts with sera from AIDS patients cannot be considered to simultaneously prove that

1. The protein is a viral antigen;

2. The antibodies in the AIDS patient sera which react with the antigen are specific for that antigen.

At present, it is known that about 80% of the proteins which band at 1.16 gm/ml, some of which react with some AIDS sera, do not constitute any of the proteins ascribed to HIV.⁴¹⁻⁴³ Most importantly, prior to the publication of the Science papers, evidence existed, confirmed since, which is at odds with the conclusion that "p24 and p41 may therefore be considered viral structural proteins":

The p41/45 protein

In AIDS research, the p41 and p45 bands are considered to represent one and the same HIV protein.

1. Like Gallo's group, Montagnier's team one year earlier, found that AIDS sera reacted with a protein p41/45 from the AIDS cultures and which in sucrose density gradients, banded at 1.16 gm/ml. However, from their data they considered that the p41 band "may be due to contamination of the virus by cellular actin which was present in immunoprecipitates of all cell extracts",⁵ that is, of "HIV infected" as well as non-infected cells and cells infected with HTLV-I. Although Gallo's group did not find such a reaction with p41 in non-infected cells, they did find a p80 protein and concluded that the reaction was "non-specific".⁸ However, at present it is known that p80 as well as two additional "HIV proteins", p120 and p160, are oligomers of p41.⁴⁴ Which proteins (bands), p41, p80, p120 or p160 are detected in a given WB depends on the culture and WB conditions, including temperature and the concentration of sodium dodecyl sulphate used to disrupt the proteins which band at 1.16 gm/ml;⁴⁵

2. Actin is an ubiquitous protein present in all cells including bacteria and several viruses. Well known retroviruses such as the mouse mammary tumour virus have also been shown to contain actin of cellular origin and it has been postulated that this protein plays a key role in both retroviral assembly and budding;^{46,47}

3. Platelets from healthy individuals also contain a p41 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".⁴⁸

4. Researchers at the Pasteur Institute have shown that sera from AIDS patients and AIDS risk groups contain high levels of antibody against calf striated muscle actin.⁴⁹

The p24/25 protein

1. Apart from a joint publication with Montagnier where they claim that the HIV p24/25 is unique, Gallo and his colleagues have repeatedly stated that the p24s of HTLV-I and HIV immunologically cross-react.⁵⁰

2. Genesca *et al*⁵¹ 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). Among the recipients of WBI blood, 36% were WBI six months after transfusion, but so were 42% of individuals who received WB-negative samples. Both donors and recipients of blood remained healthy. 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 one 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;⁵²

4. Ninety seven percent of sera from homosexuals with ITP and 94% of sera from homosexuals with lymphadenopathy or AIDS contain an antibody that reacts with a 25Kd membrane antigen found in platelets from healthy donors and AIDS patients, as well as a 2.5 Kd antigen found in green-monkey kidney cells, human skin fibroblasts, and herpes simplex cultured in monkey kidney cells. This reaction was absent in sera obtained from non-homosexual patients with either ITP or non-immune thrombocytopenia;⁴⁸

Has Gallo proven the role of HIV in AIDS?

5. Conversely, the p24 antigen is not found in all HIV positive or even AIDS patients. In one study, the polymerase chain reaction (PCR) and p24 were used to detect HIV in patients at various CDC stages from asymptomatic to AIDS. p24 was detected in 24% of patients and HIV RNA in 50%;⁵³

6. 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".⁵⁴

Thus the finding of viral particles in the AIDS cultures/co-cultures, RT and proteins which react with AIDS related sera in the material from the supernatant or cell lysates which in sucrose density gradients bands at 1.16 gm/ml, cannot be considered synonymous with the isolation or even the detection of a retrovirus. Even if a retrovirus is isolated from *in vitro* cultures/co-cultures from tissues from AIDS patients, this does not, by itself, constitute proof of the existence of the virus *in vivo*, (in AIDS patients), and even less that the retrovirus has been exogenously acquired. This is because:

1. At present, it is generally accepted that "one of the most striking features that distinguish retroviruses from all other animal viruses is the presence, in the chromosomes of normal uninfected cells, of genomes [proviruses] closely related to, or identical with those of infectious viruses". The human genome, in addition to other proviral sequences, is known to contain both HTLV-I^{55,56} and HIV⁵⁷ sequences. Depending on conditions, the proviral 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).¹⁷ In animal cultures, healthy non-virus producing cells sooner or later spontaneously release retroviruses.²⁰ The appearance and yield can be increased by (i) mitogenic stimulation;⁵⁸ (ii) co-cultivation techniques;⁵⁹ (iii) cultivation of cells with supernatant from non-virus producing cultures.⁶⁰ According to one eminent retrovirologist, George Todaro, "the failure to isolate endogenous viruses from certain species may reflect the limitation of *in vitro* cocultivation techniques".⁶¹

2. Gallo's team, like everybody else: (i) "isolated HTLV-III (HIV)" from cell cultures; (ii) "isolated HTLV-III" from mitogenically stimulated, activated cell cultures;

3. In addition, Gallo and his colleagues also used co-cultivation techniques, (H4 clone);

4. The first "HTLV-III isolation" was from the HT (H4, H9, H17) cell line. Reading Gallo and his colleagues' first paper, one surmises that the HT cell line was established in Gallo's laboratory. The Gallo inquiry revealed that the HT cell line is in fact HUT78, a cell line established in another laboratory from a patient with mature T4-cell leukaemia, a disease which Gallo claims is caused by the exogenous retrovirus, HTLV-I.³ If so, then all HT cell cultures, and the clones derived from it, "infected with HTLV-III" or non-infected, and the material from these cultures which bands at 1.16 gm/ml, should contain HTLV-I, and thus RT and retroviral particles. Furthermore, because about 25% of AIDS patients have antibodies to HTLV-I,⁴ and the immunogenic proteins of HTLV-I and HIV have the same molecular weights, then approximately 25% of the non-infected HT (H4, H9, H17) cultures in addition to RT and particles, should have, in the Western blot, the same bands as those of the "HTLV-III infected" cultures. Thus, these WBs will erroneously appear positive for HTLV-III.

Proof that HTLV-III (HIV) is causally linked to AIDS

Gallo claims that in the May 1984 Science papers he and his colleagues presented "unambiguous evidence that this [virus] and this alone was the cause of AIDS".⁶² This claim is accepted by the vast majority of AIDS researchers. A minimum requirement for making such a claim should be presentation of the following evidence:

1. That all AIDS patients are infected with HTLV-III;
2. Infection with HTLV-III leads to T4-cell depletion, given the assumption that HTLV-III leads to the clinical syndrome by its T4 cytotoxicity.

The evidence for the existence of HTLV-III was "viral isolation" and ELISA antibody tests. Even if one assumes that the data presented represents "true isolation", the virus was isolated from less than half (10/21) of AIDS

patients with opportunistic infections, and in less than one third (13/43) with Kaposi's sarcoma, then and now the two most characteristic AIDS diseases. Even if the virus could have been isolated from all patients, given the nature of retroviruses and the method used for HTLV-III isolation (cultures, mitogenic stimulation, co-cultivation) the possibility cannot be excluded that the virus did not exist *in vivo* in AIDS patients. The culture conditions may have facilitated the expression of an endogenous provirus or, alternatively, the *de novo* synthesis and expression of a retrovirus. The only method used to prove HIV infection *in vivo* was the antibody tests. Such a test can only be used only after its specificity has been proven by use of the only possible gold standard, the virus itself. This has not been done. Furthermore, the antibody test used by Gallo was ELISA, at present known to be non-reproducible and non-specific. In a study of 1.2 million healthy military applicants conducted by Colonel Donald Burke and his colleagues,⁶³ it was found that although approximately 1% of all individuals had an initial positive HIV ELISA, only 50% of repeat ELISAs were positive. Of the latter, only approximately one third were associated with two subsequent positive WBs.

In Russia, in 1990, out of 20,000 positive ELISAs "only 112 were confirmed" using the WB as a gold standard. In 1991, of approximately 30,000 positive ELISAs, only 66 were confirmed.⁶⁴ Nowhere in the four Gallo papers was HTLV-III cytotoxicity mentioned. The only reference to any cellular abnormalities or pathology in general is in the first paper where one reads: "The virus positive cultures consistently showed a high proportion of round giant cells containing numerous nuclei (Fig. 1a). These cells resemble those induced by HTLV-I and -II except that the nuclei exhibit a characteristic ring formation". [Fig. 1a is a "light microscopic examination of clone H4/HTLV-III"].

The H4 clone was obtained from the HT cell line "using irradiated mononuclear cells from peripheral blood of a healthy blood donor as a feeder". At present, it is known that the HT cell line and thus H4 are HUT78, derived in 1980 from a patient with mature T4-cell leukaemia.^{65,66} However, other cell

lines derived from patients with the same clinical syndrome are known to exhibit similar morphologies including multinucleated giant cells.⁶⁷ Thus the cellular morphological characteristics observed in the first paper may have been an intrinsic property of the HT cell line, or the result of the culture conditions, or both, and not due to HTLV-III. Finally, Gallo and his colleagues did not provide any data on the immunological status of those individuals from whom viral isolation was attempted, and no data was presented proving that HTLV-III is necessary and sufficient for T4-cell depletion and that T4-cell depletion is both necessary and sufficient for the appearance of the AIDS indicator diseases.

Conclusions

The data and arguments that have been presented by Gallo and his colleagues do not constitute proof of HIV isolation or an unambiguous role for HIV in the pathogenesis of AIDS. Although some researchers currently use methods of "viral isolation" essentially the same as that described by Gallo's group, most use less rigorous methods including singleton detection of p24 (by antibody techniques), or RT. Notwithstanding, with all of these techniques, including that described by Gallo and his colleagues, which itself seems to be greatly problematic, HIV cannot be "isolated" from 20%-70% of HIV positive and AIDS patients.^{68,69} Thus we are faced with a problem of considerable importance. The HIV antibody tests, both ELISA and WB, the only routinely used tests proving the existence *in vivo* of HIV, have yet to be verified against the only suitable gold standard, viral isolation. The available evidence suggests that this long overdue but most basic requirement of test evaluation is likely to prove an immense problem, and while the HIV antibody tests are useful prognostic markers in the high risk groups, their use as diagnostic and epidemiological tools for HIV infection is questionable.

Appendices

Appendix 1. In the Western Blot test, proteins are electrophoretically separated according to molecular weight and charge. The separated proteins are then

Has Gallo proven the role of HIV in AIDS?

transferred on to nitrocellulose strips by a process known as electroblotting. When sera are added and the strips developed, coloured bands appear representing sites of protein/antibody reactions. Each band is designated by a small "p" for the protein followed by its molecular weight in thousands.

Appendix 2. In the ELISA (Enzyme Linked Immunosorbent Assay), unseparated proteins are attached to a solid base such as the walls of plastic tubes or microplates. The serum being tested is incubated in these containers where antibody is fixed to the solid phase antigens. After washing, enzyme-labelled anti-human immunoglobulin is added and also incubated. The containers are again washed and a substrate specific for the enzyme is introduced. The resulting colour change is proportional to the amount of antibody present and is read by eye, or with a spectrophotometer.

Acknowledgements

We wish to thank all our colleagues and especially Udo Schüklenk, Barry Page, Bruce Hedland-Thomas, David Causer, Richard Fox, John Peacock, David Prentice, Ronald Hirsch, Patricia Shalala, Keith Jones, Alun Dufty, June Rider-Jones, Coronry Barrow, Dorothy Davis, Julian Smith, Mark Strahan, Vincent Turner, Wallace Turner, Gary James and Graham Drabble for their continued support and assistance.

References

1. Essex M, McLane MF, Lee TH, *et al.* Antibodies to Cell Membrane Antigens Associated with Human T-Cell Leukemia Virus in Patients with AIDS. *Science* 1985;220:859-862.
2. Gallo RC, Sarin PS, Gelmann EP, *et al.* Isolation of Human T-Cell Leukemia Virus in Acquired Immune Deficiency Syndrome (AIDS). *Science* 1983;220:865-867.
3. Gallo RC. The First Human Retrovirus. *Sci Am* 1986; 255:78-88.
4. Marx JL. Human T-Cell Leukemia Linked to AIDS. *Science* 1983;220:806-809.
5. Barré-Sinoussi F, Chermann JC, Rey F, *et al.* Isolation of a T-Lymphotropic Retrovirus from a patient at Risk for Acquired Immune Deficiency Syndrome (AIDS). *Science* 1983;220:868-871.
6. Popovic M, Samgadharan MG, Read E, *et al.* Detection, Isolation, and Continuous Production of Cytopathic Retroviruses (HTLV-III) from Patients with AIDS and Pre-AIDS. *Science* 1984;224:497-500.
7. Gallo RC, Salahuddin SZ, Popovic M, *et al.* Frequent Detection and Isolation of Cytopathic Retroviruses (HTLV-III) from Patients with AIDS and at Risk for AIDS. *Science* 1984;224:500-502.
8. Schüpbach I, Popovic M, Gilden RV, *et al.* Serological analysis of a Subgroup of Human T-Lymphotropic Retroviruses (HTLV-III) Associated with AIDS. *Science* 1984;224:503-505.
9. Samgadharan MG, Popovic M, Bruch L, *et al.* Antibodies Reactive to Human T-Lymphotropic (HTLV-III) in the Serum of Patients with AIDS. *Science* 1984;224:506-508.
10. Culliton BJ. Gallo Inquiry Takes Puzzling New Turn. *Science* 1990;250:202-203.
11. Culliton BJ. Inside the Gallo Probe. *Science* 1990;248:1494-1498.

12. Hamilton DP. What Next in the Gallo Case? *Science* 1991;254:944-945.
13. Palca J. Draft of Gallo Report Sees the Light of Day. *Science* 1991;253:1347-1348.
14. Cohen J. HHS: Gallo Guilty of Misconduct. *Science* 1993;259:168-170.
15. Gallo RC, Sarin PS, Kramarsky B. *et al.* First isolation of HTLV-III. *Nature* 1986;321:119.
16. Rous P. A Sarcoma of the Fowl transmissible by an agent separable from the Tumor Cells. *J Exp Med* 1911;13:397-411.
17. Weiss R, Teich N, Varmus H, Coffin J. RNA Tumor Viruses. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York; 1982.
18. Temin HM, Baltimore D. RNA-Directed DNA Synthesis and RNA Tumor Viruses. *Adv Vir Res* 1972;17:129-186.
19. Toplin I. Tumor Virus Purification using Zonal Rotors. *Spectra* 1973;No. 4:225-235.
20. Bader P. Reproduction of RNA Tumor Viruses. In: Fraenkel-Conrat H, Wagner RR, eds. *Comprehensive Virology* Vol 4. New York: Plenum Press, 1975:253-331.
21. Sinoussi F, Mendiola L, Chertmann, JC, *et al.* Purification and partial differentiation of the particles of murine sarcoma virus (M. MSV) according to their sedimentation rates in sucrose density gradients. *Spectra* 1973;No. 4:237-243.
22. Maddox J. More on Gallo and Popovic. *Nature* 1992;357:107-109.
23. Lee MH, Sano K, Morales FE. *et al.* Sensitive Reverse Transcriptase Assay to Detect and Quantitate Human Immunodeficiency Virus. *J Clin Microb* 1987;25:1717-1721.
24. Gallo RC, Sarin PS, Wu AM. On the nature of the Nucleic Acids and RNA Dependent DNA Polymerase from RNA Tumor Viruses and Human Cells. In: Silvestri LG, ed. *Possible Episomes in Eukaryotes*. Amsterdam: North-Holland Publishing Company, 1973:13-34.
25. Whitkin SS, Higgins PJ, Bendich A. Inhibition of reverse transcriptase and human sperm DNA polymerase by anti-sperm antibodies. *Clin Exp Immunol* 1978;33:244-251.
26. Varmus H. Reverse Transcription *Sci Am* 1987;257:48-54.
27. Hart DJ, Gogu SR, Agrawal KC, *et al.* Inhibition of RNA-dependent DNA polymerases (reverse transcriptase) of normal cells by activated azidothymidine: a possible basis for drug toxicities? In: Vol I, Abstracts VII International Conference on AIDS, Florence, 1991:110.
28. Gallo RC, Wong-Staal F, Reitz M, *et al.* Some Evidence For Infectious Type-C Virus in Humans. In: Baltimore D, Huang AS, Fox CF, eds. *Animal Virology*. New York: Academic Press Inc., 1976:385-407.
29. Panem S, Prochownik EV, Reale FR, *et al.* Isolation of Type C Virions from a Normal Human Fibroblast Strain. *Science* 1975;189:297-299.
30. Panem S, Prochownik EV, Knish WM, Kisten WH. Cell Generation and Type C Virus Expression in the Human Embryonic Cell Strain HEL-12. *J Gen Virol* 1977;35:487-495.
31. Panem S. C Type Virus Expression in the Placenta. *Curr Top Pathol* 1979;66:175-189.
32. Samgadharam MG, Robert-Guroff M, Gallo RC. DNA Polymerases of Normal and Neoplastic Mammalian Cells. *Biochim Biophys Acta* 1978;516:419-487.
33. Klatzmann D, Barr-Sinoussi F, Nugeyre MT, *et al.* Selective Tropism of Lymphadenopathy Associated Virus (LAV) for Helper-Inducer T Lymphocytes. *Science* 1984;225:59-63.
34. Montagnier L. Lymphadenopathy-Associated Virus: From Molecular Biology to Pathogenicity. *Ann Int Med* 1985;103:689-693.
35. Gendelman HE, Orenstein JM, Martin MA, *et al.* Efficient Isolation and Propagation of Human Immunodeficiency Virus on Recombinant Colony-Stimulating Factor 1-Treated Monocytes. *J Exp Med* 1988;167:1428-1441.
36. Gelderblom HR, Zel M, Hausmann EHS, *et al.* Fine Structure of Human Immunodeficiency Virus (HIV), Immunolocalization of Structural Proteins and Virus-Cell Relation. *Micron Microsc* 1988;19:41-60.
37. Lecatsas G, Taylor MB. Pleomorphism in HTLV-III, the AIDS virus. *S Afr Med J* 1986;69:793-794.
38. Hockley DJ, Wood RD, Jacobs JP, *et al.* Electron Microscopy of Human Immunodeficiency Virus. *J Gen Virol* 1988;69:2455-2469.
39. Dournashkin RR, O'Toole CM, Bucher D, Oxford JS. The presence of budding virus-like particles in human lymphoid cells used for HIV cultivation. In: Vol I, Abstracts VII International Conference on AIDS, Florence, 1991:122.
40. O'Hara CJ, Groopmen JE, Federman M. The Ultrastructural and Immunohistochemical Demonstration of Viral Particles in Lymph Nodes from Human Immunodeficiency Virus-Related Lymphadenopathy Syndromes. *Hum Pathol* 1988;19:545-549.
41. Henderson LE, Sowder R, Copeland TD, *et al.* Direct Identification of Class II Histocompatibility DR Proteins in Preparations of Human T-Cell Lymphotropic Virus Type III. *J Virol* 1987;61:629-632.
42. Lundberg GD. Serological Diagnosis of Human Immunodeficiency Virus Infection by Western Blot Testing. *JAMA* 1988;260:674-679.
43. Edwards VM, Mosley JW and the Transfusion Safety Study Group. Reproducibility in Quality Control of Protein (Western) Immunoblot Assay for Antibodies to Human Immunodeficiency Virus. *Am J Clin Pathol* 1991;91:75-78.
44. Pinter A, Honnen WJ, Tilley SA, *et al.* Oligomeric Structure of gp41, the Transmembrane Protein of Human Immunodeficiency Virus Type I. *J Virol* 1989;63:2674-2679.
45. Zolla-Pazner S, Gorny MK, Honnen WJ. Reinterpretation of Human Immunodeficiency Virus Western Blot Patterns. *NEJM* 1989;320:1280-1281.
46. Damsky CH, Sheffield JB, Tuszynski GP, *et al.* Is there a role for Actin in Virus Budding? *J Cell Biol* 1977;75:593-605.
47. Stanislawsky L, Mongiat F, Neto VM, *et al.* Presence of Actin in Oncoviruses. *Biochem Biophys Res Com* 1984;118:580-586.

Has Gallo proven the role of HIV in AIDS?

48. Stricker RB, Abrams DI, Corash L, *et al.* Target Platelet Antigen in Homosexual Men with Immune Thrombocytopenia. *NEJM* 1985;313:1375-1380.
49. Masiota P, Chamaret S, Montagnier L, *et al.* Detection of Natural Autoantibodies in the serum of Anti-HIV Positive-Individuals. *Ann Inst Pasteur/Immunol* 1987;138:223-233.
50. Wong-Staal F, Gallo RC. Human T-lymphotropic retroviruses. *Nature* 1985;317:395-403.
51. Genesca I, Jett BW, Epstein JS, *et al.* What do Western Blot indeterminate patterns for Human Immunodeficiency Virus mean in EIA-negative blood donors? *Lancet* 1989;ii:1023-1025.
52. Ranki A, Johansson E, Krohn K. Interpretation of Antibodies Reacting Solely with Human Retroviral Core Proteins. *NEJM* 1988;318:448-449.
53. Delord B, Ottmann M, Schrive MH, *et al.* HIV-1 expression in 25 infected patients. A comparison of RNA PCR, p24 EIA in Plasma and in situ Hybridization in mononuclear cells. In: Vol. I, Abstracts VII International Conference on AIDS, Florence, 1991:113.
54. Todak G, Klein E, Lange M, *et al.* A clinical appraisal of the p24 Antigen test. In: Vol. I, Abstracts VII International Conference on AIDS, Florence, 1991:326.
55. Mager DL, Freeman JD. Human Endogenous Retroviruslike Genome with Type C pol Sequences and gag Sequences Related to Human T-Cell Lymphotropic Viruses. *J Virol* 1987;61:4060-4066.
56. Banki K, Maceda I, Hurley E, *et al.* Human T-cell lymphotropic virus [HTLV]-related endogenous sequence, HRES-1 encodes a 28-kDa protein: A possible autoantigen for HTLV-I gag-reactive autoantibodies. *Proc Natl Acad Sci* 1992; 89:1939-1943.
57. Horwitz MS, Boyce-Jacino MT, Faras AJ. Novel Human Endogenous Sequences Related to Human Immunodeficiency Virus Type 1. *J Virol* 1992;66:2170-2179.
58. Aaronson SA, Todaro GJ, Scholnick EM. Induction of murine C-type viruses from clonal lines of virus-free BALB/3T3 cells. *Science* 1971;174:157-159.
59. Hirsch MS, Phillips SM, Solnik C, *et al.* Activation of Leukemia Viruses by Graft-Versus-Host and Mixed Lymphocyte Reactions In Vitro. *Proc Nat Acad Sci* 1972;69:1069-1072.
60. Toyoshima K, Vogt PK. Enhancement and Inhibition of Avian Sarcoma Viruses by Polycations and Polyanions. *Virology* 1969;38:414-426.
61. Todaro GJ, Benveniste RE, Sherr CJ. Interspecies Transfer of RNA Tumour Virus Genes: Implications for the search for "Human" Type C Viruses. In: Baltimore D, Huang AS, Fox CS, eds. *Animal Virology*. New York: Academic Press Inc.; 1976:369-384.
62. Connor S. AIDS: Science Stands On Trial. *New Scientist* 1987; Feb 12:49-54.
63. Burke DS, Brundage JF, Redfield RR, *et al.* Measurement of the False Positive Rate in a Screening Program for Human Immunodeficiency Virus Infections. *NEJM* 1988;319:961-964.
64. Voevodin A. HIV screening in Russia. *Lancet* 1992;339:1548.
65. Gazdar AF, Camey DN, Bunn PA, *et al.* Mitogen Requirements for the In Vitro Propagation of Cutaneous T-Cell Lymphomas. *Blood* 1980;55:409-417.
66. Rubinstein E. The Untold Story of HUT78. *Science* 1990;248:1499-1507.
67. Poiesz B, Ruscetti FW, Mier JW, *et al.* T-cell lines established from human T-lymphocytic neoplasias by direct response to T-cell growth factor. *Proc Natl Acad Sci* 1980;77:6815-6819.
68. Chioldi F, Albert J, Olausson E, *et al.* Isolation Frequency of Human Immunodeficiency Virus from Cerebrospinal Fluid and Blood of Patients with Varying Severity of HIV Infection. *AIDS Res Hum Retrovirology* 1988;4:351-358.
69. Learnont J, Tindall B, Evans L, *et al.* Long-term symptomless HIV-1 infection in recipients of blood products from a single donor. *Lancet* 1992;340:863-867.

[Accepted: 2 April, 1993]