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Clinical and seroepidemiological case-control study of tropical spastic paraparesis and multiple sclerosis in Barbados

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ABSTRACT

A CLINICAL AND SEROEPIDEMIOLOGICAL
CASE-CONTROL STUDY OF
TROPICAL SPASTIC PARAPARESIS AND MULTIPLE SCLEROSIS
IN BARBADOS

Cargill H. Alleyne, Jr

(Sponsored by George Nicholson, faculty of Medical Sciences, Cave Hill campus, University of the West Indies and Francis L. Black, laboratory of Epidemiology and Public Health, Yale University School of Medicine)

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Tropical spastic paraparesis (TSP) and multiple sclerosis (MS) are both chronic neurological disorders which affect thousands of persons worldwide. Recently, studies have shown an association between the human T-lymphotropic virus type I (HTLV-I) and clusters of patients with TSP in several countries worldwide. More controversial has been the proposition that HTLV-I may be an etiological factor in MS.

This age and sex matched case-control study examines the relationship between both TSP and MS and antibodies to HTLV-I in Barbados. It also examines the clinical, demographic and social characteristics of patients with TSP and MS compared with controls in an attempt to identify risk factors for these

diseases as seen in Barbados.

Four cases of TSP and 14 cases of MS were identified. Three relatives of MS cases agreed to participate. Serum HTLV-I antibodies were determined using an enzyme-linked immunosorbent assay (ELISA) and confirmed by Western blot analysis. All sera were tested for antibody to **Treponema pallidum** and for antibody to **Borrelia burgdorferi**, the agent of Lyme disease. Test results indicated that 2 TSP patients (50%) and none of the MS patients or relatives were seropositive for HTLV-I. None of the controls of either group was seropositive for HTLV-I. None of the sera from the cases was VDRL reactive while antibody to **Treponema pallidum** was confirmed by Treponema pallidum hemagglutinin assay (TPHA) in 1 MS control. IgM and IgG antibodies to the agent of Lyme disease were also tested by ELISA. No increase in titers was noted in either cases or controls. In general, computer analysis of the data revealed no significant difference in the responses and characteristics of the cases as compared with the controls.

These data suggest an association of HTLV-I with TSP but do not suggest a role of HTLV-I in the etiology of MS as diagnosed in Barbados. There is no support for an etiological role of **Treponema pallidum** or **Borrelia burgdorferi** in either MS or TSP. The absence of HTLV-I positivity in MS patients suggest that TSP cases are not being misdiagnosed as MS in Barbados.

INTRODUCTION

Historical aspects of TSP

Case reports of chronic neurological disorders of unknown etiology from a tropical area were first published in 1888 by Strachan[1] when he described a group of Jamaican patients with an ataxic peripheral neuritis, paresthesiae and defects in vision. A similar syndrome was described by Scott in 1918[2] when an epidemic began in Jamaican canecutters. It was reported to be preceded by conjunctivitis and stomatitis and characterized by numbness, tingling and burning of the feet, constipation and gait ataxia. Scott ascribed the neurological symptoms to a toxic factor, most likely sugarcane since this cyanogenic crop[3-6] comprised the bulk of the patients' diet during the sugarcane season. Another possibility is that these cases represented a post-infectious acute sensory neuronopathy syndrome[7] associated with an epidemic viral outbreak of acute hemorrhagic conjunctivitis. This 'Strachan-Scott syndrome' became known as tropical ataxic neuropathy (TAN).

TAN was soon reported throughout the tropics and has been linked to a variety of nutritional disorders including vitamin A, riboflavin, niacin, folate, B_{12} and iron deficiencies.[8-12] TAN has also been noted in World War II prisoners of war (Canadians held by the Japanese, prisoners held in Singapore,

and Dutch, British and Indian prisoners held in Malaya, Thailand and Singapore)[13-15] and in conjunction with post-infective tropical malabsorption.[16-18] In addition, cassava (**Manihot esculenta**) neurotoxicity has been implicated in TAN.[5,19,20]

In 1956 Cruickshank described a neuropathic disorder[21] which seemed clinically different from the syndrome described by Strachan and Scott. By 1964 it had become clear that this second 'Jamaican neuropathy' had become predominant in Jamaica. Whereas TAN is characterized by a sensory ataxia due to symmetrical dorsal column lesions, this syndrome, also known as tropical spastic paraparesis (TSP), is characterized by bilateral and symmetrical involvement of the pyramidal tract especially at the lumbar level. This usually leads to a gradual onset of spasticity and hyperreflexia of the legs with a positive Babinski reflex. Also common are lumbar back pain, leg weakness, dysesthesiae of the feet, bladder dysfunction, severe constipation and impotence in males. In some cases there may also be slight involvement of the dorsal columns. Mental and cranial nerve functioning are usually normal except in Jamaica where deafness and retrobulbar neuropathy have occurred.

Epidemic TSP has been linked to excessive cassava consumption during drought in Nigeria and Mozambique,[5,20,22,23] to malabsorption,[24] and to lathyrism (caused by excessive

consumption of peas of the **Lathyrus** family in India and parts of Africa).[25,26] An infectious agent may also be involved in some epidemics of TSP.[27]

Demographics of TSP

The endemic form of TSP has now been recognized in Martinique,[28,29,30] Trinidad,[31] Colombia,[32,33] the Seychelles,[34-36] Peru,[37] South India[38] and other countries, as well as in Jamaica.[39] The highest prevalence ratio of TSP to date (127.7 cases per 100,000 population) has been reported in a geographic isolate in the Seychelles by Kelly and De Mol.[34]

The sex distribution seems roughly balanced with a slight male preponderance in large studies[33,34,40] but a marked female preponderance in smaller studies.[29,35] One explanation given by Roman for this female preponderance is that it probably reflects

> a bias in the ascertainment of cases, since women may tend to seek medical attention more often than men, or since they may be more seriously affected. Both sexes would therefore seem to be equally exposed to the potential cause of TSP, although women appear to be affected more often.[41]

The mean age of onset is 42 years and symptoms begin after 30 years of age in 76-82% of patients, but in a smaller proportion of cases, most of which are female, the onset is after 60 years of age.[41] The youngest reported age of onset

is 13 years.[103]

Neuropathology of TSP

Autopsy studies performed on TSP cases in Jamaica,[40,42] South India[38] and the Seychelles[43,44] have revealed similar findings. Pathology typically includes a chronic meningomyelitis with perivascular cuffing, hyaline arteriolar thickening (involving primarily the spinal cord but with minimal changes in the midbrain, cerebellum and the cerebrum), and a reactive astrocytic gliosis. There is also demyelination and axonal degeneration, mainly in the pyramidal tracts but also in the dorsal columns, spinocerebellar tracts, and less commonly, the spinothalamic tracts. The auditory and optic nerves may also be involved.

A study by Moore et al[119] has demonstrated the presence of HTLV-I p19 core protein, a predominance of suppressor/cytotoxic T-cells, and the expression of class I major histocompatibility antigen in the spinal cord lesions of TSP. It is proposed that the mixed picture of demyelination and axonal loss with secondary tractal degeneration is a result of the lysis of the myelin sheath and eventually the axon by the action of cytotoxic T-cells, either directly or via the release of cytokines.

Etiology of TSP

In the past, various etiologic theories have been

proposed for TSP. The presence of treponemal antibodies in the sera of a high proportion of TSP patients in Jamaica[45,46] and Colombia,[47] areas hyperendemic for yaws and syphilis in the past, raised the question as to whether TSP is a late manifestation of yaws or syphilis.

There has also been an increased incidence of serum antibodies to **Borrelia burgdorferi** (the etiological agent of Lyme disease) in patients from Jamaica[48] and the Seychelles.[35] The significance of this finding is unknown.

The agent most strongly linked to TSP, however, is the human T-lymphotropic virus type I (HTLV-I). HTLV-I serum antibodies were initially reported in a high proportion (68%) of TSP patients by Gessain et al in 1985.[28] This finding was eventually confirmed[29,49] and lead to the discovery of HTLV-I-positive (in sera and cerebrospinal fluid-CSF) TSP cases in Jamaica,[39,50] Colombia, the Seychelles,[36] Trinidad,[31] the Ivory Coast,[53] Senegal,[54] Peru,[37] Chile,[55] French Guyana,[30] France,[54] the Solomon islands,[56] the Dominican Republic,[104] Panama,[105] in immigrants from the Caribbean to the UK,[57] and as the present study shows, in Barbados.

A chronic progressive myelopathy very similar to TSP, with 100% associated HTLV-I serum and CSF positivity, has been described in southwestern Japan by Osame et al.[58,59] This disease has been called HTLV-I associated myelopathy (HAM). An autopsy report of one HAM patient revealed perivascular

lymphocytic infiltration, with a prominent hyaline degeneration of the vascular media and adventitia, and leptomeningeal thickening.[58] These findings are virtually identical to those found in TSP patients. Differences between these two disorders include frequent CSF pleocytosis, good response to corticosteroids, and a younger mean age of onset in HAM (37 years as compared with 42 years).[41] More recently, however, the study of additional cases seems to have narrowed the differences and HAM and HTLV-I-associated TSP are considered to be the same disorder by some authors.[114]

HTLV-I infection is endemic in Africa,[59] southern Japan,[60,61] the southeastern United States,[62] the Caribbean basin,[63] Alaska,[64] Papua, New Guinea,[65] Spain,[66] and southern Italy.[67,68] Transmission of the virus seems to be via intact cells, and sexual contact, especially from male to female, has been implicated in its horizontal spread.[62,69,70,71] Vertical transmission transplacentally,[72] intrapartum,[69] and via breast milk[73-75] has also been suggested.

The lymphotropic effect of HTLV-I is well known. The virus was first isolated in 1978 to 1979 from two black American patients: one with cutaneous T-cell lymphoma diagnosed as mycosis fungoides[76] and one with Sézary T-cell leukemia.[77] The virus was also isolated from Japanese patients with adult T-cell leukemia/lymphoma (ATLL),[78] an aggressive T-cell leukemia first identified and characterized in Japan.[79]

Since that time HLTV-I associated ATLL has been noted in several groups of patients, including a series of patients of West Indian heritage.[80] In retrospect, the clinical features of the original two patients were more typical of ATLL. The chance of developing ATLL after seroconversion seems to be low: 0.1 to 0.01%.[81]

In addition to being lymphotropic, however, the data now suggest that HTLV-I does have neurotropic potential. This is significant, since for the first time evidence is provided for a causative link of a human retrovirus to a chronic neurodegenerative disorder in humans. This is further supported by the isolation of the virus from the sera and CSF of Jamaican and Colombian patients with TSP,[82] from the CSF of a patient with HAM,[83] and from the sera and CSF of three family members with TSP.[103]

Two other related but distinct human retroviruses, HTLV-II and HTLV-V, have been discovered. HTLV-II was isolated in 1982 from a patient with a T-cell variant of hairy cell leukemia,[116] while HTLV-V was isolated in 1987 from a patient with cutaneous T-cell lymphoma/leukemia.[117] There has been no indication to date that either virus has neurotropic potential.

Signs and symptoms of MS

Multiple sclerosis, characterized pathologically by areas of demyelination and sclerosis in the central nervous system, has been described as having dissemination of lesions in both time and place. The course of the disease is unpredictable but the usual patterns are: benign (20% of patients), characterized by few exacerbations and near complete remissions; exacerbating-remitting (25% of patients), with more frequent early exacerbations and less complete remissions; chronic-relapsing (40% of patients), with fewer remissions and increasing disability as the disease progresses; and chronic-progressive (15% of patients), characterized by gradual onset and steady progression of symptoms.[101]

Symptoms usually occur acutely and last for weeks or longer but can also last for a few minutes to hours. They include spastic muscle weakness, ocular disturbance, urinary disturbance, gait ataxia, paresthesiae, dysarthria or scanning speech, mental disturbance, pain, vertigo, dysphagia, convulsions, decreased hearing, and tinnitus. The signs include spasticity and/or hyperreflexia, Babinski sign, absent abdominal reflexes, dysmetria or intention tremor, nystagmus, impairment of position and vibration senses, impairment of pain, touch, and temperature sensation, facial weakness, and changes in state of consciousness.

The standard for evaluating disability in MS has become the Disability Status Scale by Kurtzke.[120,121] The scale was developed in 1953-4 in response to a need to evaluate the efficacy of a new treatment for MS and has since undergone modification.

Demographics of MS

MS has traditionally been regarded as a disease of temperate regions. It is rarely reported in equatorial zones and increases in frequency with latitude in both hemispheres. There is a prevalence rate of 50 to 100 cases per 100,000 population in temperate zones with the highest rates 150 cases per 100,000 being reported in the Shetland and Orkney Isles off the coast of northern Scotland. There has been a dramatic rise in MS cases in the tropical island of Barbados since the first recorded one in 1961. This is further examined in the discussion section.

MS has a predisposition for the female sex with a female to male ratio of 1.5 to 1. With later onset, however, the ratio is closer to 1 to 1. Symptoms typically occur between 20 and 40 years of age. Onset of symptoms before age 10 and after age 60 is rare.

Neuropathology of MS

There are usually several small areas of discoloration in the cerebral hemispheres particularly in the white matter and

periventricular areas. When defined with myelin sheath stains, these regions correspond to areas of demyelination. Sharply circumscribed plaques of varying sizes are scattered throughout the brain and spinal cord but tend to be grouped around the lateral and third ventricles. Lesions result from partial or complete destruction of the myelin, a lesser degree of damage to the axis cylinders or neurons, proliferation of glial cells and changes in blood vessels. Compound granular cells laden with cholesterol esters are present in the lesions, in the perivascular spaces of the vessels and in the tissue adjacent to them. Small lesions may be found in the gray matter and in the region between the gray and white matter.

The results of a study by McCallum et al[122] suggest that the ratio of T-cell subsets is important in the evolution of the plaque margins. It was found that the numbers of both helper-inducer and cytotoxic-suppressor T-cells peaked at the edges of the plaques and that fewer cytotoxic-suppressor T-cells were found in the normal appearing areas outside active plaques than equivalent areas outside inactive plaques. Their conclusions are that these findings, together with similar ones in experimental allergic encephalomyelitis suggest that plaque expansion depends on helper-inducer cell dominance at the border and that controlled disease is associated with cytotoxic-suppressor cell dominance in non-plaque areas.

Etiology of MS

Two major theories about the pathogenesis of MS have been proposed. The first relates to an infection with a long incubation period. Organisms that have been suspect in the past include spirochetes, rickettsia, protozoa, and various types of viruses including rabies, herpes simplex types I and II, corona, canine distemper, and measles virus. Ever since a report was published by Adams and Imigawa in 1962[123] indicating that the average titer against measles was elevated in a group of MS patients, this virus has been a favored etiological agent of MS. Subsequent studies including one by Brody et al[124] have shown that unaffected siblings of MS patients (including twins) also had elevated antibody titers against a variety of viral agents possibly reflecting the individual's HLA type and that the epidemiology of the measles virus was not consistent with that of MS.

Another theory implicates altered immune regulation, i.e., either increased immune responsiveness or a decreased suppression of the immune response to infection. Certain histocompatibility types associated with MS in northern populations are also believed to control immune responsiveness.

There is also controversial evidence that HTLV-I or an HTLV-I-like virus may play a role in some cases of MS. The idea was first suggested by Koprowski et al when positive

serum HTLV-I antibodies titers were found in 7 of 17 patients with MS in Key West, Florida.[84] Subsequent reports failed to corroborate this finding[85-89] but 25% of a group of 68 Japanese patients with MS were found to have low-level positive serum HTLV-I antibody titers.[90] Another study showed that the sera of 11 of 46 Japanese patients with MS contained antibodies against group specific proteins but not against envelope proteins of HTLV-I,[91] suggesting the involvement of a very similar but not identical virus. There have also been recent reports of the identification by polymerase chain reaction (PCR) of sequences homologous to HTLV-I DNA from patients with MS.[92,93] Other studies using this sensitive method to detect and amplify low concentrations of viral DNA have not reproduced these findings.[125]

The positive findings may be explained by an overly sensitive test. Assuming that the results are real, it is also possible that evidence of HTLV-I in isolated MS cases may be coincidental. As noted in the discussion section, the issue is complicated by the fact that TSP and the chronic progressive form of MS share common clinical features. Thus, there exists a real potential for misdiagnosis. Laboratory data have been incorporated into the diagnostic criteria for MS developed by Poser et al[126].

The role of HTLV-I and possibly other related retroviruses in the etiology of chronic neurodegenerative

disorders remains to be elucidated. It is probable, however, that we are on the brink of new discoveries that will lead to a much clearer understanding of the mechanisms, and eventually, the prevention and treatment of these disorders.

MATERIALS AND METHOD

Geographical and historical aspects

Barbados, at 13° 10' north latitude and 59° 35' west longitude, is the easternmost of the group of Caribbean islands called the Windwards which begin with Martinique and stretch south to Grenada (see figure 3). Unlike many of the other Caribbean islands, it is not of volcanic origin, but consists geologically of a limestone base with an overlying coral cap. The rainwater quickly percolates through this natural filter resulting in both an absence of rivers and an extremely pure water supply. The highest peak is 1,105 feet.

The weather is delightfully tropical with a high number of hours of sunshine per year tempered by northeast trade winds and with a humidity between 60 and 75%. The annual rainfall varies from about 40 inches in some coastal areas to 90 inches in the central ridge area and the temperature ranges from a low of about 70°F to a high in the 90's.

Its landscape is one of contrasts: the rugged cliffs of the east coast; the sandy beaches of the west; the expansive sugarcane fields; and the busy capital of Bridgetown (in the parish of St. Michael), home to almost 40% of the island's population.

Originally populated by the Arawaks, Barbados was

discovered by the Portuguese in the 16th century but remained uncolonized until it was claimed by the British in 1625. Independent since 1966, Barbados has a unique place in Caribbean history in that it has been colonized by only one nation, Britain, from 1625. The main industries are sugar and tourism. The population of 250,000 is mainly of black African ancestry (92% black; 4% white; 4% mixed: population census 1970) and with an area of 166 square miles Barbados has one of the highest population densities in the world.

Study population

This study was begun in June 1988 at the Queen Elizabeth hospital in Bridgetown, Barbados, and continued at the laboratory of Epidemiology and Public Health, Yale University School of Medicine.

The initial sample size was 6 persons diagnosed with tropical spastic paraparesis (TSP) and 15 persons diagnosed with either 'clinically definite' or 'probable' multiple sclerosis (MS).

Subjects were found from a pool of cases most of which were previously diagnosed by three clinicians at the hospital. Using hospital records, an energetic effort was made to contact the subjects by telephone and by mail. Owing to the inevitable loss to follow-up by death, change of address, and change of telephone number many were not contacted. Of those

contacted, two did not consent.

At the end of 9 weeks, the clinical data on the cases were reviewed by Dr. George Nicholson and the investigator, and the designations 'MS' and 'TSP' revised using specific clinical criteria (see tables 1 and 2). One TSP case and 2 MS cases (the terms 'TSP cases' and 'MS cases' refer to persons diagnosed with TSP and MS) were discarded since it was felt that there was insufficient evidence to make a diagnosis. In addition, one TSP case was reclassified 'MS'. The final sample size was thus 4 TSP cases and 14 MS cases. Three relatives of MS cases also consented to participate.

All cases were matched with controls according to sex and age (within 2 years except in 3 pairs, whose age differences were 3, 4 and 5 years respectively). Controls were sought either from the patients hospitalized in the medical and surgical wards, or from the outpatients who visited the hospital. Conditions afflicting the controls were unrelated to either TSP or MS.

Method

Appointments were scheduled after oral consent was obtained (see appendix 1 for Information Sheet) and each MS or TSP case was asked questions from a previously designed questionnaire (see appendix 7) aimed at identifying both risk factors for the diseases and characteristics and symptoms of

the cases. The degree of incapacitation of some of the neurological subjects was severe enough to preclude a hospital visit. In these instances they were visited in their homes by the investigator.

From each case, the investigator collected 20 ml of blood by venipuncture using 2 vacutainers and 19- or 22-gauge needles. One tube was drawn for the purpose of antibody testing and the other (heparinized) for the purpose of isolation and culture of peripheral blood lymphocytes.

At the end of each interview cases were asked if they would consent to having the investigator invite their close relatives (parents, if alive, and one sibling) to participate in the study.

Controls were also interviewed and one vacutainer of blood drawn by venipuncture for the purpose of antibody testing. All questionnaires and blood tubes were coded to ensure confidentiality.

Under sterile conditions the heparinized blood was added to lymphocyte separation fluid and centrifuged. The isolated lymphocytes were purified by differential centrifugation and then incubated with RPMI-1640 growth medium at 37°C. Periodically, medium was added to the cultures (see appendix 8 for lymphocyte isolation procedure).

Serology and analysis

The blood drawn for antibody testing was allowed to clot and the sera frozen for treponemal, **Borrelia burgdorferi**, and HTLV-I antibody testing (see appendix 2 for preparation of samples for antibody testing). The Venereal Disease Research Laboratory (VDRL) test, the fluorescent treponemal antibody absorbed (FTA-ABS) test and the **Treponema pallidum** hemagglutinin antigen (TPHA) test were done at the Sir Winston Scott Polyclinic in Barbados by C. Lucas, medical laboratory technologist (see appendices 3, 4 and 5 for protocols for the VDRL, FTA-ABS and TPHA tests, respectively).

Frozen samples were sent to the laboratory of Dr. Francis Black at the Epidemiology and Public Health department at Yale University School of Medicine. HTLV-I antibody screening was done using a commercial enzyme-linked immunosorbent assay (ELISA) by R. Capper, laboratory technician (see appendix 6 for ELISA protocol). Confirmatory Western blot analyses were performed in the laboratory of Dr. Clarence Gibbs at to National Institutes of Health (NIH) in Bethesda, Maryland (see Vernant et al[29] for Western blot protocol).

Tests for IgG and IgM antibodies to **Borrelia burgdorferi**, the agent of Lyme disease, were done by ELISA in the laboratory of Dr. S. Malawista at Yale University School of Medicine by S. Cretella, laboratory technician (see Craft, et al[100] for the protocol for measuring antibody to the Lyme

disease spirochete).

Clinical data from hospital records and the data from the questionnaires were coded and analyzed with the aid of the Statpak Gold computer program.

RESULTS

Age and sex distribution (see figures 1 and 2, and table 3.)

The age of onset of symptoms in TSP patients ranged from 22 to 47 years with a mean of 38.25 years. This is quite similar to the mean noted in TSP patients from Jamaica (39 years[40]) and Martinique (40 years[29]) and marginally less than that in patients from the Seychelles (42.5 years[35]) and Colombia (46.5 years[33]). In MS patients, the range of age at onset of symptoms was 19 to 39 years with a mean of 30.4 years. Again, this mean is similar to that of 32 years noted in the literature.[101] The mean age of onset of both diseases was lower in the female than the male patients: 31.5 and 45 years respectively for TSP and 28.1 and 34.6 respectively for MS. A female to male ratio of 1.8:1 was obtained for the MS patients while the sexes were equally represented in the TSP patients. The mean duration of illness was slightly less in the TSP patients than in the MS patients: 6.25 years (range 4 to 12 years) as compared with 8.2 years (range 1 to 16 years).

Residence and ethnicity

All 4 TSP patients were black Barbadians while of the 14 MS patients, 9 were black Barbadians, 2 were white Barbadians, 1 was of mixed ethnicity, and 2 were British-born whites who had been residing in Barbados for more than 6 years. With the

exception of 3 MS patients who were living in Canada, the UK and Australia respectively, all of the patients were residing in Barbados at the time of onset of symptoms. The most populous parish of St. Michael (about 40% of the population) was heavily represented, with 2 (50%) TSP cases and 6 (54% of 11) MS cases (see figure 3).

Travel history (see table 4)

Positive travel histories were obtained for almost all patients. Only 3 MS patients had never left the island of Barbados. A negative travel history was obtained in 7 MS controls. All TSP patients had travelled outside of Barbados and 1 TSP control had not. Countries visited included other Caribbean islands, the UK, Canada and the USA.

Social characteristics (see table 4)

All questions were asked in reference to the time of onset of the symptoms of the disease.

A very diverse range of occupations was noted for both groups of patients. The TSP group included a mechanic, cook, accountant and laborer, and the MS group included a plumber, seamstress, production manager in an electronics plant, engineer, cook, accountant, day-care worker, barmaid, bank teller, airline hostess, homemaker, two teachers, and one unemployed person.

Two TSP patients and all TSP controls were non-smokers, and 2 TSP patients were heavy smokers. More than half of the MS patients, 9, and 12 MS controls were non-smokers. Half of the TSP patients reported slight consumption of alcoholic beverages (two drinks or less per week) while one was a teetotaler and the other a heavy drinker (more than 10 drinks per week). Of the TSP controls, half were slight drinkers and half were moderate to heavy drinkers. Eleven (79%) of the MS patients and the same number of controls were either teetotalers or slight drinkers. The other 3 were moderate to heavy drinkers. When compared with controls, no significant difference in smoking or drinking habits was noted.

The household size for the TSP patients ranged from 2 to 5 members with a mean of 3. The mean household size for MS patients was slightly greater at 3.9, with a range of 1 to 11 members.

Past medical history

One of the women with TSP had had 5 pregnancies with 3 abortions while the other was nulliparous. The total number of pregnancies for 9 women with MS was 17 (mean 1.89) and two had had one or more abortions.

Only 2 patients reported positive blood transfusion histories: one TSP patient (who was also HTLV-I positive), and one MS patient. Past medical histories were, in general, non-

contributory.

Environmental toxins

Most patients reported only rare or occasional exposure to pesticides, disinfectants, or other potentially neurotoxic chemicals. Three (21%) MS patients, however, had had significant contact with dyes, insecticides, and battery fluids, respectively for some years.

No TSP patient reported a consumption of cassava greater than once every few weeks. Most of the MS patients reported the same, but two (14%) had been consuming cassava about once or twice a week. The cassava that was consumed was usually of the sweet and not of the bitter variety which has been associated with neurotoxicity when improperly prepared and comprising a large portion of the diet. The preparation of the bitter variety entails grating the cassava, washing away the poisonous juices, and drying the meal. There was no use of peas of the **Lathyrus** family.

There seemed to be minimal use of folk medicines and "bush teas" but one MS patient reported the frequent use of aloes and another, the frequent use of homeopathic "powders and pills."

Nutrition

All of the patients were clinically well nourished. Fish

played a significant role in the diet of most patients but other types of seafood were consumed occasionally or rarely.

Other factors

All TSP patients had had some exposure to pets and/or farm animals in the recent past (within the previous 10 years) compared with 10 (71%) MS patients. Another 3 (21%) MS patients had had some exposure in the more distant past while one MS patient reported no contact with pets or farm animals.

Data analysis

Using the Statpak Gold statistical analysis package, the data obtained from the questionnaires were analyzed. The type of subject (case, control and sibling) was cross-tabulated with the coded responses to the questions and the chi-square values obtained. In general, no significant difference was obtained in the responses and characteristics of cases as compared with controls.

One significant result ($p<0.05$) was noted in the cross-tabulation of type of subject versus the use of medications in the 6 months before onset of disease (cases) or in the 6 months previous to the administration of the questionnaire (controls). Controls were more likely to have used medications than cases. This is explicable by the fact that before the onset of TSP or MS, most of the cases were perfectly healthy, while the controls, drawn from a hospital

population, tended to have an underlying condition requiring the use of one or several types of medication. Memory may have also played a role since onset of disease sometimes preceded administration of the questionnaire by several years.

Clinical characteristics (see table 3)

TSP:

The clinical course of the disease was unremitting in 3 (75%) TSP patients and uncertain in 1 (25%). Two patients (50%) were able to walk unaided while the others required support. All patients reported leg weakness and dysesthesiae and 3 experienced constipation. Bladder dysfunction and blurred vision/diplopia were each reported by 2 patients.

MS:

The course of 10 (71.4%) MS patients was significant for remissions/relapses while 1 patient had a progressive course. The course of 3 patients was uncertain. Nine (64.3%) patients were ambulatory, 4 (28.6%) required support and 1 was incapacitated. The most common symptom, reported by 12 (85.7%) patients, was blurred vision or diplopia. Ten (71.4%) patients had lower limb weakness and 8 (57.1%) lower back pain. Lower limb dysesthesiae and bladder dysfunction were each reported by 7 (50%) MS patients while leg cramps and constipation were reported by 6 (42.9%) and 4 (28.6%) MS patients respectively.

Serology (see table 5)

HTLV-I:

Serum samples from MS and TSP cases and controls were tested with the Retro-Tek HTLV-I ELISA kit manufactured by Cellular Products, Inc., Buffalo, New York. Results of the ELISA test revealed 11 positive samples (3 TSP cases, 1 MS case, 2 TSP controls, 4 MS controls, and 1 MS relative) and 7 borderline samples (4 MS cases, 1 TSP control, and 2 MS controls). One of the samples (an MS case) with borderline results retested positive. Western blot confirmation was obtained with 2 TSP samples. Sera from these 2 patients demonstrated antibodies to the gag-encoded p19, p24, p33, p36, and the env-encoded gp46. None of the sera from MS patients or TSP and MS controls was positive in the Western blot assay.

Treponema pallidum:

The VDRL test was done using the Bacto VDRL antigen manufactured by Difco Labs., Detroit, Michigan. Only one sample, that from an MS control, was reactive (1:4). Confirmation was obtained with the TPHA test using the kit manufactured by Fujirebio Inc., Tokyo, Japan. Another sample, an MS case, was 'rough negative' on the VDRL but was non-reactive on the FTA-ABS test (Difco Labs).

Borrelia burgdorferi:

All sera were tested for IgG and IgM antibodies to the

agent of Lyme disease. None of the samples had elevated titers.

The attempt at culturing the isolated lymphocytes was unsuccessful because of contamination of the cell cultures.

DISCUSSION

Estimation of the number of TSP patients in Barbados

Prior to conducting the study, a gross estimate of the number of TSP patients in Barbados was made by using the then current prevalence ratio of Martinique. Martinique is 110 miles from Barbados and has an HTLV-I positivity rate of 4% in blood donors younger than 60 years of age[30]. This 4% rate is similar to the HTLV-I positivity rate in the general Barbadian population[94]. Thus, with a TSP prevalence of 11.8 per 100,000 in Martinique[30] (population 328,566) and with a Barbadian population of 250,000, the number of patients with TSP was estimated at 30. A sample size determination was also done using the following calculations.

Determination of sample size

For a matched case-control study, as discussed by Schlesselman,[118] for a specified α and β, the number of exposure-discordant pairs m (pairs of cases and controls that differ with respect to the risk factor - in this case, HTLV-I antibody) required to detect a relative risk R is given by:

$$m = [z_{\alpha}/2 + z_{\beta} \sqrt{P} \sqrt{(1 - P)}]^2 / (P - 1/2)^2$$

where $P \approx R / (1 + R)$

The relative risk R was first determined by calculating the odds ratio of TSP. The odds ratio can be calculated based on results obtained in Martinique. A 2x2 table can be drawn:

	HTLV-I		
	+	-	Total
Case	53	7	60
Control	154	1059	1213

The odds ratio is given by: (1059 x 53) / (154 x 7) = 52.1

Since TSP is a relatively rare disease, the relative risk can be estimated to equal the odds ratio, so

for $P = 52.1 / (1 + 52.1) = 0.98$

z_α (α error) = 1.645 (95%, one-tailed)

z_β (β error) = 1.282 (90%, one-tailed),

$$m = [1.645/2 + 1.282 \sqrt{0.98} \sqrt{(1 - 0.98)}]^2 / (0.98 - 0.5)^2$$

$$= 4.357$$

Thus 5 exposure discordant pairs are required for proper use of the McNemar statistical test.

If p_e represents the probability of an exposure-discordant pair, the total number of pairs M required to yield m discordant pairs is $M = m/p_e$ and p_e can be found from:

$$p_e \approx (p_0\, q_1 + p_1\, q_0)$$

where p_0 is the estimated proportion of exposed controls in the target population (4% of the general Barbadian population have antibodies to HTLV-I)[94], $q_0 = 1 - p_0$ and $q_1 = 1 - p_1$.

p_1 is the estimated proportion of exposed cases and is given by:

$$p_1 = p_0\, R \,/\, [1 + p_0\, (R - 1)]$$

Thus $p_1 = 0.04 \times 52.1 \,/\, [1 + 0.04\ (52.1 - 1) = 0.685$

and $p_e = (0.04 \times 0.315) + (0.685 \times 0.96) = 0.67$

and $M = 5/0.67 = 7.46$

Thus 8 case-control pairs are required to yield 4 exposure-discordant pairs.

With a total of 4 pairs of cases and controls and with 2 pairs that differed with respect to HTLV-I positivity in this Barbadian study, i.e half the required number, statistical analysis with the McNemar's will not be feasible.

Power of the study

The power of this matched study can be calculated from:

$$z_\beta = [-z_\alpha/2 + (P - 1/2)\ \sqrt{m}] \,/\, \sqrt{P}\ \sqrt{(1 - P)}$$

where $z_\alpha = 1.645$, $m = 2$ (there are 2 exposure-discordant

pairs in this study), and P = 0.98 as calculated previously. Thus,

$z_\beta = [-1.645/2 + (0.98 - 0.5)\sqrt{2}] / \sqrt{0.98}\sqrt{(1 - 0.98)} = -1.03$

and the power can be determined from the tables of normal distribution by finding the probability that z_β is not exceeded.

Hence the power of this study is P ($Z \leq -1.03$) or 0.152 and there is a 15.2% probability of finding that the sample estimate of relative risk differs significantly ($\alpha = 0.05$) from unity.

TSP in Barbados

The cases diagnosed as TSP in Barbados are not epidemic. Malnutrition, cassava neurotoxicity and lathyrism are not relevant since no subject was clinically malnourished and no one reported consumption of peas of the **Lathyrus** family or significant consumption of the bitter variety of cassava. As mentioned previously, the 4 cases of TSP reported here are similar, both with respect to the demographics of the patients and to the clinical characteristics of the disorder, to those endemic cases reported in the literature.

With an estimated TSP population in Barbados of 30 (based on data from Martinique), the yield of 4 cases in the current study suggests that some cases are unrecognized. A coordinated effort from the private physicians and the several

polyclinics throughout Barbados would probably identify cases that would otherwise not be followed at the Queen Elizabeth Hospital.

HTLV-I serology in TSP patients

Even without formal analysis, a 50% HTLV-I antibody positivity in TSP patients as compared with 0% in controls does suggest an etiological role of HTLV-I in the pathogenesis of TSP. This HTLV-I antibody positivity rate compares with one of 91% (in sera) and 84% (in CSF) in Jamaican TSP patients,[107] 88% (in sera) and 100% (in CSF) in TSP patients from Martinique,[108] 86% (in sera) and 100% (in CSF) in Trinidadian TSP patients,[109] and 82% (in sera) and 100% (in CSF) in TSP patients from the Dominican Republic.[104]

In any event, these rates raise the question: why is there not 100% HTLV-I positivity in the sera of TSP patients? One possibility is that the IgG antibodies in the sera of certain cases may be of such low titers that the sensitivity of the tests currently used may be inadequate. Fresh samples and proper storage of the samples also seem to be important as evidenced by the fact that when 23 of the most recently collected Jamaican samples were tested (within 4 weeks), positivity in sera increased from 91% to 100% and positivity in CSF increased from 84% to 96%.[107] The samples in this Barbadian study were properly stored and fresh when tested.

The possibility also exists that antibodies to HTLV-I are only produced at certain intervals during the course of the disease. In fact, it is quite likely that the symptoms of TSP are not necessarily caused by a persistent viral infection, but rather by a chronic immune-mediated encephalomyeloneuritis. This has been suggested by Vernant et al.[29] As mentioned previously, a virus-induced, cytotoxic T-cell mediated mechanism in which lysis of the myelin sheath and axon results from the effect of the suppressor/cytotoxic T-cells has been proposed by Moore et al.[119] This is based on the findings of a predominance of suppressor/cytotoxic T-cells, HTLV-I core protein and the expression of class I major histocompatibility antigen in spinal cord lesions. Localization of the core protein to inflammatory cells suggests that the virus may gain entry into the CNS via these cells. Since, however, some parenchymal cells were also positive for viral antigen, a direct primary infection of intrinsic CNS elements cannot be excluded.

An immune-mediated mechanism has also been suggested based on the presence of skin lesions suggestive of immune-complex vasculitis and lymphocytic pulmonary alveolitis in TSP patients.[110,111]

Vernant et al have also suggested that the HTLV-I-negative group of patients in their study may represent a different etiopathological syndrome since a comparison of this

group with the HTLV-I-positive group revealed a difference in the sex ratio, the mean age of onset, and the characteristics of the symptoms. Of interest is a comparison of the HTLV-I-positive group with the HTLV-I-negative group of TSP patients in the current Barbadian study which reveals a similar difference in the mean age of onset, despite the small sample sizes (2 in each group). The mean age of onset in the HTLV-I-positive group was 44 years while that of the HTLV-I-negative group was 32.5 years. This compares with a mean of 45 years in the former group and one of 35 years in the latter group in the TSP patients from Martinique.

It is well to bear in mind that the epidemic form of TSP seems to be caused by more than one factor. The TSP seen in HTLV-I negative patients may have some other as yet unidentified factor as an etiological agent.

Another important question is: why don't all persons with antibodies to HTLV-I develop TSP/HAM (or ATLL)? The answer may lie in a genetically predetermined susceptibility to HTLV-I. A preliminary study of the HLA haplotypes in 63 patients with HTLV-I-associated TSP in Martinique revealed an increase in the frequency of B7 and CW7 antigens in comparison with the HTLV-I-positive asymptomatic population.[112] However, when corrected for the number of antigens tested, this finding was not significant. A study in Japan has also reported a link between certain HLA haplotypes and HAM in contrast to the HLA

haplotypes found in the ATLL patients.[113]

These studies suggest that the development of a particular pathology in an HTLV-I-infected host is dependent on the HLA-related immune responsiveness of that host.

Ijichi et al[115] have hypothesized with respect to HAM patients, that

> the supply of infected (CD4 positive) T-cells from peripheral blood to CNS is primary for the development of CNS lesions. Following the expansion of infected T-cells with viral antigen presentation in the CNS, the propagation of immune responses, including responses of cytotoxic T-cells, B-cell activation, and microglial reaction, may occur on the basis of high-immune responsiveness against HTLV-I.

They note that one of hallmarks of HTLV-I infection is the autologous proliferation response (APR) which is characterized by the spontaneous growth of infected T-cells and reactive proliferation of T-cells responding to HTLV-I infected lymphocytes **in vitro**. In their conclusion they state that

> We demonstrated the existence of serum factor(s) that inhibit APR in HAM patients. Furthermore, cerebrospinal fluid from HAM patients did not exhibit a significant inhibition of APRs. These data suggest the impermeability or hypopermeability of the blood-brain barrier to the serum inhibitor(s) and lead us to propose the hypothesis that an APR-like phenomenon occurs **in vivo** in the central nervous system of HAM patients.

MS in Barbados

Multiple sclerosis has been diagnosed with a surprisingly high frequency in Barbados in the past decade (Sivarajan S, Evelyn SV, Fraser HS[102]). MS typically affects patients in temperate climes and has been reported with rarity in the Caribbean.[95] Sivarajan et al report with respect to Barbados, that

> there has been an eight-fold increase in the incidence of multiple sclerosis... over the last 25 years, and particularly the last decade (22 or 58% in the last six years).

In their discussion they cite several possible reasons for the increase in the incidence of MS, including increased risk through increased travel and exposure to northern environments, increased tourism with the compromise of function of MS patients in the hot climate, increased case finding due to increased interest in neurological disorders and improved referral and diagnostic practices, and overdiagnosis.

In the present study, 14 patients with MS consented to participate. Of these patients, 3 did not spend their childhood years in Barbados. With MS and TSP sharing many characteristics in common, there is some potential for misdiagnosis specifically with the chronic progressive form of MS which occurs in 15% of MS cases.[101] Both may have an

insidious onset and a slow progression with spasticity, hyperreflexia and leg weakness as major components of the disease. Supportive laboratory data (specifically examination of oligoclonal bands and increased production of immunoglobulin G) have been incorporated into the diagnostic criteria for MS published by Poser et al,[126] but even laboratory results may be unrevealing since oligoclonal bands in CSF fluid and abnormal cortical evoked responses may sometimes be seen in both disorders.[106,57]

Poser, Roman and Vernant[127] have suggested that before a diagnosis of MS be changed to HTLV-I-associated chronic myelitis at least two of the following six abnormalities be present: clinical or electrophysiologic involvement of peripheral nerve or muscle (rare in MS); the presence of oligoclonal bands in the serum (considered to be evidence against MS); the presence in blood or CSF of lymphocytes with multilobed nuclei (adult T-cell leukemia cells); a positive serologic test for syphilis; the presence of the sicca syndrome; the presence of pulmonary lymphocytic alveolitis.

In the present study, cases diagnosed as MS do not have a higher HTLV-I prevalence rate than the controls. Thus it is unlikely that the finding of a seemingly small number of TSP cases and a large number of MS cases relative to Martinique and Jamaica is due to misdiagnosis. Also, as noted of MS in Barbados by Sivarajan et al (unpublished)

> the increasing incidence and prevalence of MS in Barbados is intermediate between equatorial or tropical countries and the temperate, developed countries. The clinical pattern and the course have a similar distribution to that in the literature for northern countries -- i.e. 61% generalized compared to 50% in the literature, and 29% spastic (30-40% in the literature). There is no excess of the latter type as might occur in misdiagnosing a 'tropical spastic neuropathy' such as the Jamaican neuropathy as MS.

There is an excess of Caucasian MS patients (28.6%) in the present study as compared with the general population (4%), but this is not enough to explain the high MS rate. With the increased incidence of MS in Barbados, a tropical country with a population predominantly of African descent, and with the recent report of HTLV-I positive spastic paraparesis in patients of European, mixed Mestizo and Mapuches Indian descent in temperate Santiago, Chile,[55] the geographic and ethnic scope of these two diseases has expanded. This overlap may increase the uncertainty of many diagnoses unless rigid criteria are defined and adhered to. This is evidenced by a case report of an Haitian patient diagnosed with TSP but with magnetic resonance imaging (MRI) studies showing lesions in the white matter of the brain and spinal cord identical to those seen in MS.[96] As more physicians worldwide become aware of the widened distribution no doubt more 'atypical' cases of TSP and MS will be discovered.

HTLV-I serology in MS patients

The absence of HTLV-I antibody in sera of MS patients found in this study is similar to that in previous studies[85-89] and does not support a role of HTLV-I in the pathogenesis of MS. The screening tests were done using a commercially prepared ELISA kit and the positive sera confirmed by Western blot analysis using HTLV-I p-24 purified antigen, antibodies to which have been shown to correlate highly with HTLV-I infection.[97]

The lack of positivity must be interpreted with caution, however, since the commercially prepared ELISA has been criticized for lacking adequate sensitivity.[84,98] However Koike et al, using autoradiography to increase the sensitivity and a combination of Western blotting and autoradiography with recombinant p-24 to increase the specificity, failed to detect HTLV-I antibodies in sera of MS patients.[89] Most of these latter sera were also drawn when the patients were not in remission so as to avoid possible periods of low antibody levels as postulated by Koprowski.[84]

Lyme and Treponemal serology

As noted previously, there have been some reports of increased antibodies to **Treponema pallidum** and **Borrelia burgdorferi** in the sera of patients with TSP. The absence of serum antibodies to either of these two organisms in TSP or MS patients in the current study argues against a role for them

in the pathogenesis of these two disorders in Barbados. The presence of antibodies against both HTLV-I and **Treponema pallidum** in TSP cases in a recent report from the Dominican Republic[104] may be coincidental but the possibility must be entertained that HTLV-I infectivity or pathogenicity may be enhanced by a cofactor. **Treponema pallidum** and other non-specific agents may fulfill this role.

CONCLUSION

In conclusion, this study supports an etiological role for HTLV-I in cases diagnosed as TSP but not in cases diagnosed as MS in Barbados. **Treponema pallidum** and **Borrelia burgdorferi** do not seem to be associated with either of these two disorders as seen in Barbados. No other etiological factors are identified.

The finding of a relatively small number of TSP patients in Barbados as compared with Jamaica and Martinique and that of a surprisingly high incidence of MS in a tropical island with a predominantly black population are of interest. These cannot be explained on the basis of misdiagnosis of cases since HTLV-I serum antibodies were not found in cases diagnosed as MS. It is tempting to speculate that a separate agent, possibly a retrovirus related to, but distinct from HTLV-I, may be in some way associated with the HTLV-I-negative cases of TSP and the cases of MS in Barbados.

It is recognized that further work needs to be done in Barbados to determine the prevalence of TSP and to determined the significance of the findings of this study. Although HTLV-I is firmly established as the etiological agent of TSP there is a significant amount yet to be learned about the etiology of MS and the pathogenetic mechanisms of both TSP and

MS.

TABLES

Table 1.

Clinical diagnostic criteria for TSP (adapted from Roman et al[36])

1. A minimum of two of the following within 2 years of onset:
   - Increased urinary frequency, nocturia or retention and/or severe constipation and/or impotence (in men)
   - Leg cramps and/or low back pain
   - Weakness of the lower extremities within 6 months of onset of the disease
   - Complaints of numbness or dysesthesiae of the legs or feet
2. Absence of a history of relapses
3. Spasticity (clinical hypertonicity, hyperreflexia, clonus) of both legs, usually demonstrated by spastic gait.
4. Absence of a sensory level.

Table 2.

Clinical diagnostic criteria for MS[99]

Clinically definite:

- Consistent course
  - Relapsing, remitting course; at least two bouts separated by at least one month
  - Slow or stepwise progressive course for at least 6 months
- Documented neurological signs of lesions in more than one site, of brain or spinal cord white matter
- Onset of symptoms between 10 and 50 years
- No better neurologic explanations

Probable:

- History of relapsing, remitting symptoms but signs not documented and only one current sign currently associated with MS
- Documented single bout of symptoms with signs of more than one white matter lesion; good recovery, then variable symptoms and signs
- No better neurologic explanation

Possible:

- History of relapsing, remitting symptoms without documentation of signs
- Objective signs insufficient to establish more than one lesion of central white matter
- No better explanation

Table 3.

Characteristics of TSP and MS patients

	TSP (N=4)	MS (N=14)
Average age of onset		
by sex	M:45, F:31.5	M:34.6, F:28.1
overall	38.25 years	30.4 years
Range of onset age	22-47 years	19-39 years
Duration of disease	4-12 years (mean= 6.25)	1-16 years (mean= 8.2)
Female to male ratio	1:1	1.8 : 1
Lower limb weakness	4 (100%)	10 (71.4%)
Gait		
walks unaided	2 (50%)	9 (64.3%)
walks with support	2 (50%)	4 (28.6%)
cannot stand or walk	0 (0%)	1 (7.1%)
Lower limb cramps	0 (0%)	6 (42.9%)
Lower limb dysesthesiae	4 (100%)	7 (50%)
Lower back pain	3 (75%)	8 (57.1%)
Bladder dysfunction	2 (50%)	7 (50%)
Constipation	3 (75%)	4 (28.6%)
Blurred vision/diplopia	2 (50%)	12 (87.5%)
Course of disease		
Progressive/stable	3 (75%)	1 (7.1%)
Remissions/relapses	0 (0%)	10 (71.4%)
Uncertain	1 (25%)	3 (21.4%)

Table 4.

Travel, smoking and alcohol histories in TSP and MS cases and controls

	TSP		MS	
	Cases	Controls	Cases	Controls
Travel history*				
none	0 (0%)	1 (25%)	3 (21.4%)	7 (50%)
slight	3 (75%)	2 (50%)	3 (21.4%)	6 (42.9%)
moderate	1 (25%)	1 (25%)	2 (14.3%)	0 (0%)
heavy	0 (0%)	0 (0%)	5 (35.6%)	1 (7.1%)
Smoking history**				
none	2 (50%)	4 (100%)	9 (64.3%)	12 (85.7%)
slight	0 (0%)	0 (0%)	2 (14.3%)	0 (0%)
moderate	0 (0%)	0 (0%)	0 (0%)	0 (0%)
heavy	2 (50%)	0 (0%)	3 (21.4%)	2 (14.3%)
Alcohol history°				
none	1 (25%)	0 (0%)	5 (35.6%)	8 (57.1%)
slight	2 (50%)	2 (50%)	6 (42.9%)	3 (21.4%)
moderate	0 (0%)	1 (25%)	2 (14.3%)	0 (0%)
heavy	1 (25%)	1 (25%)	1 (7.1%)	3 (21.4%)

*Slight: 1-2 trips; moderate: 3-5 trips; heavy: > 5 trips.

**Slight: ≤ 2 cigarettes/day; moderate: 3-5/day; heavy: > 5/day.

°Slight: ≤ 2 drinks/week; moderate: 3-5/week: heavy: > 5/week.

Table 5.

Summary of serological results

	Case		Control		Relative
	TSP (n=4)	MS (n=14)	TSP (n=4)	MS (n=14)	(n=3)
Screening HTLV-I ELISA					
positive	3 (75%)	1 (7.1%)	2 (50%)	4 (28.6%)	1 (33.3%)
borderline	0 (0%)	4 (28.6%)	1 (25%)	2 (14.3%)	0 (0%)
Final positive HTLV-I ELISA°	3 (75%)	2 (14.3%)	2 (50%)	4 (28.6%)	1 (33.3%)
Positive HTLV-I Western blot	2 (50%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Positive TPHA	0 (0%)	1 (7.1%)	0 (0%)	0 (0%)	0 (0%)
Positive **B. Burgdoferi** ELISA	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

° After borderline samples were retested

Figure 1..

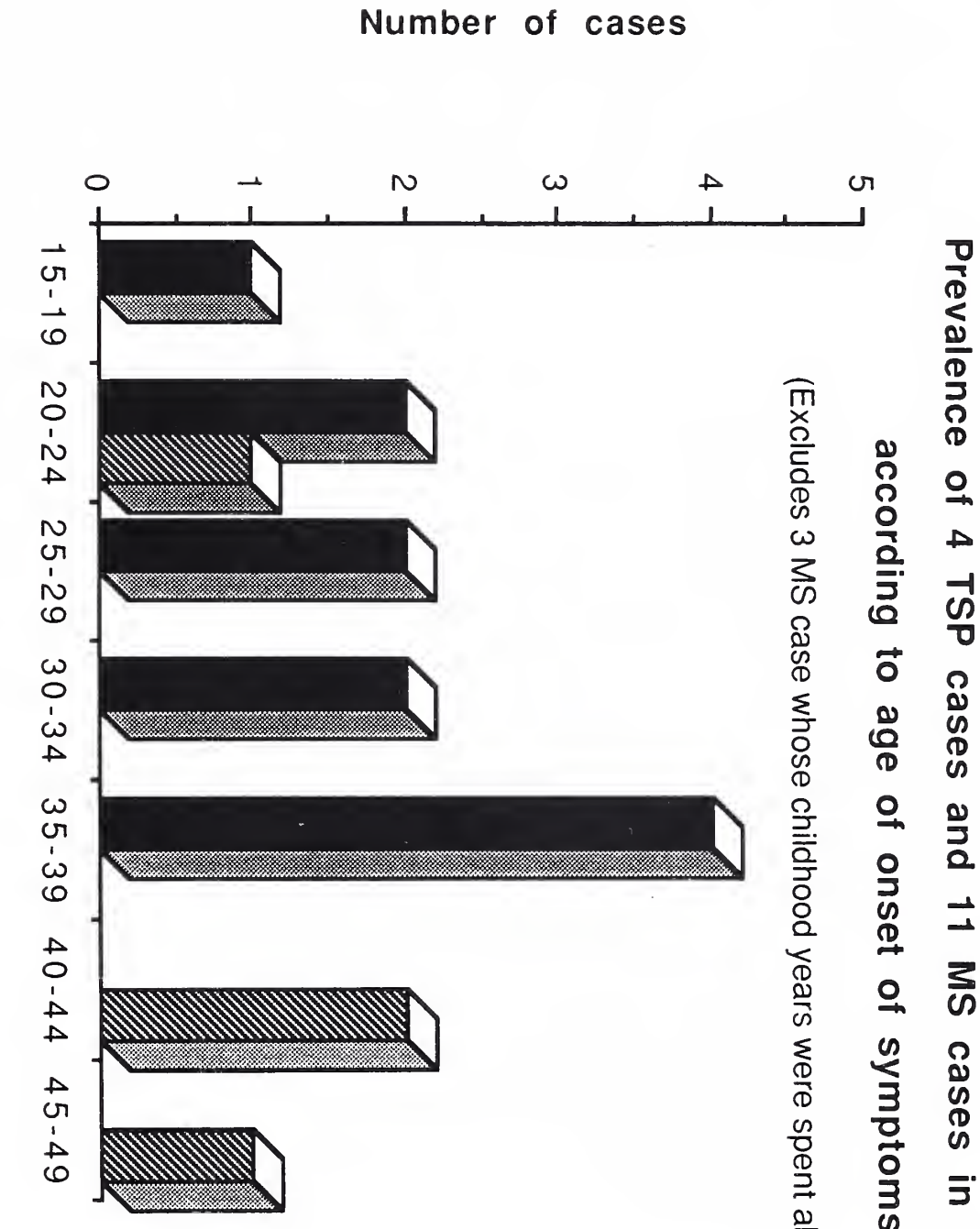
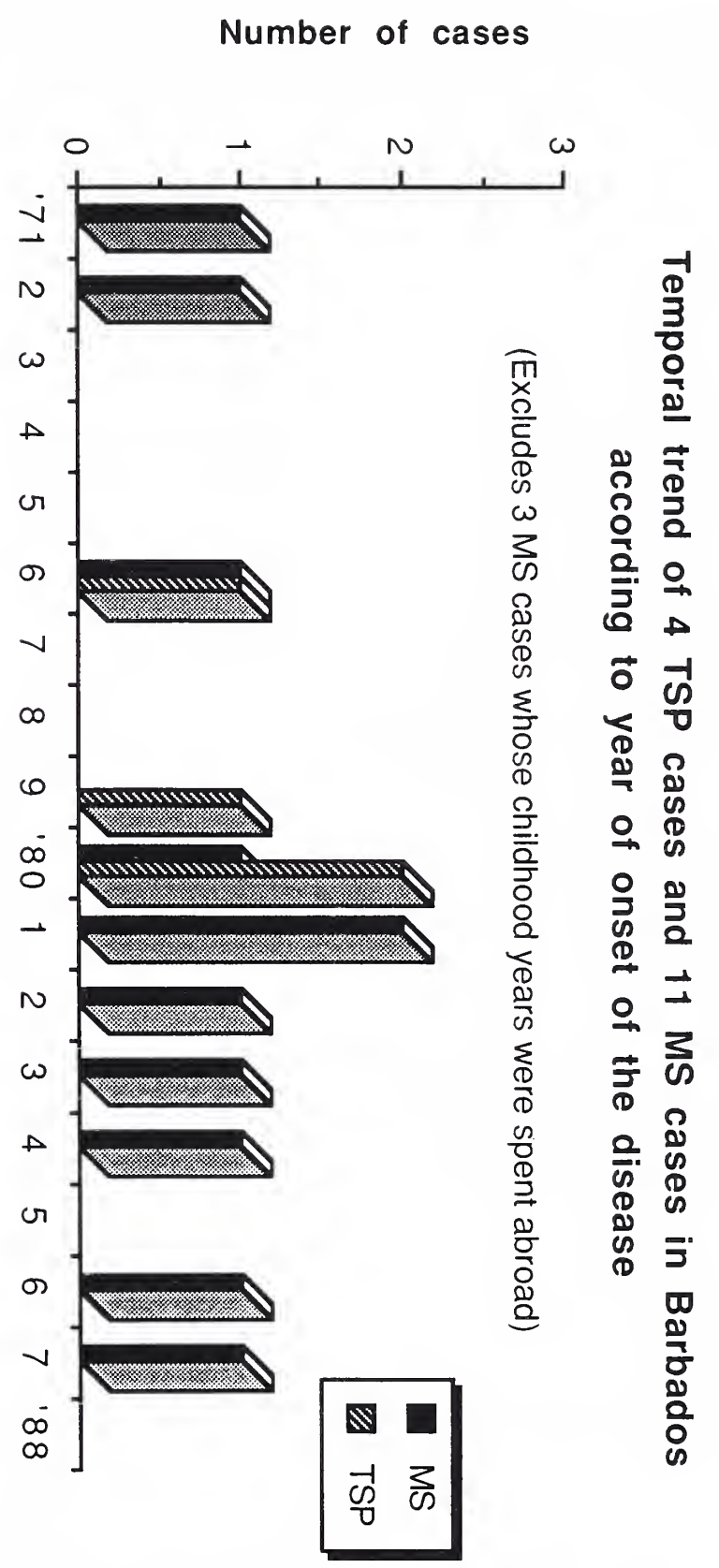
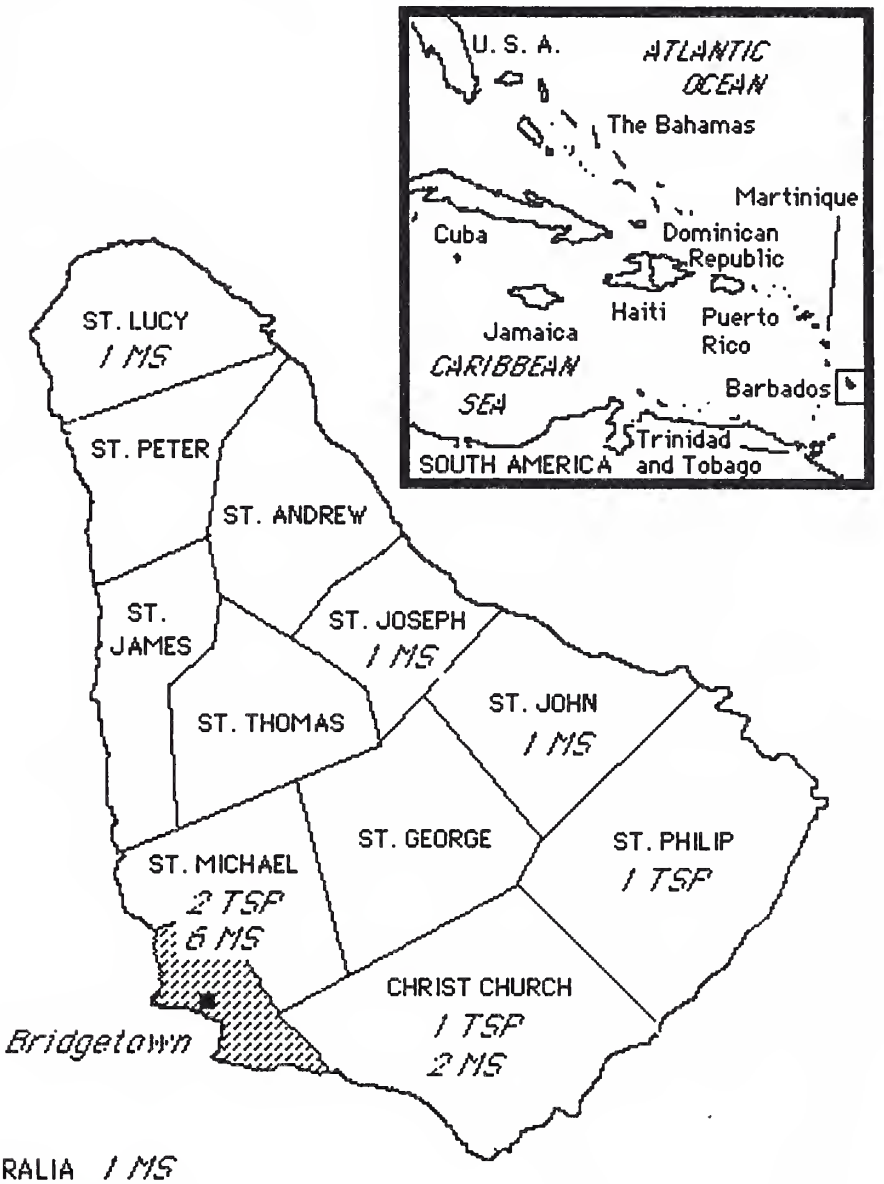

Prevalence of 4 TSP cases and 11 MS cases in Barbados
according to age of onset of symptoms
(Excludes 3 MS case whose childhood years were spent abroad)
Number of cases
0
1
2
3
4
5
15-19
20-24
25-29
30-34
35-39
40-44
45-49
Age (in years)
MS
TSP

Figure 2.


Temporal trend of 4 TSP cases and 11 MS cases in Barbados
according to year of onset of the disease
(Excludes 3 MS cases whose childhood years were spent abroad)
Number of cases
0
1
2
3
'71
2
3
4
5
6
7
8
9
'80
1
2
3
4
5
6
7
'88
Year
MS
TSP

RESIDENCE AT ONSET OF DISEASE OF 4 TSP AND 14 MS PATIENTS



AUSTRALIA 1 MS
CANADA 1 MS
UNITED KINGDOM 1 MS

MAP OF BARBADOS

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Appendix 1

INFORMATION SHEET

You are invited to participate in a study of chronic neurological diseases. The purpose of this study is to identify certain factors that may cause these diseases. You have been chosen for the study either because you or one of your relatives has such a disease, or because you are free from it.

Your participation will involve giving two small tubes of blood and being interviewed. If you are not a hospitalized patient you will be required to make a hospital visit which should take about one hour. Drawing blood may hurt slightly and you may get a small bruise.

Various tests will be done on your blood. Your name will not be used to identify your sample and will not be used in records of the study. You will be identified only by a code number and your signature on this sheet will not be required. Because we will not have your name, you will not receive results of the tests.

This study may be of no direct benefit to you but it may help us find out more about what causes certain neurological diseases and how they may be prevented.

You are free to chose not to participate. You may not be familiar with some of the terms used on this sheet. Please feel free to ask about them or about anything else you are not sure of. You are welcome to take as much time as you need to decide.

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Signature of Principal Investigator Telephone

Appendix 2

PREPARATION OF SAMPLES FOR ANTIBODY TESTING

- Collect blood (~10 ml) in a vacutainer
- Allow blood to clot by standing for at least 6 hours at room temperature
- Centrifuge at 2000 rpm for 5 minutes
- Remove ~1 ml of serum (supernatant) and freeze in vials for treponemal antibody testing
- Freeze remainder of serum in serum tube for future antibody tests

VDRL TEST REAGENTS
BACTO VDRL ANTIGEN
BACTO VDRL TEST CONTROL SERUM SET

Bacto VDRL Antigen is used for the VDRL Slide (qualitative, quantitative) Test procedures as described in the *Manual of Tests for Syphilis 1969*

VDRL antigen, along with others, is defined as a nontreponemal antigen.[2] As discussed in *Syphilis a Synopsis*, reactive nontreponemal tests confirm the diagnosis in the presence of early or late lesion syphilis, offer a diagnostic clue in latent subclinical syphilis, are an effective tool for detecting cases in epidemiologic investigations and are superior to the treponemal test for following the response to therapy.[1]

Nontreponemal antigen tests are not entirely specific for syphilis nor do they have satisfactory sensitivity in all stages of syphilis. Whenever the results of a nontreponemal antigen test disagree with the clinical impression, a treponemal antigen test such as the FTA-ABS[1] or HATTS should be performed.

TEST SUMMARY

VDRL antigen is a cardiolipin-lecithin-cholesterol antigen. Spinal fluid or inactivated serum and VDRL antigen emulsion prepared from VDRL antigen and VDRL buffered saline are mixed with the aid of a rotating machine for a prescribed period of time. During the mixing time the cardiolipin-lecithin-coated cholesterol particles flocculate in the presence of an antibody-like substance (reagin) that is present in sera from syphilitic persons and occasionally in sera of persons with other acute and chronic conditions.[1]

Quantitative testing is performed first on all specimens. Each serum that produces a Reactive, Weakly Reactive or Rough, Nonreactive result in the qualitative VDRL Slide Test is retested quantitatively, to an end point titer.

REAGENTS

Bacto VDRL Antigen contains 0.03% cardiolipin and 0.9% cholesterol dissolved in absolute alcohol with sufficient purified lecithin (0.21 ± 0.01%) to produce standard reactivity. It is prepared according to the directions given by Harris, Rosenberg and Riedel.[3] Cardiolipin and lecithin are prepared according to directions given by Pangborn.[4] Cardiolipin is prepared under license from the New York State Department of Health.

Bacto VDRL Buffered Saline (pH 6.0 ± 0.1) for the preparation of the VDRL Antigen emulsion contains:

0.037 g secondary sodium phosphate, anhydrous, (A.C.S.)
0.170 g primary potassium phosphate, (A.C.S.)
10.0 g NaCl, (A.C.S.)
1.0 liter distilled water

Bacto VDRL Buffered Saline is a slight modification of the formulation given by Harris, Rosenberg and Riedel.[3] The formaldehyde has been omitted.

Bacto Nontreponemal Antigen Reactive Serum is a human serum standardized to provide a reactive reading when tested according to the USR or VDRL test procedures.

Bacto VDRL Weakly Reactive Serum is a human serum standardized to provide a weakly reactive reading when tested according to the VDRL test procedure.

Bacto Nontreponemal Antigen Nonreactive Serum is a human serum standardized to provide a non reactive reading when tested accoroing to the USR or VDRL test procedure.

Bacto Aliquant Vial is an empty serum vial fitted with a split stopper and screw cap. It is for receiving and storing aliquants of rehydrated control sera in amounts sufficient for one testing period.

Precautions

Each human serum used in preparing the control serum contained in Bacto VDRL Test Control Serum Set has been screened for the presence of hepatitis B surface antigen. Only sera exhibiting a negative reaction are used in preparing these reagents. As an additional precaution, the user should employ proper and aseptic techniques in handling all human sera.

Before performing this test the user should be familiar with the contents of sections "Introduction" and "Appendix" of the *Manual of Tests for Syphilis 1969*.

For In Vitro Diagnostic Use.

Rehydration and Storage

On receipt, store the package of Bacto VDRL Antigen and Bacto VDRL Buffered Saline at room temperature. When ready to use the Bacto VDRL Antigen, pour the entire contents of the ampul into the Bacto VDRL Antigen Storage Vial provided in the box. Cap the bottle tightly and store it in the dark at 15 - 30 C. Withdraw antigen as required. After the bottle of Bacto VDRL Buffered Saline is opened, it should be stored at 2 - 8 C between the periodic withdrawals of saline for preparing the emulsion. Discard the Buffered Saline if mold or turbidity appears.

The three control sera contained in Bacto VDRL Test Control Serum Set are stored at 2 - 8 C. Rehydrate the sera with 3 ml sterile distilled or deionized water. Transfer rehydrated sera that are in excess of the first day's use into a Bacto Aliquant Vial and maintain at −20 C for up to one month. Do not thaw and refreeze. The expiry date of the aliquant is not to exceed the expiry date of the original container.

SPECIMEN COLLECTION AND PREPARATION

1. Obtain blood specimen and allow to clot. Separate serum from the clot by centrifugation (1500 - 2000 rpm for 5 minutes).
2. Inactivate serum specimen at 56 C for 30 minutes.
3. Centrifuge sera containing particulate debris prior to testing.
4. Reinactivate at 56 C for 10 minutes, all sera to be tested subsequent to 4 hours after original inactivation.
5. Cool all sera to room temperature (23 - 29 C) prior to testing.

PROCEDURE

Procedures as described here are essentially those described in the *Manual of Tests for Syphilis*, US Department of Health, Education and Welfare.[2]

Materials Provided

Bacto VDRL Antigen with Bacto VDRL Buffered Saline
Bacto VDRL Test Control Serum Set

Materials Required But Not Provided

Rotating machine, adjustable to 180 rpm circumscribing a circle ¾" in diameter on a horizontal plane
Hypodermic needles, without bevels - for serum test 18 gauge, for spinal fluid test 21 or 22 gauge
2" x 3" glass slide with 12 paraffin or ceramic rings, approximately 14 mm in diameter for serum test
Agglutination slide approximately 2" x 3" with concavities measuring 16 mm in diameter and 1.75 mm in depth for spinal fluid
Syringe, Luer type 1 or 2 ml
VDRL antigen emulsion bottles, 30 ml round, glass stoppered, narrow mouth, approximately 35 mm in diameter with flat inner bottom surface
Serological pipettes, 5.0 ml, 1.0 ml and 0.2 ml
Water bath 56 C
Light microscope with 10X ocular and 10X objective
Distilled water
0.9% saline solution
10% saline solution
Absolute alcohol and acetone
Interval timer
pH meter

Preparation of Specific Glassware

Syringes, with needles and emulsion bottles. Wash by hand in the following manner:

1. Pre-rinse with tap water.
2. Soak and wash thoroughly in a glassware detergent solution.
3. Rinse with tap water 6 - 8 times.
4. Rinse with unused distilled or demineralized water.
5. Rinse with absolute alcohol.
6. Rinse with acetone.
7. Air dry until acetone odor is completely eliminated.
8. Remove needles from syringes for storage.

Ceramic ringed slides:

1. Pre-rinse with tap water.
2. Wash with a glassware detergent solution.
3. Rinse with tap water 3 - 4 times.
4. Rinse with unused distilled or demineralized water.
5. Wipe dry with clean lint free cloth. If cleaned slides do not allow serum to spread evenly within inner surface of circle, treat the slides as follows:
6. Scrub the slides with a non-scratching cleanser.
7. Dry and polish with a clean lint-free cloth.

NOTE: Avoid prolonged soaking of ceramic-ringed slides in detergent solution since the ceramic rings will become brittle and flake off.

Preparation of VDRL Antigen Emulsion

1. Pipette 0.4 ml of VDRL buffered saline directly to the bottom of a VDRL antigen emulsion bottle.
2. Gently tilt bottle so that VDRL buffered saline will cover entire inner surface of bottom.
3. From the lower half of a 1.0 ml pipette graduated to the tip, add 0.5 ml of VDRL antigen as follows:
   a. Keep the pipette in the upper 1/3 area of the bottle. Do not let it touch the saline.
   b. While rotating the bottle around a circle approximately 2" in diameter, allow the antigen to be added dropwise to the VDRL buffered saline.
   c. Allow approximately 6 seconds to add antigen, then blow out remaining antigen from pipette.
4. Continue rotation of bottle for 10 seconds.
5. Add 4.1 ml of VDRL buffered saline to bottle, allowing the VDRL buffered saline to flow down the side of the bottle.

6. Place the glass stopper in the bottle and shake the bottle from bottom to top and back approximately 30 times in 10 seconds.
7. Let VDRL antigen emulsion stand without further disturbance for 10 minutes.
8. VDRL antigen emulsion is ready to use. Swirl gently prior to using. Antigen emulsion may be used during one day.

NOTE: Check pH of VDRL buffered saline prior to preparing VDRL antigen emulsion. VDRL buffered saline outside the range of pH 6.0 ± 0.1 should be discarded.

Allow VDRL antigen and VDRL buffered saline to reach 23 - 29 C before preparing VDRL antigen emulsion.

Use only emulsion bottles with flat inner bottom surfaces that allow the initial VDRL buffered saline to evenly cover the inner bottom surface of the bottle. If the VDRL buffered saline beads or does not spread evenly to cover the bottom of bottle, rewash bottle.

For reproducible results the VDRL antigen emulsion must be checked daily for proper reactivity by testing with Bacto VDRL Test Control Serum Set. Only those VDRL emulsions producing the established reactivity pattern of the control serum should be used.

NOTE: Mix VDRL emulsion prior to using in test by gentle swirling of emulsion bottle. Do not mix emulsion by forcing back and forth through the syringe and needle.

Calibration of Delivery Needles without Bevels

1. With 18 gauge needle attached to 1.2 ml Luer type syringe, fill syringe with antigen emulsion.
2. Hold the syringe with needle in a vertical position and expel the emulsion dropwise into the emulsion bottle.
3. Count the number of drops delivered per ml of emulsion. The needle should deliver 60 drops ± 2 drops per ml of antigen emulsion.
4. Adjust drops per ml by either narrowing the open end of the needle to allow more drops per ml to be delivered or opening the end of the needle to allow fewer drops per ml to be formed.

Standardization of VDRL Antigen Emulsion

1. Rehydrate each control serum with 3 ml distilled water and rotate in an end-over-end motion to dissolve completely.
2. Inactivate serum dilutions at 56 C for 30 minutes.
3. Cool to room temperature.
4. Perform qualitative VDRL Slide Test on all serum dilutions with VDRL antigen emulsion. Test serum dilutions within 1 hour after inactivation.
5. Read test immediately as follows:

Reactive Serum	Medium or large clumps
Weakly Reactive Serum	Small clumps
Nonreactive Serum	No clumping with complete dispersion of emulsion particles

Select a VDRL antigen emulsion which produces the specified reactivity pattern with each control serum.

Perform the VDRL Slide Test procedures within the temperature range 23 - 29 C since lower temperatures decrease test reactivity and higher temperatures increase test reactivity.

VDRL SLIDE QUALITATIVE TEST ON SERUM

1. With 1.0 ml pipette, mix inactivated serum several times, then using same pipette add 0.05 ml of serum into 1 ring of the paraffin-ringed or ceramic-ringed glass slide. Do not use glass slides with concavities, wells, or glass rings for the VDRL Qualitative or Quantitative Test Procedures on serum. Do not use glass slides with ceramic rings that have begun to flake off or that permit spillage of the antigen serum mixture when slides are rotated at the prescribed speed.
2. Spread the serum with a circular motion of the pipette tip so that the serum covers the entire inner surface of the paraffin or ceramic ring. Use only clean slides that allow serum to evenly cover entire surface within ceramic or paraffin ring.
3. While holding the syringe with an 18 gauge needle in a vertical position, carefully add 1 drop of antigen (1/60 ml) to the serum. Do not allow needle to touch the serum.
4. Place the paraffin- or ceramic-ringed slides containing the serum and antigen on a rotator and rotate for 4 minutes at 180 rpm circumscribing a ¾" diameter circle.
5. Prevent evaporation of slides during rotation in a dry climate by covering slides with a moisture chamber (box lid containing a moistened blotter).
6. Observe ringed slides microscopically with a 10X ocular and a 10X objective immediately after rotation.
7. Read test as follows:

Medium and large clumps	R	Reactive
Small clumps	W	Weakly Reactive
No clumping or very slight roughness	N	Non-Reactive

NOTE: Quantitatively retest each serum producing Weakly Reactive or rough Non-Reactive results when tested qualitatively since prozone reactions are encountered occasionally.

VDRL SLIDE QUANTITATIVE TEST ON SERUM

1. Prepare a two-fold serial dilution of inactivated serum in 0.9% saline as follows: (1:2, 1:4, 1:8, 1:16, 1:32).
2. Test each serum dilution using Qualitative Test Procedure.
3. Report results in terms of the greatest serum dilution that produces a Reactive (not Weakly Reactive) result in accordance with the following examples:

UNDILUTED SERUM (1:1)	1:2	1:4	1:8	1:16	1:32	REPORT
R	W	N	N	N	N	Reactive, undiluted only or 1 dilution
R	R	W	N	N	N	Reactive, 1:2 dilution or 2 dilutions
R	R	R	W	N	N	Reactive, 1:4 dilution or 4 dilutions
W	W	R	R	W	N	Reactive, 1:8 dilution or 8 dilutions
Nitrought W		R	R	R	N	Reactive, 1:16 dilution or 16 dilutions
W	N	N	N	N	N	Weakly Reactive undiluted only or 0 dilutions

If Reactive results are obtained through dilution 1:32, prepare further two-fold serial dilutions in 0.9% saline (1:64, 1:128 and 1:256) and retest using Qualitative Test Procedure.

VDRL SLIDE TESTS ON SPINAL FLUID

VDRL antigen and VDRL buffered saline may be used for spinal fluid testing in accordance with the VDRL Slide Tests on spinal fluids as stated on pages 40, 41 and 42 in the *Manual of Tests for Syphilis 1969*.[1]

LIMITATIONS OF THE PROCEDURE

VDRL antigen slide tests are non-specific tests for syphilis. All sera and spinal fluids expressing a Reactive or Weakly Reactive VDRL test in the absence of clinical evidence of syphilis should be subjected to other syphilis serology tests such as FTA-ABS[2,3] or HATTS.

Reactive or Weakly Reactive VDRL test results should not be considered as conclusive evidence that the patient is syphilitic. Conversely a Non-reactive VDRL test by itself does not rule out the diagnosis of syphilis.

SPECIFIC PERFORMANCE CHARACTERISTICS

In various stages of untreated syphilis, the likelihood of obtaining a Reactive VDRL is as follows:

Stage of Untreated Syphilis	% Reactive
Primary	76
Secondary	100
Early Latent	95
Late Latent	72
Late (Tertiary)	70

REFERENCES

1. U.S. Department of Health, Education, and Welfare, National Communicable Disease Center, Venereal Disease Program. 1964. Manual of tests for syphilis. U.S. Government Printing Office: Washington, D.C.
2. U.S. Department of Health, Education, and Welfare, National Communicable Disease Center, Venereal Disease Program. 1969. Manual of tests for syphilis. U.S. Government Printing Office: Washington, D.C.
3. U.S. Department of Health, Education, and Welfare, National Communicable Disease Center, Venereal Disease Program. 1968. Syphilis, a synopsis. U.S. Government Printing Office: Washington, D.C.
4. Kraus, S.J., J.R. Haserick, and M.A. Lantz. 1970. Fluorescent treponemal antibody-absorption test reactions in lupus erythematosis, atypical beading pattern and probable false-positive reactions. N. Engl. J. Med. 282:1287-1290.
5. Harris, A., A.A. Rosenberg, and L.M. Riedel. 1946. A microflocculation test for syphilis using cardiolipin antigen. J. Ven. Dis. Infor. 27:169-174.
6. Pangborn, M.C. 1941. A new serologically active phospholipid from beef heart. Proc. Soc. Exp. Biol. and Med. 48:484-486.
7. Pangborn, M.C. 1944. Acid cardiolipin and an improved method for the preparation of cardiolipin from beef heart. J. Biol. Chem. 153:343-348.
8. Pangborn, M.C. 1945. A simplified preparation of cardiolipin, with a note on purification of lecithin for serologic use. J. Biol. Chem. 161:71-82.

PACKAGING

Bacto VDRL Antigen with Bacto VDRL Buffered Saline	0388-56-5 0388-49-5	5 ml 10 x 0.5 ml
Bacto VDRL Test Control Serum Set	3520-32-7*	1 set

*Store at 2 - 8 C

FLUORESCENT TREPONEMAL ANTIBODY ABSORBED (FTA-ABS) TEST REAGENTS

Bacto - FTA Antigen	Code 2344
Bacto - FTA Serum Reactive	Code 2439
Bacto - FTA Sorbent Control	Code 3266
Bacto - FTA Sorbent	Code 3259
Bacto - FA Human Globulin Antiglobulin (Rabbit)	Code 2449
Bacto - FA Mounting Fluid	Code 2329
Bacto - FA Buffer Dried	Code 2314
Tween 80®	Code 3118

The fluorescent treponemal antibody absorbed test performed with the above listed reagents is an indirect FA procedure for detecting human antibody against *Treponema pallidum*, the causative agent of syphilis. Since its introduction in 1957 by Deacon, Falcone and Harris[1], the FTA Test has increased in popularity. However, certain difficulties with respect to sensitivity versus specificity have been encountered.

In its original form the test utilized a 1:5 dilution of patient's serum. At this dilution, a large number of false positive reactions were encountered. There seemed to be a cross reaction of the treponemal antigen with antibodies to group antigens which are common to all treponemes. The titer of serums containing the non-specific group antibodies ranged from 1:5 - 1:100.

In 1960 Deacon, Freeman and Harris[2] introduced a modification of the FTA Test. This procedure was called the FTA-200 Test and utilized a 1:200 dilution of patient's serum. By increasing the dilution of the serum, the non-specific antibodies were diluted beyond their titer and could no longer interfere with the test. However, by testing a high dilution of serum, the sensitivity of the test is decreased in that the low titered primary cases of syphilis are not detected. This has been substantiated by Eng, Nielsen, Wercide and Wilkinson[3,4].

It has been the trend since the introduction of the FTA-200 Test to return to the 1:5 dilution and eliminate the non-specific antibodies in the serum. Deacon and Hunter[5] demonstrated that by using appropriate absorption procedures the reactivity of non-specific antibodies can be eliminated or blocked. This absorptive procedure has developed into an improved test, the FTA-ABS Test[6].

Treponemal Antigen Tests such as the FTA-ABS Test are used primarily as confirmatory tests in diagnostic problem cases; e.g. patients in which the clinical, historical or epidemiological evidence of syphilis is equivocal. The FTA-ABS Test is more sensitive than the VDRL in Primary Syphilis and Late Latent and Tertiary Syphilis.

The persistant reactivity of the FTA-ABS Test to a treated case of syphilis, sometimes for life, minimizes its use for following the response to therapy as well as making it unreliable for detecting new untreated cases in epidemiologic investigations.

REAGENTS

Bacto - FTA Antigen is a desiccated, standardized killed suspension of the Nichols strains of *T. pallidum*. Unreconstituted antigen is stored at 2-8 C. To rehydrate, add 1 ml sterile distilled water per vial and rotate to effect complete solution. The antigen suspension should be thoroughly mixed with a disposable pipette and rubber bulb. Drawing the suspension into and expelling from the pipette 8 - 10 times, will break the treponemal clumps and insure an even distribution of treponemes.

Treponemal antigen smears may be prepared in the following manner:

1. On clean grease free slides inscribe 2 circles 1 cm in diameter with a diamond point stylus. Remove any free glass particles by wiping slides with a piece of clean gauze.
2. Within the inscribed circles, smear 1 loopful of Bacto - FTA Antigen using a standard 2 mm, 26 gauge, platinum wire loop. Allow to dry at least 15 minutes at room temperature.
3. Immerse the dry slides into acetone for 10 minutes. This fixes the treponemes to the slide so they will not wash off during the rinsing procedures later in the test. Not more than 50 slides should be fixed with 200 ml of acetone.
4. It is recommended that a freshly rehydrated bottle of Bacto - FTA Antigen be used in its entirety for making Treponemal antigen smears on the same day. These slides may then be stored at -20 C or lower after acetone fixation. The frozen slides are usable as long as satisfactory results are obtained with the controls. Thawed slides should not be refrozen.

Bacto - FTA Serum Reactive is a standardized, desiccated human serum for use as a positive control in the FTA-ABS Test. To rehydrate this serum, add 5 ml or 1 ml good quality distilled water as indicated by the package size. When diluted 1:5 in FA buffer or sorbent a 4+ fluorescence will be obtained. To obtain a 1+ fluorescence this serum must be diluted further in Bacto - FA Buffer. This dilution will vary with each lot and is provided in the instructions received with each vial. The undiluted serum may be dispensed in 0.4 ml aliquots and frozen until needed. Do not refreeze thawed aliquots. This serum should be heated at 56 C for 30 minutes before using.

Bacto - FTA Sorbent is a standardized extract of the non-pathogenic Reiters treponeme containing group Treponemal antigens. It is designed to be used in the FTA-ABS Test for blocking the action of non-specific antibodies in the serum. The sorbent comes ready to use and is supplied in vials of 5 ml and boxes of 6 x 5 ml. It is recommended that Bacto - FTA Sorbent be stored either at 2 - 8 C or in the frozen state at -20 C after it is received in the laboratory. A 1:5 dilution of the serum is made by placing 0.05 ml of heated serum into 0.2 ml sorbent and mixing thoroughly.

Bacto - FTA Sorbent Control is a desiccated standardized human serum demonstrating at least a 2+ fluorescence in the FTA-ABS Test at a dilution in Bacto - FA Buffer of 1:5. At a dilution of 1:5 in Bacto - FTA Sorbent no reactivity will be observed. This serum is supplied in 6 x 0.5 ml vials and is good for 1 week after rehydration if refrigerated at 2-8 C or longer if stored in the frozen state.

Tween 80®

A 2% solution of Tween 80 may be prepared as follows:

1. Heat the bottle of Tween 80 and 98 ml Bacto - FA Buffer in a 56 C water bath until both solutions are at 56 C.
2. Using a 2 ml pipette graduated to the tip, add 2 ml Tween 80 to the Bacto - FA Buffer and rinse the pipette thoroughly.
3. The 2% solution should have a pH of 7.0 - 7.2. The solution keeps well in the refrigerator at 2 - 8 C, but should be discarded if the pH changes or if a precipitate is noticed.

Bacto - FA Human Globulin Antiglobulin (Rabbit) is a fluorescein conjugated anti-human globulin designed for use in the FTA-ABS Test. It is used as an indicator system to detect the presence of human syphilitic antibodies on the treponemal antigen. It is a desiccated product and is supplied in 5 ml and 1 ml quantities. In rehydration, use the amount of good quality distilled water indicated by the package size. The rehydrated conjugate may be stored in the refrigerator at 2 - 8 C or in 0.4 ml aliquots at -20 to -40 C.

Each vial is supplied with a dilution titer. This titer has been confirmed in several laboratories. With each new lot of conjugate used, the titer should be confirmed with the fluorescent assembly in use at the time. This may be done in the following manner:

1. Prepare serial dilutions of the conjugate with the 2% Tween 80 solution. The serial dilutions should include the titer specified on the vial.
2. Each conjugate dilution is tested with the Bacto - FTA Serum Reactive diluted 1:5 in Bacto - FA Buffer and a non-specific staining control (conjugate + antigen).
3. A known conjugate is set up with the Bacto - FTA Serum Reactive (4+), a minimally reactive (1+) serum, and a non-specific staining control to control reagents and test conditions.
4. The dilution to be used in the test is taken as 1 doubling dilution lower than the 4+ end point (the highest dilution of conjugate giving a 4+ fluorescence with the 4+ control serum).

For In Vitro Diagnostic Use.

PREPARATION of the SPECIMEN

Test and control serums should be heated at 56 C for 30 minutes before being tested. Previously heated serum should be reheated for 10 minutes on the day of testing.

PROCEDURE

Procedure steps are taken essentially from Pub. Hlth. Serv., Pub. No 411, 1969[6]

Materials provided:
- Bacto - FTA Antigen
- Bacto - FTA Serum Reactive
- Bacto - FTA Sorbent Control
- Bacto - FTA Sorbent
- Tween 80
- Bacto - FA Human Globulin Antiglobulin (Rabbit)
- Bacto - FA Mounting Fluid
- Bacto - FA Buffer, Dried

Materials required but not provided:
- Diamond point stylus (not needed if pre-etched slides are used)
- Plain slides or 1 x 3 inch frosted end approximately 1 mm thick or slides with circles already inscribed
- Cover slips, No. 1, 22 mm square
- 2 mm, 26 gauge, platinum wire loop
- Acetone
- Distilled water
- Water bath at 56 C
- Interval timer
- Staining dish with removable slide carriers
- Moisture chamber
- 0.2 ml serological pipette
- 5 ml serological pipette
- 1 ml serological pipette
- Bibulous paper
- Applicator sticks
- Fluorescent microscope assembly

1. Identify previously prepared slides to correspond to the serums and control serums to be tested.
2. Before using, slides taken from the freezer must be completely thawed and thoroughly dry.
3. Prepare the proper dilutions for the 4+ control, the 1+ control, the non-specific serum controls and for the unknown serums to be tested. The 1:5 dilutions may be prepared by adding 0.05 ml serum (using a 0.2 ml pipette graduated to the tip and measuring from the bottom) to 0.2 ml sorbent or Bacto - FA Buffer, whichever is required. Mix the dilution no less than 8 times. All unknown serums are diluted in FTA Sorbent only.
4. Do not dilute the control and unknown serums more than 30 minutes before they are to be placed on the antigen smears.

   Controls
   a. Reactive 4+ Control
      Bacto - FTA Serum Reactive diluted 1:5 in Bacto - FA Buffer and in Bacto - FTA Sorbent. A 4+ fluorescence should be observed with both dilutions.
   b. Minimally Reactive (1+) Control
      A dilution of Bacto - FTA Serum Reactive to give a 1+ fluorescence. This control is used as a reading standard to indicate the minimal degree of fluorescence that can be reported as reactive.
   c. Non-Specific Serum Control
      1. Antigen smear treated with 0.03 ml of Bacto - FA Buffer.
      2. Antigen smear treated with 0.03 ml of Bacto - FTA Sorbent.

   Test runs in which the proper control results were not obtained are considered unsatisfactory and should not be reported.

5. Cover each identified antigen smear with the corresponding serum dilution and the proper non-specific staining controls with Bacto - FA Buffer or Bacto - FTA Sorbent. Make certain the entire smear is covered. The shaft of an applicator stick held parallel to the surface of the smear is an effective tool for this purpose.
6. Place the slides into a moist chamber to prevent evaporation and incubate at 35 - 37 C for 30 minutes.
7. Place the slides in a slide carrier and rinse them in running Bacto - FA Buffer for 5 seconds and then soak in Bacto - FA Buffer for 5 minutes. Agitate the slides by dipping them in and out of the buffer 10 times. Repeat the soaking and agitation process for another 5 minutes in fresh Bacto - FA Buffer.
8. Rinse the slides in running distilled water for 5 seconds and blot dry with bibulous paper.
9. Dilute the conjugate to its working titer using FA Buffer containing 2% Tween 80.
10. Cover each smear with approximately 0.03 ml diluted conjugate. Make certain the entire smear is covered.
11. Repeat steps 6, 7 and 8.
12. Mount slides immediately using a small drop of Bacto - FA Mounting Fluid and a cover slip. Be careful not to entrap air bubbles in the mounting fluid between the slide and cover slip.
13. Examine slides microscopically as soon as possible using mercury arc illumination and a high power dry objective. A combination of BG 12 exciting filter, not greater than 3 mm thickness and OG 1 barrier filters or their equivalents has been found to be satisfactory. Slides may be stored in a darkened room and read within 4 hours.
14. Non-reactive smears should be checked using illumination from a tungsten light source in order to verify the presence of treponemes.

RESULTS

Using the 1+ control serum as a reading standard, record the intensity of fluorescence and report as follows:

READING	INTENSITY OF FLUORESCENCE	REPORT
2+ – 4+	Moderate to strong	Reactive
1+	Equivalent to 1+ control	Reactive
less than 1+	Weak but definite	Borderline
–	None or vaguely visible	Non-reactive

Retest all specimens with a fluorescence of 1+ or less. When a specimen initially read as 1+ gives a 1+ or better fluorescence, it is reported as reactive. All other results are reported as borderline. It is not necessary to retest non-reactive specimens.

LIMITATIONS of the PROCEDURE

There is a degree of difficulty in determining whether a reading is weak or vaguely visible. The ability to make this distinction is critical since a non-reactive (vaguely visible to none) serum is not retested.

The test cannot be used to follow the response to therapy nor can it be relied upon to detect new, untreated cases in epidemiological investigations.

SPECIFIC PERFORMANCE CHARACTERISTICS

In various stages of untreated syphilis the likelihood of obtaining a reactive FTA-ABS is as follows:[7]

STAGE of UNTREATED SYPHILIS	% REACTIVE
Primary	86
Secondary	100
Early latent	99
Late latent	96
Late (Tertiary)	97

REFERENCES

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2. *Ibid.*, 103:827,1960
3. Wld. Hlth. Org. Document WHO/VDT/314 WHO/VDT/RES/29, Mar. 1963
4. Proc. Royal Soc. Med., 56:478,1963
5. Proc. Soc. Exp. Biol. and Med., 110:352,1962
6. Publ. Hlth. RPTS., 79:410,1964
7. Publ. Hlth. Service Publ. No. 1660, 1968
8. Publ. Hlth. Service Publ. No. 411, 1969

PACKAGING

Bacto - FTA Antigen	2344-50-0*	1 ml
Bacto - FTA Serum Reactive	2439-50-6*	1 ml
	2439-56-0*	5 ml
Bacto - FTA Sorbent	3259-56-5*	5 ml
	3259-57-4*	6 x 5 ml
Bacto - FTA Sorbent Control	3266-49-6*	6 x 0.5 ml
Bacto - FA Human Globulin Antiglobulin (Rabbit)	2449-50-4*	1 ml
	2449-56-8*	5 ml
Bacto - FA Mounting Fluid	2329-57-2*	6 x 5 ml
Bacto - FA Buffer, Dried	2314-33-8	6 x 10 g
	2314-15-0	100 g
Tween 80®	3118-15-6*	100 g

* Store 2 - 8 C.
® Atlas Chemical Co.

DIFCO LABORATORIES
1333 DETROIT MICHIGAN USA August 1974

TPHA TEST KIT

FD101 FD106
FD102 FD103

A HEMAGGLUTINATION TEST FOR SYPHILIS USING ERYTHROCYTES SENSITIZED WITH PATHOGENIC TREPONEMA PALLIDUM EXTRACTS

MICRO HEMAGGLUTINATION ASSAY TECHNIQUE:

FD101 Qualitative 100 tests or Quantitative 30 tests
FD102 Qualitative 50 tests x 2 or Quantitative 15 tests x 2
FD106 Qualitative 25 tests x 4 or Quantitative 7 tests x 4
FD103 Qualitative 250 tests x 2 or Quantitative 75 tests x 2

CHARACTERISTICS:

TPHA (Treponema pallidum hemagglutination) test kit is prepared using a component of Treponema pallidum which has been adsorbed onto fixed sheep red blood cells. In the presence of antibodies to T. pallidum, the causative agent of syphilis, the sensitized red blood cells will be hemagglutinated, making possible a simple, easily performed and readable test. A special absorption diluent is included in the kit to eliminate false positive reactions, therefore this TPHA test has a high degree of specificity and eliminates a high percentage of the Biological False Positives (BFPs).

REAGENTS:

	FD101	FD102	FD106	FD103
A. **Reconstituting Solution** This consists of distilled water.	1 x 10 mℓ	1 x 10 mℓ	1 x 10 mℓ	1 x 28 mℓ
B. **Absorbing Diluent** This liquid consists of sonicated cell membranes from sheep and ox erythrocytes, normal rabbit testicular extract, sonicated Reiter treponemes, normal rabbit serum, Tween 80 and acacia powder in phosphate buffered saline (PBS), pH 7.2. This reagent is used to preabsorb and dilute sera, and to prepare the working dilutions of the Sensitized and Unsensitized Cells.	2 x 28 mℓ	2 x 28 mℓ	2 x 28 mℓ	2 x 170 mℓ
C. **Sensitized Cells** (Lyophilized) a. The rehydrated antigen is a 2.5% suspension of formalinized, tanned sheep erythrocytes which have been sensitized with sonicated T. pallidum. b. Prepare a working dilution of antigen by adding one part of the rehydrated suspension to 5.5 parts of Absorbing Diluent (1:6.5 dilution).	1 x 2.5 mℓ	2 x 1.3 mℓ	4 x 0.8 mℓ	2 x 7.5 mℓ
D. **Unsensitized Cells** (Lyophilized) When rehydrated, this is a 2.5% suspension of formalinized, tanned sheep erythrocytes (not sensitized with T. pallidum antigen). A working dilution of 1:6.5 is prepared, allowing 0.075 mℓ for each test serum.	1 x 1.5 mℓ	2 x 1.0 mℓ	4 x 0.5 mℓ	2 x 4.5 mℓ
E. **Reactive Control** (Lyophilized) Reconstitute with 1 mℓ of Reconstituting Solution. Upon reconstitution, this Control is PREDILUTED TO 1:80. Titer of this Control is 1:2560 plus or minus one doubling dilution.	1 x 1.0 mℓ	1 x 1.0 mℓ	1 x 1.0 mℓ	2 x 1.0 mℓ

EQUIPMENT NECESSARY FOR PERFORMING ASSAY

1. Microdiluters (0.025 mℓ)
2. Calibrated pipet droppers 0.025 mℓ/drop (included in kit)
3. Go no-go delivery testers (blotters)
4. Rigid or permanent type U-shaped microplate. Make sure that the wells of the plate are free from dust or lint before dropping reagents.

PRECAUTIONS

1. Lyophilized and reconstituted reagents should be stored at 2~10°C and discarded if they become contaminated or do not demonstrate the proper reactivity with the Controls.
2. The reagents must be reconstituted with Reconstituting Solution and mixed thoroughly to ensure a homogenous suspension. We would suggest that the rehydrated reagents be LEFT AT ROOM TEMPERATURE FOR AT LEAST 30 MINUTES BEFORE USE.
3. Reconstituted reagents should be stored at 2~10°C and used within 5 days. Working dilutions of Sensitized and Unsensitized Cells should be stored at 2~10°C and used within one day.
4. Each batch of reagents has been standardized to produce the proper reaction, therefore, reagents should not be interchanged with those of other batches.
5. The product or their residues must not be allowed to come into contact with ruminating animals or swine.

SHELF LIFE

18 months from production date (refer to outer package for expiry date)

CONTROLS

1. Test Serum Control
   The 1:20 dilution of the test serum is tested using 0.075 mℓ of the working dilution of the Unsensitized Cells (Well 3). No hemagglutination should occur.
2. Reagent Controls
   The Absorbing Diluent is tested using both the Sensitized and the Unsensitized Cells as shown in Table 3. There should be no reaction.
3. Reactive Control
   When rehydrated, this is a 1:80 dilution of Reactive Control Serum and is ready for use. Titer of this Control is 1:2560 and the result may vary plus or minus one doubling dilution.

PREPARATION OF MICRODILUTING EQUIPMENT

1. Microdiluter Preparation
   a. Rotate microdiluters in distilled water to clean.
   b. Flame microdiluters over a Bunsen burner and then quench in cold water.
2. Go No-go Delivery Test (delivery test and prewetting are accomplished in the same operation).
   a. Fill each microdiluter by touching it to the surface of PBS or saline solution. Do not wet the canopy (top) of the loop.
   b. Touch the microdiluter to the center of one of the circles on the Go No-go delivery tester (blotter), and observe the dampened area within the circle.
   c. A properly prepared microdiluter will deliver all contained fluid which will be sufficient to immediately dampen the entire area within the circle.
3. The calibrated pipet dropper must be clean. To assure proper delivery of fluid, wipe the exterior of the dropper tube after filling and hold the dropper in a vertical position when adding fluid to the tray cups.

CLEANING PROCEDURE

Sufficient care should be taken in cleaning the equipment since this process has a great influence on the reaction.
Microplates, pipets, droppers and diluters should be rinsed in running water and then soaked in a neutral compound detergent solution for 4 or 5 hours. Rinse again in running water, then rinse in purified water and dry in incubator (37°C) or in the open air.

QUALITATIVE ASSAY ON SERUM

1. Using a calibrated pipet dropper (0.025 mℓ), place Absorbing Diluent in the wells as shown in Table 1.
   (Note: 5 wells will be used for each test serum.)
2. Place 0.025 mℓ of test serum into Well 1. Using microdiluters, mix the contents of Well 1. Remove the diluters and place into Well 2. Rotate to mix and move the diluters to Well 4 (omit Well 3). Mix again and move diluters to Well 5. Mix and discard the fluid in the diluters. Place clean diluters (Refer to Cleaning Procedure) into Well 2. Rotate to mix and transfer diluters to Well 3. Rotate again and then discard remaining fluid in the diluters. (Refer to Table 1.)
3. After completing the dilutions in each tray, cover and incubate at room temperture (15~25°C) for at least 30 minutes.
4. Using one of the pipet droppers (enclosed in kit), add 0.075 mℓ (3 drops) of Unsensitized Cells to Well 3.
5. Using the other pipet dropper, add 0.075 mℓ (3 drops) of Sensitized Cells to Wells 4 and 5.
6. Reactive Control (Refer to Table 3.)
   Drop 0.025 mℓ Absorbing Diluent into the wells of a microplate as shown in Table 3. Place 0.025 mℓ of Reactive Control Serum (PREDILUTED TO 1:80) in Well 1. Make a serial dilution in Wells 1 through 5. Add 0.075 mℓ Sensitized Cells to Wells 1 through 5.
7. Reagent Controls (Refer to Table 3.)
   Place 0.025 mℓ Absorbing Diluent in Well 7, add 0.075 mℓ Sensitized Cells. Place 0.025 mℓ Absorbing Diluent in Well 8, add 0.075 mℓ Unsensitized Cells.
8. Shake the trays gently, cover with an empty tray.
9. Incubate the trays at room temperature (15~25°C) for at least 2 hours. The incubation period may be extended overnight with no change in patterns.
10. Read the settling patterns of the red blood cells using a tray viewer to visualize the patterns.
11. Readings are scored on a scale of (−) to (4+) and the degree of hemagglutination is judged according to the following criteria:

Degree of Hemagglutination	Reading	Interpretation
Smooth mat of cells covering entire bottom of well, edges sometimes folded	4+	Reactive
Smooth mat of cells covering less area of well	3+	Reactive
Smooth mat of cells surrounded by red circle	2+	Reactive
Smooth mat of cells surrounded by smaller red circle	1+	Reactive
Button of cells having small "hole" in center	±	***Retest
Definite compact button in center of well or may have a very small "hole" in the center	−	Nonreactive

12. The results of the Controls should conform to the criteria outlined in the CONTROLS section.
13. Interpretation
    a. Report as REACTIVE a serum showing hemagglutination of (1+) or higher with Sensitized Cells, provided there is no hemagglutination with the Unsensitized Cells.
    b. Report as NONREACTIVE a serum showing no hemagglutination (−) with Sensitized Cells and Unsensitized Cells.
    ***c. Reading of (±) may occur because of several technical factors. Therefore it is suggested that specimens giving (±) readings be retested. If repeat (±) reading are shown, take another serum sample (after several days) and then perform the

qualitative test again, or test the serum by a different method (FTA-ABS, etc).

14. If non-specific hemagglutination occurs with the Unsensitized Cells, absorption with HA Absorbent and retesting is recommended (HA Absorbent available separately).

QUANTITATIVE ASSAY ON SERUM (Refer to Table 2.)

If serum endpoints titers are desired, quantitatively test all sera that show a REACTIVE result in the Qualitative Assay:

1. As shown in Table 2, place the Absorbing Diluent in each well required for dilutions.
2. Place 0.025 mℓ of test serum in Well 1, perform serial dilutions with diluters. Rotate diluters in Well 1 and move to Well 2. Rotate and move to Well 4 (omit Well 3). Continue rotating and transfering diluters from one well to the next and discard the excess from the last well. Place clean diluters in Well 2, rotate to mix and move to Well 3. Mix well and discard the excess. The final serum dilution in Wells 3 and 4 is 1:80.
3. After completing the dilutions in each tray, incubate at room temperature (15–25°C) for at least 30 minutes.
4. Add 0.075 mℓ Unsensitized Cells to Well 3.
5. Add 0.075 mℓ Sensitized Cells to each of Wells 4 to the final well.
6. Set up Reagent Controls (see Step 7 in "QUALITATIVE ASSAY ON SERUM").
7. Complete tests and read as described in the Qualitative Assay (see Steps 8 through 12 in "QUALITATIVE ASSAY ON SERUM").
8. Quantitative tests showing NONREACTIVE results in the Control are reported in terms of the highest dilution giving a REACTIVE result (1+, 2+, 3+ or 4+) as illustrated below:

Final Serum Dilution						
1:80	1:160	1:320	1:640	1:1280	1:2560	Report
4+	4+	3+	1+	±	–	R 1:640
3+	2+	1+	–	–	–	R 1:320
1+	–	–	–	–	–	R 1:80
±	–	–	–	–	–	*Retest
–	–	–	–	–	–	N
2+	4+	4+	4+	2+	±	R 1:1280

*It is suggested that specimens giving (±) reading at 1:80 be retested. Repeat (±) readings are reported as NONREACTIVE.

9. If non-specific hemagglutination occurs with Unsensitized Cells (Test Serum Control), retest using one of the two methods below:
   A. Absorb the non-specific factors with HA Absorbent and retest (HA Absorbent is available separately).
   B. Prepare dilutions in two rows of wells as shown in Table 2.
      [1] Add 0.075 mℓ of Sensitized Cells to each well of one row.
      [2] Add 0.075 mℓ of Unsensitized Cells to the other row.
10. Report as REACTIVE without reference to titer, if:
    [1] The hemagglutination with Sensitized Cells is at least two doubling dilutions greater than with Unsensitized Cells;
    [2] The first dilution showing hemagglutination with Unsensitized Cells has a (3+) or (4+) reaction with Sensitized Cells.
11. Report as "Inconclusive, Non-specific Hemagglutination in Serum Control", if:
    [1] The hemagglutination with Sensitized Cells is only one doubling dilution greater than with Unsensitized Cells;
    [2] The hemagglutination with Sensitized Cells is at the same dilution as the Sensitized Cells.

Table 1 Outline of Qualitative Test

Cup No.	Test Serum + ml	Absorbing Diluent ml	= Serum Dilution	Diluted Unsensitized Cells, ml	Diluted Sensitized Cells, ml	Final Serum Dilution
1	0.025	0.025	1:2			
2	0.025	0.1	1:10			
3	0.025	0.025	1:20	0.075		1:80
4	0.025	0.025	1:20		0.075	1:80
5	0.025	0.025	1:40		0.075	1:160

Table 2 Outline of Quantitative Test

Cup No.	Test Serum + ml	Absorbing Diluent ml	= Serum Dilution	Diluted Unsensitized Cells, ml	Diluted Sensitized Cells, ml	Final Serum Dilution
1	0.025	0.025	1:2			
2	0.025	0.1	1:10			
3	0.025	0.025	1:20	0.075		1:80
4	0.025	0.025	1:20		0.075	1:80
5	0.025	0.025	1:40		0.075	1:160
6	0.025	0.025	1:80		0.075	1:320
7	0.025	0.025	1:160		0.075	1:640
8	0.025	0.025	1:320		0.075	1:1280
9	0.025	0.025	1:640		0.075	1:2560
10	0.025	0.025	1:1280		0.075	1:5120

Table 3 Controls

Cup No.		Absorbing Diluent ml	Control Serum Dilution	Diluted Unsensitized Cells, ml	Diluted Sensitized Cells, ml	Final Serum Dilution
	Reactive Control Serum, ml					
1	0.025(1:80)	0.025	1:160	–	0.075	1:640
2	0.025(1:160)	0.025	1:320	–	0.075	1:1280
3	0.025(1:320)	0.025	1:640	–	0.075	1:2560
4	0.025(1:640)	0.025	1:1280	–	0.075	1:5120
5	0.025(1:1280)	0.025	1:2560	–	0.075	1:10240
	Reagent Controls					
1		0.025	–	–	0.075	–
2	–	0.025	–	0.075	–	–

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Manufactured by
FUJIREBIO INC.
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Revised Oct. 1985

HUMAN T-LYMPHOTROPIC VIRUS TYPE I
(HTLV-I)

Retro-Tek™ HTLV-I ELISA

An Enzyme Linked Immunosorbent Assay for the Detection of Antibody to Human T-Cell Lymphotropic Virus Type 1 (HTLV-I)

FOR RESEARCH USE ONLY

Manufactured by
CELLULAR PRODUCTS, INC
Buffalo, New York 14202

Form #043
May 19, 1988

Retro-Tek™ HTLV-I ELISA

An Enzyme Linked Immunosorbent Assay for the Detection of Antibody to Human T-Lymphotropic Virus Type I (HTLV-I)

NAME AND INTENDED USE

Retro-Tek™ HTLV-I ELISA is an in vitro qualitative enzyme linked immunosorbent assay for the detection of antibodies to HTLV-I in research specimens. The test may be employed to detect antibodies in human serum or plasma specimens.

IT IS RECOMMENDED THAT THE PRODUCT INSERT BE READ IN ITS ENTIRETY BEFORE PROCEEDING WITH THE TEST PROCEDURE.

STORAGE

Assembled test kits and all components within the test kit must be stored at 2-8°C.

SUMMARY AND EXPLANATION OF THE TEST

The ELISA format has been utilized to detect antibodies to viruses and the Retro-Tek™ HTLV-I ELISA can provide highly specific and sensitive results when performed as recommended in this Product Insert. Each test kit is supplied with HTLV-I Microplates, Positive and Negative Controls, and all reagents required to detect the presence of antibodies to HTLV-I in human serum or plasma.

The HTLV-I is propagated in a permissive cell line and supernatants obtained from the cell cultures are purified to yield active viral particles. After disruption by chemical means, a lysate of the HTLV-I is used to coat the wells of 96-well microplates. Potential protein binding sites within the microplates are "blocked" with non-specific proteins and the plates are dried and supplied to users along with all necessary reagents and controls.

Serum or plasma samples are pre-diluted and added to the HTLV-I Microplate wells. Antibodies specific for HTLV-I proteins, which may be present in the sample, bind to the viral proteins which are bound to the microplate wells. A subsequent washing step will remove any non-HTLV-I specific antibodies and a goat anti-human IgG antibody (heavy and light chain specific) conjugated to alkaline phosphatase is added to the same wells. If human antibodies have been bound to the viral proteins coating the wells, the alkaline phosphatase conjugate will bind to the antigen/antibody complex. An enzyme substrate (p-nitrophenylphosphate) is added and the colorless compound will be converted to a detectable yellow substance in proportion to the amount of human antibody to HTLV-I which is bound to the well.

MATERIALS SUPPLIED

1 HTLV-I Microplate. 96-well (12x8 well) microplate coated with HTLV-I viral proteins. Storage: 2-8°C (desiccated)

2 Positive Control. Heat-inactivated human source material containing antibody to HTLV-I proteins and negative for Hepatitis B surface antigen. Preservative: 0.005% Thimerosal and 0.1% sodium azide. Storage: 2-8°C.

3 Negative Control. Human source material negative for antibody to HTLV-I proteins and Hepatitis B surface antigen. Preservative 0.005% Thimerosal and 0.1% sodium azide. Storage 2-8°C

4. Sample/Conjugate Diluent. Used to dilute test specimens, Positive and Negative Controls, and Conjugate. (Contains PBS with 20% heat-inactivated goat serum, 0.05% Tween-20, and 1.0% BSA) Preservative 0.005% Thimerosal and 0.1% sodium azide Storage 2-8°C.

5 Conjugate. Goat Anti-Human IgG (heavy and light chain specific) conjugated with alkaline phosphatase, supplied in a lyophilized form. Storage 2-8°C.

6 Substrate Tablets: p-Nitrophenylphosphate (pNPP) substrate tablets. Storage 2-8°C.

7 Substrate Diluent. Buffer used to solubilize Substrate Tablets for use in ELISA. (Contains 1.0M diethanolamine (DEA) with 0.5 mM $MgCl_2$) Preservative: 0.1% sodium azide Storage 2-8°C

8. 10X Plate Washing Buffer. After dilution, this buffer is utilized to wash the HTLV-I Microplate where noted in the Test Procedure Section. (Contains 10X PBS with 0.5% Tween-20) Preservative: 1.0% chloroacetamide. Storage 2-8°C

9 Stop Solution. 3N NaOH used to stop alkaline phosphatase reaction. (NaOH is a known caustic reagent, use care in handling) Storage: 2-8°C.

10 Microplate Covers

11 Plate Storage Bag

MATERIALS REQUIRED BUT NOT SUPPLIED

Micropipettes: To deliver 15 ul, 45 ul, 300 ul, and 900 ul
Multiple (8 or 12) Channel to deliver 50 ul and 200 ul

Multichannel pipette reagent reservoir, 30 ml capacity

Assorted beakers or flasks to dilute concentrates supplied

Test tubes (12 x 75 mm) for pre-dilution of sample

Timer

Disposable gloves

Non-metallic forceps

Deionized or distilled water

37°C dry air incubator

ELISA plate washer (optional)

ELISA plate reader (405 nm) or spectrophotometer

Vacuum pump system (for optional ELISA plate washer)

Multiple (8 or 12) channel vacuum device for manual wash

Automatic sample dilutor (optional)

Autoclave

Refrigerator 2-8°C

Retro-Tek™
HTLV-I ELISA TEST KIT FOR DETECTION OF HTLV-I ANTIBODY

Catalog Number	0801022	Retro-Tek™ HTLV-I ELISA Test Kit 576 Determinations
Catalog Number:	0801021	Retro-Tek™ HTLV-I ELISA Test Kit 192 Determinations

0801022	0801021	Component Description
6 plates	2 plates	HTLV-I Microplate 96 wells in 12 x 8 well strips
1 0ml	0.5ml	Positive Control
1 0ml	0 5ml	Negative Control
0 1ml	0 1ml	Conjugate (lyophilized powder)
2x185ml	125ml	Sample/Conjugate Diluent
150ml	60ml	Substrate Diluent
500ml	2x100ml	10X Plate Wash Buffer
50ml	15ml	Stop Solution (Contains NaOH, a known caustic reagent)
31 Tablets	11 Tablets	Substrate Tablets
Storage Bag	Storage Bag	Storage Bag for storing unused Microplate strips
18 Plate Covers	6 Plate Covers	Disposable Microplate Covers

WARNINGS AND PRECAUTIONS FOR USERS

1. TheRetro-Tek™ HTLV-I Microplates must be treated as if capable of transmitting HTLV-I Disposable gloves must be worn at all times Used or outdated microplates plates must be discarded by autoclaving. The HTLV-I Microplate has been coated with HTLV I proteins which have been inactivated by detergent lysis

2 Both the Positive and Negative Controls have been found to be negative for Hepatitis B surface antigen The Positive Control has been heated at 56°C for sixty minutes to inactivate any HTLV-I which might be present However, care should be taken to handle each of the controls as potential biohazards

3. All potentially biohazardous materials in this assay kit and samples must be disposed of in a manner that will insure inactivation of infectious virus. Solid wastes and vials, HTLV-I Microplates, Positive and Negative Controls must be autoclaved for 60 minutes at 121°C Liquid wastes may be inactivated by the addition of sodium hypochlorite (household bleach) to achieve a final concentration of 1 0% sodium hypochlorite for a period of at least thirty minutes The inactivated material may then be discarded down the sink drain followed by copious amounts of water

4 The components of this kit have been assembled and tested as a unit Do not interchange components from this kit with others manufactured by Cellular Products or other manufacturers

5 An expiration date is clearly stamped on outer kit labeling Do not use the kit or components beyond the labeled expiration date

6 Do not handle Substrate Tablets (pNPP) with bare fingers or permit contact with exposed skin Use non-metallic forceps to transfer the tablets The tablets and diluted pNPP reagent must be protected from direct light.

7 DO NOT MOUTH PIPETTE under any circumstances Use of pipetting aids is required.

8 Stop Solution is 3N NaOH, a known caustic substance and irritant to mucous membranes Wear gloves and eye protection when working with this reagent

9 Avoid microbial contamination of kit reagents Use aseptic technique when withdrawing component aliquots from primary containers

10 Color developement is inhibited by residual amounts of diluted Plate Wash Buffer left in test wells prior to the addition and incubation of Substrate Plates must be blotted thoroughly prior to the addition of Substrate

REAGENT PREPARATION

1 The Sample/Conjugate Diluent is used directly as supplied No dilution is required

2 The Positive and Negative Controls are prepared exactly as described for the test sera Pre-dilute controls by adding 45 ul of the control to 900 ul of Sample/Conjugate Diluent and then introducing 200 ul of diluted sample to each of the appropriate wells The diluted Positive Control is analyzed in quadruplicate and the diluted Negative Control is analyzed in duplicate

3 The lyophilized Conjugate must be reconstituted by the addition of 1.0 ml of Sample/Conjugate Diluent to provide a Stock Solution of Conjugate which is diluted as described below to provide a working solution of Conjugate as needed Reconstitution of the lyophilized Conjugate with Sample/Conjugate Diluent should be performed at least 18 to 24 hours prior to use in preparing the working solution The resolubilized Stock Solution is stable for at least 3 months when stored at 2-8°C Any working solution of Conjugate should be discarded at the end of the day

No of Tests*	Vol. of Stock Sol. of Conjugate	Vol of S/C Diluent	Final Volume of Working Solution
0-20	10 ul	5 0 ml	5 01 ml
21-40	20 ul	10 0 ml	10 02 ml
41-60	30 ul	15 0 ml	15 03 ml
61-80	40 ul	20 0 ml	20 04 ml
81-96	50 ul	25 0 ml	25 05 ml

* Including all control samples

For the sake of accuracy, it is recommended that the preparation of a Working Solution of Conjugate utilize the above schedule so that quantities less than 10 ul of Stock Conjugate Solution are not pipetted

4 The Substrate Tablets are dissolved in Substrate Diluent according to the following schedule

CAUTION PREPARE SUBSTRATE SOLUTION NO SOONER THAN 30 MINUTES PRIOR TO USE ANY SUBSTRATE SOLUTION NOT USED WITHIN 30 MINUTES OF PREPARATION MUST BE DISCARDED

Number of Test Wells*	Number of Substrate Tablets	Volume of Diluent
0-20	1 0	5.0 ml
21-40	2 0	10 0 ml
41-60	3 0	15 0 ml
61-80	4 0	20 0 ml
81-96	5 0	25 0 ml

*Including all control samples

5 The 10X Plate Wash Buffer Concentrate must be diluted ten-fold prior to use with distilled or deionized water The following dilution schedule supplies sufficient diluted Plate Wash Buffer for use in manual washing procedures

Number of Test Wells*	Vol. of 10X Concentrate	Volume of Water	Final Volume
0-24	6 5 ml	58 5 ml	65 0 ml
25-48	13 0 ml	117 0 ml	130 0 ml
49-72	20 0 ml	180 0 ml	200 0 ml
73-96	25 0 ml	225 0 ml	250 0 ml

*Including all control samples

If automated plate washers are employed, prepare the appropriate amount of diluted Plate Wash Buffer as required to fill and prime all feed lines

Diluted Plate Wash Buffer can be held at room temperature and used up to 2 weeks after preparation

6 Stop Solution is used directly as supplied No dilution is required

SPECIMEN PREPARATION

CAUTION: All research specimens analyzed in this assay should be handled as though capable of transmitting infectious agents

For optimal performance of the Retro-Tek™ HTLV-I ELISA, serum or plasma samples should be tested within 24 hours of collection. If samples are not analyzed immediately, store at 2-8°C until ready to test. If testing is delayed longer than 24 hours, storespecimens at -20°C or colder.

Avoid multiple freeze-thaw cycles

TEST PROCEDURE

CAUTION: ALL RESEARCH SPECIMENS TO BE ANALYZED MUST BE HANDLED AS IF CAPABLE OF TRANSMITTING INFECTIOUS AGENTS. FURTHERMORE, ALL POSITIVE SERUM CONTROLS AND HTLV-I MICROPLATES CONTAINED IN THE Retro-Tek™ HTLV-I ELISA TEST KIT MUST ALSO BE HANDLED AS IF CAPABLE OF TRANSMITTING INFECTIOUS VIRUSES.

1. Allow all test components to warm to room temperature before starting test procedure. If the entire 96-well microplate is not used, remove only the strips of wells that will be used and reseal the remaining unused wells in the Plate Storage Bag along with the desiccant. Label the Plate Storage Bag with lot number and expiration date of the kit and store the resealed microplate at 2-8°C. The remaining test wells may be used until the expiration date.
2. Pre-dilute all test and control specimens 1/21 using the following protocol:

THE FOLLOWING DILUTIONS PROVIDE SUFFICIENT VOLUME TO PERMIT FOUR (4) WELLS OF THE POSITIVE CONTROL, TWO (2) WELLS OF THE NEGATIVE CONTROL AND ONE (1) WELL OF THE TEST SPECIMEN TO BE ANALYZED

Positive Control: Add 45 ul of Positive Control to 900 ul of Sample/Conjugate Diluent in a test tube

Negative Control: Add 45 ul of Negative Control to 900 ul of Sample/Conjugate Diluent in a test tube

Test Specimens: Add 15 ul of Test Specimen to 300 ul of Sample/Conjugate Diluent in a test tube

Alternatively, an automated dilutor may be used to achieve the delivery of 200 ul of a 1/21 dilution of sample in Sample/Conjugate Diluent to the test well.

3. Using a micropipettor adjusted to 200 ul, place 200 ul of the pre-diluted Positive Control into each of four separate wells of the HTLV-I Microplate. (If an automated dilutor is employed, steps two (2) and three (3) are performed simultaneously)
4. Using a micropipettor adjusted to 200 ul, place 200 ul of the pre-diluted Negative Control into each of two separate wells of the HTLV-I Microplate. (If an automated dilutor is employed, steps two (2) and four (4) are performed simultaneously).
5. Using a micropipettor adjusted to 200 ul, place 200 ul of the pre-diluted serum or plasma into a single well ofthe HTLV-I Microplate. Each sample evaluated in the Retro-Tek™ HTLV-I ELISA test must be placed into a separate well of the HTLV-I Microplate. A separate pipette tip should be used for each individual specimen. (If an automated dilutor is employed, steps two (2) and five (5) are performed simultaneously)

IN ORDER TO PROVIDE A REAGENT BLANK WELL, BE CERTAIN TO PLACE 200 ul OF SAMPLE/CONJUGATE DILUENT TO ONE WELL. THE ABSORBANCE VALUE OF THE REAGENT BLANK WELL MUST BE SUBTRACTED FROM THE ABSORBANCE VALUE OF EACH WELL USED TO ANALYZE THE POSITIVE AND NEGATIVE CONTROLS AND THE UNKNOWN TEST SPECIMENS. ALTERNATIVELY, ELISA PLATE READERS SO EQUIPPED SHOULD BE PROGRAMED TOAUTOMATICALLY SUBTRACT THE ABSORBANCE VALUE OF THE REAGENT BLANK WELL FROM ALL OTHER WELLS

6. Cover the Microplate with a plate cover to prevent evaporation and incubate for 60 minutes (+/- 5 minutes) at 37°C +/-1°C. DO NOT STACK PLATES IN THE INCUBATOR IF MORE THAN ONE PLATE IS ANALYZED
7. Aspirate the entire contents of each well with a vacuum device
8. Wash each well of the Microplate being analyzed six times by adding a quantity of diluted 10X Plate Wash Buffer to each well and then aspirating. When using multichannel pipettes for the wash step, add 200 ul diluted wash buffer per well. When using automated or semi-automated plate wash equipment, use 300 ul diluted plate wash buffer per well
9. Thoroughly blot microplate by sharply striking the inverted microplate on a pad of absorbent towels placed on the benchtop. Continue striking until no droplets remain in the wells
10. Add 200 ul of Working Solution of Conjugate to each well of the microplate being analyzed
11. Cover the microplate with a plate cover and incubate for 60 minutes (+/- 5 minutes) at 37°C +/- 1°C. DO NOT STACK PLATES IN THE INCUBATOR IF MORE THAN ONE PLATE IS ANALYZED

12. Aspirate the entire contents of each well with a vacuum device.

13. Wash each well of the microplate being analyzed six times exactly as described above in Step 8 and 9

    CAUTION: RESIDUAL PLATE WASH BUFFER IN TEST WELLS WILL INHIBIT COLOR DEVELOPMENT. EXAMINE WELLS AND REBLOT, IF NECESSARY, TO REMOVE ANY RESIDUAL BUFFER.

14. Add 200 ul of previously reconstituted Substrate (i.e., Tablets in Substrate Diluent) to each well of the microplate being analyzed. The Substrate Solution should be formulated within 30 minutes prior to its use. Any unused Substrate Solution should be discarded.

15. Cover the microplate with a plate cover and incubate it for 30 minutes (+/- 5 minutes) at 37°C +/- 1°C. DO NOT STACK PLATES IN THE INCUBATOR IF MORE THAN ONE PLATE IS ANALYZED

16. Stop the ELISA reaction by adding 50 ul of Stop Solution to each well of the microplate being analyzed

17. Read the absorbance of each well within 60 minutes of stopping the reaction of the microplate being analyzed by placing the plate into an ELISA plate reader equipped with a 405 nm filter. A single wavelength (i.e. 405 nm) or dual wavelength (i.e. using a 405 nm primary filter) may be used to read the HTLV-I Microplate. **ELISA microplate readers so equipped should be programed to read all absorbance readings against the blank well. Alternatively, read all wells against air and subtract the absorbance of the blank well from all other control and specimen-containing wells.**

CALCULATION OF RESULTS

(A) Determination of the mean of Negative Serum Control:

After adjusting for the blank, add the two separate ELISA readings of the Negative Serum Control together and divide the result by two (i.e. calculate the average absorbance of the two separate Negative Serum Control wells). The mean of the Negative Serum Control ELISA readings must be in the range of 0.000 O.D. to 0.200 O.D.

(B) Determination of the mean of the Positive Serum Control

1. At least three of the four absorbance readings of the Positive Serum Control must be in the range of 0.800 O.D. to 2.100 O.D. after subtracting of the blank well, AND

2. At least three of the four absorbance readings meeting the above requirement must be within 30% of the mean absorbance reading of all wells meeting the above criteria

3. Calculate the mean of the absorbance readings meeting both criteria listed above

EXAMPLE:
Absorbance values of four Positive Serum Controls

Sample 1: 1.400
Sample 2: 1.502
Sample 3: 0.803
Sample 4: 1.376

1. All Absorbance values meet criteria (1) above:
2. a. Determination of mean: 1.270
   b. 30% of mean: 0.381
3. Only Sample 1, Sample 2, and Sample 4 meet criteria (2) above, therefore, the mean of Sample 1, Sample 2, and Sample 4 is: 1.426. This value represents the mean of the Positive Serum Control

(C) Determination of Cutoff Value

In order to determine the cutoff value, divide the mean of the Positive Control as determined in (B) by 4. This value is the cutoff value between non-reactive and reactive readings.

INTERPRETATION OF RESULTS:

Specimens which yield absorbance values less than or equal to the Cutoff Value should be considered negative for antibodies to HTLV-I while specimens which yield absorbance values greater than the Cutoff Value should be considered positive for antibodies to HTLV-I

Appendix 7

QUESTIONNAIRE

(Note: A few questions or parts thereof were deleted in the final analysis after they were felt to be non-contributory or ambiguous).

Please answer these questions as honestly as you can. If there is any question you don't understand feel free to ask me to explain.

1. Name: (on separate sheet) I.D:
2. Has anyone in your family, living or dead, ever had a nerve disease? 1.Yes 2.No Who?_____
3. Has anyone in your family, living or dead, ever had unexplained problems in walking? 1.Yes 2.No Who?_____
4. What were the first signs of your disease?_____
   When did they occur?_____
5. Where were you living when you first noticed signs of the disease? 1.St. Andrew 2.Christ Church 3.St. George 4.St. James 5.St. John 6.St. Joseph 7.St. Lucy 8.St. Michael 9.St. Peter 10.St. Philip 11.St. Thomas 20.Other West Indian island 30.England 40.Other
6. How long were you living in above when you first noticed signs of the disease?_____years.
7. When you first noticed signs, were you:
   1.Married 2.Divorced 3.Separated 4.Widowed 5.Never married

8. Spastic gait: 1.Present 2.Absent

9. Degree of disability: 1.Walks unaided 2.Walks with support
3.Unable to stand or walk or does so rarely.

10. Do you ever have bladder problems? 1.Never 2.Rarely
3.Sometimes 4.Fairly often 5.Very often

What problems? 1.Frequency 2.Retention 3.Both

When did you first notice them? Month and year_____

11. Do you ever have leg cramps? 1.Never 2.Rarely 3.Sometimes
4.Fairly often 5.Very often

When did you first notice?_____

12. Do you ever get back pains? 1.Never 2.Rarely 3.Sometimes
4.Fairly often 5.Very often

When did you first notice?_____

13. Do your legs ever feel weak? 1.Never 2.Rarely 3.Sometimes
4.Fairly often 5.Very often

When did you first notice?_____

14. Do your feet ever tingle or burn? 1.Never 2.Rarely
3.Sometimes 4.Fairly often 5.Very often

When did you first notice?_____

15. Do you ever have difficulty with bowel movements
(constipation)? 1.Never 2.Rarely 3.Sometimes
4.Fairly often 5.Very often

When did you first notice?_____

16. Did you ever have blurred vision or lose your sight in one or
both eyes? 1.Yes 2.No

Did you ever have double vision? 1.Yes 2.No

17. Has anyone in your family, living or dead, ever had leukemia (blood cancer) 1.Yes_____ 2.No

18. How many pregnancies have you had?_____

19. How many abortions have you had?_____

20. Were you using any prescribed drugs or medications in the 6 months before the first signs? 1.Yes 2.No

21. What health problems do you have?_____

_____

22. How many blood transfusions, if any, did you have before you noticed the first signs?____ When?_____

23. How many injections did you receive in the year before you noticed the first signs? 1. 0 2. 1 or few 3. Several Who gave them to you?_____ When?_____

24. Were you breast-fed as a baby? 1.Yes 2.No 3.Don't know

25. Did you ever have....? When? (If not sure say don't know)

Lyme disease_____ Conjunctivitis_____

Unexplained skin rashes_____ Diphtheria_____

Syphilis_____ Yaws_____ Urinary infection?_____

Gonorrhea_____ Head injuries_____ Other_____

_____

26. How many cigarettes did you smoke per day? 1. 0 2. 1-2 3. 3-5 4. 6-10 5. More than ten.

27. How many alcoholic drinks did you drink per week? 1. 0 2. 1-2 or less 3. 3-5 4. 6-10 5. More than 10.

28. Did you eat seafood? How often? (1.Never etc.) Conch_____

Clams____ Crabs____ Shrimp____ Other shellfish _____

29. How often per week did you eat fish_____

peas_____ mushrooms_____

cassava_____ (type and frequency) 1. 0 2.Once every few weeks or less 3. 1-2 4. 3-5 5. 6 or more

Did you prepare your own cassava? 1.Yes 2.No

30. Did you use folk medicine? How often? (1.Never etc.)_____

How did you take it? 1.By mouth 2.Ointment 3.Injection 4.Other_____

31. What unprescribed drugs did you use?_____

32. Did you use bush teas? How often? (1.Never etc.)_____

33. Did you raise any farm animals? 1.Yes, within the last 10 years 2.Yes, more than 10 years ago 3.No

34. Did you keep any pets? 1.Yes, within the last 10 years 2.Yes, more than 10 years ago 3.No

35. Did you work with...? How often? (1.Never etc.)

Insecticides_____ Pesticides_____

Disinfectants_____ Other chemicals or poisonous materials_____

36. Gender: 1. M 2. F

37. Date of birth: Month_____Day_____Year_____

38. Race: 1.Black 2.White 3.Mixed 4.Indian 5.Other_____

39. Where were you born? (Options as in question 5.)

40. Where do you live now? (Options as in question 5.)

41. Are you currently : 1.Married 2.Divorced 3.Separated 4.Widowed 5.Never married

Before you noticed the first signs:

42. What was your job?_____

1.Service 2.Trade 3.Professional 4.Unemployed

43. What was your highest level of education? 1.No formal education 2.Primary school 3.Secondary or high school 4.University 5.Post-graduate education

44. How many persons lived in your home besides you? Include all children_____

45. What were their relationships to you and what were their ages?_____

46. Is your sex drive decreased? 1.No, it is as usual 2.Yes, a little 3.Yes, very much

When did you first notice?_____

47. How often did you have sex before you noticed the first signs? 1.Never 2.Once every few years 3.Once every few months 4.Once every few weeks 5. 1-2 times per week 6. 3-5 times per week 7. More than 5 times per week

48. How many different sexual partners did you have in the 6 months before you noticed the first signs?

1. None 2. One 3. Two 4. Three 5. Four to eight 6.More than eight

49. Before you noticed the first signs did you and your partners use... How often? (1.Never etc.) Condom_____ Diaphragm_____ Pill_____ Other type of contraception_____

50. Do you have any problems in getting erections? 1.Never 2.Rarely 3.Sometimes 4.Fairly often 5.Very often

51. Have you ever taken by needle...How often? (1.Never etc.) Heroin_____ Cocaine_____ Other drugs_____

52. Have you ever had sex with someone who has taken by needle... How often? (1.Never etc.) Heroin_____ Cocaine_____ Other drugs_____

53. Have you ever had sex with a member of the same sex? How often? 1.Never 2.Rarely 3.Sometimes 4.Fairly often 5.Very often

54. Have you ever travelled abroad? 1.Never 2.Slight (1 or 2 times) 3.Moderate (3-5 times) 4.Extensive (more than 5 times) Where?_____

55. What are the names and ages of your brothers and sisters, your parents, and your children? _____

_____

Appendix 8

ISOLATION OF LYMPHOCYTES

. Collect blood in a sterile heparinized vacutainer

(The following 2 steps are done in a flow hood)

. Pipette at least 2.5 ml of Ficoll into 2 sterile 30-40 ml tubes

. Add delicately ~5 ml of blood on top of the Ficoll

. Centrifuge 30 minutes at 3000 rpm

. Remove top plasma layer which may be discarded or frozen for eventual antibody detection

. Collect leukocytes in buffy coat using sterile Pasteur pipettes

. Place in sterile container and add 10 ml of RPMI-1640 medium

. Centrifuge at 2000 rpm for 10 minutes

. Discard supernatant

. Resuspend the cell pellet in 5 ml of RPMI-1640 medium containing 10% fetal calf serum, 2-mercaptoethanol (10^{-5} M), antibiotics, phytohemagglutinin (10 ug/ml), anti γ-interferon serum and polybrene (2 ug/ml); tap tube to mix

. Incubate for 3 days at 37°C

. After stimulation, centrifuge cells, discard supernatant and resuspend in 5 ml of complete medium without phytohemagglutinin; tap tube to mix

. Add 5 ml growth medium twice a week until container is half-full, then pipette off medium and start over

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