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TRANSFER FACTOR DERIVED FROM WHOLE BLOOD AND ITS USE

IN THE TREATMENT OF MULTIPLE SCLEROSIS

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TRANSFER FACTOR DERIVED FROM WHOLE BLOOD AND ITS USE IN THE TREATMENT OF MULTIPLE SCLEROSIS

By

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ABSTRACT

TRANSFER FACTOR DERIVED FROM WHOLE BLOOD AND ITS USE IN THE TREATMENT OF MULTIPLE SCLEROSIS

By

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Transfer factor (TF) derived from whole blood appears to have some properties similar to that of TF derived from leukocytes. It is a non-protein, non-antigenic substance which will stimulate lymphocytes *in vitro*. This was demonstrated by apparently nonspecific responses by lymphocytes in active rosette and blastogenesis studies. Chemical data suggest a low molecular weight polynucleotide.

Transfer factor specificity for immunoprophylaxis for multiple sclerosis (MS) cannot be determined until a specific antigen related to the disease is identified and purified.

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INTRODUCTION

Transfer factor (TF) is one of many substances or lymphokines that are produced by lymphocytes as part of the cellular immune response to foreign antigen. In 1949 Lawrence (29) discovered a method to recover this factor from leukocytes. Its mechanism of action and biochemical structure is yet unknown. In the past ten years researchers have tried using TF to treat some cancerous, autoimmune, and infectious diseases in which impaired immunity may be involved. Many of the clinical trials have had promising results while others have been disappointing.

Recently transfer factor therapy has been tried in the treatment of multiple sclerosis (MS), a disabling neurologic disease which afflicts young people. The etiology of the disease is unknown and no cure is available, although many treatments have been tried with only limited success.

The Human Immunology Foundation in Red Bank, New Jersey (J. Angers, M.D., Director) has tried treating MS patients with a transfer factor derived from whole blood rather than leukocytes. They report a favorable response in 62 out of 80 patients, or over 80%.

Edward W. Sparrow Hospital in Lansing, Michigan, has begun a similar program for treating multiple sclerosis under the direction of John Kateley, Ph.D., Director of Immunology. The transfer factor is obtained from whole blood donated by household contacts of the

MS patients following the protocol of Angers et al. (5). This TF is then administered to the patients by themselves or their own private physicians.

The purpose of this study was to attempt to identify some of the chemical properties of the transfer factor derived from whole blood and to determine its effect on lymphocytes using *in vitro* techniques such as blastogenesis and rosette formation.

LITERATURE REVIEW

Transfer Factor

In humans the immune response to foreign antigens such as bacteria and viruses is a complex system involving lymphocytes. There are at least two types of lymphocytes: B-cells and T-cells. B-cells produce the specific humoral antibodies which are responsible for antibody mediated immunity; T-cells initiate the events of cell-mediated immune reactions either through 1) direct cell-to-cell interactions or 2) production of lymphokines (48). Some of these lymphokines include: macrophage inhibition factor (MIF), chemotactic factor (CF), lymphotoxin (LT), blastogenic factor (BF), interferon, and transfer factor (TF). All of these lymphokines are currently being evaluated for use in controlling disease. Within the last ten years one of these, TF, has been used as therapy for diseases which are either caused by or associated with defects in the cell mediated immune (CMI) system. Transfer factor is a dialyzable substance which can initiate, as well as increase, delayed hypersensitivity (DH) and, therefore, CMI.

Historically, in 1906, Pirquet (20) described a patient who demonstrated skin sensitivity to tuberculin after that patient recovered from a tuberculous infection. This reaction (red welt at injection site), elicited by very small quantities of tuberculin,

remained positive long after the infection. Pirquet suspected this response was related to immunity.

In the early 1940's Landsteiner and Chase (20,31) discovered that tuberculin hypersensitivity in guinea pigs was transmitted by leukocytes. They also found that this reactivity could be transferred from one animal to another by injecting intact leukocytes from the donor into the recipient. After injection of the leukocytes the recipient also had a positive response where previously there had been no response. Investigators have observed that, in humans, a smaller dose of cellular material can elicit a similar response and that the sensitivity lasts longer.

Landsteiner and Chase realized that this transfer of reactivity could have great significance in the treatment of immune deficiency diseases. However, there was a serious problem in the use of intact leukocytes, particularly in the immune suppressed patient who could develop graft-versus-host (GVH) disease.

In 1949 H. Sherwood Lawrence (20,28,36) reported that cell-free leukocyte extracts could transfer the same reactivity from donor to recipient as intact leukocytes. He was the first to name the responsible agent "transfer factor", based on the fact that he could transfer immunity from a sensitized donor to an unsensitized patient, and that patient in turn became sensitized in a short time. For about 15 years Lawrence's discovery was largely ignored; however, increasing clinical use in the 1970's has brought TF back into immunological research.

Lawrence's method for producing TF utilized frozen-thawed leukocytes from a sensitized donor. The cells were treated with DNAse and dialyzed against distilled water to remove serum proteins. Transfer

factor remains in the cell-free dialysate. Many modifications of Lawrence's method have been described for preparation of TF. These modifications make comparisons between different preparations difficult to interpret.

Molecular weight estimates indicate TF to be approximately 10,000 daltons. Transfer factor is a non-antigenic, polynucleotide-polypeptide complex which is resistant to DNAse, pancreatic RNAse, and trypsin but not to pronase (35). It is not an immunoglobulin nor does it effect changes in the immunoglobulin levels of the recipient.

The presence of TF in a leukocyte extract is usually demonstrated indirectly by the use of an *in vivo* or *in vitro* immunoassay. If TF is made from a donor who is known to be sensitized to a specific antigen such as tuberculin (PPD) and that TF is given to a recipient who is PPD negative, that recipient will exhibit a positive PPD skin test within hours. This method of testing has also proved that transfer factor is immunologically specific. When TF is derived from a donor sensitized to tuberculin but not diphtheria toxoid and is then given to a recipient who is not sensitized to either substance, that recipient will become sensitized to tuberculin only.

Recently, other methods for testing the response to TF therapy *in vitro* have been reported. They include a) an increase in the number of active T-lymphocyte rosettes, b) an increase in macrophage inhibition factor (MIF), and c) an increase in lymphocyte blastogenesis. Lawrence and colleagues have utilized the latter procedure extensively. For example, if TF prepared from tuberculin sensitive cells is incubated with nonsensitive lymphocytes, no immunologically

specific events are observed. However, if tuberculin is then added to the culture, a small number of lymphocytes will undergo transformation to lymphoblasts. These cells act as if they are now sensitized to the antigen (31). All the *in vitro* testing methods provide easier ways of testing patient responses and the reactivity of individual TF preparations.

The mechanism by which TF works *in vivo* is a question as yet unanswered, although there are many reasonable hypotheses available. Kirkpatrick (36) suggests that TF allows cells which previously could not respond to a specific antigen to produce lymphokines such as MIF or chemotactic factor. Fudenberg (36) projects that TF increases the number of specifically sensitized lymphocytes by recruiting previously uncommitted cells. Possibly TF induces macrophages to act on lymphocytes to uncover new antigen receptor sites or it causes precursor cells to become immunocompetent T-cells. Lawrence (28) suggests that TF may be an informational molecule acting as an antigen specific receptor on T-lymphocytes or that TF may act as a derepressor of normal lymphocytes. In any case, small populations of normal circulating lymphocytes become antigen responsive, transform and undergo clonal proliferation (29,35). Further research in this area is required and more will be possible when animal models are found who respond in a similar fashion as humans. At this time results in this area are inconclusive (7).

Therapeutically, transfer factor has been used in the treatment of various diseases. Many bacterial, parasitic and fungal diseases such as disseminated coccidioidomycosis, tuberculosis and schistosomiasis have been treated with TF therapy, usually in conjunction with

antibiotics, with favorable responses in most patients. One disease, disseminated mucocutaneous candidiasis, appears to react the best. Many of these patients whose disease has been resistant to protracted amphotericin therapy have responded with clinical improvement or even total remission. In any infectious disease TF appears to reverse the course of an overwhelming infection, reduces the need for toxic antibiotics, and gives the immune deficient patient capability to respond to pathogens in his environment (26).

Specific immunodeficiency diseases have been treated with transfer factor also. The Wiscott-Aldrich syndrome, a sex-linked (x) recessive deficiency state in children characterized by thrombocytopenia, eczema, and recurrent infections, has been successfully treated with TF. Following TF therapy many children with this disease can lead amost normal lives without continual illnesses.

Treatment of cancer is another area of TF research. Malignant melanomas in some patients have reduced in size following injection of TF. In any cancer, however, immunotherapy alone cannot destroy large tumor nodules but, if used as an adjunct to surgery, chemotherapy and radiation, TF could increase the chances of long term survival (32).

For the studies reported above, the donors for TF can often be screened for specific cell mediated immunity. Frequently, the donors have had the disease themselves.

There are essentially no side effects in patients given TF. It causes no inflammatory response (except perhaps a small localized reaction at the site of injection). No hematological, biochemical, or enzymatic abnormalities have been detected even when large doses are given (30). One possible side effect could be the transfer of

unwanted hypersensitivities from donor to recipient (for example, to poison ivy or bee stings). However, taking careful donor histories should make this possibility negligible.

Clearly, the use of transfer factor therapy shows promising results, although many problems and inconsistencies have been reported. Patients and their diseases are heterogeneous. Other variables result from the many different methods of TF production and the variations in dosages used. Clinicians agree that the time has come for more controlled trials where uniform TF is given in uniform doses under uniform protocols. Hopefully, some of these trials will begin soon.

Multiple Sclerosis

Multiple sclerosis (MS) is a disabling neurological disease which usually affects young adults between the ages of 20 and 40. Its symptoms are caused by a localized inflammatory reaction in which the myelin surrounding the nerve fibers disintegrates and is replaced by scar tissue which forms plaques. This occurs primarily in the white matter of the central nervous system (CNS), particularly the periventricular areas, optic nerves, and the cerebellum. The plaques distort or block nerve impulses which control such functions as gait, sight, speech, and memory. The disease is characterized by alternating periods of attacks (exacerbations) and remissions. Some patients may have only one or two attacks and can lead relatively symptom-free lives. Others will suffer from progressive exacerbations leading to total disability in only a few years from onset. No two patients follow the same pattern and, although most patients will show some improvement after each attack, none will ever recovery completely.

The diagnosis of MS is difficult. There is no symptom or clinical test which clearly defines the disease. Currently, the only way to prove the existence of MS in a patient is to study the nervous system lesions at autopsy; however, some reasonable standards have been developed (37,44). Multiple sclerosis is classified as clinically definitive if:

1) There is a history of remitting and relapsing symptoms with two or more episodes.

2) There are documented neurological lesions at two or more separate sites in the CNS white matter.

3) The age of onset of symptoms is between ten and fifty.

4) The history of signs or symptoms is six months or longer.

5) There is no better neurologic explanation for the observed symptoms.

Other classifications of probable or possible MS are limited variations of the above criteria.

One laboratory test which is helpful in supporting the diagnosis of MS is the identification of immunoglobulin G present in the cerebrospinal fluid (CSF). Sixty to eighty percent of MS patients exhibit an oligoclonal pattern of IgG which may be present even when the amount of immunoglobulin in the CSF is normal. This pattern is found more often in patients with evidence of a currently active disease process and may signify immunological activity in the brain (16). The finding of oligoclonal bands in the CSF is not diagnostic itself, but it does provide strong supporting evidence when syphilis, sarcoidosis, chronic infections of the CNS and certain other rare diseases are excluded (37,44).

Racial, genetic, and environmental factors appear to play a major role in determining susceptibility to multiple sclerosis. Approximately

ten percent of MS patients will have a closely related member of the family affected. Identical twins may have both or only one twin affected. Women have MS slightly more often than men (ratio 1.4 to 1) and it is particularly prevalent in caucasians of European descent. Worldwide distribution shows the disease to be more common in the higher latitudes on both sides of the equator. Multiple sclerosis is rare in the Japanese (and probably other orientals) and in African Blacks. Migration from a high risk area to a low risk area during childhood will confer low risk to the individual. Migration at a later age will modify the risk to a lesser degree. Migrants from a low risk area to a high risk area will acquire the risk factor of the new area, especially if they are susceptible (genetically predisposed) to the disease (1).

Increasing evidence shows that histocompatibility antigens (HLA) may be involved in the susceptibility to MS, particularly HLA-A3, HLA-B7, and HLA-Dw2 (formerly LD-7a). These antigens are significantly more common in MS patients than in control populations (13). They are not diagnostic for MS, but possibly allow patients to be predisposed to the agents which cause the disease. It is also possible that another gene closely linked to an HLA gene may be responsible.

Histocompatibility genes may also influence the progress of MS. Jersild et al. (22) has reported that a rapid progression of the demyelinating disease is more prevalent in patients with HLA-Dw2.

The etiology of MS is not known, but several areas of investigation are open. The primary consideration now is that MS may be due to a viral infection. Three features of the disease, 1) the

demyelination, 2) the intermittent course, and 3) the lengthy duration, are each characteristic of some known virus diseases and each can be associated with virus infections of the central nervous system (CNS) (39). The virus most often implicated is the measles virus, although other paramyxoviruses may be involved.

In 1976, Alter pointed out that measles occurs later in childhood in industrialized than in primitive societies and suggested that MS may be a rare response to measles in late childhood. Multiple sclerosis patients tend to have had measles later in life than controls (2).

In 1962, Adams and Imagawa (16) first demonstrated slightly increased titers of measles antibody in patients with MS when compared to normal populations. Antibodies to other viruses have been found by some workers, but the measles antibodies are the most consistent. In those patients who have the abnormal band of IgG in the CSF, only a small part of this immunoglobulin is measles specific. By contrast, in subacute sclerosing panencephalitis (SSPE), a disease known to be caused by the measles virus, the CSF IgG is almost all measles specific (16). In approximately one-third of MS patients, there is an IgM which is specific for an antigen on the surface of measles infected test cells. Clearly, more testing needs to be done, particularly with purified antigens, to determine the specificity of the antibodies present in the serum and CSF of MS patients.

Additional evidence that a virus is involved was first reported by Carp et al. (10) in 1972 and confirmed by Koldovsky and Henle (23) in 1975. When materials from the brain, serum, spleen, or CSF of MS patients were injected into mice, there was a decrease in circulating

polymorphonuclear neutrophils (PMN's) within 16 to 48 hours. This PMN depression was serially transmitted to normal mice by inoculation of sera from the infected mice. Multiple sclerosis material added to tissue cultures of transformed mouse fibroblasts (PAM cells) which contain an RNA tumor virus resulted in decreased cell yields. Cell-free lysates from these cultures also produced PMN reduction in mice (38). The effect of the transmissible agent was neutralized by sera from MS patients and their relatives who did not have the disease (16,38). Although these studies were promising, they have not been confirmed by others, nor can Carp and Henle repeat their results (45).

Immunologically, there is evidence that cell-mediated immune mechanisms may be involved in multiple sclerosis. Lymphocytes are present at lesion sites. In the peripheral blood there are changes in lymphocyte subpopulations and their functions, and there are similarities between MS and some known autoimmune diseases in humans. Unfortunately, cell-mediated immune functions in MS patients are difficult to assess due to many technical variables in the testing, particularly the complexity of reactions and the impurity of test antigens.

It has been demonstrated that lymphocytes are present around the CNS lesions of MS patients indicating an immune response. Whether this response causes the original damage or develops as a result of damage induced by viruses or other causes is unknown.

Using cell-surface markers, T- and B-cells in the peripheral blood are generally found to be in different proportions when compared to normal populations. Many patients have shown a decrease in T-cells,

particularly during acute exacerbations (Oger et al.; Lisak et al.) (25). In others, however, the number may be unchanged. In addition, some investigators have shown reduced T-cell blast transformation with plant lectins, although others cannot confirm this finding (25). Most workers (25) agree that the number of B-cells is slightly increased, which may explain the increase in viral antibody titers in some patients. These same results are also found in autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (25).

At present there are no animal models available to study multiple sclerosis. It is possible to induce experimental allergic encephalomyelitis (EAE) in animals which is characterized by demyelination, one of the most prominent pathologic features of MS. Antibodies specific to the basic protein of myelin can be demonstrated in animals with EAE; however, these antibodies cannot be demonstrated in humans, which suggests some other antigen is involved in MS.

Current treatment of multiple sclerosis lies in relief of symptoms through the use of drugs to control spasticity and physical therapy. All have only modest results (6). Steroids may shorten the disability resulting from an exacerbation, but chronic steroid therapy does not significantly alter the outcome of the disease (42).

Because MS is of such long duration and is subject to spontaneous exacerbations and remissions, and because there is a wide variation in symptoms between individuals, the effectiveness of any single form of treatment is difficult to evaluate in a controlled fashion.

Transfer Factor - Multiple Sclerosis

One of the diseases for which transfer factor therapy has been tried is multiple sclerosis, but only a few of the clinical trials have been reported. Because the etiologic agent of MS is unknown, an adequate method of determining the immunity of a donor to MS is unavailable. Therefore, the reactivity of the TF to MS when given to the patient is unknown and can only be assessed by patient response.

Behen et al. (8) published a study in which 30 MS patients received TF prepared from household contacts. It was felt that relatives would have more chances of producing an immunity to MS than random donors. No substantial improvement was noted in these patients who received two injections over a six-month interval.

Platz et al. (20) reported improvement of three out of eleven patients who received high doses of TF over a two-year period. Fog and Zabriskie reported no improvement in their studies (15,50).

The most promising report was published by Angers et al. (4) in which TF prepared from household contacts and given at weekly intervals induced improved clinical response in greater than 50% of MS patients. Clinical signs which were improved included bladder function, mobility, coordination, stamina, and balance.

The wide variation in the results of the studies reported are probably due to donor selection and the variable status of the disease itself. In addition, most studies use dialysates from frozen-thawed leukocytes (TF_D), while Angers used frozen-thawed whole blood (TF_{WB}). Moreover, the dosage schedules differed in each study. Angers' protocol used TF_{WB} at weekly intervals, while other investigators administered TF less frequently.

Until the etiologic agent of MS is known, adequate donor selection is impossible to control. However, continued efforts can be made to determine the structure and the action of TF and to continue to monitor effects of TF therapy by patient response.

MATERIALS AND METHODS

Transfer Factor

Donors

The majority of the donors of the whole blood required to prepare the TF were relatives of MS patients. Some were friends and co-workers. Every donor was required to meet the age, health, and physical requirements set forth in the Standards of the American Association of Blood Banks (1976) for routine blood donation as well as closer scrutiny relative to viral diseases and allergies. In addition, the following laboratory tests were performed to insure the donor's health: complete blood count (CBC), erythrocyte sedimentation rate (ESR), VDRL, HBsAg (for hepatitis) and a screening test for infectious mononucleosis. Any abnormal results were reviewed by a pathologist to determine acceptability of using the donated blood.

After meeting these requirements, one unit of blood (450 ml) was drawn into a plastic bag containing heparin as the anticoagulant.[a] The blood was immediately frozen (without addition of cryopreservatives) at -70°C and maintained at that temperature until used.

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[a]Fenwal Corporation, Chicago, IL.

Transfer Factor Preparation

Whole blood was thawed at 4°C and placed in a sterile beaker. A 1/200 volume of Tween 80[b] (25% solution) was added and the mixture was stirred for 5-10 minutes at room temperature (25°C). During this time dialysis tubing[c] (6 ft. length) which had been soaking for 3-5 days in distilled water was prepared. Immediately before use it was rinsed in reagent grade denatured alcohol (95%)[d] and then rinsed 5-6 times in sterile distilled water. A double knot was made in one end of the tubing. The blood was poured into the other end, the tubing tied and placed in a 3800 ml wide-mouthed Erlenmeyer flask filled with cold pyrogen-free distilled water. Both ends of the tubing were placed outside the flask and secured with masking tape. The flask was then placed at 4°C for 24 hours while the water was gently agitated using a stir bar and magnetic stir rod. After 24 hours the dialysate was concentrated using an Amicon Ultrafiltration apparatus with filters having a 1000 M.W. cutoff (High Output UF Cell #2000).[e] The blood was redialyzed with cold, sterile water for another 24 hours and the dialysate concentrated. The concentration of both dialysates was achieved by positive pressure using an inert nitrogen propellant at 30-60 psi. The ultrafiltration was performed in the cold and a flow rate of 60-80 ml/hr is optimal. The ultrafiltration units and filters were sterilized by rinsing with 10% alcohol in

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[b] Tween 80 - #T-164, Fisher Scientific Co., Livonia, MI.

[c] Dialysis Membrane Size 30FO, Union Carbide Corp., Chicago, IL.

[d] Mallinckrodt #7008, Scientific Products, Allen Park, MI.

[e] Amicon Corp., Lexington, MA.

sterile distilled water. The units are periodically screened for bacterial contamination. All of the above work was carried out in the laboratory walk-in refrigerator, monitored at 4°C ± 2°.

The 20-25 ml of TF recovered after filtration was diluted to a 50 ml volume with 1.5% NaCl and was then filtered through a 0.22 μ Milex filter[f] and dispensed in 5 ml volumes (10 vials) and stored at -20°C until needed for use. Transfer factor was released to the patient's physician only when the sterility screen had been completed and the results were negative.

Dosage Schedule

Each dose of 5 ml was administered subcutaneously. Each patient received a dose three times during the first week of therapy. Thereafter, only one injection per week was given. This dosage may have been modified later depending upon patient response.

Biochemical Studies

The following tests were done to determine some of the chemical characteristics of TF_{WB}.

pH

The pH of 19 samples was determined using the Beckman[g] Model 3560 Digital pH Meter which was first standardized with buffers of pH 7.40 and 6.86.

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[f] Millipore Corp., Bedford, MA.

[g] Beckman, Fullerton, CA.

Osmolality

Five microliters of TF_{WB} was placed onto filter paper which then fit inside the vapor chamber of the #5100 Vapor Pressure Osmometer.[h] The osmolality results were read from a digital readout.

SMAC

Samples of TF_{WB} identical to that used by the patients were analyzed on the SMAC (Sequential Multiple Analyzer Computerized).[i] The following parameters were determined:

Albumin	CO_2
Alkaline phosphatase	CPK
ALT (SGP-T)	Creatinine
AST (SGO-T)	Glucose
Bilirubin, total	LDH
Bilirubin, direct	Phosphorus
BUN	Potassium
Calcium	Protein, total
Cholesterol	Sodium
Chloride	Uric acid

260/280 Ratios

A 1:20 dilution of TF_{WB} was made with distilled water. These diluted samples were then scanned on the Beckman DB Spectrophotometer[j] over a range of 240nm-300nm. The optical densities at 260 nm and 280 nm were determined and a ratio obtained. It was previously determined that diluting the sample would not alter the ratio.

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[h] Wescor, Inc., Logan, UT.

[i] Technicon Corp., Tarrytown, NY.

[j] Beckman, Fullerton, CA.

Limulus Amebocyte Lysate (LAL)[k]

This test was developed to detect endotoxins of gram-negative bacteria.

Glass vials were provided which already contained lyophilized lysate (0.1 ml/vial). This lysate was previously standardized to detect at least 1.0 ng/ml *E. coli* 0111:B4 endotoxin. Using a fresh pipette for each sample, 200 µl of transfer factor was added to each vial of lysate. Pyrogen free distilled water served as the negative control, while *E. coli* 0111:B4 endotoxin was the positive control. Each vial was mixed by tilting and gentle rolling until the contents were in solution. All the vials were then left *undisturbed* in a 37°C water bath for one hour. After incubation each vial was carefully placed in an inverted position on a flat surface. A positive test was characterized by the formation of a solid gel. A negative test was characterized by the absence of a gel or clot.

Several vials containing 0.1 ml lysate already treated with endotoxin also had TF added to them using the testing procedures already listed. These vials were used to determine whether TF was inhibitory to the reaction. A positive reaction was expected. If a negative reaction was observed, the entire test was considered invalid.

Agglutinins

Two drops of TF_{WB} and one drop of appropriate cells were combined in a 10 x 75 mm glass tube.

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[k]Microbial Associates, Bethesda, MD.

Agglutinin	Test Cell
Isoagglutinins	
Anti-A	a cells[l]
Anti-B	b cells
Alloagglutinins	
i.e., Anti-D	0 cells[m]

Two drops of 22% bovine albumin[n] were added to the tubes containing the 0 cells. All the tubes were centrifuged and then observed for hemolysis and/or agglutination. The tubes testing for alloagglutinins were then incubated for 30 minutes at 37°C. Again they were centrifuged and read. The cells were washed three times in 0.85% NaCl. After the third wash the supernatant was decanted and two drops of anti-human globulin (Coombs reagent)[n] were added to the cells remaining. The tubes were again centrifuged and then observed for hemolysis and/or agglutination.

Immunoglobulin Levels

Levels of IgG, IgM and IgA were determined in the sera of MS patients both before and after receiving TF. Five microliters of patient serum, standards and controls were placed in the wells of specific Tri-Partigen radial diffusion plates.[o] The plates were allowed to stand at room temperature for a minimum of 50 hours before the diameters of the precipitin rings were measured using a calibrated

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[l]Affirmagen, Ortho Diagnostics, Raritan, NJ.

[m]Selectogen, Ortho Diagnostics, Raritan, NJ.

[n]Ortho Diagnostics, Raritan, NJ.

[o]Behring Diagnostics, Somerville, NJ.

magnifier. The immunoglobulin level was then determined from a standard curve.

Lymphocyte Studies

Lymphocyte Preparation

Peripheral blood was collected in heparinized tubes and 0.1 ml dextran coated carbonyl iron (DCCI) was added to every 2.0 ml of the anticoagulated blood. The tubes were placed in a 37°C water bath for 20 minutes and the contents mixed every five minutes. The blood was then transferred to a Ficoll-Hypaque (F-H) gradient (1.0 ml of blood to 1.0 ml of gradient). The cell suspensions were centrifuged on the gradient at 1800 rpm for 25 minutes after which the upper layer of plasma was removed and discarded. The lymphocytes at the interface of the F-H gradient were collected with a Pasteur pipette and transferred to another centrifuge tube. To remove residual gradient, the lymphocyte suspension was washed with Hanks' Balanced Salt Solution (HBSS)-Incomplete by centrifuging at 1200 rpm for 10 minutes. Two washes were generally sufficient to remove all gradient components. Platelets were removed from the lymphocyte suspensions by a third wash at 500 rpm for five minutes. The supernatants were discarded following each centrifugation step. The lymphocytes were resuspended in Roswell Park Memorial Institute 1640 (RPMI 1640) supplemented with 2% human AB plasma for blastogenesis studies or Hanks' Balanced Salt Solution (HBSS) if rosette analyses or histocompatibility typing tests were required. The lymphocyte suspensions were adjusted to 2×10^6 cells per ml with the appropriate media.

Histocompatibility Testing

The standard National Institutes of Health (NIH) technique of microlymphocytotoxicity was used for determining the histocompatibility antigens on the lymphocytes of patients with multiple sclerosis. The antisera were supplied by NIH. Please see the Appendix for the complete procedure.

Rosette Analysis

Preparation of neuraminidase-treated erythrocytes (E_N cells) for identification of thymus-derived (T) lymphocytes: Sheep erythrocytes (SRBC) were washed with Hanks' Balanced Salt Solution (HBSS) three times. The SRBC's were adjusted to a 5% suspension with HBSS. Four-tenths milliliter vibriocholera neuraminidase (VCN) was added to 2.0 ml of the 5% SRBC's and incubated at 37°C for 60 minutes. After incubation, the VCN treated SRBC's were washed three times with HBSS and then readjusted to a 5% suspension. The working suspension for the assay was 0.5% SRBC's.

Preparation of human erythrocytes with added complement (HEAC cells) for bone-marrow derived (B) lymphocytes: Group O human erythrocytes (RBC) were washed five times with HBSS. The cells were adjusted to a 2.5% suspension and then 2.0 ml of the human RBC's were incubated with 2.0 ml of a 1:20 dilution of rabbit anti-human RBC serum for 40 minutes at 37°C. Then the cells were centrifuged and the supernatant removed. Two-tenths milliliter of mouse complement was added and the cells were incubated for an additional 30 minutes. After being washed four times with HBSS the cells were adjusted to a 5% suspension. The working suspension for the assay was 0.5% RBC's.

Rosette assay: One-tenth milliliter lymphocyte suspension (2×10^6/ml) was mixed with 0.1 ml of E_N or HEAC cells in a 10 x 75 mm glass tube. The tube was centrifuged at 600 rpm for eight minutes. Approximately 0.1 ml of supernatant was removed from the tube and replaced with 0.1 ml of a 0.01% gentian violet solution. The pellet was *gently* resuspended with a Pasteur pipette and one drop was transferred to a hemacytometer. All the lymphocytes in the four large corner squares were counted, both rosettes and lymphocytes with no RBC's attached. Lymphocytes with three or more E_N or HEAC cells attached were considered rosettes. The percentage of rosette forming cells (RFC) was determined by dividing the number of rosettes by the total number of lymphocytes counted.

Active T-Rosettes: One-tenth milliliter of lymphocyte suspension (2×10^6/ml) was mixed with 0.2 ml inactivated fetal calf serum (FCS) and 0.2 ml of 0.5% suspension of SRBC's which had been washed four times in HBSS. This mixture was incubated in a 10 x 75 mm glass tube for 60 minutes at 37°C and then centrifuged at 400 rpm for 7 minutes. Approximately 0.3 ml of supernatant was removed from the tube and replaced with 0.1 ml of 0.01% gentian violet suspension. The pellet was gently resuspended and one drop was transferred to a hemacytometer. All the lymphocytes in the four large corner squares were counted and the percentage of rosette forming cells was determined.

Active T-Rosettes with TF: Lymphocytes (5×10^6 ml) from both MS patients and controls were incubated with 0.5 ml TF at 37°C for 30 minutes. The cells were then washed three times with Medium 199 and resuspended to a 2×10^6/ml suspension. Then the percentage of active T-rosettes was determined as above.

Lymphocyte Blastogenesis

The lymphocytes were prepared and adjusted to a suspension of 2×10^6 cells/ml with RPMI 1640 medium with 2% FCS. Mitogen concentrations were adjusted to give a wide dose range (Leucoagglutinin - 20 µg/ml to 0.5 µg/ml). Control cultures (no mitogen) and 2 or 3 mitogen doses were usually performed for each leukocyte culture. One-tenth milliliter cells and 0.1 ml mitogen were added to microliter plates with an Eppendorf pipette. Cultures were set up in duplicate and triplicate if enough cells were available. The cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere and the blastogenic response was determined approximately 72-80 hours after culture initiation. Fifty microliters of tritiated thymidine (^{3}H-Tdr) was added about 18 hours prior to harvesting the cultures using a MASH-II. The filter paper disks were placed in scintillation vials and 10 ml of Scintiverse was added. The cultures were dark adapted in the scintillation counter for two hours and were then counted for two minutes. The stimulation index (SI) was then calculated:

$$\frac{\text{(cpm cultures with mitogen) - (cpm background cultures)}}{\text{(cpm background cultures)}}$$

Lymphocyte Blastogenesis with TF

Two milliliters of prepared lymphocytes (5×10^6/ml) from both MS patients and controls were incubated with 1.0 ml TF at 37°C for different time intervals. The cells were then washed three times with RPMI 1640 and resuspended to a 2×10^6/ml suspension. The blastogenesis cultures were then set up as outlined above.

RESULTS

The results obtained are listed in the following tables.

Table 1. Percentage comparison of 16 major histocompatibility antigens between 44 multiple sclerosis patients and a normal population (49)

HLA Antigen	% MS Patients	% Normal Caucasian Population
HLA-A1	27	28
HLA-A2	36	50
HLA-A3	30	26
HLA-A9	20	20
HLA-A10	14	12
HLA-A11	9	11
HLA-A28	2	9
HLA-A29	7	9
HLA-B5	11	11
HLA-B7	48	24
HLA-B8	18	21
HLA-B12	14	29
HLA-B13	2	4
HLA-B14	11	8
HLA-B18	11	8
HLA-B27	2	6

Table 2. Chemical evaluation of whole blood transfer factor using the Sequential Multiple Analyzer-Computerized (SMAC)

Parameter	Mean	Range	Standard Deviation	Normal Serum Values
Albumin	0.0			3.5-5.0 g/dl
Alkaline phosphatase	0.0			30-105 U/L
ALT (SGPT)	0.0			2-45 U/L
AST (SGOT)	0.0			10-40 U/L
Bilirubin - total	0.0			0.2-1.0 mg/dl
Bilirubin - direct	0.0			0.0-0.3 mg/dl
BUN	0.0			6-23 mg/dl
Calcium	8.4	2.1-15	3.2	8.5-10.5 mg/dl
Cholesterol	8.5	4-22	3.9	150-250 mg/dl
Chloride	>130			96-107 mEq/L
CO_2	0.0			24-33 mM/L
CPK	0.0			25-145 U/L
Creatinine	0.6	0.1-1.3	0.2	0.6-1.1 mg/dl
Glucose	5.7	0-15	4.9	65-110 mg/dl
LDH	0.0			100-225 U/L
Phosphorus	4.9	0.6-9.9	3.2	2.5-4.5 mg/dl
Potassium	7.1	1.7-10.0	2.9	3.3-4.6 mEq/L
Protein - total	0.0			6-8 g/dl
Sodium	>160			135-145 mEq/L
Uric acid	0.0			2.5-8.0 mg/dl

Table 3. Spectrophotometric ratio, osmolality, and pH of whole blood transfer factor

Parameter	Mean	Range	Standard Deviation
260/280 ratio	6.7	1.2-12.9	1.8
Osmolality	320	213-469	46
pH	7.39	6.73-7.68	1.67

Table 4. Evaluation of whole blood transfer factor for isoagglutinins and alloagglutinins

No isoagglutinins (anti-A, anti-B) or alloagglutinins were demonstrated in 20 samples of transfer factor.

In 2 cases in which anti-D was known to exist in the donor blood, the antibody was not demonstrated in the transfer factor prepared from that blood.

No unexpected antibodies were demonstrated in 20 multiple sclerosis patients up to 6 months following transfer factor therapy.

Table 5. Immunoglobulin levels of 9 MS patients before and after TF therapy

Ig	Pre (mean)	Post (mean)	T-test Values	Normal Values
G	958	996	0.40	548-1768 mg/dl
M	153	151	0.08	45-153 mg/dl
A	152	158	0.20	78-322 mg/dl

Using the Student's t-test, there is no significant difference between the pre and post values at P=0.05.

Table 6. Lymphocyte populations (percent) in peripheral blood of controls and MS patients following TF therapy

Group	Number	B-cells	T-cells	Active T-cells
Controls	20	18.1±1.9	69.4±3.7	20.6±3.8
MS patients	25	17.0±3.6	67.8±4.7	20.1±5.1
T-test values		1.31	1.19	0.32

Using the Student's t-test, there is no significant difference between these two populations at P=0.05.

Table 7. *In vitro* effect of TF on active T-rosettes of controls and MS patients (percent rosettes)

Group	No TF	With TF	T-test Values
Controls	19.7±1.7	24.1±3.4	2.28
MS patients	18.2±3.0	23.4±3.0	2.20

Using the Student's t-test, there is a significant difference between the number of active T-rosettes with and without TF at P=0.05.

Table 8. Results of Limulus Amebocyte Lysate (LAL) tests on 20 TF samples plus the pH and osmolality of each

Sample No.	LAL Results	pH	Osmolality
1	-	6.73	413
2	+	7.38	375
3	+	6.95	226
4	-	7.01	346
5	+	7.55	261
6	+	7.48	256
7	+	7.41	325
8	-	7.43	469
9	+	7.53	403
10	+	-	289
11	+	7.62	302
12	+	7.52	367
13	+	7.49	443
14	-	7.45	365
15	+	7.15	320
16	+	7.68	275
17	-	-	345
18	-	7.35	313
19	-	-	-
20	+	7.60	234

Positive control = +
Negative control = -
All inhibition controls = +

Table 9. Lymphocyte blastogenesis stimulation indices of MS patients and controls (Mitogen - Leukoagglutinin)

		Leukoagglutinin (µg/culture)				
		2.0	1.0	0.5	0.1	0.05
MS Patient	#1	8.11	8.23	8.89	6.63	5.56
	#2	9.73	10.30	11.44	15.81	9.52
	#3	9.81	9.74	10.92	9.07	5.30
	#4	7.60	7.80	7.78	8.98	5.99
	#5	43.55	52.30	47.66	3.23	9.00
Controls	#1	41.51	41.99	45.78	28.78	13.55
	#2	26.17	26.89	29.30	7.85	2.99
	#3	26.00	27.26	28.67	14.85	7.33
	#4	77.47	86.56	93.18	25.05	4.44

Table 10. Blastogenesis stimulation indices of lymphocytes incubated with TF thirty minutes, washed three times, and then cultured with Leukoagglutinin (0.5 µg/culture)

		With TF	Without TF
MS patient	#1	101.72	102.13
	#2	111.55	103.28
	#3	127.81	121.14
Controls	#1	118.82	101.11
	#2	107.31	113.36
	#3	100.98	131.88

Table 11. Blastogenesis stimulation indices of lymphocytes incubated with TF twenty-four hours, washed three times, and then cultured with Leukoagglutinin

	Leukoagglutinin (µg/culture)				
	2.0	1.0	0.5	0.1	0.05
MS patient #1					
with TF	393.48	432.02	440.77	175.83	96.85
without TF	118.21	115.05	111.19	4.67	2.33
MS patient #2					
with TF	291.52	326.19	333.12	131.15	74.44
without TF	96.94	103.28	98.43	28.06	3.52
Control #1					
with TF	329.38	312.35	266.98	68.39	14.91
without TF	160.37	146.74	121.19	6.75	1.08
Control #2					
with TF	247.84	257.12	247.49	72.99	24.08
without TF	89.13	156.34	167.28	72.20	37.82

DISCUSSION

Studies of immune competence in patients with multiple sclerosis are numerous and many give conflicting results (25). Dysfunctions in humoral as well as cell mediated immunity have been noted.

Sixty to eighty percent of MS patients will have an abnormal oligoclonal IgG in their CSF while serum immunoglobulin levels remain within normal ranges. Reduced mixed lymphocyte reactions, blastogenic responses to phytolectins and impaired delayed hypersensitivity reactions to microbial and fungal skin test antigens have been reported. Other studies, however, have failed to duplicate these results (25).

Virus-specific immune responses in MS patients have also been variable. Sever and Kurtzke (46) reported delayed hypersensitivity in skin reactions to measles and mumps antigen preparations in MS patients. A reduced blastogenic response of these patients to measles viral antigens was observed by Dau and Peterson (11); however, Knowles and Sanders (26) found no significant differences. Utermohlen and Zabriskie and Ciongoli et al. (25) demonstrated a diminished capacity of MS peripheral blood lymphocytes (PBL) to synthesize migration inhibition factor (MIF) when stimulated *in vitro* with measles antigen. In general, there appears to be a tendency toward a reduced activity of CMI responses against measles and paramyxoviruses, as compared with other antigens.

Because of the demyelinating nature of MS the response to neural antigens has also been studied. Lisak et al. (25) observed no delayed hypersensitivity to CNS antigens in MS patients and also were unable to show the production of MIF with MS cells exposed to myelin basic protein. Other workers, however, have reported otherwise (25). Both positive and negative results for lymphocyte transformation in response to basic protein have been reported (25).

It has been proposed that altered cellular immunity in MS patients could result from changes induced by viruses or by responses to viral antigens cross-reacting with neural antigens.

This study confirms many of the observations already reported.

Of the histocompatibility antigens generally associated with MS, HLA-A3 and HLA-B7, only one, HLA-B7, was significantly increased. It was found in twice as many of the MS patients as would normally be expected. There was only a very slight increase in the HLA-A3 antigen. (The HLA-Dw2 antigen was not included in this testing.) Two antigens, HLA-A2 and HLA-B12, were expressed less often than in the normal caucasian population. (All the patients tested were caucasian.) (Table 1)

The lymphocyte populations (T- and B-cells) in the peripheral blood of the MS patients occurred in proportions similar to those of normal controls tested at the same times. Other workers have reported a decrease in T-cells with a relative increase in B-cells (25). However, the MS patients used in this study were already using TF for therapy. It is possible that the TF brought their T-cells back up to normal levels but pre and post TF studies need to be done to prove this (Table 6).

It has also been reported that blastogenic response is reduced in MS patients and this study tends to confirm this report (Table 9). Four out of five of the patients studied had stimulation indices significantly lower than normal controls when their lymphocytes were cultured with varying amounts of Leukoagglutinin.

The major objective of this study was to evaluate the influence of TF_{WB} on lymphocytic activities and several observations can be made.

Antibody production, either active or passive, is not stimulated by TF_{WB}. No irregular antibodies were found in twenty MS patients up to six months following the onset of TF therapy (Table 5). Neither were there any changes in the serum immunoglobulin levels in a group of patients studied before and after TF therapy. This suggests that TF_{WB} does not stimulate the humoral immune system, a fact which has also been demonstrated with TF_{D} (29).

Isoantibodies (anti-A and anti-B) and alloantibodies (anti-Rh_{o}[D]) known to exist in donor blood were not found in TF_{WB} (Table 4). Because antibodies are immunoglobulins they are molecules too large to pass the dialysis membrane.

One of the more interesting results of this study was the influence of TF_{WB} on the active-rosette population in MS and control volunteers (Table 7). Transfer factor derived from whole blood stimulated both patient and normal control active T-lymphocytes. This suggests a non-specific response of the cells to the TF. The active rosettes are generally considered to be T-cells which are actively engaged in the immune response at the time of testing. Yu (21) concluded that the active cells are a subpopulation with

different numbers of membrane sulfhydryl and sialic acid groups which lead to a different surface distribution of SRBC receptors. Holtzman and Lawrence (21) have demonstrated that TF_D will increase the number of active rosettes *in vitro* in the normal population following a three hour incubation. They concluded that this was a non-specific response. This study shows that TF_{WB} also will increase the number of active rosettes following only a thirty minute incubation.

It should be noted here that inconsistencies arose in the active rosette testing procedure when RBC's from different sheep were used from day to day. Results became reproducible when the red cells from the same sheep (#20 from Microbiological Associates) were used throughout the experiment.

Blastogenic procedures were tried using Leukoagglutinin as the mitogen and various combinations of TF_{WB}. The results were variable. The TF_{WB} was not mitogenic, nor was it cytotoxic. The percentage of viable control cells and TF treated cells was 90% at 24 hours, 85% at 48 hours, and 75% at 72 hours incubation at 37°C.

Lymphocytes from both MS patients and normal controls were incubated with TF_{WB} for thirty minutes, washed three times, and then cultured with Leukoagglutinin (0.5 µg/culture). A sub-optimal dose of the mitogen was used so that elevation in the stimulation index would be possible. In all cases there was essentially no change in lymphocyte stimulation by incubating for a short time with TF_{WB} (Table 10).

However, when lymphocytes from patients and controls were incubated with TF_{WB} for 24 hours before culture, there was a marked change

in the blastogenic response. The two MS patients had a 3- to 4-fold increase in stimulation and the normal controls had a 2- to 3-fold increase.

As with the active rosettes, there appears to be a non-specific response by the cells to the TF_{WB} since both the patient and normal control cells responded to TF_{WB} incubation. This only points out the need for further characterization of the active molecules of transfer factor and their separation into adjuvant and antigen-specific activities.

The chemical studies show that transfer factor derived from whole blood appears to have properties similar to those reported for TF derived from leukocytes. It is a non-immunogenic substance that contains no protein (Table 2). The sodium and chloride levels are high due to the addition of normal saline in the preparation of the product. The other molecules present in significant amounts are ions (calcium, potassium, and phosphorus) which have molecular weights less than 10,000.

Spectrophotometrically, TF_{WB} exhibited a significant absorption peak at 254 nm. This observation suggests that nucleotides are present in TF_{WB} preparation since absorbance in the range of 260 nm is usually due to nucleotides while absorbance at or near 280 nm is usually due to protein. Little, if any, protein is contained in TF_{WB} which was determined by the SMAC analysis. All the TF_{WB} preparations tested by this method had a peak absorbance near 260 nm which gave a 260/280 ratio greater than 1.0 (Table 3). Groust et al. (18) reported 260/280 ratio values from 1.03 to 1.35 on TF derived from leukocytes (TF_D). The mean value in this study using

TF_{WB} was 6.7 with a range of 1.2 to 12.9. It therefore is possible that there are more nucleotides in TF_{WB} than in TF_{D} as a direct result of either the erythrocytes or something in the plasma. It is also possible that something other than nucleotides is causing the absorbance at that wavelength. Further studies using different separation techniques would be helpful in the determination. This also might indicate where the active moiety of TF lies - whatever that may be.

Another test for the existence of nucleotides in TF_{WB} is the Limulus Amebocyte Lysate (LAL) test for endotoxin of gram-negative bacteria. Elin and Wolff (14) and Levin et al. (33) have demonstrated that some compounds such as polynucleotides, heavy metals, blood products, or high osmolality may give false positive or false negative results and that the pH range of the samples must be 6.8 to 7.5 for maximum efficiency of the reaction. It is highly unlikely due to aseptic techniques and cold temperatures throughout all procedures that endotoxin is present in TF. Moreover, each preparation exhibited no bacterial growth on culture. However, as noted in Table 8, thirteen out of twenty samples of TF_{WB} tested demonstrated a positive reaction to LAL. The pH and osmolality of each sample is within range for appropriate reaction and all controls gave expected results. If no endotoxin is present, something else is responsible for the non-specific positive reaction. Again, separation techniques or even dilution studies will give more answers to this question.

It is difficult to fully assess TF_{WB} produced for multiple sclerosis because a specific antigen for the disease has not yet been found. Donor selection is purely hypothetical and patient response

is inherently variable. However, more specific testing needs to be done. Possibilities may include blastogenesis using other mitogens or more extensive macrophage inhibition studies, etc. There is so much more yet to be learned.

SUMMARY AND CONCLUSIONS

Transfer factor derived from whole blood appears to have some properties similar to that of TF derived from leukocytes. It is a non-protein, non-antigenic substance which will stimulate lymphocytes *in vitro*. Its specificity for immunoprophylaxis for multiple sclerosis cannot be determined until a specific antigen related to the disease is identified and purified. Its clinical effects on MS patients is very difficult to evaluate due to the highly variable nature of the disease. Long-term exacerbation-free intervals in several patients at risk for relapses would provide positive information on its clinical usefulness.

Clearly, further studies are necessary to purify and characterize the active moieties of TF, but these studies must wait until more specific information is known about the etiologic agent in multiple sclerosis.

APPENDIX

Histocompatibility Testing

Preparation of antisera trays - All histocompatibility typing sera (supplied by NIH) were dispensed into polystyrene micro-histocompatibility typing trays. Liquid petroleum (0.004 ml) was added to each well with a 250 lambda Terisaki dispenser. Typing serum (0.001 ml) was added to each wall under the oil with a 50 lambda Terisaki dispenser. Known positive and negative sera were added as controls and the trays were stored at -70°C until needed.

Test procedure - Lymphocytes were prepared as on page and adjusted to a suspension of 2×10^6 cells/ml in HBSS. The mixed lymphocytes (0.001 ml) were added to each of the tray wells with a 50 lambda Terisaki dispenser making sure the antisera and cells mixed thoroughly. The tray was then incubated at room temperature (22-26°C) for thirty minutes. Rabbit complement (0.005 ml) was added to each well and the tray was incubated at room temperature for sixty minutes. Five percent eosin solution (0.003 ml) followed by 0.005 ml buffered formaldehyde were added to each well. A 50 x 75 mm microscope slide was lowered onto the wells in order to flatten the tops of the droplets. The wells were then observed with an inverted phase contrast microscope for cell viability. Living lymphocytes were small and refractile while dead reactive lymphocytes

were larger and stained red. A positive reaction was noted when a majority of the cells were killed.

Dextran-Coated Carbonyl Iron (DDCI)

Dextran-T-70 (70,000 M.W.) - Pharmacia, Piscataway, NJ
Carbonyl iron (3 μ particles) - GAF Corp., NY

One gram carbonyl iron is mixed with 12 ml of 5% dextran in 0.85% NaCl and placed in a 37°C water bath for 10 minutes. Mix occasionally. Store at 4°C.

Ficoll-Hypaque Gradient (F-H)

Ficoll (400,000 M.W.) - Pharmacia, Piscataway, NJ (9% solution)
Hypaque (sodium diatrizoate) - Winthrop Laboratories, NY (33% solution)

For the gradient combine 24 parts Ficoll to 10 parts Hypaque. The specific gravity should be 1.08.

The following may be obtained from GIBCO - Grand Island Biological Corporation, Grand Island, NY

RPMI 1640 with 25 mM Hepes Buffer (with glutamine - without antibiotics)

HBSS - Hanks' Balanced Salt Solution (1X)

Hanks' BSS (10X) - Hanks' Incomplete without calcium, magnesium, sodium bicarbonate

Medium 199

Vibrio cholera neuraminidase (VCN)

1.0 ml VCN is mixed with 1.0 ml HBSS and then the pH is adjusted to 7.0-7.4 with 0.1 N NaOH. VCN can be stored at 4°C.

Leukoagglutinin - Pharmacia, Piscataway, NJ

Fetal Calf Serum (FCS) - Reheis #50-204; absorbed on ice with sheep and human erythrocytes

Tritiated Thymidine (^{3}H-Tdr) - New England Nuclear

6.7 Ci/mM, #NET-027 Thymidine: 1.0 mCi, 0.036 mg stock solution
0.1 ml H-Tdr + 9.9 ml RPMI 1640

Scintillation Cocktail - Scintiverse - Fisher Scientific (#SO--X-1)

MASH - Multiple Automated Sample Harvester

Liquid Petroleum - Paraffin oil (mineral oil) - White, Light
Fisher Scientific Co., Silver Spring, MD

Terisaki Dispenser - Hamilton Co., Inc., Whittier, CA

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