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TRANSFER FACTOR AND EXPERIMENTAL
ALLERGIC ENCEPHALOMYELITIS

by

Dorothy Ellen Lewis

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DEPARTMENT OF MICROBIOLOGY AND MEDICAL TECHNOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY
WITH A MAJOR IN MICROBIOLOGY

In the Graduate College
THE UNIVERSITY OF ARIZONA

1978
I hereby recommend that this dissertation prepared under my direction by Dorothy Ellen Lewis entitled Transfer Factor and Experimental Allergic Encephalomyelitis be accepted as fulfilling the dissertation requirement for the degree of Doctor of Philosophy.

As members of the Final Examination Committee, we certify that we have read this dissertation and agree that it may be presented for final defense.

Final approval and acceptance of this dissertation is contingent on the candidate's adequate performance and defense thereof at the final oral examination.
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SIGNED: Dorothy Ellen Lewis
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ABSTRACT

Transfer factor prepared from Strain 13 guinea pigs sensitized to basic protein (BP) plus heat killed *Mycobacterium tuberculosis*, *M. tuberculosis* alone, or BP in Freund's incomplete adjuvant was found to have a significant prophylactic effect on the clinical severity of Experimental Allergic Encephalomyelitis in challenged recipients. Transfer factor prepared from uninoculated animals had no effect on the course of the disease. Histologic examination of brain sections correlated with the clinical observations.

Animals that received only the transfer factor preparations were skin tested 2 days after transfer factor with BP or tuberculin. In most cases, delayed-type skin reactivity to the sensitizing antigen was passively transferred.

A group of animals that survived the encephalitogenic challenge was skin tested 28 days after sensitization. A significant negative correlation was found between skin test diameter and clinical severity of the disease. Those with the least severe disease demonstrated larger skin test responses.

Lymphocyte transformation in vitro was used to monitor the disease in both the transfer factor treated groups
and control animals. Results indicate that those subjects that survived the disease had greater blastogenic responses to BP in vitro. In addition, the Concanavalin A response in those that survived was increased over the course of the disease, whereas the Phytohemagglutinin responses remained stable in all animals.

Total white blood cell and differential counts were made over the course of the disease. The white blood cell counts increased as a function of time after sensitization. The differential counts indicated that in those who survived the disease, the number of mononuclear cells was significantly elevated compared with those dying of the disease.

The data support the hypothesis that delayed-type hypersensitivity is dissociated from disease induction. Transfer factor preparations induced delayed-type hypersensitivity and caused suppression of the disease.
INTRODUCTION

Experimental Allergic Encephalomyelitis (EAE) is an autoimmune disease of the central nervous system (CNS) induced in a variety of experimental animals by injection of CNS tissue or myelin basic protein (BP) in Freund's complete adjuvant (FCA) (1-10). The disease in the guinea pig is characterized by onset within 14-17 days, rapid loss of weight, hind leg paralysis, incontinence, impacted feces, and death. Histologically, EAE is characterized by perivascular infiltration of mononuclear cells, predominantly lymphocytes, in the brain (11).

Although EAE has been studied extensively for 30 years, its pathogenesis remains unclear. Most investigators exclude antibody to BP as the mediator since the disease cannot be passively transferred with serum (12-14) and levels of antibody do not correlate with the severity of the disease (13, 15). Cell mediated immunity has been implicated, since the disease can be passively transferred with lymphoid cells (16, 17). The cells involved have been identified as T lymphocytes. However, it is not clear how these T cells mediate the disease (18, 19). Several investigators (15, 20) have shown that delayed-type skin reactivity to BP,
lymphocyte transformation (20-22), and macrophage migration inhibition (23, 24) all develop during the disease.

The antigen responsible for EAE production, myelin basic protein, has been purified and amino acid sequences determined for the human, bovine, guinea pig, and rat proteins. BP has a weight of 18,000 daltons, is composed of 169 amino acids, has a high proportion of basic amino acids, and has very little helical structure (25-27). BP, when emulsified with FCA and injected into animals, is encephalitogenic and induces both antibody and delayed-type skin reactivity. BP in saline or in Freund's incomplete adjuvant (FIA) does not produce the disease, but can be used as a sensitizing agent to induce antibody formation or delayed-type hypersensitivity to BP. If BP in FIA is given prior to an encephalitogenic challenge, it prevents EAE. If BP in FIA is given after an encephalitogenic challenge it can suppress the clinical signs of the disease and may decrease the severity of the histologic lesions in the brain (28).

Peptides derived from BP have been isolated and characterized as to their encephalitogenic activity. In the guinea pig, the encephalitogenic peptide consists of amino acid sequence 114-122, which contains a tryptophan residue that is essential for encephalitogenic activity (29). Another peptide derived from BP, peptide 44-89, induces delayed-type hypersensitivity only (30-32).
Lymphocyte Transformation Assay

Lymphocyte transformation in vitro is used to determine the responsiveness of an animal to specific antigens or to mitogens (33-38). The majority of normal peripheral blood lymphocytes do not rapidly synthesize deoxyribonucleic acid (DNA) unless stimulated in some manner. Upon stimulation with mitogens or with specific antigens, small lymphocytes undergo morphological enlargement concomitant with increased DNA synthesis. Prior to the use of radiolabeled precursors, the most common techniques used to determine increased DNA synthesis were determination of increased numbers of mitotic figures and enumeration of blast cells using autoradiography (33-35). The most widely used method today is that of measuring increased incorporation of radio-labeled thymidine ($^3$H TdR) into the DNA of transforming cells.

When the lymphocyte transformation assay is applied to the study of EAE, increased uptake of $^3$H TdR has been shown to occur in EAE sensitized lymphoid cells using either nervous tissue antigens or BP as a stimulus in vitro. The first study, reported by Warnatz, Scheiffarth, and Kuntz (21) used sensitized guinea pig lymph node cells incubated with whole brain antigens. The cells were incubated for five days, prepared for autoradiography, and the labeled cells counted microscopically. The authors found an increased
number of labeled cells over the course of the disease, with those checked at 18 days after sensitization showing the highest percentage of labeled cells. In addition, the authors attempted to correlate the number of labeled cells with the clinical severity and histological lesions. The results were inconclusive, with some of the severely affected animals showing greater numbers of labeled cells, whereas some animals showed increased numbers of labeled cells with no clinical signs.

In 1972, Fowler (22) reported the use of buffy coat peripheral rabbit leukocytes from animals sensitized to EAE to study the response in vitro to whole brain homogenate and to BP. He demonstrated that a change could be detected one week after sensitization. The blastogenic response continued until 5-6 weeks after sensitization when the animals became severely paralyzed. In addition to studying the responsiveness of animals sensitized to BP in FCA, he also studied the effect of pretreatment with BP in FIA on the lymphocyte transformation assay. The rabbits were pretreated with BP in FIA and then given a normal encephalitogenic emulsion of BP in FCA. Fowler demonstrated that pretreatment with BP in FIA (and thus protection from disease) rendered the lymphocytes incapable of responding to BP in vitro.
More recently, Lisak and Zweiman (39) and Lisak et al. (20) reported in vitro reactivity to BP in guinea pig peritoneal exudative lymphocytes (PEL). In the first study, these investigators collected peripheral blood and peritoneal exudative cells at 10, 13, and 17 days after sensitization. The peripheral blood lymphocytes were separated using methylcellulose-sodium diatrozoate and then cultured at a concentration of $1 \times 10^6$ lymphocytes per ml. The PEL were separated on a coil cotton column and then used for culture at the same cell concentration. The authors failed to detect a response to BP or to Old Tuberculin with the peripheral blood lymphocytes at any time period, but detected a response using the PEL. They also showed a positive correlation between the level of lymphocyte proliferation and the degree of CNS pathologic abnormalities, as well as a correlation between BP skin reactivity and the lymphocyte proliferative response. The second study (20) also reported negative peripheral blood responses and positive PEL responses 13 days after sensitization.

The lymphocyte transformation assay has not been used extensively in studying EAE in the guinea pig. There is only one report on the use of the whole blood assay in this system (40). The whole blood assay has the advantage of considerably less manipulation of cells as well as the ability to follow the course of sensitization in an individual animal.
Suppression of EAE

Prevention or suppression of EAE can be accomplished in a variety of ways, including BP treatment (28, 41, 42), mycobacterial pretreatment (43, 44), antilymphocyte serum administration (45), and cyclophosphamide or methotrexate treatment (46-48). The most studied is the inhibition produced by BP. Prevention of EAE is usually accomplished by injections of BP in saline or FIA, typically 6-7 biweekly injections of 100 μg BP or 7 injections of BP in FIA over a 2 week period before an encephalitogenic injection (49). Suppression of the disease can be accomplished with injections of BP begun as late as 11 days after sensitization (15, 28), whereas studies indicate that reversal of clinical signs (28) can be accomplished in most guinea pigs if the disease is not severe. Recently, BP, with the tryptophan in the encephalitogenic region (amino acids 114-122) chemically blocked, has been used to inhibit the disease (29). Fragments of the BP molecule distinct from the encephalitogenic region (amino acids 44-89) have also been used to inhibit the disease (30). The protection induced by pretreatment is long lasting (up to 52 weeks) (50, 51) and passive transfer of EAE can be prevented if cell donors are treated with BP (52).

The mechanism of antigen-induced inhibition of EAE remains to be elucidated. The two major hypotheses are the
formation of protective antibody or the induction of un-responsiveness at the cellular level. All the available evidence indicates that antibody is not responsible (28, 48, 49, 52, 53), whereas recent reports indicate that suppressor T cells are probably involved. Swierkosz and Swanborg (54) report that lymph node cells from BP pretreated, sensitized Lewis rats are incapable of transferring EAE. However, these lymph node cells are capable of transferring unresponsiveness to an encephalitogenic challenge. The ability of such cells to render recipient rats unresponsive to challenge is abrogated by in vitro treatment of the lymph node cells with antithymocyte serum plus complement. Further experiments indicate that the cells do not adhere to glass wool (55). The investigators, on these limited data, suggest that the immunoregulatory cells in EAE are suppressor T lymphocytes. This finding has been corroborated using lymphocytes from mice (56), but not from guinea pigs.

Another means of suppression which has been explored is that induced by mycobacterial treatment (43, 44, 57-59). Lisak and Kies (43) first reported the effect of mycobacterial treatment on antibody levels and the development of delayed-type hypersensitivity. They found that pretreatment with mycobacteria or the use of excessive amounts of mycobacteria in the sensitizing emulsion not only prevented EAE, but had no effect on antibody levels and suppressed the
development of delayed-type skin reactivity to BP. These findings were confirmed and extended in a later report (44) where not only skin reactivity was suppressed, but also in vitro macrophage migration inhibition to BP was reduced.

**Transfer Factor**

Transfer factor is a dialyzable material, obtained from extracts of lymphoid cells from sensitized donors, which transfers specific delayed-type skin reactivity to naive recipients. Jeter, Tremaine, and Seebohm (60), in 1954, first described the transference of chemical hypersensitivity in guinea pigs using cellular extracts. This was followed by Lawrence's description of sub-cellular transfer of tuberculin and streptococcal sensitivities in humans (61, 62). Since this material is known to transfer a variety of delayed-type hypersensitivities (63-67) in the guinea pig and because EAE is thought to be mediated by delayed-type hypersensitivity, we thought that transfer factor might be responsible for the pathogenesis in EAE. Accordingly, lymphoid extracts from EAE sensitized guinea pigs were prepared and checked for their ability to transfer the disease. These attempts proved unsuccessful (68, 69).

Since leukocytic extracts failed to transfer the disease, a major question arose: Is there a role for transfer factor in EAE? Recent evidence suggests the dissociation of delayed-type hypersensitivity from encephalitogenic
activity in the disease (31, 32, 70-72). Swanborg (30) has shown that pretreatment with peptide 44-89 can suppress EAE in animals challenged with BP in FCA. In addition, Hashim and Schilling (31) and Hashim, Hwang, and Schilling (32) have shown that peptide 44-89 induces delayed-type hypersensitivity to BP and can suppress EAE in animals challenged with BP in FIA. Since transfer factor is associated with delayed-type hypersensitivity and yet failed to transfer the disease, it is conceivable that transfer factor is produced from lymphocytes sensitized to the delayed-type determinants in the BP molecule. Therefore, the suppression of the disease as described by Swanborg (30) and Hashim et al. (31, 32) may be mediated by transfer factor. Several recent reports indicate the efficacy of transfer factor therapy in a variety of experimental and clinical situations (73-80). Experiments were therefore designed to investigate the possibility that transfer factor might serve as a prophylactic agent in EAE.
MATERIALS AND METHODS

Animals

Wright Strain 13 guinea pigs (Southwestern Research Animals) were used in all experiments. The transfer factor donors weighed 600-800 g and recipient animals weighed 400-600 g. Original breeding stock was obtained from Dr. Denis R. Burger. Brother-sister matings maintained the inbred nature of the animals, which was monitored by occasional skin grafting crosses. The animals were housed in separate cages and maintained on Wayne guinea pig diet, fresh cabbage daily, and water ad libitum.

Antigen Preparation and Sensitization Methods

Bovine Basic Protein (BP) was prepared by the method of Kies (9). A dose response was done with each preparation to determine the best encephalitogenic dose for use. In most cases, 0.5 to 1 mg BP in saline plus 2-2.5 mg heat killed, dry Mycobacterium tuberculosis, strain H37RV, in Freund's incomplete adjuvant (FIA) was used to sensitize recipients. The emulsions were made by combining an equal volume of the appropriate concentration of BP in saline with a solution (FIA) consisting of 8.5 parts sterile light mineral oil (American Drug and Chemical Co.), 1.5 parts Arlacel A (Atlas Chemical Industries, Inc.), and the appropriate
concentration of *M. tuberculosis*. The mixture was combined slowly and forced through a syringe until a stable emulsion resulted. Recipient animals were sensitized intradermally on the nape of the neck in 3 sites, each site receiving 0.1 ml of the emulsion.

**Sensitization Method for Each Transfer Factor**

Transfer factor was prepared from strain 13 guinea pigs after animals were sensitized with different emulsions. Transfer factor designated BPTB was prepared by sensitizing donors with 2 mg BP plus 2 mg *M. tuberculosis* in FIA. TB and BP transfer factors were prepared by sensitizing with 2 mg *M. tuberculosis* and 2 mg BP, respectively. Normal transfer factor was prepared by either injecting donors with FIA or from uninoculated animals. In all cases, the preparation of the antigen and the sensitization was carried out as described above. Transfer factor was collected 6 days after sensitization.

**Collection of Peritoneal Exudative Cells**

Each transfer factor donor received 20 ml of sterile light mineral oil intra-abdominally 48 hours prior to collection of peritoneal exudative cells. The injection site was cauterized to prevent leakage. To collect the cells, animals were anesthetized with ether, exsanguinated by
cardiac puncture, and the abdominal wall opened aseptically. The cells were collected in two washes of the abdominal cavity with sterile Hanks' Balanced Salt Solution (HBSS) containing 10 units per ml of heparin and 20% normal guinea pig serum. The wash fluids were pooled, much of the oil removed with a separatory funnel, and the cells centrifuged at 500 x g for 15 minutes at room temperature. The cells were washed twice in HBSS plus 20% serum to remove any additional oil and were resuspended in HBSS.

Collection of Lymph Node Cells

Suprascapular, axillary, and cervical lymph nodes were dissected from each animal and trimmed of fat, minced, and the cells expressed through a stainless steel screen (20 gauge, 60 mesh). The cells were centrifuged at 500 x g for 15 minutes at room temperature and washed twice in HBSS plus 20% normal serum. The peritoneal exudative cells and lymph node cells were combined. Total and viable white blood cell counts were estimated by trypan blue exclusion (81).

Preparation of Transfer Factor

Pooled peritoneal exudative and lymph node cells were diluted so that 1 x 10⁹ viable white blood cells were contained in 7.5 ml of HBSS. This mixture was incubated for 4 hours at 37 C with frequent agitation. After the incubation period, cells were centrifuged at 500 x g for 15
minutes at room temperature. The supernatant fluid was harvested and placed in dialysis tubing (Van Waters and Rogers, average pore radius permeability of 24 angstroms, which has been soaked in double distilled water). The fluid was vacuum dialyzed for 24 hours at 4 C and either used immediately or stored at -20 C. One dose of transfer factor was defined as the volume of the dialyzed supernatant that had contained $1 \times 10^9$ white blood cells.

**Experimental Design**

The experimental design used to determine the effect of the transfer factors on the development of EAE is as follows. The transfer factor preparations (designated BPTB, TB, BP, or Normal) were injected intraperitoneally at a dosage of 1-2.5 doses either two days before or two days after inoculation of a sensitizing encephalitogenic emulsion. Controls included those animals receiving only the transfer factor and those receiving only the encephalitogenic emulsion. All animals were checked daily for signs of EAE. Body weight and other clinical observations were recorded beginning 10 days after sensitization. Clinical severity was scored by the method of Stone (17). Four plus indicated death; three plus was characterized by severe clinical signs (weight loss, paralysis, incontinence, impacted feces) exclusive of death; two plus indicated weight loss and one other clinical sign (paralysis, incontinence
or impacted feces); one plus was weight loss, and zero indicated that no signs were observed in the animal. Brains were removed for histological studies in three experiments and placed in 10% neutral buffered formalin. The brains were collected at a time when the animals were moribund or at the termination of the experiment. Animals were exsanguinated and killed by ether overdose. Histology was scored as to the methods of Alvord and Kies (11). Two plus indicated that there was marked perivascular inflammation, easily seen in almost every field. One plus was characterized by definite inflammation about many vessels in the central nervous system, easily seen in many fields with 30 x magnification. Plus/Minus meant there were a few small foci of leukocytes about blood vessels within brain or cord substance, and zero indicated no abnormality. The slides were prepared and read by Dr. R. E. Reed, Professor of Veterinary Science, Department of Veterinary Science, University of Arizona.

**Skin Testing**

Animals receiving only the transfer factor preparations were skin tested intradermally 48 hours after the material was given. One tenth ml of saline containing 10 or 50 µg BP (20) or 0.1 ml 1:50 dialyzed Old Tuberculin (OT) was used, depending on the type of transfer factor given. Skin reactions were read at 0, 6, and 24 hours after skin
testing and expressed as mean diameter in millimeters of erythema and induration. In some experiments, animals surviving the experiment were skin tested prior to termination of the experiment. In a few cases, skin biopsies were obtained and histology scored by Dr. R. E. Reed to ascertain the nature of the skin reaction.

**In Vitro Experiments**

In addition to the clinical observations described above, the lymphocyte transformation assay, essentially that of Han and Pauley (82) and Lisak et al. (20), was used over the course of sensitization to determine if differences between treated and control animals could be detected. Lymphocyte cultures were made at all time periods (days 0, 7, 14, and if possible day 21 after sensitization) by adding 1 ml of heparinized (Heparin, preservative free, Grand Island Biological Co., Grand Island, New York, 10 units per ml) guinea pig cardiac blood to 29 ml of Roswell Park Memorial Institute 1640 medium containing 200 mM L-glutamine (Grand Island Biological Co., Grand Island, New York). The medium was supplemented with penicillin (100 units per ml), streptomycin (100 μg per ml), N-2-hydroxyethylpeperazine-N-2-ethane-sulfonic acid (HEPES) (25 mM), and sodium bicarbonate (2 g per liter). The medium was sterilized by filtration (0.22 μ), stored at 4 C until use, and adjusted to pH 7.4 prior to the addition of the blood.
The blood-medium mixture was placed on a magnetic stirrer and 2 ml volumes dispensed into 17 x 100 mm polypropylene culture tubes (Falcon, No. 2059). The tubes either contained no addition or 0.1 ml amounts of mitogen or antigen diluted in Cell Balanced Salt Solution (CBSS) (83). Phytohemagglutinin-P (PHA-P, Difco Laboratories, Detroit, Michigan) was used at a concentration of 50 μg/ml, Concanavalin A (Con A, Calbiochem, San Diego, California) was used at a concentration of 20 μg/ml and BP concentrations included 50 and 150 μg/ml. Mitogen and antigen concentrations were pretested to find an optimal level. Control and antigen or mitogen treated cells were set up in triplicate. All cultures were incubated at 37 C in an humidified, 5% CO₂-95% air atmosphere for five days, then pulsed with 1 μCi in 0.1 ml of tritiated thymidine (³H-TdR, specific activity five Ci/mM, New England Nuclear) for 6 hours. To determine the uptake of tritium into deoxyribonucleic acid (DNA), the cultures were harvested for scintillation counting by collection of glass fiber filters contained in a Millipore sampling manifold (No. 1225, Bedford, Massachusetts). The method employed a saline wash, followed by 3% acetic acid, a second saline wash, and finally a methanol wash. The filters were dried and placed in a toluene base scintillation cocktail and counts per minute measured by the use of the Packard-Tri-Carb Liquid Scintillation Spectrometer (Model 3320, La Grange, Illinois).
White Blood Counts and Differentials

Total white blood counts and smears for Wright-stained differentials were prepared at each time period. The smears were counted and the number of mononuclear cells to neutrophils \( \frac{M}{N} \) obtained. This ratio was used in further analysis of the data. Also, as the disease progressed, atypical lymphocytes appeared in the blood. These cells were large, had abnormally dark blue cytoplasm, appeared to have an active Golgi apparatus, had prominent nucleoli, and the chromatin was dense and pyknotic. A tabulation was therefore made for the percentage of these atypical lymphocytes counted in each of the differential smears.

Statistical Analysis

Data from all studies were analyzed statistically by several different methods. The first method involved tabulation of the clinical severity according to the different treatments, placing comparisons in two way tables, and performing \( f \ln f \) transforms on the data. In this analysis, a Chi square table was used to determine significance on the basis of goodness of fit. In this way, each of the treatment groups and control group could be compared as to the clinical severity.

The lymphocyte transformation data were prepared for analysis by taking the geometric means of triplicate cultures.
and transforming these to $\log_{10}$ counts/minute. The stimulation indices were calculated as the log counts/minute in test cultures minus the log counts/minute in control cultures. Stimulation indices were also calculated by dividing the mean counts per minute (cpm) of the test cultures by the mean cpm of the control cultures without conversion to $\log_{10}$.

Data that included the clinical observations (severity, change in body weight, date of death) and the lymphocyte transformation studies, white blood cell counts and differentials were analyzed by the use of multiple regression and multiple discriminant analysis on the Control Data Computer Model 6400, using programs provided by Dr. L. M. Kelley, Department of Microbiology, University of Arizona.
RESULTS

Since leukocytic extracts from EAE sensitized donors were unable to transfer EAE to naive recipients, the question was asked, "What effect, if any, might transfer factor preparations have on the development of EAE?" Transfer factor was prepared from Strain 13 donor guinea pigs which had been sensitized to basic protein (BP) plus *Mycobacterium tuberculosis* (BPTB), *M. tuberculosis* alone (TB), or from animals sensitized to BP in incomplete Freund's adjuvant (BP). Transfer factor was also prepared from uninoculated animals or animals inoculated with incomplete Freund's adjuvant.

The transfer factor preparations, at a dosage of \(1-2.5 \times 10^9\) white blood cell equivalents, were administered either 2 days before or 2 days after a sensitizing encephalitogenic emulsion. Control animals received either the encephalitogenic emulsion or a transfer factor preparation.

BPTB, TB, and BP transfer factor preparations were found to have a significant (G-test for goodness of fit) prophylactic effect on the development of EAE in sensitized recipients. Transfer factor prepared from uninoculated animals or animals inoculated with FIA had no effect on the disease.
Results depicting the clinical severity for each type of transfer factor used are presented in Table 1. The severity scoring system is that of Stone (17). A score of four indicates death, three denotes disease exclusive of death, two is weight loss plus one other clinical sign, one is weight loss only, and zero indicates no clinical signs of EAE. As can be seen, 50% of the animals receiving BPTB transfer factor had no clinical signs of the disease, whereas 45% of those receiving TB transfer factor and 31% of those receiving BP transfer factor had no clinical signs. Ten percent of the animals that received transfer factor from uninoculated animals had no clinical signs of EAE, which compares with 7% of the control animals. As is also apparent from Table 1, the number of animals that died in the transfer factor treated groups and the control groups is similar (clinical severity score of four). Forty-six percent of the BPTB treated animals, 36% of the TB transfer factor treated animals, 31% of the BP treated animals, 40% of those treated with transfer factor from normal animals, and 51% of the untreated animals died of the disease. The transfer factor preparations (BPTB, TB, and BP) were effective in altering the number of animals without clinical signs, but had no significant effect on the number that eventually died of the disease.

The data in Table 1 represent several experiments for each transfer factor preparation. A typical experiment
Table 1. Comparison of transfer factor regimens in Experimental Allergic Encephalomyelitis.

<table>
<thead>
<tr>
<th>Group</th>
<th>Transfer Factor Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Time Administered&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Clinical Severity&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Without Clinical Signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>BPTB</td>
<td>before</td>
<td>6 0 1 2 8</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>after</td>
<td>2 0 3 3 9</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>TB</td>
<td>before</td>
<td>3 0 1 1 6</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>after</td>
<td>5 0 0 2 4</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>BP</td>
<td>before</td>
<td>2 0 0 4 3</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>after</td>
<td>3 1 1 0 3</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Normal</td>
<td>before</td>
<td>1 1 3 1 0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>after</td>
<td>3 1 0 1 1</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>None</td>
<td>N.A.</td>
<td>23 6 7 6 3</td>
<td>7</td>
</tr>
</tbody>
</table>

<sup>a</sup> BPTB, transfer factor from animals sensitized to BP plus M. tuberculosis; TB, transfer factor from animals sensitized to M. tuberculosis; BP, transfer factor from animals sensitized to BP in FIA; Normal, transfer factor from unsensitized animals. All animals were sensitized with an encephalitogenic emulsion.

<sup>b</sup> Transfer factor preparations were given either 48 hours before or 48 hours after an encephalitogenic emulsion. N.A., not applicable.

<sup>c</sup> Clinical severity scored as follows: 4, death; 3, disease exclusive of death; 2, weight loss and one other clinical sign; 1, weight loss only; 0, no clinical signs. Data were analyzed using the G-test for goodness of fit. Groups A, B, and C were significantly different from Groups D and E (99.95%). Groups A, B, and C, and groups D and E were not significantly different from each other.
included 3 animals per group and in some experiments all 3 were protected from the disease, in others only 1 of 3 was protected from EAE. Statistical analysis using f ln f transforms for the goodness of fit test (G-test) using the Chi square table revealed a significant difference between the transfer factor treated groups (BPTB, TB, and BP) and the animals receiving only the encephalitogenic emulsion. Those receiving normal transfer factor were not significantly different from the emulsion alone group. In addition, this analysis showed no difference between the time of administration of the transfer factors or between the BPTB, TB, and BP transfer factor treatments.

Histologic examination of the brains was made on materials from three experiments. The results are shown in Table 2. As can be seen, there is good agreement between the clinical severity and the histologic examination, so that 22% of the animals receiving BPTB transfer factor before the encephalitogenic emulsion had clinical signs and these same 22% had histologic lesions. Of the animals that received BPTB transfer factor after the sensitizing emulsion, 22% showed clinical signs and 33% had histologic lesions. In contrast, 93% of the animals receiving the encephalitogenic emulsion alone showed clinical signs and 77% demonstrated histologic lesions. In addition, the animals that received the BPTB transfer factor only showed no clinical signs of EAE and demonstrated no histologic lesions. A
Table 2. Correlation of clinical severity and histologic examination in control animals and in animals treated by transfer factor.

<table>
<thead>
<tr>
<th>Treatment a</th>
<th>Number Tested</th>
<th>Clinical Signs</th>
<th>% With</th>
<th>Histology b</th>
<th>% With</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPTB before induction</td>
<td>9</td>
<td>22</td>
<td>7 zero</td>
<td>1 plus</td>
<td>22</td>
</tr>
<tr>
<td>BPTB after induction</td>
<td>9</td>
<td>22</td>
<td>6 zero</td>
<td>1 plus-minus</td>
<td>33</td>
</tr>
<tr>
<td>BPTB only</td>
<td>5</td>
<td>0</td>
<td>5 zero</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Emulsion only</td>
<td>13</td>
<td>93</td>
<td>3 zero</td>
<td>1 plus-minus</td>
<td>77</td>
</tr>
</tbody>
</table>

a. BPTB, transfer factor from animals sensitized to BP plus M. tuberculosis. Transfer factor was given either 48 hours before or 48 hours after a sensitizing encephalitogenic emulsion.

b. Histology was scored as follows: zero, no abnormality; plus-minus, a few small foci of leukocytes about blood vessels within the substance of the brain; plus, definite inflammation about many fields in the Central Nervous System, easily seen in many fields with 30 x magnification; 2-plus, marked perivascular inflammation, easily seen in almost every field; ND, not done, animals died before samples could be taken. Data were analyzed using the G-test for goodness of fit. Transfer factor treated animals were significantly different than those that received only the encephalitogenic emulsion.
G-test was performed on the data and a significant difference was found between the transfer factor treated groups and those that received encephalitogenic emulsion alone.

In addition to the protection experiments, each transfer factor preparation was tested for its ability to transfer skin reactivity to BP or to tuberculin. Animals were given the transfer factor intraabdominally and skin tested 2 days later with either 10 μg BP or 1:50 Old Tuberculin (OT). Table 3 shows the skin test results in the transfer factor recipients. As can be seen, 9 of 11 of those receiving BPTB transfer factor demonstrated skin reactivity to BP. In a few instances OT was tested in addition to BP and the reactions were significant (5 x 5 mm of erythematous, indurated area). In the transfer factor recipients that received TB transfer factor, 6 of 9 demonstrated delayed reactivity when skin tested with OT. In addition, 2 of the animals (see animals 8 and 9) were skin tested with both OT and BP and reacted only to BP. The BP transfer factor caused 5 of 6 recipients to demonstrate delayed reactivity to BP. However, no positive delayed skin reactions were observed in the animals skin tested with OT. All of the animals that received normal transfer factor were skin test negative to both BP and OT. Histologic examination of the skin reactions that were biopsied showed a perivascular infiltrate of mononuclear cells, predominantly lymphocytes, with a few neutrophils.
Table 3. Skin test reactions of animals receiving transfer factor preparations.

<table>
<thead>
<tr>
<th>Type of Transfer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Animal #</th>
<th>Skin Tested With&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor Given</td>
<td></td>
<td>BP</td>
</tr>
<tr>
<td>BPTB</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7</td>
</tr>
<tr>
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<td>6</td>
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<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> BPTB, transfer factor from animals sensitized to BP plus M. tuberculosis; TB, transfer factor from animals sensitized to M. tuberculosis; BP, transfer factor from animals sensitized to BP in FIA; Normal, transfer factor from unsensitized animals.

<sup>b</sup> Animals were skin tested 48 hours after transfer factor with 0.1 ml of 10 μg BP or 1:50 OT. Reactions are expressed as a mean diameter in millimeters of erythematous, indurated area. ND, not done.
In addition to skin testing those animals that received the transfer factor preparations, a group of animals that survived the encephalitogenic challenge was skin tested with 10 μg BP in saline 28 days after sensitization. The relationship of the skin test diameter to clinical severity is plotted in Figure 1. A bivariant analysis showed a significant negative correlation between skin test diameter and clinical severity. Those with the most severe disease showed smaller skin reactions, whereas, those with no clinical signs of EAE demonstrated larger skin test diameters.

In addition to monitoring the course of the disease using clinical severity and skin testing, lymphocyte transformation in vitro was employed in an attempt to determine if differences between the encephalitogenic emulsion alone group and the transfer factor treated groups could be detected. Peripheral blood was drawn at various times after sensitization (0, 7, 14, and 21 days) and the uptake of \(^{3}\)H thymidine into newly synthesized DNA after 5 days in vitro was determined. The uptake in unstimulated cultures was compared with cultures stimulated by BP and with cultures stimulated with the mitogens phytohemagglutinin (PHA) and Concanavalin A (Con A).

Multivariant discrimination analysis showed that the in vitro responses to mitogens and to antigen did not discriminate the animals that received encephalitogenic emulsion alone from the transfer factor treated animals. However,
Figure 1. Relationship of skin test diameter to clinical severity of disease. Animals were skin tested with 10 μg BP 28 days after sensitization with an encephalitogenic emulsion. Severity was scored as follows: 3, disease exclusive of death; 2, weight loss plus one other clinical sign; 1, weight loss only; 0, no clinical signs. Data was analyzed using a bivariate analysis and a significant negative correlation found.
Further analysis showed that a difference in the in vitro response to BP could be demonstrated if the animals were divided on the basis of those that survived the encephalitogenic challenge and those that succumbed to this challenge, regardless of treatment. Figure 2 shows the response in vitro to BP at 5 µg and 15 µg over time in the animals that survived (36 animals) and in those that died of the disease (17 animals). The results are expressed as a stimulation index which is the mean of the counts per minute (cpm) in the cultures with antigen divided by the mean of the cpm in the cultures without antigen. A stimulation index over 2 is considered significant (82, 84). The cpm in the cultures without antigen or mitogen were found to vary little over time in both the "lived" and "died" groups (range 2000-2400 cpm). As can be seen in Figure 2, all the animals at days 7, 14, and 21 have a significant response to both concentrations of BP. Fourteen days after sensitization, a difference between those that survived and those that succumbed emerged, with those surviving an encephalitogenic challenge showing higher stimulation indices to both concentrations of BP. At 5 µg BP the mean stimulation index for those that survived was 4.1 ± 0.75 standard error (S.E.) compared to 2.4 ± 0.52 S.E. for those that died of the disease. At 15 µg BP the mean stimulation index for those that lived was 5.3 ± 1.01 S.E., whereas, in the animals that died
Figure 2. Mean stimulation indices over time to 5 μg and 15 μg basic protein in animals that survived an encephalitogenic challenge and in those that eventually died of EAE.
the mean stimulation index was 2.9 ± .55 S.E. At day 21 all animals in the "died" group had succumbed, however, animals from the "lived" group demonstrated a response to both concentrations of BP.

Figure 3 shows the mean stimulation indices to PHA and Con A over time in the "lived" and "died" groups. The PHA response after 5 days in vitro in both groups varied little over the course of sensitization and the standard errors were found to overlap (Figure 3B). In contrast, the Con A response at 5 days in culture showed a definite divergence 14 days after sensitization, with the animals that survived the encephalitogenic challenge responding somewhat better (Figure 3A). The mean stimulation index at day 14 in the "lived" group was 2.4 ± .31 S.E., while in those that succumbed, the mean stimulation index was 1.79 ± .25 S.E. In addition, the response to Con A, 21 days after sensitization in the animals that lived is significantly different than the day 0 response. The day 0 response was 1.91 ± .16 S.E. and the day 21 response was 2.87 ± .44 S.E.

White blood counts and differentials were obtained at each time period over the course of sensitization. The results for the white blood counts in the "lived" and "died" groups are shown in Figure 4. As can be seen, there is little difference between the groups. However, there is a small but significant elevation of white blood cells as a
Figure 3. Con A and PHA mean stimulation indices over time in animals that survived an encephalitogenic challenge and in those that eventually died of EAE.
Figure 4. White blood cells per cubic millimeter of blood as a function of time in animals that survived an encephalitogenic challenge and those that eventually died of EAE. — Shaded area indicates normal range observed in this study.
function of time, with the day 14 counts higher than the day 0 counts. The differentials were tabulated to obtain a ratio of mononuclear cells to neutrophils. The results of this measurement over the course of the disease in those that survived the encephalitogenic challenge and in those that died of the disease are shown in Figure 5. An elevation in numbers of mononuclear cells can be seen as early as 7 days after sensitization in the animals that survived, although the standard errors between the groups overlap. Fourteen days after sensitization the difference between the "lived" and "died" groups is most pronounced, with the ratio of mononuclear cells to neutrophils in the "lived" group 4.9 ± .74 S.E. and in those that succumbed 2.3 ± .60 S.E. Thus, even though the white blood count was elevated in both groups over the course of the disease, only the "lived" group demonstrated a dramatic increase in the numbers of mononuclear cells.

In addition to the differential counts of mononuclear cells and neutrophils, increased numbers of atypical, large blast mononuclear cells were observed as the disease progressed in all animals tested.
Figure 5. Ratio of the number of mononuclear cells to neutrophils in animals that survived an encephalitogenic challenge and in those that eventually died of EAE.
DISCUSSION

Transfer factor, prepared from animals sensitized with bovine basic protein (BP) plus heat killed Mycobacterium tuberculosis and administered before the onset of signs, caused a significant reduction in the clinical severity of EAE. Likewise, transfer factor from animals sensitized to M. tuberculosis alone or to BP alone had a similar effect. Transfer factor from unsensitized animals did not alter the development of EAE. Transfer factor preparations were effective if given either 2 days before or 2 days after injection of encephalitogenic emulsion. In three experiments, positive histologic findings in brain sections correlated with the clinical severity scoring system used.

The prophylactic effect of the transfer factor preparations was not 100% however, as was the case when treatment with BP or pretreatment with mycobacteria was given (28, 43, 44). This could be due to variation in the potency or purity of the transfer factor preparations used, timing of the transfer factor administration, variation in an individual animal's response to transfer factor, or the fact that only a single dose of transfer factor was given and this was insufficient to provide protection from the disease. Multiple injection times might have produced a greater alteration in the clinical severity of the disease.
In addition, the time of collection of the transfer factors was 6 days after sensitization; other collection times might produce a more or less potent transfer factor preparation or a preparation containing fewer amounts of interfering factors. Since the mode of action of transfer factor is unknown in any system, the presence of other factors could reduce the effectiveness of a given transfer factor preparation.

The efficacy of all three transfer factors (BPTB, TB, and BP) in the prophylaxis of EAE may be associated with the known dermal cross reactivity between BP and mycobacterial antigens. Vandenbark et al. (85), in 1975, reported that guinea pigs sensitized to human or guinea pig basic protein in Freund's incomplete adjuvant demonstrated dermal reactivity to tuberculin purified protein derivative (PPD) when skin tested 5-8 weeks after sensitization. In addition, guinea pigs sensitized with mycobacteria demonstrated dermal reactivity to both guinea pig (100 μg) and human (100 μg) basic proteins. Thus, the fact that TB and BP transfer factors were able to reduce the clinical severity of the disease suggests that the cross reactive antigens, BP and mycobacteria, may also stimulate cross reactive transfer factors.

Each transfer factor preparation was also tested for its ability to transfer delayed-type hypersensitivity
to naive recipients. In most cases, delayed skin reactivity to the sensitizing antigen was passively transferred, and although the reactions were greater than 5 x 5 mm of erythema and induration, none was above 10 x 10 mm. These less than optimal skin reactions might be explained by the early time of collection of the transfer factors (6 days after sensitization). This time period was chosen because it was before clinical signs appeared in the BPTB transfer factor donors, and because initial experiments pointed to the efficacy of material from this collection time in the prophylaxis of the disease. TB and BP transfer factors were also prepared 6 days after sensitization so that a comparison of prophylactic ability could be made with the BPTB transfer factor preparations. In addition, there is evidence for positive delayed-type reactivity 6 days after EAE induction, although the peak skin reactivity was reported to occur 10 days after sensitization (15).

Other investigators have harvested transfer factor relatively early after sensitization. Olson and Drube (86) prepared transfer factor from influenza sensitive guinea pigs 10 days after sensitization. They demonstrated both protection from disease and transference of delayed-type hypersensitivity with their preparations. Shifrine et al. (87) collected transfer factor from coccidiodomycosis sensitive dogs 9 days after sensitization and passively
transferred dermal reactivity with these preparations. On the other hand, the skin reactions might have been larger in diameter if the transfer factor was harvested at a later time period. Most investigators prepare transfer factor from tuberculin and coccidiodomycosis sensitive animals 4-6 weeks after initial sensitization (88, 89). In the dinitrochlorobenzene system, on the other hand, transfer factor is collected 12-16 days after sensitization (90). The problem of determining the optimal time for collection of transfer factor is compounded by the fact that in many systems it is not known whether delayed-type hypersensitivity correlates directly with protection from disease. For example, Cantanzaro and Spitler (76) used transfer factor therapy in human systemic coccidiodomycosis and demonstrated that there was not a strict correlation between conversion of immunologic parameters (including delayed-type skin reactivity) and clinical improvement.

Some cross reactivity of tuberculin and basic protein was also noted in this study. Three animals that received BPTB transfer factor demonstrated positive delayed skin reactions to both BP and Old Tuberculin (OT). In addition, 2 animals that received TB transfer factor responded only to BP. However, no positive tuberculin reactions were obtained in the animals that received only BP transfer factor. These results are difficult to explain,
since Vandenbark et al. (85) demonstrated that the cross reactivity between BP and mycobacteria was reciprocal. However, these investigators used guinea pig and human basic protein, rather than bovine basic protein, as well as a much higher concentration of basic protein for skin testing (100 µg versus 10 µg used in this study). In addition, we tested only a few animals with both antigens, so further testing might alter the results obtained.

A group of animals that survived the encephalito-genic challenge was skin tested 28 days after sensitization with 10 µg basic protein. The results were intriguing, in that a significant negative correlation was found between skin test diameter and clinical severity. Those with no clinical signs of EAE demonstrated larger skin test diameters. Previous investigators have skin tested with homologous basic protein 10 days after sensitization and demonstrated that the larger the skin test diameter, the more severe the clinical signs of the disease (15). Skin testing with heterologous basic protein (bovine) 10 days after sensitization was shown not to correlate with clinical severity. In contrast, our results indicate that skin testing with heterologous basic protein 28 days after sensitization distinguishes between those with no clinical disease and those most severely affected.
Lymphocyte transformation in vitro in whole blood cultures was used in this study to determine if increased incorporation of $^3$H TdR into newly synthesized DNA in cultures stimulated with basic protein over the course of sensitization could be detected. It was also used to determine if differences in the basic protein response in vitro in the transfer factor treated groups and the control animals could be observed. Most investigators have stated that, although a response could be detected in vitro to BP in peritoneal exudative lymphocytes (20, 39), no response was detected in peripheral blood as a function of time. Griffith et al. (40), however, have recently shown that the whole blood assay can be used to study the course of the disease.

Our results indicate that a response to 5 μg and 15 μg BP in vitro can be observed beginning 7 days after sensitization and peaking at day 14. However, the assay did not discriminate between the transfer factor treated groups and the control group. Further analysis showed that a difference between animals in the study could be shown if the animals were divided to include those that survived an encephalitogenic challenge and those that died of the disease. The surviving animals showed significantly greater responses to basic protein in vitro than those dying of the disease. The reasons for this phenomenon are not clear.
One possibility is that in animals dying of the disease, the lymphocytes capable of responding to the antigen are not in the peripheral blood, but have migrated to the central nervous system to mediate the disease. Another explanation may be that if lymphocyte transformation to a specific antigen is indeed a correlate of delayed hypersensitivity, then those showing a better response to BP in vitro have developed a stronger delayed hypersensitivity to the antigen. Support for this hypothesis may be found in the observation that in those that survived the disease and were skin tested 28 days after sensitization, the animals with the largest skin reactions had the least severe disease. In addition, these animals had the greatest blastogenic response to BP 14 days after sensitization.

In addition to studying the response in vitro to the antigen BP, the mitogens PHA and Con A were tested. These cultures were harvested after 5 days in vitro, rather than at the optimal time for mitogens (2-3 days). This may account for the low stimulation indices observed. The 5 day results indicate that the PHA response changes little over the course of sensitization. However, the Con A response in the animals that survived the disease showed a small but definite increase in the Con A stimulation index as a function of time. Other investigators have reported a similar observation in other situations, suggesting the
possibility that this reflects changes in subpopulations of T cells in the peripheral blood (91-94).

Reports of hematological observations in EAE are few. Behan et al. (95) showed an elevated white blood cell count over the course of the disease in monkeys with EAE. They also reported an increase in the number of atypical lymphocytes. Both of these observations were supported by our results. In addition, Wright-stained differentials revealed a significant increase in the numbers of mononuclear cells in those that survived an encephalitogenic challenge, whereas in those that died of the disease, the numbers remained stable. These may be the cells that are responding to both concentrations of BP in vitro in those that survived the encephalitogenic challenge.

The ability of the transfer factor preparations to reduce the clinical severity of EAE is difficult to explain. The consensus of evidence indicates that EAE is mediated by delayed hypersensitivity. The disease is passively transferred by T lymphocytes, and the passive transfer can be abrogated by treatment of the cells with anti-Thy 1 plus complement in rats and mice (55, 56). Recently, a cell free extract containing a labile factor was shown to transfer passively histologic lesions in rats (96). A correlation has been shown to exist between the diameter of the skin reaction 10 days after sensitization and the clinical
severity of the disease (15). In addition, the histologic manifestations in the brain are characteristic of delayed hypersensitivity responses. The lesions contain an infiltration of mononuclear cells, predominantly lymphocytes. The in vitro correlates of delayed hypersensitivity, positive macrophage migration inhibition and lymphocyte transformation responses also develop during the disease (20-24). Additional evidence for the mediation of the disease by delayed hypersensitivity comes from the reports of Swanborg (29, 30, 54), that whereas passive transfer of cells from animals pretreated with BP suppresses EAE in challenged recipients, it also inhibits the development of delayed hypersensitivity. Since transfer factor is associated with delayed hypersensitivity and cells from EAE sensitized animals passively transfer EAE, why does transfer factor not mediate the passive transfer of the disease, but rather cause significant reduction in the severity of EAE?

The data presented in this study indicate that in most cases the transfer factor preparations passively transferred delayed reactivity to naive recipients. In addition, a group of animals was skin tested 28 days after sensitization, and those with the least severe disease had larger skin test diameters to the sensitizing antigen. Finally, although the lymphocyte transformation data could not be used to discriminate between the transfer factor treated groups and the encephalitogenic emulsion control group, the
animals that survived an encephalitogenic challenge had better blastogenic responses to both concentrations of BP. These three pieces of information suggest that delayed hypersensitivity may not be solely responsible for the disease.

There are recent reports that different regions in a sensitizing agent can give rise to different responses (97, 98). For example, in the antibody response to lysozyme, investigators (97) have shown that different regions in the molecule induce either suppressor T lymphocytes or helper T lymphocytes. When these cells are added to an in vitro antibody synthesizing system, they alter the antibody response to intact lysozyme. In addition, there is evidence (98) that in the anti-insulin antibody response in Strain 2 and Strain 13 guinea pigs, these strains respond to different polypeptide chains (A and B) of the insulin molecule.

In the EAE model, there is evidence that modified BP or peptides derived from BP can cause suppression of the disease, if administered prior to an encephalitogenic challenge. Some claim that the presence of the encephalitogenic region in the BP molecule is essential for this suppression (99), others state that other peptides distinct from this region are capable of suppressing the disease (30). In most of these studies, there is not only suppression of disease, but also inhibition of delayed hypersensitivity. This is clearly not what our results suggest.
Recently, several investigators have reported suppression of the disease with peptide 44-89, which does not contain the encephalitogenic region. Of importance is the fact that this suppression was accompanied by delayed-type hypersensitivity to both the peptide and to intact BP (31, 32). This is significant, since our data suggest that transfer factor both causes suppression of the disease and induces delayed hypersensitivity. The evidence points to the hypothesis that transfer factor preparations from lymphoid cells sensitized to BP peptide 44-89 (or a similar sequence of mycobacteria) in some way sensitizes cells in the recipient so that if challenged 2 days before or 2 days after the administration of transfer factor, they are better able to deal with this encephalitogenic challenge. Since some of the animals in this study did develop the disease after transfer factor treatment, there may be a difference in the commitment of effector cells toward the transfer factor and the encephalitogenic emulsion.
LITERATURE CITED


