DEMONSTRATION OF A BLOCKING FACTOR IN THE PLASMA AND SPINAL FLUID OF PATIENTS WITH SUBACUTE SCLEROSING PANENCEPHALITIS

I. Partial Characterization*

By AFTAB AHMED, DOUGLAS M. STRONG, KENNETH W. SELL, GARY B. THURMAN, RICHARD C. KNUDSEN, RICHARD WISTAR, JR., and WILLIAM R. GRACE

(From the Experimental Immunology Division, Clinical Medical Sciences Department, Naval Medical Research Institute, Bethesda, Maryland 20014)

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Since the description of a case with "diffuse encephalitis with sclerosing inflammation of the hemisphere white matter" by Bodechtel and Guttman (1), there have been numerous reports that have indicated that measles virus is associated with this disease. Subacute sclerosing panencephalitis (SSPE)[1] occurs mostly in children and young adults as a slow progressive inflammatory disorder of the central nervous system (2). There have been reports that the SSPE virus is not identical (3, 4) to the wild or attenuated strain of the measles virus. One characteristic laboratory finding in clinical cases of SSPE has been the presence of high concentration of measles antibody levels in the serum and spinal fluid of nearly all known cases of this progressive neurological disease (5, 6). Still, the presence of these high levels of humoral antibody does not alter the course of the disease in patients with SSPE. Horta-Barbosa et al. (7) have isolated the measles virus in mixed cultures of lymph node cells and HeLa cells and identified it by hemagglutination inhibition, immunofluorescence, and neutralization tests. Dayan and Stokes (8) identified measles virus antigen by immunofluorescence in cells from cerebrospinal fluid in four patients with SSPE.

Immunologic abnormalities have been reported (9, 11) in patients with SSPE. Saunders et al. (12) studied the in vitro response of an 8½-year old boy with SSPE to measles antigen and found specific immunocompetent lymphocytes in the patient and postulated that the destructive interaction between such lymphocytes and host cells in the brain may lead to SSPE. Mizutani et al. (13) confirmed the presence of cellular immunity to measles antigen in a 9-year old girl with SSPE, using skin testing and macrophage migration inhibition as an index. On the other hand, Moulias et al. (14) tested 20 cases of SSPE and found that none of them responded to measles antigen as judged by blastic transformation and leukocyte migration tests. Lischner et al. (15)

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[1] *Abbreviations used in this paper:* ADV, autoclaved, dialyzed measles vaccine; CMV, cytomegalovirus suspensions; LT, lymphotoxin; MASH, multiple automated sample harvester; MIF, migration inhibition factor; SSPE, subacute sclerosing panencephalitis; TMVP, tobacco mosaic virus protein.

found results similar to that of Moulias et al. (14). Dayan and Stokes (16) and Philips (17) state that antigen-antibody complexes might be involved in the pathogenesis of the disease.

Gatti (18) has reviewed the presence of inhibitory factors that have been detected in the serum and plasma of patients with a wide range of disorders. He found inhibitors of allogeneic lymphocyte responses to PHA in the plasmas of 16 of 21 patients with various forms of nonlymphoid malignant disease, including two of five with breast cancer. Plasma inhibitors have been reported in diseases like ataxia telangiectasia (19) and multiple sclerosis (20).

Scheurlen et al. (21) characterized an inhibitor present in the plasma of patients with Hodgkin's disease and cancer. This plasma inhibited specific lymphocyte transformation with tuberculin and also nonspecific stimulation by agents such as phytohemagglutinin in normal donors. Allen et al. (22) described a labile inhibitory factor of mitogen-induced lymphocyte transformation in the fresh plasma of a patient in stage 3 of SSPE.

In the present study, using the migration inhibition test, the lymphocyte transformation and the lymphotoxin assays, we found that plasma and spinal fluid from four cases of SSPE blocked the in vitro response of their lymphocytes to measles-like virus isolated from a known case of SSPE. This blocking was not seen by the use of measles sero-negative plasma. Attempts were made to characterize this blocking factor.

Materials and Methods

Measles Antigen—Measles virus vaccine, live attenuated (Schwarz strain), which was from a chick embryo tissue culture (Bio no. 185, Dow Chemical Co., Indianapolis, Ind.), was used initially as a source of antigen. This vaccine was also used after autoclaving at 121°C for 30 min. Both the antigens were dialyzed with 200 × volume of 0.15 M NaCl before use to remove preservatives. The suspension was diluted with RPMI 1640 medium containing penicillin, 100 U/ml; streptomycin, 100 μg/ml and 200 mM L-glutamine (all from Grand Island Biological Co., Grand Island, N. Y.). Live measles virus isolated from a known case of SSPE was prepared by Dr. L. Horta-Barbosa, National Institute of Mental Health, NIH, Bethesda, Md., by the following procedure. This virus was passaged three times in approximately $10^{7.7}$ HeLa cells with a multiplicity of ± two infective particles per cell. After adsorption for 1 h at 37°C, the infected HeLa cells were suspended in 500 ml Joklik medium enriched with penicillin 75 U/ml, and streptomycin 50 U/ml, with 5% fetal bovine serum, and cultivated in suspension (stirring flask) at 37.5°C. Samples of this culture were analyzed daily for hemagglutinins. After 6 days, the hemagglutinin (HA) titer was 1:128. The culture was then frozen and thawed twice and clarified by centrifugation (200 rpm for 30 min). The infective titer of this preparation was $10^{6.5}$/ml. This antigen preparation was concentrated 100 × by centrifugation as follows: 6% gelatin (1 ml per 100 ml of antigen preparation) was initially added, and the material was centrifuged at 30,000 rpm for 90 min in a Spinco Model L ultracentrifuge (Beckman Instrument Co., Inc., Palo Alto, Calif.). The sedimented material was resuspended in Hank's media containing penicillin and streptomycin to $\frac{1}{10}$ the initial volume. This procedure was repeated to achieve a 100 × antigen concentration. This concentrate was frozen and thawed three times and centrifuged at 1,500 rpm for 10 min to remove the gelatin and cellular debris. The hemagglutinin titer of this 100 × antigen concentrate was 1:12,800.

Measles Antibody Titers—Antibody titers were performed by the Serology Laboratory,

Naval Hospital, Bethesda, Md., and by Dr. Dale E. Dietzman, NIMH, NIH. An ultraviolet treated measles virus vaccine was supplied by Dr. Jeffrey Allen, NIMH, and was used in one of the experiments as a source of antigen. Hemagglutination, complement fixing gel immunoprecipitation, and hemagglutinin inhibition titers were determined on the serum and spinal fluid of the patients and control subjects used in this investigation.

Preparation of Leukocyte Suspensions—Blood was drawn from the patients and control subjects in sodium heparin (preservative free, Medical Chemicals Corporation, Chicago, Ill.) and allowed to settle in the syringe in the inverted position. Leukocyte-rich plasma was drawn off and centrifuged at 150 *g* for 15 min. The plasma was saved, and the cells washed two times with 50 volumes of the media, counted by means of an Autocytometer (Autocytometer II, Fisher Scientific Co., Pittsburgh, Pa.) and resuspended to 10^7 cells/ml.

The Migration Inhibition Test—This was performed essentially according to the method of David and David (23) with a few modifications. Peripheral leukocytes from the patients, the positive control and the negative control, were washed and resuspended in media to contain 3×10^6 leukocytes/ml. Measles antigen in various concentrations was then added. Control cultures received either media or the supernates of uninfected HeLa cell cultures. Lymphocyte cultures were incubated for 48–72 hr in 2-ml suspensions in 15 × 75-mm snap-top culture tubes (Falcon Plastics, Oxnard, Calif.) at 37°C in a 5% CO_2, humidified air atmosphere Cultures were centrifuged and the supernatant fluid collected. Macrophages obtained by oil induction from guinea pigs were loaded into plain, blue tip, 75-mm capillary tubes sealed at one end. This was accomplished by means of a Hamilton repeating dispenser and syringe unit (Lawshe Instruments Company, Bethesda, Md.) and a sterile, disposable, 26 gauge spinal needle (Becton, Dickinson & Co., Rutherford, N. Y.), such that each capillary contained 50 μl of macrophage suspension containing 50×10^6 cells/ml. The capillary tubes were centrifuged at 150 *g* for 10 min and the capillary tube cut at the cell fluid interface. The short piece of capillary tube was affixed to the inside of a migration inhibition chamber (Mini Lab Co., Ville De Laval, P.Q., Canada) with silicone grease. Each chamber contained two tubes and was sealed at the top by means of a cover slip and silicone. Fluid (0.5 ml) from the lymphocyte cultures was injected into the sealed chambers by means of a tuberculin syringe. The injection ports were sealed with plastic tape to prevent evaporation. The chambers were incubated at 37°C for 24–48 h. The image of the cover slip, capillary, and cell sheet was projected by means of a Bausch and Lomb projecting prism (Bausch and Lomb, Inc., Rochester, N. Y.) onto a sheet of paper and the outline of the migrated cells was traced. The area of migration was determined by planimetry and the percent migration inhibition was determined by the following formula:

$$\% \text{ migration inhibition} = 1 - \frac{\text{Average area of migration with antigen}}{\text{Average area of migration without antigen}} \times 100.$$

A second method for determining production of migration inhibition factor (MIF) was also employed. This consisted of adding 1 vol of the leucocyte suspension (10×10^6 cells) from the patient to 1 vol of guinea pig macrophage suspension (30×10^6/ml). This mixture was loaded into capillary tubes and placed into the chambers as previously described. Instead of adding the supernates of lymphocyte cultures, antigen suspensions of various dilutions in media were added to the chambers. Migration inhibition was determined after 18–24 h.

The Lymphocyte Transformation Test:—Leukocyte suspensions in media were layered onto Ficoll-hypaque gradients (sp gr 1.078) and centrifuged at 400 *g* for 45 min according to the method of Boyum (24). The lymphocyte-rich band was aspirated by means of a pasteur pipet and washed with 50 vol of media. The cells were resuspended to 2×10^6/ml in media containing either 15% autologous or homologous plasma. The lymphocyte transformation test was performed using either the virus suspensions or SSPE virus infected HeLa cells as described in a previous report (25).

When virus suspensions were used, 100 μl of the lymphocytes (2×10^6/ml) were added to each well of a microtiter plate and 50 μl of varying concentrations of the virus suspension added. Control wells received media. Sero-positive, sero-negative, and patients' plasma were added in varying concentrations in 50 μl.

Triplicate cultures were prepared in Microtest II microtiter plates (Falcon Plastic). Rapid dispensing of lymphocytes, cultured cells and culture additives were accomplished with Hamilton repeating dispensers (Hamilton Company, Reno, Nev.). Cultures were incubated 72 h at 37°C in a 5% CO_2 humidified atmosphere. 18 h before harvesting, 0.02 ml of RPMI 1640 containing 1 μCi of [methyl-^{3}H]thymidine (sp act 1.9 Ci/mmol, Schwarz Mann, Div. Becton, Dickinson & Co., Orangeburg, N. Y.) was added. Harvesting was accomplished using a multiple automated sample harvester (MASH) as previously described (26–28) and the tritiated thymidine incorporation was determined by scintillation spectrophotometry. Data from the scintillation counter was recorded on paper tape and analyzed using a Wang 700C advanced programming calculator (Wang Laboratories, Inc., Tewksbury, Mass.).

Lymphotoxin Assay.—Sensitive lymphoid cells in the presence of the sensitizing antigen or normal cells, when cultured with nonspecific mitogenic agents, cause lymphocyte transformation. The cell-free medium of such activated cultures has been shown to possess a lymphokine-termed lymphotoxin (LT) (29). LT activity has been demonstrated by the ability of cell-free supernatant fluids to cause the destruction of mouse "L" fibroblasts, and the secretion of LT has been shown to be a thymic-dependent function (30). This lymphotoxin assay was performed using a new microassay system.[2] In this assay system, the LT activity was measured as the decrease in the uptake of [^{3}H]thymidine by L cells. Briefly, lymphocytes from the sero-negative controls, the sero-positive controls, and the SSPE patients (2×10^6/ml), in a total volume of 5 ml in 15% autologous or 15% sero-negative plasma and 1% final concentration of SSPE virus, were incubated at 37°C, 5% CO_2, 95% humidified air for 72 hr. Supernatant fluid from these cultures was used for the lymphotoxin assay. The ID_{50} levels of lymphotoxin activity were calculated as the dilution of the supernatant fluid being tested for LT activity which gave 50% of the counts per minute of the media control values. HeLa cells were similarly used as another source of target cells for the LT assay.

Characterization of the Blocking Factor.—Plasma from SSPE patients (1 ml each) was treated by standard biochemical techniques with various enzymes such as neuraminadase, ribonuclease, and deoxyribonuclease. The enzyme-treated plasma was centrifuged at 600 *g* and the supernatant fluid was passed through a 0.22 μ Swinnex filter separately and each tested for its ability to block the in vitro response of leukocytes from patients with SSPE in the presence of 1% SSPE virus suspensions. 1-ml samples of SSPE plasma were also treated with trypsin-EDTA and 0.1 M 2-mercapto-ethanol, dialyzed, filtered, and used as above. Ammonium sulfate precipitation was carried out on SSPE plasma and three cuts were obtained, the 0.25%, 25–50%, and > 50% fractions. These were also dialyzed and filtered before use.

Plasma and spinal fluid from the SSPE patients were applied to a G-200 Sephadex ascending column and three major fractions collected. Using markers of known molecular weight, peak A of both the plasma and spinal fluid was judged to have a mol wt greater than 150,000.

Other Antigens Used.—To test for specificity of the blocking factor, other viral and bacterial agents were used. These consisted of cytomegalovirus suspension grown in WI-38 cell line, PPD, histoplasmin, Streptokinase Streptodornase, tetanus toxoid, mumps, and vaccinia vaccines. All the antigens were dialyzed against RPMI 1640 media to remove preservatives before use.

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[2] Knudsen, R. C., A. Ahmed, and K. W. Sell. 1974. A microassay for lymphotoxin using the multiple automated sample harvester. Manuscript in preparation.

RESULTS

Serology:—The diagnosis of SSPE was made clinically in all four cases studied, and supported by the finding of significant antimeasles titers in both the serum and cerebrospinal fluid. The case histories and clinical findings will be discussed in a separate paper. M. C. and R. S. were patients at the Naval Hospital, T. C. was a patient at the Clinical Center, NIH, and S. O. was a patient at St. Vincent's Hospital, New York.

M. C., T. C., and S. O. were in stage 3 of SSPE when these studies began, whereas R. S. was in stage 1 at the beginning of the study and progressed to stage 3 during the course of this investigation. The measles antibody titers are seen in Table I. All of them were confirmed to be rubeola specific by gel immunoprecipitation. The positive controls used in these studies (J. W. and S. P.) had antimeasles titers of 1:128. The negative controls (A. A. and B. B.) had no measles antibody titers (<1:10) by either complement fixation, hemagglutination inhibition, and gel immunoprecipitation techniques. Spinal fluid used as a control (J. M.) was obtained from a normal healthy volunteer which also had a negative titer against measles. In some cases, the spinal fluid titer was higher than the serum titer, which is unusual, but the assays were performed on different days. In general, if performed together, the titer was always higher in the serum.

Migration Inhibition Test.—Various antigen preparations were tried to deter-

TABLE I

Measles Antibody Titers in the Serum and Spinal Fluid of Four Cases of SSPE

Patient	Date performed	Reciprocal of the antibody titer*	
		Serum	Spinal fluid
M. C.‡	9/27/71	256	256
	10/10/71	256	256
	5/12/71	1,024	128
T. C.§	2/11/71	512	128
R. S.‡	4/24/72	32	32
	5/02/72	128	256
	5/15/72	128	2,048
	5/31/72	128	2,048
	6/12/72	512	1,024
S. O.‖	8/29/72	>2,048	16
	10/27/72	ND	4
	2/21/73	>128	16
	3/05/73	>8,000	32

* Serum and antibody titers determined by complement fixation test and confirmed to be rubeola specific by gel immunoprecipitation techniques.

‡ Patients at the National Naval Medical Center, Bethesda, Md.

§ Patient at the Clinical Center, NIH.

‖ Patient at St. Vincent's Hospital, New York.

mine which would give optimal results in vitro. Commercial, live attenuated vaccine was dialyzed against 200 × of 0.15 M sodium chloride and concentrated by means of an Amicon UM-2 membrane filter Amicon Corp., Lexington, Mass. Either the dialyzed preparation or a dialyzed, autoclaved preparation (121°C, 30 min) were used undiluted or diluted in media to a final concentration of 1:10, 1:100, or 1:1000. The results of the MIF assays were obtained using these two preparations and the SSPE virus (courtesy of Dr. Horta-Barbosa, NIMH). Cells used in these tests were obtained from one SSPE patient (M. C.), and one positive control (J. W.), and two negative controls (A. A. and B. B.). Table II shows a comparison between two types of MIF assays used. It can be seen that the SSPE patient and the sero-positive control produce MIF in the presence of a 1:100 dilution of SSPE virus and a 1:10 dilution of the autoclaved, dialyzed measles vaccine (ADV). The sero-positive control also produced MIF in the presence of a 1:100 dilution of the ADV in the direct assay but not in the indirect assay, indicating an increased sensitivity of the direct assay. Both sero-negative controls showed lack of reactivity to either antigen preparations. ADV, used in similar concentrations, produced negative results. The ADV was unstable in storage and gave inconsistent results upon repeated testing. Therefore, the SSPE virus preparation was used for subsequent experiments at a final concentration of 1:100 (1%) in media.

Since the direct assay was more consistent and produced results in a shorter

TABLE II

Effects of Various Measles Antigen Preparation on the Direct and Indirect Migration Inhibition Assays

	% Migration inhibition*							
	M. C. cells‡		J. W. cells§		B. B. cells‖		A. A. cells‖	
	Indirect¶	Direct**	Indirect	Direct	Indirect	Direct	Indirect	Direct
SSPE virus								
1:100	83	72	95	94	2	7	10	12.5
1:1000	6	3	11	32	9	6	0	1.5
Autoclaved, dialyzed measles vaccine								
1:10	95	61.5	93	77	0	0	ND	
1:100	2	4.5	4	98.5	0	0	ND	
1:1000	0	0	0	19.5	0	0	ND	

* $\%\ \text{Migration inhibition} = 1 - \frac{\text{Average area of migration with antigen}}{\text{Average area of migration without antigen}} \times 100.$

‡ M. C. SSPE patient.

§ J. W., sero-positive measles control.

‖ B. B. and A. A., sero-negative measles control, 7 mo baby and adult, respectively.

¶ Indirect assay using supernatant fluid from lymphocyte-viral antigen culture.

** Direct assay using lymphocyte-macrophage migration in culture with viral antigen.

period of time than the indirect assay, it was used throughout the remainder of the investigation.

Since the SSPE patients were demonstrating good in vitro cellular reactivity by the production of MIF in response to 1% SSPE virus and also had significant amounts of antimeasles antibody titers both in their serum and spinal fluid, it was decided to search for factors that would give rise to abnormal cellular reactivities in vivo. Since all the MIF tests were run in media or media with fetal calf serum, it was the purpose of the next experiment to determine the effect of SSPE plasma and spinal fluid in the MIF test. Figs. 1 and 2 show the results of the use of various concentrations of SSPE plasma and spinal fluid on the production of MIF by leucocytes from R. S. (SSPE patient), measles sero-negative and measles sero-positive controls. The sero-negative cells showed negligible MIF production in the presence of either SSPE plasma or spinal fluid. The patient's cells (R. S.) and the sero-positive control cells (S. P.) produced MIF in the presence of various concentrations of sero-negative plasma but demonstrated a marked decrease in response upon increasing

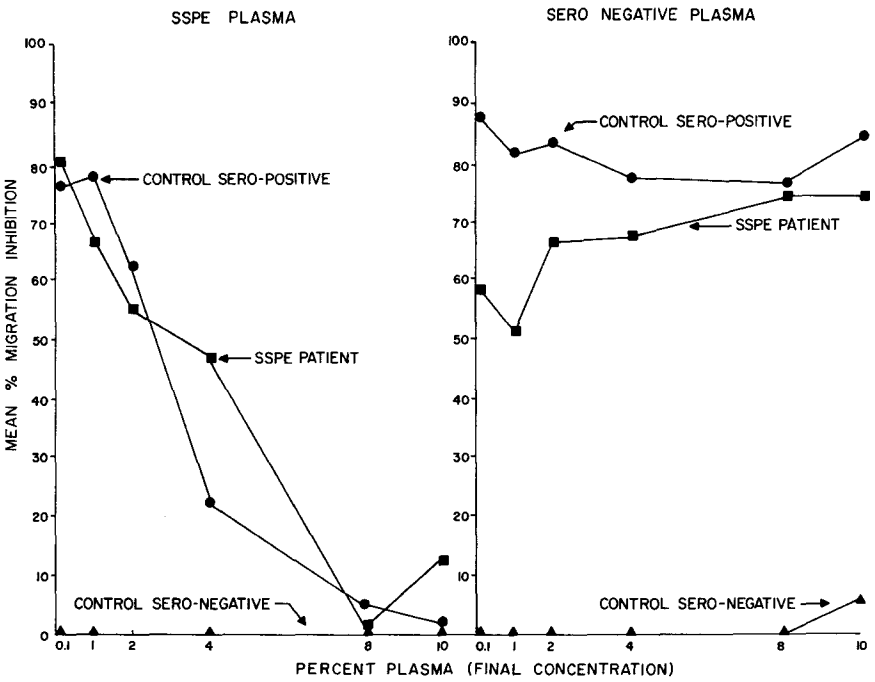

FIG. 1. Effect of varying concentrations of plasma from a measles sero-negative and a patient with SSPE on the release of migration inhibition factor by lymphocytes in response to 1% SSPE virus. (▲—▲), measles sero-negative lymphocytes response; (●—●), measles sero-positive lymphocyte response; (■—■), SSPE patient's (R. S.) lymphocyte response.

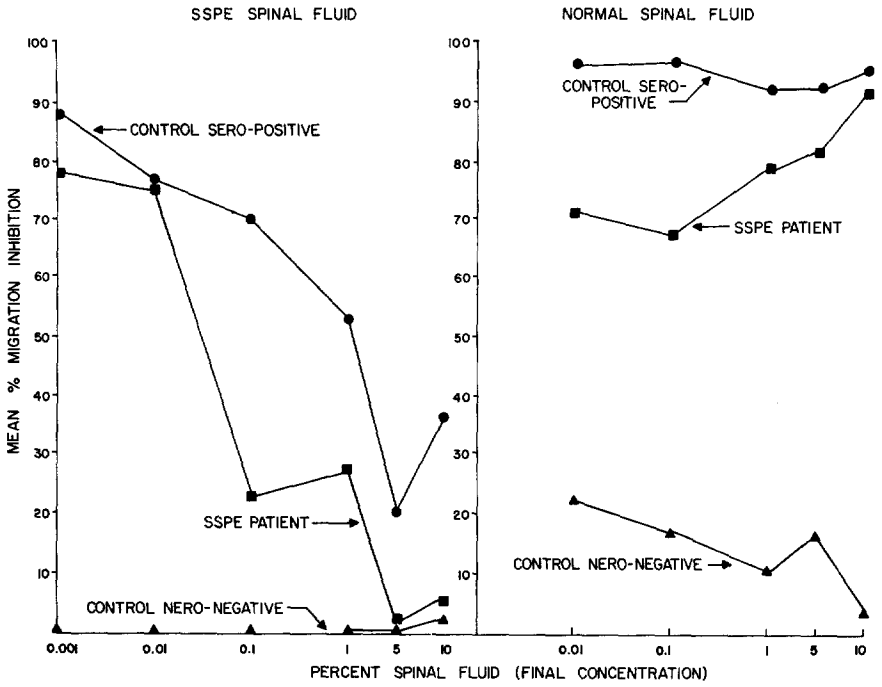

FIG. 2. Effect of varying concentrations of spinal fluid from a measles sero-negative and a patient with SSPE on the release of migration inhibition factor by lymphocytes in response to 1% SSPE virus. (▲—▲), measles sero-negative lymphocyte response; (●—●), measles sero-positive lymphocyte response; (■—■), SSPE patient's (R. S.) lymphocyte response.

concentrations of SSPE plasma (Fig. 1). The production of MIF was completely blocked at concentrations of SSPE plasma higher than 8%. When spinal fluid was tested for such "blocking factors", it was found (Fig. 2) that cells from the SSPE patients and sero-positive cells again showed decreased production of MIF with increasing concentrations of SSPE spinal fluid. This blocking factor was not found in normal (J. M.) spinal fluid. Generally, the amount of the blocking factor was in higher concentration in the SSPE spinal fluid than in the SSPE plasma.

In efforts to determine whether this type of blocking factor occurs in the plasma of other cases of SSPE, the experiment was repeated using various concentrations of plasma and spinal fluid from other SSPE cases studied. Tables III and IV show that such a blocking factor also occurs in plasma and spinal fluid of all of the SSPE cases studied but does not occur in measles sero-positive plasma and spinal fluid as demonstrated by the response of both the SSPE cells and the sero-positive cells. The sero-negative cells did not produce MIF. This showed that the blocking factor in the SSPE plasma and spinal

TABLE III

Inhibition of Release of Migration Inhibition Factor by Cells from Patients with SSPE by Their Plasma

Plasma concentration	% Migration inhibition*					
	Control		M. C.‖	R. S.‖	T. C.‖	S. O.‖
	Negative‡	Positive§				
1% control negative	1.9	59.4	53.7	72.4	69.8	59.8
10% control negative	4.4	53.7	81.3	81.6	74.4	64.6
25% control negative	5.7	64.5	80.4	69.4	60.7	51.8
1% control positive	5.3	51.8	ND	56.9	ND	52.7
10% control positive	4.9	55.7	ND	64.1	ND	61.1
25% control positive	9.8	68.4	ND	59.8	ND	58.3
1% M. C.	8.5	53.4	87.1	85.5	52.3	64.8
10% M. C.	2.8	14.0	14.7	−0.8	4.1	−3.3
25% M. C.	3.6	4.1	11.2	4.6	−7.8	6.4
1% R. S.	7.8	44.1	54.9	56.6	66.6	61.7
10% R. S.	5.9	8.4	12.8	5.1	5.8	8.8
25% R. S.	11.3	1.7	−0.9	−3.7	11.4	4.0
1% T. C.	4.1	ND	49.4	64.3	47.9	ND
10% T. C.	5.3	ND	8.4	13.8	12.6	ND
25% T. C.	11.1	ND	12.9	2.2	7.3	ND
1% S. O.	3.7	ND	ND	ND	ND	77.1
10% S. O.	2.1	ND	ND	ND	ND	15.3
25% S. O.	−0.8	ND	ND	ND	ND	−0.2

* $\%\ \text{Migration inhibition} = 1 - \frac{\text{Average area of migration with antigen}}{\text{Average area of migration without antigen}} \times 100.$

‡ A. A., measles sero-negative donor.

§ S. P., measles sero-positive donor.

‖ M. C., R. S., T. C., and S. O., clinically diagnosed cases of SSPE.

fluid was cross-reactive in its effect on cells from either SSPE patient or measles sero-positive controls.

Lymphocyte Transformation Test:—Purified peripheral blood lymphocytes from R. S. (SSPE patient) showed a significant response to SSPE virus-infected HeLa cells (Tables V and VI). R. S. lymphocytes cultured with noninfected HeLa cells gave 354 (±20) cpm. When cultured with SSPE virus infected cells, they produced 4,656 (±1197) cpm, which gives a stimulation index of 13.9. When 10% R. S. plasma (Table V) or 1% R. S. cerebrospinal fluid (Table VI) was present, the response dropped to control values indicating a blocking of the blastogenic response of the lymphocytes. 1% R. S. plasma (Table V) or 0.1% R. S. cerebrospinal fluid (Table VI) was not sufficient to eliminate the response.

In the MIF results on these same individuals, the measles sero-positive control (S. P.), as well as the SSPE patient, produced MIF when exposed to the purified SSPE virus. However, Table V indicates that in this transforma-

TABLE IV

Inhibition of Release of Migration Inhibition Factor by Cells from Patients with SSPE by Their Cerebrospinal Fluid

Spinal fluid concentration	% Migration inhibition*					
	Control		M. C.‖	R. S.‖	T. C.‖	S. O.‖
	Negative‡	Positive§				
0.1% J. M.	5.8	43.6	50.1	34.9	42.8	47.5
1% J. M.	9.1	49.8	56.4	51.1	56.9	64.3
10% J. M.	8.1	40.4	44.7	49.6	48.3	56.9
0.1% M. C.	2.7	49.6	52.8	56.7	41.8	39.9
1.0% M. C.	5.1	11.3	5.8	9.6	8.7	1.8
10% M. C.	4.8	4.6	4.4	11.4	9.3	−2.8
0.1% R. S.	5.1	38.5	55.3	47.5	ND	53.7
1.0% R. S.	9.9	2.1	7.7	−16.5	ND	2.8
10% R. S.	5.3	0.8	1.1	1.4	ND	4.9
0.1% S. O.	4.1	ND	ND	ND	ND	49.9
1.0% S. O.	3.7	ND	ND	ND	ND	2.7
10% S. O.	9.2	ND	ND	ND	ND	−5.9

* % Migration inhibition in response to 1% SSPE virus =

$$1 - \frac{\text{Average area of migration with virus}}{\text{Average area of migration without virus}} \times 100.$$

‡ A. A., measles sero-negative control.
§ S. P., measles sero-positive control.
‖ M. C., R. S., T. C., and S. O., four cases of SSPE.
¶ J. M., healthy human volunteer.

tion test, the measles sero-positive lymphocytes did not show a blastogenic response to the SSPE virus infected HeLa cells, whereas the SSPE patient's lymphocytes did. None of the lymphocytes responded to the noninfected HeLa cells, and the blastogenic responses to 0.1% PHA-P of lymphocytes from all SSPE patients and controls were within the normal range.

These results were confirmed using lymphocytes from another SSPE patient (S. O.). Lymphocytes from S. O. cultured with noninfected HeLa cells gave 599 (±66) cpm (Tables V and VI). When cultured with SSPE virus infected HeLa cells, they responded with 7,571 (±291) cpm, giving a stimulation index of 12.6. Again, 10% plasma or 1% cerebrospinal fluid from S. O. was sufficient to nullify the blastogenic response. No response to noninfected HeLa cells was seen, and the nonspecific (PHA-P) mitogenic responses were within the normal range.

Lymphotoxin Assay:—This microlymphotoxin assay uses only 0.05 ml of supernatant fluid of lymphocyte cultures. Supernatant fluids from activated lymphocyte cultures were tested for LT activity using L cells and HeLa cells as target cells. When lymphocytes from SSPE patients and measles sero-positive controls were incubated with 1% SSPE virus suspensions for 48 h, low

TABLE V

Inhibition of Lymphocyte Response to SSPE Virus Infected HeLa Cells by Plasma from Patients with SSPE

Lymphocytes*	Concentration of plasma	Uptake of [^{3}H]thymidine (cpm ± SE)		
		0.1% PHA-P‡	SSPE virus infected HeLa cells§	Noninfected HeLa cells§
None	—	—	320 ± 100	227 ± 26
Control negative‖	10% control negative	50976 ± 5127	281 ± 71	397 ± 18
	10% control negative	63918 ± 6043	356 ± 28	411 ± 29
	10% R. S.	49498 ± 4351	429 ± 34	396 ± 36
	10% S. O.	55984 ± 6359	327 ± 27	138 ± 18
Control positive¶	10% control negative	42975 ± 3723	174 ± 19	287 ± 18
	10% control positive	56385 ± 4810	331 ± 18	ND
	10% R. S.	48193 ± 5331	153 ± 32	ND
	1% R. S.	56616 ± 4218	443 ± 56	ND
R. S.**	10% control negative	43388 ± 4481	4656 ± 1197	334 ± 28
	10% control positive	ND	ND	ND
	10% R. S.	59139 ± 4813	225 ± 61	308 ± 27
	1% R. S.	453199 ± 3124	5609 ± 280	460 ± 41
S. O. **	10% control negative	92184 ± 5918	7571 ± 291	588 ± 65
	10% control positive	101563 ± 7271	7735 ± 440	511 ± 29
	10% R. S.	88591 ± 4289	412 ± 59	548 ± 46
	10% S. O.	123141 ± 9346	230 ± 25	539 ± 141
	1% S. O.	94333 ± 5120	7638 ± 294	384 ± 31

* 200,000 lymphocytes per 0.2 ml culture.

‡ Final concentration of phytohemagglutinin-P in each culture measles.

§25,000 HeLa cells (treated with mitomycin-C) per 0.2 ml culture.

‖ Measles sero-negative donor.

¶ Measles sero-positive donor.

** SSPE patients.

levels of LT were obtained (ID_{50} range from 1:2 to 1:4.5) by both cell types but only in measles sero-negative plasma. SSPE plasma completely blocked the production of LT. Measles sero-negative lymphocytes did not produce any LT in response to the SSPE virus.

When SSPE virus infected HeLa cells were used as a source of antigen, interestingly, much higher levels of LT (1:12 using HeLa target cells and 1:32 using L target cells) were produced by lymphocytes from SSPE patients in 10% measles sero-negative plasma. This response was again completely blocked using 10% SSPE plasma (Table VII). This data clearly shows that plasma from SSPE patients blocks the production of LT by SSPE lymphocytes by both direct activation by the virus or by the use of SSPE viral infected HeLa cell and lymphocyte cell coculture techniques.

Characterization of the Blocking Factor:—Plasma from a SSPE patient was

TABLE VI

Inhibition of Lymphocyte Response to SSPE Virus Infected HeLa Cells by Spinal Fluid from Patients with SSPE

Lymphocytes*	Final concentration of spinal fluid	Uptake of [^{3}H]thymidine (cpm ± SE)	
		SSPE virus infected HeLa cells‡	Noninfected HeLa cells‡
None		361 ± 43	389 ± 22
	0.1% J. M.§	289 ± 24	312 ± 53
	1.0% J. M.	573 ± 12.5	288 ± 38
Control negative‖ cell	0.1% R. S.	434 ± 19	239 ± 11
	1% R. S.	479 ± 33	615 ± 69
	1% S. O.	440 ± 36	543 ± 44
	0.1% J. M.	4,818 ± 239	512 ± 46
R. S.¶ cells	1% J. M.	4,656 ± 1197	334 ± 21
	0.1% R. S.	3,754 ± 80	218 ± 22
	1.0% R. S.	464 ± 58	257 ± 51
	0.1% J. M.	8,981 ± 384	596 ± 73
S. O.¶ cells	1% J. M.	7,192 ± 201	610 ± 64
	1% R. S.	552 ± 58	514 ± 56
	1% S. O.	198 ± 34	533 ± 40

* 200,000 lymphocytes per 0.2 ml culture.

‡ 25,000 HeLa cells (blocked with mitomycin-C) per 0.2 ml culture.

§ Normal adult human volunteer.

‖ Measles sero-negative donor.

¶ SSPE patients.

TABLE VII

Inhibition of Release of Lymphotoxin by Lymphocytes from Patients with SSPE in Response to SSPE Virus Infected HeLa Cells in the Presence of SSPE Plasma

Supernatant from:*	Plasma source (10%)	Lymphotoxin level‡ (ID_{50})	
		HeLa cells	L cells
SSPE cells + SSPE-infected HeLa cells	SSPE plasma	0	0
Normal cells§ + SSPE-infected HeLa cells	SSPE plasma	0	0
SSPE cells + SSPE-infected HeLa cells	Normal plasma§	1:12	1:32
Normal cells + SSPE-infected HeLa cells	Normal plasma	0	0

* Supernatant fluid from cultures of lymphocytes (2×10^6/ml) and SSPE virus infected HeLa cells (50,000 cells/ml) after 66 h of incubation at 37°C in a 5% CO_2, 95% air humidified atmosphere.

‡ ID_{50} level of lymphotoxin calculated to be the dilution of lymphotoxin that showed 50% reduction in the uptake of [^{3}H]thymidine by L cells or HeLa cells.

§ Measles sero-negative donor.

incubated at 45°C or 56°C for varying periods of time. As is seen in Fig. 3, the blocking factor was inactivated by heating at 56°C for greater than 20, min. Plasma from SSPE patients stored at −20°C for greater than 6 mo still retained its activity in blocking the in vitro release of MIF.

When plasma from SSPE patients was treated with various enzymes, it was found (Table VIII) that it was sensitive to neuraminadase and trypsin but not

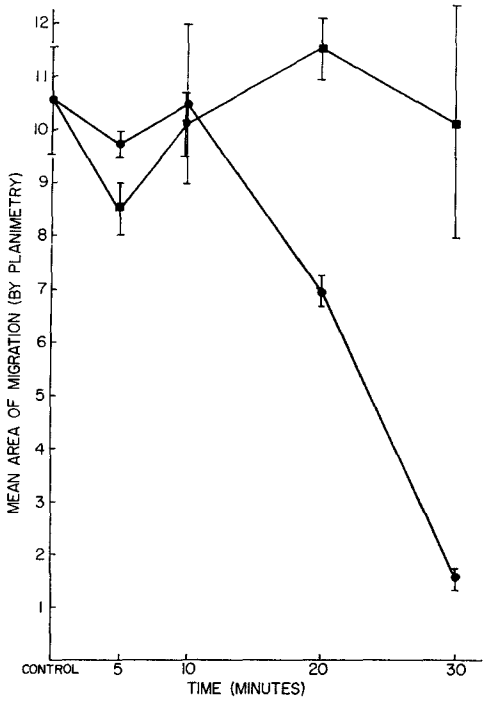

FIG. 3. Effect of temperature on the ability of blocking factor in the plasma from patients with SSPE to inhibit the response of SSPE leukocytes to a purified SSPE virus (1%) suspension using the migration inhibition test. (●—●) 56°C; (■—■) 45°C.

to ribonuclease and deoxyribonuclease. The blocking factor was not reduced by 2-mercapto-ethanol treatment and possessed most of its activity in the 25–50% $(NH_4)_2SO_4$ fraction.

Plasma or spinal fluid from the SSPE patients was applied to a Sephadex G-200 column and fractions collected using 0.1 M Tris-HCl buffer at pH 7.4. It can be seen (Fig. 4) that generally three major fractions were obtained with plasma. Using markers of known molecular weight, such as hemoglobin, cytochrome *c*, and rabbit IgG, it was determined that the mol wt of peak A was greater than 150,000. Tubes under each peak were pooled, concentrated to the original volume in each case, and termed peak A, B, and C. These fractions were then tested in the migration inhibition test to determine the active frac-

TABLE VIII

Effect of Various Enzymes and Chemical Treatment on the Ability of Blocking Factor in Plasma from Patients with SSPE to Inhibit the Response of SSPE Leukocytes to a Purified SSPE Virus Suspension Using the Migration Inhibition Test

SSPE plasma treated with:	% Migration inhibition*
Control‡	69.2
Untreated§	8.7
Neuraminadase	75.4
Ribonuclease	26.9
Deoxyribonuclease	−3.0
Trypsin-EDTA	73.9
2-mercapto-ethanol (0.1 M)	6.3
Ammonium sulfate (0–25 gm %)	4.3
Ammonium sulfate (25–50 gm %)	73.5
Ammonium sulfate (>50 gm %)	−10.0

* $\%\ \text{Migration inhibition} = 1 - \frac{\text{Average area of migration with antigen}}{\text{Average area of migration without antigen}} \times 100.$

‡ Control had measles sero-negative plasma.

§ 10% SSPE plasma untreated.

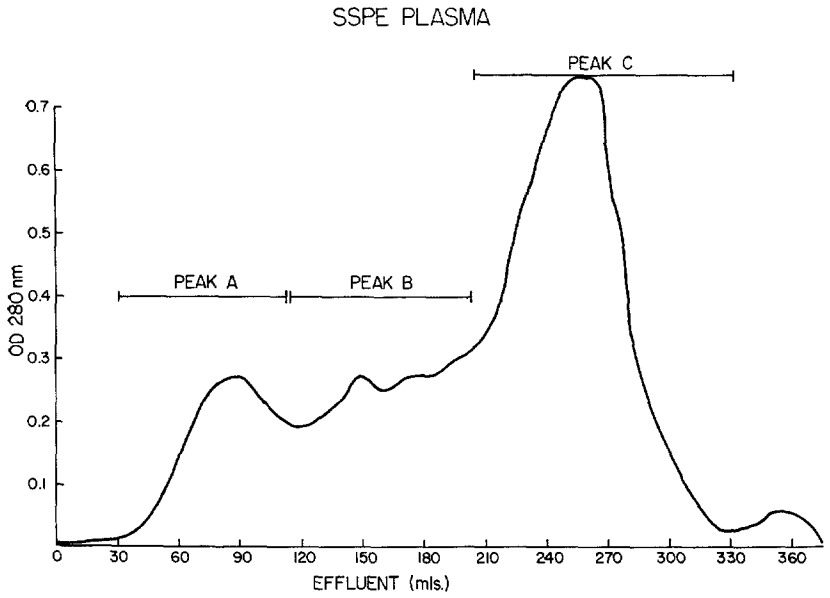

FIG. 4. Fractionation of plasma from a patient with SSPE by gel filtration with Sephadex G-200 (column 3.0 × 60 cm, ascending flow rate 12 ml/h) and 0.1M Tris-HCl buffer, pH 7.4.

tion. It is seen in Fig. 5 that peak A of both the plasma and spinal fluid possessed the ability to block the release of MIF from leukocytes of both the SSPE patient and the measles sero-positive control. The measles sero-negative control did not show any response.

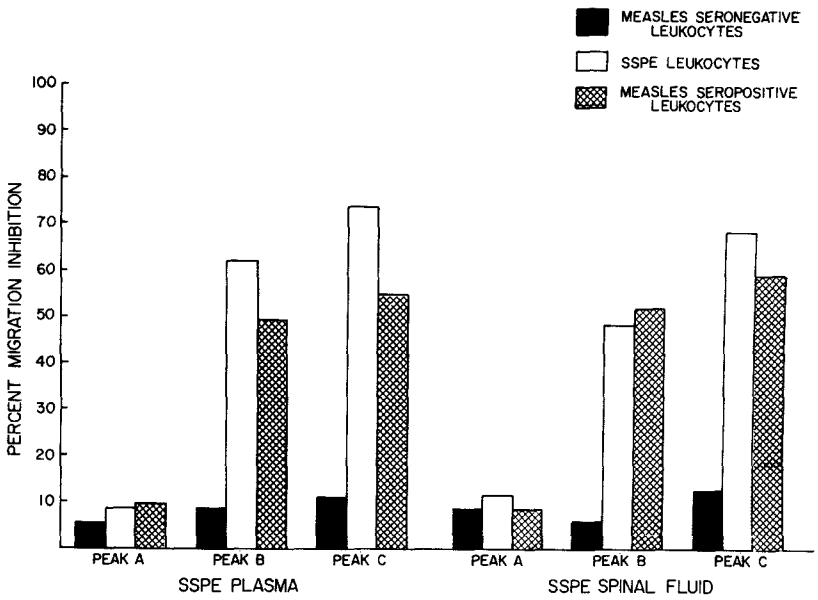

FIG. 5. Migration inhibition patterns of leukocytes from SSPE patient, measles seropositive and measles sero-negative donors in response to 1% SSPE virus in the presence of 10% Sephadex fractions A, B, and C from the plasma and spinal fluid of SSPE patients.

Blocking Factor Specificity.—In order to determine the specificity of the blocking factor in the plasma of SSPE patients, experiments were performed using lymphocytes from donors sensitive to a variety of antigens. Leukocytes from the various antigen donors and normal guinea pig macrophages were incubated with their donor specific sensitizing antigens in the presence of either 10% normal plasma or 10% SSPE plasma. The direct MIF test was used in these experiments and the results showed (Table IX) that using either bacterial or viral antigens, the SSPE blocking factor was only effective with the SSPE leukocytes response to SSPE virus. It did not have any effect on the other antigens used.

DISCUSSION

Conflicting reports on the cellular immune capacity of patients with SSPE have been reported. Gerson and Haslam (10) reported that none of the four cases of SSPE that they studied responded to six common skin-test antigens which included measles antigen. These cases rejected skin allografts slowly, and they did not develop skin sensitivity to dinitrochlorobenzene. Similar observations have been reported by Kolar (31). Moulias et al. (14), after studying 20 cases of SSPE, reported that the cell-mediated immunity to antigens other than measles was normal in these patients. These in vitro and in vivo

TABLE IX

Specificity of the Blocking Factor in the Plasma from Patients with SSPE

Source of leukocytes	% Migration inhibition in response to respective* antigen‡	
	10% Normal plasma	10% SSPE plasma
SSPE patient 1	69.6	8.1
SSPE patient 2	70.9	−10.2
SSPE patient 3	69.1	3.6
Cytomegalovirus-infected patient	69.2	61.1
PPD-sensitive donor	87.8	84.5
Histoplasmin-sensitive donor	48.4	42.7
Streptokinase-streptodornase-sensitive donor	72.6	68.3
Tetanus-toxoid-sensitive donor	49.0	58.6
Mumps-sensitive donor	42.0	40.2
Vaccinia-sensitive donor	47.4	43.5

* Respective antigen to which patient was sensitized was used as a predetermined optimal concentration.

‡ % Migration inhibition

$$= 1 - \frac{\text{Average area of migration with respective antigen}}{\text{Average area of migration without antigen}} \times 100.$$

results were similar to those obtained by Sharma et al. (32) and others (12, 33). However, Moulias et al. (14) reported that lymphocytes from their patients with SSPE did not respond to measles antigen. This observation has been reported by others (15, 34). Contrary to these data, Mizutani et al. (13) and Saunders et al. (12), report that there was no evidence of impairment of the cell-mediated function to measles antigen in patients with SSPE, as judged by skin tests and the macrophage migration inhibition test. We have previously reported results (25, 35, 36) that clearly demonstrate the presence of cellular immunity to measles antigen in patients with SSPE.

The reasons for the discrepancies obtained could be (a) the antigen used for skin testing or for in vitro testing; (b) the assay system involved; (c) the stage of the disease when testing is done; (d) the clinical treatment of the patient (immunosuppression) when testing is performed which might obscure results; (e) infection of the patient with viral agents other than SSPE virus and other medical complications.

While the measles etiology of SSPE has been documented by several reports (6, 37–39), there have been other reports that suggest that either the SSPE virus might be slightly different from measles virus (3, 4, 40, 41) or that there are other viruses which might act as helper viruses or their presence is unexplained (42, 43). In our preliminary experiments, measles virus vaccine used gave inconsistent results. It was only after using SSPE virus preparation that consistency was obtained. This, therefore, suggests one of the major sources of

variation of results obtained from several laboratories. This further amplifies the need for a standard virus suspension for use in such in vitro and in vivo testing.

The assumption, therefore, that native measles virus is the major cause of this encephalopathy becomes debatable. It is very possible that there are two or more types of virus, the wild type and the SSPE type virus, and that they probably all belong to the myxovirus group, but the antigenic variation between them could cause one to be extremely pathogenic. This would also account for the cross-reactivity to the measles antigen and subsequent failure of the immune response. On the other hand, it is also possible that the measles virus is activated by yet another virus, an arbovirus or a papovavirus which act as helper viruses by the activation of the infected cell genome after fusion with susceptible cells in the presence of a myxovirus. This association of two viruses has also been noted in cases of rabies and LCM and other diseases such as those produced by Epstein-Barr virus. This would therefore entail a two-step theory of SSPE where live attenuated measles virus, when used as an immunization, can be reactivated by some other paramyxovirus or papovavirus or arbovirus and change its antigenic structure sufficiently to cause fusion of the genome of the measles virus into the DNA of the glial cells and thereby give rise to the encephalopathy. The blocking substance could be produced by these superinfected glial cells which release this substance into the spinal fluid and which then travel into the vascular system.

In addition to skin testing with measles antigen, various in vitro assays have been employed to determine the cell-mediated immune capability of patients with SSPE. Again, variable results have been obtained using the lymphocyte transformation and the production of MIF assays. Although direct stimulation with the many virus suspensions that we have used gave results that were difficult to interpret (4,000 cultures), use of virus-infected cells as stimulating cells provided consistent data showing the presence of cellular immunity in these patients (25). The MIF assay gave very consistent results in our laboratory, but only using SSPE virus suspensions. Measles sero-positive individuals also showed MIF production in response to the SSPE virus; however, there was no response of sero-positive lymphocytes to SSPE infected HeLa cells in the transformation studies. This dichotomy can be explained by the assumption that either there are different antigens involved in stimulating the different responses; that is, viral envelope antigens stimulating MIF production and viral-induced cell surface antigens causing lymphocyte blastogenesis, or that MIF is a preformed product which is released by cells on recognition of homologous sensitizing antigen and lymphocyte transformation is an active process, whereby presensitized cells undergo DNA synthesis in response to specific antigens. The lack of response of lymphocytes from measles sero-positive individuals in the tests where lymphocytes were stimulated by virus-infected cells would seem to indicate nonsensitization of the lymphocytes to the

cell surface antigens induced in the infected HeLa cells by the SSPE viral genome. MIF was measured at 24 h, whereas blastogenesis was measured at 72 h after the contact of viral antigens with sensitized cells. It is possible that viruses are neutralized by in vitro interferon production by sensitized lymphocytes and therefore not available for stimulation, whereas using virus-infected cells, antigens are so provided that cells do not respond by producing interferon but respond to the antigens expressed on the infected cell surface. Such a difference between in vitro transformation and MIF assays has been previously reported by Spitler et al. (44) using tobacco mosaic virus protein (TMVP). Lymphocytes from TMVP-sensitized animals produced MIF in response to isolated peptides from TMVP but did not show any capacity for in vitro stimulation as measured by the uptake of [^{14}C]thymidine. They explain their data by suggesting that they did not use high enough concentrations of the peptides or that there was low binding capacity of the peptides to lymphoid receptor sites, or that to stimulate lymphocyte activation a multivalent antigen may be required. Similar differences between lymphocyte transformation and MIF assay has been shown by other workers (45, 46).

Rosenberg et al. (47) have demonstrated specific in vitro transformation using sensitized rabbit spleen lymphocytes by suspensions of ultraviolet light-inactivated Herpes-Simplex and vaccinia viruses. Similarly, Gerber and Lucas (48), using ultraviolet, irradiated, noninfectious Epstein-Barr virus, were able to induce activation in sensitized human lymphocytes. On the other hand, Thurman et al. (25) could not demonstrate stimulation of sensitized lymphocytes using cytomegalovirus suspensions (CMV), whereas CMV-infected WI-38 cells specifically activated the sensitized cells. Thus, depending on the antigen used and the assay employed, certain viruses can be used for in vitro stimulation. The other assay performed was the LT assay. Here it appeared that LT was produced by measles sero-positive cells and SSPE cells in the presence of sero-negative plasma and that this effect was blocked by SSPE plasma.

Although cell-mediated immunity against the SSPE virus could be demonstrated by the various assay systems, an important finding of this investigation was the demonstration of blocking factors present in the plasma and spinal fluid of all SSPE patients studied. The concentration of the blocking factor was found to be about 10 times greater in the spinal fluid as compared to the plasma in all four SSPE cases studied. It is possible that the blocking factor was made by the glial cells of the brain and gradually passed into the vascular system. Others have reported the presence of inhibitory factors in the serum and plasma from patients with a wide range of disorders (18, 19, 21, 49–52). An inhibition of lymphocyte transformation to antigens and nonspecific mitogens was found in the plasma of one of three SSPE patients studied by Allen, Oppenheim, and Brody (22). The blocking factor reported here was specific for the response to SSPE virus and did not block mitogen or antigen responses. The possibility that the blocking factor is hyperimmune levels of specific antibody in the

plasma of patients with SSPE cannot be completely ruled out. However, the fact that the blocking activity is found in Fraction A of the Sephadex gel chromatography (Fig. 4) of the plasma and that anti C'3 neutralizes the blocking activity argues against this possibility. Also the mol wt was found to be greater than 150,000. The nonsensitivity to 2-mercapto-ethanol rules out the presence of IgM type antibodies as the blocking agent. Preliminary data also show that if plasma of patients with SSPE is incubated with commercially available measles virus and centrifuged, and the supernatant fluid examined for blocking activity, there is a very slight decrease in blocking ability. This still does not rule out the possibility that there is an antigenic difference between the commercially available measles virus and the SSPE virus and that the antibody is directed against that specific SSPE antigen. Further studies are currently being undertaken to further define the nature of this blocking factor.

It is difficult to determine if the blocking factor present in patients with SSPE occurs before the disease or one of the manifestations of the disease. The only way this can be determined is by animal models and, to date, the models which have been successfully used have been ferrets (53), hamsters (54), calves, and lambs (55). The investigators employing these models have as yet not addressed themselves to the immunological implications (56). The importance of blocking factors and their role in the immunological response of lymphocytes from patients with a wide group of disorders, including hepatitis, multiple sclerosis, cancer, and slow virus diseases such as Kuru, Jacob-Kreutzfeldt, etc., remains to be elucidated.

SUMMARY

Conflicting reports on the immune responsiveness of patients with subacute sclerosing panencephalitis (SSPE) have been reported. This report shows that the leucocytes from four SSPE patients exhibited strong sensitivity to both measles and SSPE virus preparations as measured by the macrophage migration inhibition test, mixed lymphocyte virus infected cell culture test, and the lymphotoxin assay. Earlier suggestions that a factor may be operating to suppress cellular reactivity are confirmed by the demonstration that the response of lymphocytes from SSPE patients could be blocked by the addition of SSPE spinal fluid or plasma. It was determined that the blocking factor was stable at −20°C, heat labile at 56°C for 30 minutes, trypsin and neuraminadase sensitive, and had a mol wt greater than 150,000 as determined by Sephadex G-200 gel chromatography. The blocking factor appeared to be specific for SSPE virus and did not block the response of lymphocytes to nonspecific mitogenic agents and other viral and bacterial agents.

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REFERENCES

1. Bodechtel, G., and E. Guttman. 1931. Diffuse encephalitis mit sklerosierender. Entzundung des Hemispharenmarkes. *Z. Gesamte Neurol. Psychiat.* **133**:601.
2. Jabbour, J. T., D. A. Duenas, J. L. Sever, H. M. Krebs, and L. Horta-Barbosa. 1972. Epidemiology of subacute sclerosing panencephalitis (SSPE): a report of the SSPE registry. *JAMA J. Amer. Med. Assoc.* **220**(7):959.
3. Oyanagi, S., V. ter Meulen, M. Katz, and H. Koprowski. 1971. Comparison of subacute sclerosing panencephalitis and measles virus: An electron microscopic study. *J. Virol.* **7**(1):176.
4. ter Meulen, V., M. Katz, Y. Kackell, G. Barbante-Brodano, H. Koprowski, and E. H. Lennette. 1972. Subacute sclerosing panencephalitis: *in vitro* characterization of viruses isolated from brain cells in culture. *J. Infect. Dis.* **126**(1):11.
5. Sever, J. L., and E. Zeman, editors. 1968. *Neurology.* **18**(1, Pt. 2). 1-379.
6. Horta-Barbosa, L., R. Hamilton, B. Witteg, D. A. Fuccello, and J. L. Sever. 1971. Subacute sclerosing panencephalitis: isolation of suppressed measles virus from lymph node biopsies. *Science* (*Wash. D. C.*). **173**:840.
7. Horta-Barbosa, L., H. Krebs, A. Ley, T. C. Chen, M. R. Gilkeson, and J. L. Sever. 1971. Progressive increase in cerebrospinal fluid measles antibody level in subacute sclerosing panencephalitis. *Pediatrics.* **47**:782.
8. Dayan, A. D., and M. I. Stokes. 1971. Immunofluorescent detection of measles virus antigens in cerebrospinal fluid cells in subacute sclerosing panencephalitis. *Lancet.* **1**:891.
9. Reinert, Ph., R. Moulias, J. M. Goust, J. Harpey, and F. Cathala. 1971. Etude qualitative de l'immunite contre le virus de la rougeole an cours de la Less. *Presse Med.* **79**:1634.
10. Gerson, K. L., and R. H. A. Haslam. 1971. Subtle immunologic abnormalities in four boys with subacute sclerosing panencephalitis. *N. Engl. J. Med.* **285**:78.
11. Mizutani, H. 1972. Immunologic abnormalities in subacute sclerosing panencephalitis. *N. Engl. J. Med.* **256**(14):786.
12. Saunders, M., M. Knowles, M. E. Chambers, and E. A. Caspary. 1969. Cellular and humoral responses to measles in subacute sclerosing panencephalitis. *Lancet.* **1**:72.
13. Mizutani, H., S. Saito, K. Nihei, and T. Izuchi. 1971. Cellular hypersensitivity in subacute sclerosing panencephalitis. *JAMA J. Amer. Med. Assoc.* **216**(7):1201.
14. Moulias, R. L., Ph. Reinert, and J. M. Goust. 1971. Immunologic abnormalities in subacute sclerosing panencephalitis. *N. Engl. J. Med.* **285**:1090.
15. Lischner, H. W., W. D. Grover, and A. DeForest. 1971. Proliferative response of lymphocytes to measles antigen in tissue culture in subacute sclerosing pan-

encephalitis (SSPE). *In* Proceedings 4th Annual Leucocyte Culture Conference. O. R. McIntyre, editor. Appleton-Century-Crofts, New York. 565.

16. Dayan, A. D., and M. I. Stokes. 1972. Immune complexes and visceral deposits of measles antigens in subacute sclerosing panencephalitis. *Br. Med. J.* **2**:374.
17. Philips, P. E. 1972. Immune complexes in subacute sclerosing panencephalitis. *N. Engl. J. Med.* **286**(17):949.
18. Gatti, R. A. 1971. Serum inhibitors of lymphocyte responses. *Lancet.* **1**:1351.
19. McFarlin, D. E., and J. J. Oppenheim. 1969. Impaired lymphocyte transformation in ataxia-telangiectasia in part due to a plasma inhibitory factor. *J. Immunol.* **103**:1212.
20. KNOWLES, M., D. HUGHES, E. A. Caspary, and E. J. Field. 1968. Lymphocyte transformation in multiple sclerosis. Inhibition of unstimulated thymidine uptake by a serum factor. *Lancet.* **2**:1207.
21. Scheurlen, P. G., W. Schneider, and A. Pappas. 1971. Inhibition of transformation of normal lymphocytes by plasma factor from patients with Hodgkins disease and cancer. *Lancet.* **2**:1265.
22. Allen, J., J. J. Oppenheim, J. A. Brody, and J. Miller. 1973. Labile inhibitor of lymphocyte transformation in plasma from a patient with subacute sclerosing panencephalitis. *Infect. Immun.* **8**(1):80.
23. David, J. R., and R. David. 1971. Assay for inhibition of macrophage migration. *In In Vitro* Methods in Cellular Immunity. B. R. Bloom and P. R. Glade, editors. Academic Press, Inc., New York. 249.
24. Boyum, A. 1968. Separation of leucocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* **21**(Suppl. 97):1.
25. Thurman, G. B., A. Ahmed, D. M. Strong, R. C. Knudsen, W. R. Grace, and K. W. Sell. 1973. Lymphocyte activation in subacute sclerosing panencephalitis virus and cytomegalovirus infections: in vitro stimulation in response to viral infected cell lines. *J. Exp. Med.* **138**:839.
26. Hartzmann, R. J., M. L. Bach, F. H. Bach, G. B. Thurman, and K. W. Sell. 1972. Precipitation of radioactively labelled samples: A semiautomatic multiple sample processor. *Cell. Immunol.* **4**:182.
27. Thurman, G. B., D. M. Strong, A. Ahmed, S. S. Green, K. W. Sell, R. J. Hartzmann, and F. H. Bach. 1973. Human mixed lymphocyte cultures: evaluation of a microculture technique utilizing the multiple automated sample harvester. *Clin. Exp. Immunol.* **15**:279.
28. Strong, D. M., A. A. Ahmed, G. B. Thurman, and K. W. Sell. 1973. *In vitro* stimulation of murine spleen cells using a microculture system and a multiple automated sample harvester. *J. Immunol. Methods.* **2**:279.
29. Granger, G. A., and W. P. Kolb. 1968. Lymphocyte *in vitro* cytotoxicity: mechanism of immune and non-immune small lymphocyte mediated target L-cell destruction. *J. Immunol.* **101**:111.
30. Shacks, S. J., J. Chiller, and G A. Granger. 1973. Studies on *in vitro* models of cellular immunity: the role of T and B cells in the secretion of lymphotoxin. *Cell. Immunol.* **7**:313.
31. Kolar, O. 1968. Measles and subacute sclerosing panencephalitis. *Lancet.* **2**:1242.
32. Sharma, M. K., W. D. Grover, and D. S. Huff. 1971. Studies of immunologic competence in subacute sclerosing panencephalitis (SSPE). *Clin. Res.* **19**:730.

33. Jabbour, J. J., J. A. Roane, and J. L. Sever. 1969. Studies of delayed dermal hypersensitivity in patients with subacute sclerosing panencephalitis. *Neurology*. **19**:929.
34. Vandvik, B. 1970. Immunologic studies in subacute sclerosing panencephalitis. *Acta Neurol. Scand. Suppl.* **46**(Suppl. 43):232.
35. Sell, K. W., A. Ahmed, G. B. Thurman, and D. M. Strong. 1973. Plasma and spinal fluid blocking factor SSPE. *N. Engl. J. Med.* **288**:215.
36. Ahmed, A., K. W. Sell, G. B. Thurman, and D. M. Strong. 1973. Demonstration of a blocking factor in the plasma and spinal fluid of two patients with subacute sclerosing panencephalitis. *Fed. Proc.* **32**:1026.
37. Payne, F. E., and J. V. Baublis. 1971. Measles virus and subacute sclerosing panencephalitis. *In* Perspectives in Virology, Vol. 7. M. Pollard, editor. Academic Press, Inc. New York. 179.
38. ter Meulen, V., G. Enders-Ruckle, D. Muller, and G. Joppich. 1969. Immunohistological, microscopical and neurochemical studies on encephalitides. III. Subacute progressive panencephalitis. Virological and immunological studies. *Acta Neuropathol.* **12**:244.
39. Connolly, J. H., I. V. Allen, L. J. Hurwitz, and J. H. D. Miller. 1967. Measles virus antibody and antigen in subacute sclerosing panencephalitis. *Lancet*. **1**:542.
40. Barbanti-Brodano, G., S. Oyanagi, M. Katz, and H. Koprowski. 1970. Presence of two different viral agents in brain cells of patients with subacute sclerosing panencephalitis. *Proc. Soc. Exp. Biol. Med.* **134**:230.
41. Muller, D., V. ter Meulen, M. Katz, and H. Koprowski. 1971. Cytochemical evidence for the presence of two viral agents in subacute sclerosing panencephalitis. *Lab. Invest.* **25**(4):337.
42. Dayan, A. D. 1971. Encephalitis due to simultaneous infection by herpes simplex and measles viruses. *J. Neurol. Sci.* **14**:315.
43. ter Meulen, V., M. Katz, and D. Muller. 1972. Subacute sclerosing panencephalitis: a review. *Curr. Top. Microbiol. Immunol.* **57**:1.
44. Spitler, L., E. Benjamini, J. D. Young, H. Kaplan, and H. H. Fudenberg. 1970. Studies on the immune response to a characterized antigenic determinant of the tobacco mosaic virus protein. *J. Exp. Med.* **131**:133.
45. Godfrey, H., H. Baer, and S. D. Chaparas. 1969. Inhibition of macrophage migration by skin reactive polysaccharide from BCG culture filtrates. *J. Immunol.* **102**:1466.
46. Baer, H., and S. D. Chaparas. 1964. Tuberculin reactivity of a carbohydrate component of unheated BCG culture filtrate. *Science* (*Wash. D. C.*). **146**:245.
47. Rosenberg, G. L., P. A. Farber, and A. L. Notkins. 1972. *In vitro* stimulation of sensitized lymphocytes by herpes simplex and vaccinia virus. *Proc. Natl. Acad. Sci. U. S. A.* **69**:756.
48. Gerber, P., and S. J. Lucas. 1972. *In vitro* stimulation of human lymphocytes by Epstein-Barr virus. *Cell Immunol.* **5**:318.
49. Silk, M. R. 1967. Effect of plasma from patients with carcinoma on *in vitro* lymphocyte transformation. *Cancer*. **20**:2088.
50. Cooperband, S. R., H. Bondevik, K, Schmid, and J. A. Mannick 1968. Transformation of human lymphocytes: inhibition by homologous alphaglobulin. *Science* (*Wash. D. C.*). **159**:1243.

51. Youtanahukhorh, V., and P. Matangkasmbuti. 1973. Specific plasma factors blocking human material cell-mediated immune reaction to placental antigens. *Nat. New Biol.* **242**:110.

52. Hellstrom, K. E., and I. Hellstrom. 1970. Cytotoxic lymphocytes, blocking serum factors and "unblocking" antibodies in cancer patients. *In* Membranes and Viruses in Immunopathology. S. B. Day and R. A. Good, editors. Academic Press, Inc., New York. 523.

53. Katz, M., L. B. Rorke, W. S. Masland, H. Koprowski, and S. H. Tucker. 1968. Transmission of an encephalitogenic agent from brains of patients with subacute sclerosing panencephalitis to ferrets. *N. Engl. J. Med.* **279**:793.

54. Lehrick, J. R., M. Katz, L. B. Rorke, G. Barbanti-Bordano, and H. Koprowski. 1970. Subacute sclerosing panencephalitis viruses: induction of experimental encephalitis in hamsters. *Trans. Am. Neurol. Assoc.* **95**:51.

55. Thein, P. A. Mayr, V. ter Meulen, H. Koprowski, M. Y. Kackell, D. Muller, and R. Meyermann. 1972. Subacute sclerosing panencephalitis: Transmission of the virus to calves and lambs. *Arch. Neurol.* **27**:540.

56. Allison, A. C., W. I. B. Beveridge, W. C. Cockburn, *et al.* 1972. Virus associated immunopathology: animal models and implications for human disease. II. Cell-mediated immunity, autoimmune diseases, genetics and implications for clinical research. *Bull. W. H. O.* **47**(2):265.