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Multiple Sclerosis-Associated Agent: Transmission to Animals and Some Properties of the Agent

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In confirmation and extension of observations by Carp and his associates, brain tissue and sera from patients with multiple sclerosis (MS) were found to harbor an agent which induces a transitory depression in polymorphonuclear leukocytes (PMN) in mice as well as in rats, hamsters, and guinea pigs. All of eight MS brains contained this agent at titers as high as 10^{-9}/g of brain tissue. The agent was found in MS sera at titers up to 10^{-3}/ml of serum, but its presence depended to some extent on the clinical status of the patients; it was observed more frequently in sera of patients with active disease (73%) than in sera of patients with quiescent disease (31%). Control brain tissues or sera failed to induce PMN depressions. The apparently MS-associated agent (MSAA) passed through 50-nm but not 25-nm membrane filters (Millipore Corp.) and was largely sedimented at 105,000 × g but not at 50,000 × g for 1 h. It multiplied to high titers in the central nervous tissue of the inoculated animals and could be serially transmitted from animal to animal by passage of brain homogenates. Various observations and considerations appear to preclude that MS-associated agent represents an indigenous animal virus. Although its role in MS remains to be determined, it should be considered a candidate for the etiology of this disease.

It has long been suspected that multiple sclerosis (MS) might be caused by a virus. Whereas several viruses have been implicated on a serological basis or by isolation from involved central nervous tissue, none have been proven to be etiologically related to the disease (cf. 4). Recently, Carp and his associates (2) reported that intraperitoneal or intracerebral injection of specimens from MS patients into inbred mice rapidly induced persistent changes in the differential counts of circulating leukocytes, i.e., a decrease in the percentage and absolute number of polymorphonuclear leukocytes (PMN). The factor causing the PMN depression was found in all MS specimens tested, i.e., three brains, one spleen, three sera, and two cerebrospinal fluids from a total of nine patients. It was not demonstrable in similar numbers of specimens of each type derived from patients with other diseases. The factor was present in MS brain homogenates at concentrations of up to 10^{12} U/g of tissue, was passable through 50-nm but not 25-nm membrane filters (Millipore Corp.), clearly multiplied in the brain of the injected animals, and could be passed to other mice.

These observations indicated that the factor causing the PMN depression was a small virus with unusual biological activities, either derived from the MS specimens or activated by them in the injected mice. It was essential to confirm these findings, to exclude a latent murine virus, and to relate the agent to man, and in particular to MS. Since total and differential leukocyte counts in mice are notoriously variable and influenced by many factors (cf. 5), the findings of Carp and his co-workers could not be confirmed in a small experimental series (1). As will be shown below, the essential observations of Carp and his group could be reproduced, although with considerable difficulties. For convenience, the agent present in MS specimens will be referred to as MS-associated agent (MSAA).

MATERIALS AND METHODS

MS and control specimens. A few of the MS and control specimens were obtained from occasional sources, but most were collected in an organized manner at the Universitäts-Nervenklinik, Cologne, Germany, at the Department of Neurology, Temple University School of Medicine Philadelphia,

and under the auspices of the National Multiple Sclerosis Society by W. Tourtelotte. Specimens from the last source were obtained by R. I. Carp and kindly transmitted by him to us.

(i) Brains. Samples of a total of eight frozen brains from MS patients became available and were stored in small portions in a Revco freezer at −70 C until used. Portions of brains obtained at autopsy or surgery from patients with Alzheimer's disease (one), Creutzfeld-Jacob disease (two), astrocytomas (four), or nonneurological conditions (four) served as controls. The brain specimens were homogenized in a tissue grinder with sufficient phosphate-buffered saline (PBS) solution, made with pyrogen-free water (Grand Island Biological Co.), to yield 20% suspensions (wt/vol). After clarification by low-speed centrifugation, the suspensions were injected within a few hours, in varying dilutions, into animals, using PBS as diluent.

(ii) Cerebrospinal fluids. A total of eight specimens from MS patients and 10 from patients with other diseases were tested. They were injected, either fresh or after storage, at −70 C.

(iii) Sera. Sera were obtained from MS patients with active or resting disease, relatives (spouses, siblings, parents, or offspring), nursing personnel, and other healthy donors. All were frozen at −70 C soon after separation, and those from abroad were received by air express under dry-ice refrigeration. Sera from Hodgkin's disease patients and appropriate controls were selected from an available collection supplied by Henry S. Kaplan, Stanford University Medical Center, and maintained at −20 C. The sera were injected after fivefold dilution in PBS, and further fivefold dilution steps were used for titrations.

Viruses. The following viruses were obtained from the American Type Culture Collection: measles (Edmonston strain); parainfluenza type 1 (HA-2 strain); H-1 rat virus (Toolan); RV-13 (Kilham); and pneumonia virus of mice (strain 15).

Experimental animals. Mice were purchased from various dealers. The strains included Balb/c, C57 black/6, C3H/HE, C3BF1, BC3F1, and outbred albinos. With few exceptions, only female animals were used, since the percentages of PMNs may differ, not only among strains of mice, from as low as 20% to as high as 65%, but also to some extent between sexes, males showing lower values and more variations than females (cf. 5). Such differences were noted also in the present study as evident from Table 1, which shows in addition that injection of various control materials, among them sera from six different healthy donors, failed to cause significant changes in the distribution of leukocytes. If anything, there was at times a slight increase of the percentages of PMNs over those observed in noninjected mice. The differences noted with different

TABLE 1. *Normal percentages of PMNs in various strains of mice from different suppliers after injection of control materials (four mice/group)*

Strain of mice	Sex	Supplier	Control materials injected									
			None		PBS		Brain homogenates				Human sera[a]	
							Human		Murine			
			% PMN	SE[b]	% PMN	SE	% PMN	SE	% PMN	SE	% PMN	SE
Balb/c	F	A			32.2	3.374			30.4	1.418	34.8	3.359
	F	B	24.2	1.396	23.3	1.101			22.5	2.037	25.0	2.182
C57 bl/6	F	B			30.4	2.421					38.0	2.562
	F	B			23.1	2.785					30.4	3.940
	M	B			19.6	0.959			18.5	1.309	25.6	1.753
	F	C			35.1	6.734					33.3	4.160
	F	E	20.0	2.393	27.2	5.120	30.4	2.961				
C3H/He	F	A			42.5	4.438						
	F	B			50.2	2.476	47.9	4.072	46.0	2.371		
	F	C			51.8	3.416			44.8	4.434		
	F	E	20.5	0.703	33.7	4.642			30.7	2.172		
C3BF1	F	B	30.0	2.565								
	F	B	29.4	1.360	30.0	3.999	26.1	1.706	29.6	3.554		
	F	B	30.5	2.815								
BC3F1	F	B	27.5	0.914	25.3	1.000						
	F	B	30.0	2.268								
Outbred	F	A			20.6	1.344			24.4	2.380		
Albino	F	D			19.6	1.539			19.9	1.909		

[a] Different sera from healthy donors were employed in each test.
[b] SE, Standard error.

strains of mice, or even with the same strain from different dealers or with different batches from the same supplier, did not interfere with detection of PMN depressions after injection of MS specimens. However, batches of mice were received in the course of these studies that showed unusually large variations in PMN percentages without inoculation or after injection of control materials. In these instances, the animals were generally in an unsatisfactory condition, which necessitated changes in dealers and/or in the strains of mice. Thus, a number of experiments could not be evaluated because of gross variations in the control groups or, with growing experience, scheduled tests were cancelled because of the unhealthy appearance of the available mice.

Since there exist diurnal variations in total and differential leukocyte counts and such assays are known to be influenced by undue agitation of the animals and the bleeding procedure (see below), the animals were finally kept in strict isolation in constant-temperature rooms to which only two persons had access for daily care and experimental procedures. Blood smears were prepared at a set time of day, i.e., between 1 and 3 p.m.

Rats (Lewis strain), guinea pigs (strain 13), and Syrian hamsters (Lakeview Farm) were handled under the same conditions as the mice.

Inoculation of animals and preparation of blood smears. MS and control materials were injected usually intraperitoneally (0.2 ml), rarely intracerebrally (0.03 ml). Blood smears were prepared at varying times thereafter depending on the strain of mice, since some were found to respond faster than others. Bleeding of the mice from the heart, orbital sinuses, or axillary blood vessels yielded lower percentages of PMNs and also more variable results than bleeding from the severed tip of the tail, which was adopted as standard procedure. Only the first free-flowing drops of blood were used for smears. This procedure permitted repeated testing of mice, but the intervals had to exceed 2 weeks to avoid irregular results due to the healing process or possibly infections. Rats were bled in the same manner. Blood from guinea pigs and hamsters was obtained by cardiac puncture under light ether anesthesia.

The air-dried blood smears were fixed with methanol and stained with Giemsa solution by standard methods. The smears were coded by one person and read by others without knowledge of their identity to avoid bias. At least 100 leukocytes were counted and classified as granulocytes or mononuclear cells. In the initial experiments groups of three, later four, and up to eight mice received the same inoculum. The mean percentages of PMNs recorded in the experimental groups were compared with those of the controls, and the differences were expressed as percentage of reduction of the control values. The significance of these results was determined by calculation of the P values by the two-tailed test, with $P \leq 0.05$ taken as significant.

Passage of MSAA. At varying times after injection of MS or control materials, animals were sacrificed, the brains were removed aseptically and homogenized as described, and the suspensions, after appropriate dilution, were injected into new groups of mice. Blood smears were obtained at given intervals, and the animals were subsequently sacrificed for further passages in the same manner. Sufficient numbers of serial passages were carried out to assure that the estimated cumulative dilution factors far exceeded the titers of MSAA in the MS specimens used for initiation of the passage series.

Filtration procedures. Ten- or 100-fold-diluted mouse passage brain homogenates were clarified at 10,000 × g for 1 h and passed successively through 450-, 220-, 100-, 50-, and 25-nm membrane filters (Millipore Corp.). MS sera were filtered in the same manner after 5- or 25-fold dilution in PBS. Samples of the original materials and the various filtrates were then injected into mice, and blood smears were prepared and analyzed 1 to 2 weeks later as described.

High-speed centrifugation. Test specimens, prepared as for the filtration experiments, were subjected to 50,000 × g or 105,000 × g in a Spinco preparative centrifuge, and the starting materials, the supernatant, and the pellets, resuspended in PBS to the original volumes, were tested in mice by the usual procedure.

RESULTS

It has been emphasized in Materials and Methods that for demonstration of PMN depressions after injection of MS specimens it is essential to have mice in a uniformly healthy state. This condition was not always met by the dealers, and some experiments failed on that account. Other reasons for experimental failures will become apparent later. Yet, satisfactory results were obtained in many tests.

Inoculation of MS brain homogenates. Table 2 presents in detail several experiments in which mice were injected intraperitoneally with MS and control brain homogenates. The percentages of PMNs observed in individual mice after injection of MS brain suspensions were usually all lower than the lowest value found in mice injected with control brain homogenates. The reductions in the mean percentages of PMNs, as compared to the controls, were as high as 66% and highly significant.

Several MS brain homogenates were titrated in serial 10- or 100-fold dilution steps and highly significant PMN depressions were induced with dilutions as high as 10^{-9}. Figure 1 shows the results of two separate titrations with the means and the spread of PMN percentages. The figures for the two control brain homogenates used as a 10^{-1} dilution matched those obtained with dilutions of the MS brain suspensions beyond the end point of activity. As will be shown later, mouse passage brain suspensions may yield similarly high titers.

Inoculation of MS sera. Sera from healthy donors generally failed to cause significant

TABLE 2. *Induction of PMN depressions in mice after inoculation of MS brain homogenates*

Inoculum	Strain of mice	Day	PMN (%)						Reduction (%)	P
			1[a]	2	3	4	Mean	SE[b]		
PBS	C3H	3	39.0	39.2	40.6		39.6	0.503		
Control brain-1 (10^{-1})			35.8	37.2	42.5		38.5	2.040	2.6	
MS brain-A (10^{-1})			12.5	13.2	15.1		13.6	0.776	64.6	<0.001
Control brain-3 (10^{-1})	Outbred	22	33.3	34.2	46.2		37.9	4.158		
MS brain-A (10^{-1})	Albino		13.4	14.9	16.5		14.9	0.895	60.6	0.025-0.01
PBS	C3H	3	30.4	32.2	33.3		31.8	0.845		
Control brain-2 (10^{-1})			32.8	35.1	40.5		36.1	2.282	0	
MS brain-G (10^{-1})			10.2	13.0	13.4		12.2	1.060	66.2	<0.001
PBS	C3BF1	13	23.2	24.3	24.7	25.9	25.8	0.7736		
			26.0	27.8	29.1					
Control brain-4 (10^{-4})			10.1	20.5	22.6	35.5	22.1	5.214	14.3	0.4-0.2
MS brain-H (10^{-4})			11.0	13.6	20.4	23.2	17.0	2.851	34.1	0.005-0.0025

[a] Mouse number.
[b] SE, Standard error.

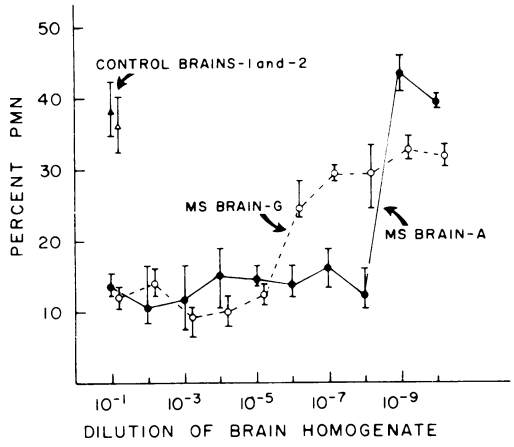

FIG. 1. *Results of titrations of MS brain homogenates in two separate experiments. Control brain-1 (solid triangle) was used with MS brain-A, and control brain-2 (open triangle) was used with MS brain-G.*

changes in the distribution of leukocytes (Table 1). Table 3 summarizes a representative experiment in which 11 MS sera were tested simultaneously in 1:5 dilution in groups of three to five mice each. With seven of the sera, all the individual PMN percentages were below those of the controls, whereas with the other sera one or two values fell within the range of the control percentages. The reductions in the mean percentages ranged from 12.6% (insignificant) to 51.8% ($P < 0.001$). Whereas in general the significance increased with an increase in the percentage of reduction, one nonresponding mouse among four can change a mean decline in PMNs of 33% to borderline significance.

A number of MS sera were tested repeatedly in the same or different strains of mice. Table 4 presents examples which show that the results were on the whole reproducible, but the degrees of significance varied to some extent. These variations could have occurred for two reasons: (i) the mice at times were bled possibly before PMN depressions had fully developed, which might occur especially with low-titered sera; and (ii) the activity of sera had declined on prolonged storage or after repeated freezing and thawing.

Table 5 shows representative titrations of positive MS sera in fivefold dilution steps. Some could be diluted as much as 1:625 and still cause significant PMN depressions and other could be diluted no more than 25- or even 5-fold. With a number of sera, such as HA-1, the lowest serum dilution was noted to produce a somewhat less striking reduction in PMN percentages than the next higher dilution(s). This might not be entirely due to change but could be a reflection of the presence of antibodies, as will be discussed in the accompanying paper (3).

The time of appearance of PMN depressions and their durations have not been accurately determined, since serial blood smears from the severed tip of the tail cannot be obtained at short intervals without introducing variations in the differential leukocyte counts due to the healing process. The time required for induction of significant PMN depressions seemed to depend, however, not only on the dose of MSA but also on the strain of mice used, with outbred albino mice being relatively resistant. The most pronounced reductions in PMNs were observed usually between 5 and 28 days after injection of MS specimens, whereas blood

TABLE 3. *Induction of PMN depressions in C57 black mice by MS sera in a representative experiment*

Sera	No. of mice	PMN (%) (range)	Mean (day 5)	Reduction (%)	P
PBS control	4	30.5–37.2	33.0		
GE-55	4	14.7–22.8	19.5	40.9	0.005–0.001
GE-57	5	22.8–35.6	28.8	12.6	0.2–0.1
GE-58	4	23.8–34.4	27.0	18.2	0.2–0.1
GE-59	4	15.1–34.3	22.0	33.3	0.1–0.05
GE-64	4	16.2–30.4	23.9	27.5	0.05–0.025
GE-65	4	9.1–20.5	15.9	51.8	<0.001
GE-67	4	20.3–31.0	26.0	21.2	0.05–0.025
GE-70	4	18.6–29.0	23.1	30.0	0.01–0.005
GE-71	4	15.0–19.8	16.9	48.7	<0.001
GE-72	3	15.0–25.1	21.6	34.5	0.025–0.01
HA-16	4	10.7–25.2	20.5	37.8	0.025–0.01

TABLE 4. *Reproducibility of PMN depressions induced in mice by MS sera*

Test no.	Inoculum (serum)	Strain of mice	No. of mice	Day	PMN (%)		Reduction (%)	P
					Range	Mean		
1	Control[a]	Balb/c	3	8	18.5–28.9	25.1		
	HA-1	Balb/c	3	8	13.2–19.1	16.7	33.6	0.1–0.05
2	Control	Balb/c	3	8	41.1–52.8	45.8		
	HA-1		3	8	24.7–32.1	27.9	39.0	0.025–0.01
3	Control	C57 bl	3	6	25.6–30.7	38.6		
	HA-1	C57 bl	3	6	13.4–21.0	17.3	39.4	0.025–0.01
1	Control	Balb/c	3	8	41.2–52.8	45.8		
	GE-2	Balb/c	3	8	29.0–35.0	31.2	31.8	0.025–0.01
2	Control	Balb/c	3	6	29.6–55.0	43.7		
	GE-2	Balb/c	3	6	26.0–30.0	27.4	37.2	0.1–0.05
3	Control	C57 bl	3	4	21.0–29.0	25.0		
	GE-2	C57 bl	3	4	7.0–15.0	10.6	57.5	0.05–0.025
1	Control	C57 bl	8	6	15.3–29.5	23.2		
	GE-55	C57 bl	5	6	7.5–27.6	18.5	20.2	0.4–0.2
2	Control	C57 bl	4	5	30.5–37.2	33.0		
	GE-55	C57 bl	4	5	14.7–22.8	19.5	40.9	0.005–0.001
3	Control	C3BF1	8	9	24.3–38.6	34.1		
	GE-55	C3BF1	4	9	21.3–24.4	22.8	30.4	0.001
1	Control	C57 bl	6	6	20.8–40.1	30.7		
	GE-57	C57 bl	3	6	15.3–44.4	28.0	8.7	0.5
2	Control	C57 bl	5	5	30.5–37.2	33.0		
	GE-57	C57 bl	5	5	22.8–35.6	28.8	13.6	0.2–0.1
3	Control	C57 bl	4	4	21.0–29.0	25.0		
	GE-57	C57 bl	4	4	22.0–24.0	22.6	9.4	0.4

[a] PBS.

TABLE 5. *Titration of MS sera for induction of PMN depression*

Strain of mice	Serum	Dilution	PMN (% day 6–8)		Reduction (%)	P
			Range	Mean		
C3BF1 (4/group)	Control (A.B.)[a]	1:5	30.6–34.3 34.6–38.6	34.1		
	MS-GE-87	1:5	9.9–24.4	16.1	52.8	<0.001
		1:25	19.0–26.9	22.5	34.0	<0.001
		1:125	13.4–23.0	17.5	48.6	<0.001
		1:625	14.5–28.6	19.9	41.6	<0.001
	MS-GE-71	1:5	9.7–19.2	14.7	56.9	<0.001
		1:25	14.8–24.1	19.9	41.6	<0.001
		1:125	14.5–19.7	17.0	50.1	<0.001
		1:625	21.6–25.5	23.4	31.4	0.001–0.001
		1:3125	28.7–39.6	32.9	3.5	NS[b]
Balb/c (3/group)	Control (G.J.)[a]	1:5	35.6–49.0	43.7		
	MS-GE-2	1:5	26.0–30.0	27.4	37.2	0.025–0.01
		1:25	22.8–30.0	26.9	38.4	0.025–0.01
		1:125	32.4–35.2	33.6	23.1	0.1–0.05
		1:625	35.8–48.1	41.3	5.5	NS
C57 bl (3/group)	Control (G.J.)[a]	1:5	25.6–30.7	28.2		
	MS-Ha-1	1:5	13.4–19.0	16.7	40.8	0.01–0.005
		1:25	3.6–16.0	9.0	68.1	0.01–0.005
		1:125	21.3–27.6	24.3	13.8	0.2–0.1

[a] Healthy donors.
[b] NS, Not significant.

smears obtained 6 or more weeks after inoculation revealed only minor or no changes in the distribution of leukocytes as compared to the controls. Thus, the PMN depressions were found to be transitory.

As mentioned, not all tests turned out satisfactory nor were results uniformly reproducible. Thus, some MS sera, which previously had caused significant PMN depressions, occasionally yielded in subsequent tests doubtful or negative results. Conversely, several sera from healthy donors, which had regularly been negative, were noted in one experiment to produce on titration erratic PMN depressions in some groups of the mice without evident correspondance to the serum concentration injected. Whereas tests yielding such unexpected results were usually among those considered unsatisfactory because of the condition of the mice, it seemed important nevertheless to verify or disprove the questioned data. Consequently, brains were collected from mice, which had shown such unexpectedly negative or positive responses, for homogenization and passage to new groups of mice. Brain homogenates from mice which had not been injected or had received PBS served as controls. Passages of brain suspensions from mice which had responded with significant PMN depressions after injection of MS specimens will be discussed later.

The control brain homogenates did not cause PMN depressions (Table 6). In contrast, brain homogenates from mice which unexpectedly had failed to respond to injection of previously positive MS sera caused PMN depressions on passage. Failures were noted only with brain homogenates from mice injected with too dilute MS serum (GE-87, 1:125) or when the brain suspensions were diluted beyond their end points. All brain homogenates from mice which had responded irregularly with PMN depressions to injection of varying dilutions of sera from healthy donors failed to yield positive results, with one exception of borderline significance. Passages thus are useful to confirm or disprove questionable results.

Summary of results obtained with MS and control materials. Table 7 summarizes the overall results obtained to date. All eight MS brain homogenates tested have given in one or more tests significant PMN depressions ($P \leq$

TABLE 6. *Clarification of irregular results obtained in PMN depression assays by passage of brain homogenates from the inoculated mice*

Inocula	Initial test: PMN result	Initial test: Reduction (%)	Initial test: P	Passage of brain homogenates from initial test: At day:	Dilution of brain homogenates	Day	PMN (%) range (no.)	Mean	Reduction (%)[a]	P	Result
None				29	10^{-3}	14	21.4–31.2 (5)	28.6	8.6	>0.5	−
PBS				33	10^{-4}	13	20.3–29.2 (8)	23.1	0.9	>0.5	−
PBS				40	10^{-4}	10	14.4–39.4 (4)	31.3	0	>0.5	−
MS sera[b]											
GE-63 1:8	−	0		40	10^{-4}	10	11.5–23.0 (4)	14.9	51.9	<0.001	+
GE-71 1:8	−	0		40	10^{-4}	10	10.2–17.5 (4)	14.6	52.9	<0.001	+
GE-78 1:8	−	0		40	10^{-4}	10	13.3–22.0 (4)	17.6	43.2	<0.001	+
GE-55 $10^{-4.2}$	±	23.3	0.5–0.25	33	10^{-4}	13	12.2–23.5 (3)	18.7	19.6	0.005–0.001	+
GE-87 1:25	−	0		29	10^{-3}	14	8.8–23.4 (5)	15.7	49.8	<0.001	+
	−			29	10^{-4}	14	24.0–34.8 (4)	28.7	0		−
GE-87 1:125	−	0		33	10^{-4}	13	24.0–28.0 (4)	25.8	0		−
Control sera[b, c]											
EL 1:5	+	21.3	0.025–0.001	33	10^{-4}	13	15.8–25.0 (4)	21.0	8.8	0.4	−
EL 1:625	+	29.1	0.01	33	10^{-4}	13	16.2–31.2 (4)	22.6	5.6	>0.5	−
EL 1:25	−	0	0.5	29	10^{-3}	14	14.7–35.5 (5)	28.0	10.5	>0.5	−
CR 1:25	+	16.7	0.1–0.05	33	10^{-4}	13	12.1–24.0 (4)	20.7	10.9	>0.5	−
CR 1:25	−	22.3	0.025	29	10^{-3}	14	25.2–34.8 (5)	31.0	2.5	>0.5	−
JU 1:25	+	21.9	0.05–0.025	33	10^{-4}	13	17.5–22.4 (4)	19.9	14.4	0.1–0.05	±
JU 1:625	+	44.3	0.01	33	10^{-4}	13	17.2–27.8 (4)	22.6	3.7	>0.5	−

[a] Based on PBS control of given tests.
[b] The MS sera had yielded positive, and the control sera negative, results in other tests.
[c] Dilutions not listed (1:5, 1:125, or 1:625) had yielded negative results.

TABLE 7. *Summary of PMN depressions obtained by injection of mice with various specimens*

Material injected	No. tested	No. positive ($P < 0.05$)	% Positive
Human brain suspensions			
MS	8	8	100
Tumors[a]	4	0	0
Slow virus diseases[b]	3	0	0
Nonneurological diseases	4	0	0
Human sera			
MS, active disease	22	16	73[c]
MS, inactive disease	13	4	31
MS, stage unknown	8	5	62
Healthy donors[d]	24	0	0[e]
Cerebrospinal fluid			
MS	8	0	0
Other diseases	10	0	0
Viruses			
Parainfluenza type 1 (HA-2 strain)		0	
Measles (Edmonston strain)		0	
Pneumonia virus of mice (strain 15)		0	
H-1 (Toolan)		0	
RV-13 (Kilham)		0	

[a] Astrocytomas.
[b] Alzheimer's disease (1); Creutzfeld-Jacob's disease (2).
[c] Three doubtful results are excluded.
[d] Relatives, nursing personnel of MS patients or laboratory technicians.
[e] One doubtful result is excluded; passage in mice was negative or of borderline significance.

0.05). In contrast, none of the control brain suspensions caused significant changes in the percentages of PMNs. Of the 43 MS sera examined, 25 induced highly significant reductions in PMNs. When the patients were grouped according to their clinical status, sera from patients with active disease were more frequently positive (73%) than sera from patients with quiescent disease (31%). Of the 24 sera from healthy donors, only one from a laboratory technician was questionably positive (the serum shown in Table 6). Neither the eight spinal fluids from MS patients nor the 10 specimens from other patients induced PMN depressions. All of the MS spinal fluids were found to have the capacity to neutralize MSAA, as will be reported subsequently (3). Finally, none of the viruses listed in the table induced PMN depressions. They were chosen because they either had been implicated in the etiology of MS or are common, latent animal viruses.

Serial passages of the PMN depressing factor. Serial passages in mice, initiated with MS brain homogenate or MS serum, are shown in Table 8. In the course of these experiments, several strains of mice had to be used, and evaluation of the PMN reductions as well as the time of sacrifice of the animals varied considerably for logistic reasons. Yet, the agent was clearly transmissible in series at high dilutions of the brain homogenates obtained at each passage level. The PMN depressions observed between 5 and 27 days after injection were all of statistical significance. In the passage series shown, the original MS brain homogenate and the MS serum had ultimately been diluted by a factor of at least 10^{-30} and $10^{-12.4}$, respectively, i.e., far beyond the titers of MSAA in the starting materials.

PMN depressions induced in species other than mice. In as yet limited efforts, it was noted (Table 9) that PMN depressions may be induced not only in mice, but in rats, guinea pigs, and hamsters as well. An MS brain homogenate was injected into guinea pigs, MS sera were injected into rats, and mouse passage brain homogenates from animals that had responded positively to injection of an MS serum or MS brain were injected into rats and hamsters, respectively. The reductions in the mean percentages of PMNs as compared to the controls ranged from 40 to 78%. Passage of a brain homogenate from two groups of rats induced PMN depressions in mice.

Some properties of the PMN depressing agent. Table 10 shows that the agent in MS sera passes through 50-nm but not 25-nm membrane filters (Millipore Corp.) as described by Carp et al. (2). Similar results were obtained with mouse passage brain homogenates. It was noted repeatedly that the 50-nm filtrates of MS sera may cause more severe depressions of PMNs than the specimens before or after passage through larger filters, as, for example, serum GE-75. Sera from healthy donors, e.g., serum J. H. in the table, gave negative results before as well as after filtration through 50-nm membrane filters.

MS brain homogenates and MS sera were also subjected to high-speed centrifugation. There was no detectable sedimentation of the agent when centrifuged at 50,000 $\times g$ for 1 h. It was extensively or totally sedimented from MS brain suspensions by centrifugation at 105,000 $\times g$ (Table 11). When MS sera were subjected to 105,000 $\times g$, the activity was usually divided between the supernatants and the resuspended pellets. Compared to earlier assays, two of the sera (GE-55 and GE-87) showed a decline in activity when tested in 1:25 or 1:5 dilutions, respectively. However, after centrifugation serum GE-55 caused significant PMN depressions. It is conceivable that the small quantities of MSAA present in sera do not sediment as readily under the condition employed as the

TABLE 8. *Serial passages of MS brain or MS serum in mice*

Passage no.	Mouse strain	Inoculum	Dilution		Day	PMN (%)				Reduction (%)[a]	P
			Passage	Total		1	2	3	Mean		
1	C3H	MS-brain-A	10^{-2}	10^{-2}							
2-C[b]	Balb/c	None			5	47.0	48.5	50.8	48.8		
2		P-2-brain (day 42)[c]	10^{-4}	10^{-6}	5	26.1	26.5	27.0	26.5	45.7	0.01–0.005
3-C	Outbred	P-2-brain (day 13)	10^{-5}	10^{-5}	7	26.3	30.1	35.4	30.6		
3	Albino	P-2-brain (day 13)	10^{-5}	10^{-11}	7	15.7	18.6	21.3	18.5	39.5	0.025–0.01
4-C	Albino	P-3-brain (day 34)	10^{-5}	10^{-10}	27	25.5	26.2	42.4	31.4		
4	Albino	P-3-brain (day 34)	10^{-5}	10^{-16}	27	11.1	12.6	22.2	15.3	51.3	0.1–0.05
5-C	Albino	P-4-brain (day 29)	10^{-7}	10^{-17}	19	34.1	39.7	51.4	41.7		
5	Albino	P-4-brain (day 29)	10^{-7}	10^{-23}	19	18.0	19.2	29.1	22.1	47.0	0.05–0.025
6-C	Albino	P-5-brain (day 14)	10^{-7}	10^{-24}	14	26.6	30.3	31.0	29.3		
6	Albino	P-5-brain (day 14)	10^{-7}	10^{-30}	14	4.2	6.6	9.0	6.6	77.5	<0.001
1	Balb/c	PBS			6	35.6	46.6	49.0	43.7		
		MS serum GE-2	$10^{-1.4}$	$10^{-1.4}$	6	22.8	28.0	30.0	26.9	38.6	0.025–0.01
2	Balb/c	PBS			7	44.5	46.5	47.5	46.2		
		P-1-brain (day 29)	10^{-5}	$10^{-6.4}$	7	25.0	27.8	34.7	29.2	36.8	0.005–0.001
3	C3BF1	PBS			13	23.3 27.8	24.3 26.0	24.7 29.1	25.9		
		P-2-brain (day 11)	10^{-6}	$10^{-12.4}$	13	8.3 17.4	15.7	16.9	14.6	43.6	<0.001

[a] Inocula of the C passage series failed to cause PMN depression when compared in several passages to PBS-injected mice. The data obtained in this series were used to determine the significance of the PMN depressions observed in the passages initiated with MS brain.

[b] The letter C refers to the control passage series initiated with normal mouse brain homogenates.

[c] P-1 (day 47), etc. denotes the passage number and the day after injection at which brains were collected from the mice for the next passage.

TABLE 9. *Induction of PMN depressions in animals other than mice*

Species	Inoculum	Day	PMN (%)						Reduction (%)	P
			1	2	3	4	5	Mean		
Guinea pig	None	10	37.5	42.1	48.0			42.5		
	MS brain-A (10^{-1})		3.4	11.5	12.6			9.2	77.6	0.005–0.001
Hamster	None	26	23.6	26.9	29.0			26.5		
	Mouse passage (MS brain-A) (10^{-3})		11.4	15.0	21.8			16.0	39.6	0.05–0.025
Rat	PBS	8	13.3	16.2	27.0	32.0	34.4	26.6		
	MS serum-GE-55 1:5[a]		8.9	11.8	12.1			10.3	58.5	0.1–0.05
	MS serum-GE-57 1:5[a]		11.8	12.8	14.6			13.6	48.8	0.1–0.05
	Mouse passage brain (MS serum) (10^{-4})		6.3	12.3	13.8	16.0	23.8	14.4	45.8	0.1–0.05
Passage to mice, C3BF1	PBS	6	26.3	27.0	27.4	31.4		28.0		
	Mouse passage brain (MS brain-A) (10^{-4})		14.0	15.5	16.7	18.8		16.2	42.3	<0.001
	Rat passage brain[a] (MS serum GE-55) (10^{-4})		18.8	20.0	22.4	23.3		21.1	24.6	0.005–0.001
	PBS	14	23.3	23.8	25.2	28.3		25.1		
	Rat passage brain[a] (MS serum GE-57)									
	10^{-3}		14.1	16.5	18.6	23.6		18.2	27.4	0.025–0.01
	10^{-6}		8.2	14.1	17.0	18.1		14.3	43.0	0.005–0.001
	10^{-9}		10.7	12.2	13.3	15.4		12.9	48.5	<0.001
	10^{-11}		19.8	23.3	24.8	26.7		23.6	6.0	>0.5

[a] Brains were collected from these groups of rats 32 days after inoculation, and the homogenates were passed in varying dilutions to mice.

TABLE 10. *Filterability of PMN depressing agent in MS sera*

Inoculum	Day	PMN (%; mean)	Reduction (%)	P
PBS (control	13	33.3		
MS serum GE-89 (1:10)	13	20.9	37.2	0.1–0.05
50-nm filtrate	13	20.8	37.5	0.05–0.025
25-nm filtrate	13	33.7	0	
MS serum GE-78				
100-nm filtrate (1:5)	13	21.8	34.5	0.025–0.01
50-nm filtrate	13	17.6	47.1	0.01–0.005
25-nm filtrate	13	29.2	11.9	0.5–0.4
MS serum GE-75 (1:5)	13	30.0	9.9	NS[a]
100-nm filtrate	13	24.2	27.3	0.05–0.025
50-nm filtrate	13	19.4	41.6	0.025–0.01
25-nm filtrate	13	32.7	1.7	
Control serum (JH) (1:5)	11	25.4		
50-nm filtrate	11	27.5	0	

[a] NS, Not significant.

TABLE 11. *Sedimentation of the PMN depressing agent by high-speed centrifugation*

Inoculum		Day	PMN (%; mean)	Reduction (%)	P
None		5	32.8		
MS brain-A	(10^{-1})		13.4	59.1	<0.001
Supernatant, 105,000 × *g*	(10^{-1})		27.1	17.3	0.1–0.05
Sediment	(10^{-1})		14.3	56.4	0.025–0.01
Control brain 1	(10^{-1})	4	47.4		
MS brain-A	(10^{-2})		21.7	54.2	<0.001
Supernatant, 105,000 × *g*	(10^{-1})		56.8	0	
	(10^{-3})		58.2	0	
Sediment	(10^{-1})		25.2	47.2	<0.001
	(10^{-3})		25.7	45.7	<0.001
PBS		14	31.3		
MS serum GE-2	(1:10)		18.2	41.8	0.01–0.005
Supernatant, 105,000 × *g*	(1:10)		15.3	51.0	0.025–0.01
Sediment	(1:10)		21.4	31.5	0.05
MS serum GE-87	(1:5)		23.5	24.9	0.1–0.05
Supernatant, 105,000 × *g*	(1:5)		23.2	25.8	0.1–0.05
Sediment	(1:5)		25.2	19.4	0.2
PBS		10	30.6		
MS serum GE-55	(1:25)		25.8	15.7	0.5
Supernatant, 105,000 × *g*	(1:25)		15.4	49.6	0.01–0.005
Sediment	(1:25)		19.3	36.3	0.025

large concentrations found in MS brain homogenates, or components in the sera may counteract sedimentation.

In the course of the studies it was noted that the PMN depressing agent was unstable even in low dilutions of MS brain homogenates or of MS sera. When such diluted preparations were reused after overnight storage at 4 C they generally failed to yield significant PMN depressions. This was confirmed experimentally in that considerable declines were observed after 8 h and complete loss of activity was observed after 24 h at 4 C. It is necessary, therefore, to use freshly prepared inocula within a few hours. No dependable stabilizer has as yet been found.

DISCUSSION

The data presented largely confirm and extend the observations presented by Carp and his associates (2). MS brain homogenates and

MS sera may contain an agent which, under appropriate conditions, induces a depression in the relative concentration of PMNs in mice and also, as shown here, in rats, guinea pigs, and hamsters. The reduction in PMNs, however, was not as persistent as reported, and normal percentages were found generally when the animals were retested 2 or more months after inoculation. The responsible agent passed through 50-nm but not 25-nm membrane filters and was largely sedimentable at 105,000 × g and not at 50,000 × g. It was found to be labile even at 4 C, rather than stable as previously described (2). The agent clearly replicated in the brains of injected mice and was serially transmissible from animal to animal. It is presently unknown whether the PMN depressions are due to a direct action of MSAA on these cells or their precursors or due to a by-product of the interactions of the agent with other tissues, such as the central nervous system.

All of the eight MS brain homogenates tested induced PMN depressions, but none of 11 control brain suspension did. Although not all MS sera revealed the presence of the agent, these differential results appeared to be referable to some extent to the clinical status of the patients; that is, sera from patients with active disease induced PMN depressions more frequently then sera from patients with quiescent disease. None of eight spinal fluids from MS patients caused reductions in the percentages of PMNs. In fact, these spinal fluids, as well as some of the negative MS sera, were found to neutralize the agent, as will be reported in the accompanying paper (3).

The agent appears to be a virus, since it replicates in the injected animals and can be serially transmitted. It seems unlikely that the agent represents an indigenous animal virus for the following reasons: (i) injection of control materials from patients with other neurological or nonneurological diseases failed to cause PMN depressions; (ii) passages of brain homogenates from mice, either uninjected or injected with control specimens, likewise gave negative results; (iii) the factor in MS specimens is retained by 25-nm but not 50-nm membrane filters, as is the agent in mouse passage brain homogenates; (iv) PMN depressions were induced in all strains of mice tested as well as in three other species of animals, and it would be surprising to find the same latent agent in all; and, finally, (v) as will be shown (3), certain human sera were found to neutralize the agent as present in MS specimens or mouse passage material. According to these observations and considerations, the agent appears to be associated with MS and therefore has been called MSAA, but its role in this disease remains to be determined. Whereas leukocyte counts in MS are within normal range, PMN depressions might occur well before diagnosis since they are seen early and are transient in animals.

The present assay for MSAA, the induction of PMN depression in mice, is unfortunately beset with all the inherent vagaries of leukocyte counts in experimental animals (cf. 5). Irregular results are often observed if the mice are not in a prime state of health, if the blood smears are not prepared at a uniform time of day, and if the animals become unduly agitated during handling. Furthermore, the evident lability of the agent during prolonged storage and on repeated freezing and thawing of specimens has been a cause of experimental failures. This has been especially apparent with MS sera, in which the concentration of MSAA is relatively low. It is probable, too, that with low-titered specimens only part of the animals will receive a dose of MSAA sufficient to cause PMN depressions (as in any virus titration at end point dilutions), and thus the mean PMN percentages may not reflect significant reductions when compared to the control values. In addition, some sera may have components which actually cause slight increases in the percentages of PMNs which could counteract the effects of MSAA. Finally, there is an indication that some MS sera may contain both MSAA and neutralizing antibodies, partly as complexes, (3), which may account for the occasionally greater PMN depressions observed with diluted MS sera or with 50-nm membrane filtrates than with more concentrated or unfiltered sera.

From the above remarks it is evident that the test procedure applied to sera from MS patients is beset with difficulties and thus cannot be considered a dependable tool. It is possible, and this deserves further exploration, that passages of brain homogenates from the initially inoculated mice might improve the reliability of the test. It would be far more desirable, however, to develop more convenient and dependable means for detection of MSAA and, in turn, for measuring neutralizing activity in human sera. Limited attempts to transmit the agent to cultures of various types of cells have failed to induce detectable cytopathic effects. However, several cell lines were established from brains of animals inoculated with MS specimens, but not from brains of control animals. The continuous cultures obtained were found to harbor MSAA, as will be reported later (P. Koldovsky, U. Koldovsky, G. Henle, and W. Henle,

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