DECREASED PERCENTAGE OF POLYMORPHONUCLEAR NEUTROPHILS IN MOUSE PERIPHERAL BLOOD AFTER INOCULATION WITH MATERIAL FROM MULTIPLE SCLEROSIS PATIENTS*

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At present multiple sclerosis (MS) presents a very complex medical problem. There are three fundamental reasons for this: first, the diagnosis of MS is extremely difficult (1); secondly, the etiology of the disease is unknown (2); and finally, a problem that is the keystone to the analysis of the other problems is the lack of a system, other than man, in which the disease can be studied. In experiments initiated to investigate the etiology of MS, Palsson et al. (3) and Field (4) described a disease in sheep and mice, respectively, that had been inoculated with MS brain homogenate. In each instance the disease was characterized by spongiform degeneration of the central nervous system (CNS). The significance of these findings is difficult to assess because: (a) these results have not been confirmed by others despite several attempts, and (b) it is not possible to exclude the possibility of contamination of the MS brain material or the test animals with scrapie, an agent with known potential for causing spongiform degeneration in these animals (5).

An important limitation of the above studies was the fact that the only criteria for infection were clinical signs and/or histopathological changes. We have been searching for less overt alterations at the cellular and subcellular level after inoculation with MS material. The results reported here describe an alteration in the percent polymorphonuclear neutrophils (PMN) in clinically normal mice after inoculation with material from MS patients.

Materials and Methods

Specimen Sources.—All specimens were kindly supplied by Dr. W. W. Tourtellotte of Wadsworth General Hospital, Los Angeles, Calif. This material came from cases that had been clinically diagnosed and histopathologically confirmed as MS. The sample materials included brain, spleen, cerebrospinal fluid (CSF), and serum. Each sample came from a different MS case. Normal controls were tissues and fluids obtained from individuals with no history of CNS disease.

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Abbreviations used in this paper: CNS, central nervous system; CSF, cerebrospinal fluid; EE, Eagle's medium in Earle's balanced salt solution; MS, multiple sclerosis; PMN, polymorphonuclear neutrophils; RBC, red blood cells.

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Preparation of Solid Tissue Material.—Brain and spleen sections were minced, made up to a 20% (w/v) suspension in Eagle's medium in Earle's balanced salt solution (EE) containing penicillin (100 units/ml) and streptomycin (100 µg/ml), and homogenized in a Ten-Broek tissue grinder (Fisher Scientific Co., Springfield, N. J.) using 20 strokes per sample. The homogenate was then centrifuged for 15 min at 1500 g and the supernatant removed and frozen. The remaining pellet was frozen and thawed three times and resuspended to the original volume, then centrifuged as before. The two supernatants were pooled and stored at -20°C until needed. Samples were tested for sterility before use.

Brain Pools Used for Inoculation.—Coronal sections from two normal human brains were prepared as above, pooled, and used as the normal human brain homogenate unless otherwise specified. Coronal sections having pathological lesions from three different MS brains were prepared as above and pooled. This comprised the MS brain homogenate inoculum unless otherwise specified.

Mouse Inoculations.—Male, weanling C57BL/6J mice were inoculated either intraperitoneally with 0.2 ml of material or intracerebrally with 0.03 ml.

Total Leukocyte Counts.—Total leukocyte counts were done by mixing a 20 µl sample of freshly drawn blood with "ZAP" (Coulter Electronics, Hialeah, Fla.), a red blood cell (RBC) lysing agent, in 10 ml of physiological saline. The number of leukocytes was determined with a Coulter electronic cell counter, Model B (Coulter Electronics). Settings were 12.5 for the lower and 100 for the upper thresholds. Amplification was set at 14 while the aperture current was at 34. The resultant count was equal to the number of leukocytes per cubic millimeter of whole blood.

Differential Leukocyte Counts and Staining.—Peripheral blood smears were made using heparinized blood obtained from the brachial artery and vein of ether-anesthetized mice. After 24 hr, smears were stained by flooding the slides with Wright's staining solution for 7 min. Then a few drops of Giordano's buffer (pH 6.5) were added for an additional 6 min. The slides were rinsed with distilled water and air-dried. All slides were coded such that the experimenter did not know the origin of the material. The slides were read under oil immersion (×1250) for the distribution of white blood cell types.

Filtration of MS Brain Material.—Extracts of MS brain material were passed through a series of membrane filters (Millipore Corp., Bedford, Mass.) of decreasing size (220, 100, 50, 25 nm). The filtrates at each stage were inoculated intracerebrally into groups of mice.

RESULTS

Altered Leukocyte Distributions in MS-Inoculated Mice.—Differential leukocyte counts were performed on mice after intraperitoneal or intracerebral inoculation of brain homogenate prepared from normal individuals or MS patients. Three mice from each group were tested every 2 or 3 wk for 43 wk after intraperitoneal and 37 wk after intracerebral inoculation. At 2 and 4 wk after inoculation, mice inoculated with MS material showed an altered distribution of leukocyte cell types which was characterized by a decrease in the percent PMN with a concomitant increase in the percent lymphocytes. In Fig. 1, the average (three mice/point) PMN percentages are expressed as a function of time after inoculation. The lower percentages of PMN in MS-inoculated mice were manifest throughout the entire period of the experiment for both intracerebral and intraperitoneal mice. Although most of the PMN values for MS mice were lower than normal, a few (7.5%) had values that were higher than those found for mice inoculated with normal brain homogenate (see a in
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Fig. 1). It is not clear whether these few high values bear any relation to the inoculation of MS material. For intracerebrally inoculated mice, the difference in PMN percentages between normal and MS groups were statistically significant (P of no difference ≤0.05 by the Mann-Whitney U test) at all times except at 18 and 32 wk after inoculation. For intraperitoneal inoculation, the differences were also statistically significant (≤0.05) except at 2 wk and at those points marked a. To further evaluate the results, all of the individual PMN values in each treatment group were pooled across the entire time period. When arranged as frequency distributions the PMN percentages obtained among the four groups showed virtually no overlapping (Fig. 2). Almost all values for MS mice were lower than those for normal mice, and the few exceptional values noted above were higher than most normal values.

As additional controls, we performed differential leukocyte counts on the peripheral blood of mice in the following groups: (a) uninoculated and (b) inoculated with EE, the diluent used for the preparation of brain homogenates. These mice were tested at ages comparable to those used for the above studies. The average percentage of each leukocyte type was computed for each of the six treatment groups (Table I). Those values marked with a in Fig. 1 were not included in the summary. The values obtained for mice inoculated with EE or normal human brain and for uninoculated mice are similar to those that have been described for C57BL/6J mice (6). The differences in the average PMN (or lymphocyte) percentages between MS mice and any control group were highly statistically significant (Student's t test, P ≤ 0.001). All other comparisons failed to reveal any statistically significant differences.

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Fig. 1. Average per cent PMN in mice as a function of time after inoculation with MS brain homogenate (●), or normal human brain homogenate (○); intracerebrally inoculated, A; intraperitoneally inoculated, B. (a) One value that was markedly different from the usual value for normal or MS-inoculated mice. This value was not included in the average for that particular point.
Cell Type Responsible for the Alteration in Leukocyte Differential Counts.—From observations of the blood cell smears it was obvious that the alteration in the distribution of the leukocyte cell types after MS inoculation cannot be accounted for by the appearance of unusual or abnormal cell types, e.g., atypical lymphocytes or juvenile PMN. Rather, the change in differential counts represents an altered distribution of normal cell types. Secondly, the alteration in differential counts cannot be accounted for by increases in either eosinophils or

![Graph showing distribution of per cent PMN among mice inoculated with A, normal human brain, and B, MS brain inoculated intracerebrally; C, normal human brain, and D, MS brain inoculated intraperitoneally.](image)

**Fig. 2.** Distribution of per cent PMN among mice inoculated with A, normal human brain, and B, MS brain inoculated intracerebrally; C, normal human brain, and D, MS brain inoculated intraperitoneally.

**TABLE I**

*Per Cent Leukocyte Types in MS-Inoculated and Control C57BL/6J Mice*

| Inoculum and route | No. tested | Leukocyte distribution* |
|--------------------|------------|-------------------------|
|                    |            | Polymorphonuclear neutrophils | Lymphocytes | Eosinophils | Monocytes |
| None               | 36         | 13.4(2.9) | 85.6(3.1) | 0.8(1.2) | 0.2(0.4) |
| Eagle's medium (i.c.) | 18       | 15.0(3.2) | 83.9(3.5) | 0.9(1.2) | 0.1(0.3) |
| Normal human brain (i.c.) | 73       | 13.9(2.9) | 84.9(3.4) | 0.9(1.5) | 0.2(0.5) |
| MS brain (i.c.)    | 72         | 5.9(2.2)  | 93.1(2.7) | 0.6(0.9) | 0.1(0.4) |
| Normal human brain (i.p.) | 58       | 14.6(2.8) | 83.9(3.5) | 1.1(1.6) | 0.4(1.0) |
| MS brain (i.p.)    | 49         | 6.0(2.5)  | 92.9(3.2) | 0.8(1.3) | 0.2(0.6) |

* Average; (sd).
‡ $t = 18.54$, $P$ of no difference <<0.001.
§ $t = 16.09$, $P$ of no difference <<0.001.
¶ $t = 16.60$, $P$ of no difference <<0.001.
¶¶ $t = 13.88$, $P$ of no difference <<0.001.
monocytes, since they occur at very low frequencies in all of the treatment groups. The third and fourth possibilities are that either there is an increase in the number of lymphocytes or a decrease in the number of PMN. These alternatives can be distinguished by their effect on total leukocyte counts. A decrease in the percentage of PMN from 14.0 per cent noted in normal mice to 5.5 per cent noted in MS mice would require an increase in the absolute number of lymphocytes of approximately threefold. This, in turn, would cause a 2.5-fold increase in the total leukocyte counts. In contrast, a decrease in the absolute number of PMN would hardly affect the total leukocyte count. To test these alternate possibilities, total leukocyte counts and per cent PMN were determined on each of 19 MS-inoculated mice as well as on 29 control mice (inoculated with normal human or normal mouse brain). The results (arranged as a scatter diagram in Fig. 3) show that: (a) there is no obvious association between high total leukocyte counts and low PMN percentages; and (b) the range in total leukocyte counts is approximately the same for MS- and normal brain-inoculated mice. These conclusions are supported by statistical analysis in that (a) the correlation coefficient r for MS mice is 0.001 and that for normal mice is 0.008, and (b) the mean total leukocyte counts among the MS-inoculated mice is not statistically different from that of normal mice (Student's t test). Therefore, the altered leukocyte distribution in MS-inoculated mice is caused by a decrease in the absolute number of PMN.

Early Appearance of the Decrease in PMN Percentages.—To determine how soon after inoculation the PMN percentages changed differential counts were performed early after intracerebral inoculation with MS material (Table II). The per cent PMN at 4 and 8 hr postinoculation were higher than the values in normal human brain-inoculated mice. The significance of these data is not clear,
but they may represent a response to the inoculation per se. From 16 hr onward, however, the decrease in per cent PMN in mice was well established and statistically significant. After intraperitoneal inoculation (Table III), the decrease

| TABLE II | Effect of MS and Normal Brain Homogenate on the Per Cent PMN As a Function of Time after Intracerebral Inoculation |
|----------|--------------------------------------------------------------------------------------------------|
| Time postinoculation | Average* of PMN percentages | Normal | MS |
| 4 hr | 8† | 21 |
| 8 hr | 9† | 18 |
| 12 hr | 13 | 11 |
| 16 hr | 15 | 5† |
| 20 hr | 16 | 8† |
| 24 hr | 14 | 6† |
| 26 hr | 13 | 5† |
| 48 hr | 14 | 6† |
| 52 hr | 11 | 7† |
| 3 days | 11 | 4† |
| 4 days | 12 | 6† |
| 5 days | 18 | 8† |
| 10 days | 12 | 6† |

* Average of three animals.
† P of no difference ≤ 0.05 (Mann-Whitney U test).

| TABLE III | Effect of MS and Normal Brain Homogenate on the Per Cent PMN As a Function of Time after Intraperitoneal Inoculation |
|----------|--------------------------------------------------------------------------------------------------|
| Time postinoculation | Average* of PMN percentages | Normal | MS |
| 12 hr | 9 | 8 |
| 24 hr | 10 | 9 |
| 48 hr | 12 | 7† |
| 3 days | 13 | 9† |
| 4 days | 12 | 7† |
| 5 days | 15 | 5† |
| 7 days | 16 | 6‡ |
| 14 days | 12 | 7‡ |

* Average of three animals.
‡ P of no difference ≤ 0.05 (Mann-Whitney U test).

in per cent PMN was apparent 48 hr postinoculation and the difference remained statistically significant during the rest of the experiment.

Titrations of the PMN Factor.—To quantitate the amount of factor causing the decreased PMN that was present in the MS brain homogenate, mice were inoculated intracerebrally with decimal dilutions (in EE) of the homogenate.
Differential leukocyte counts were done 3, 4, 5, and 9 wk after inoculation. The results are summarized in Table IV. The highest dilution that still caused the decreased PMN percentage was $10^{-11}$. This would correspond to a titer of $3 \times 10^{12}/\text{ml}$ in the original homogenate. During the interval between 3 and 4 wk postinoculation there was a 100-fold increase in the highest dilution causing the PMN decrease ($10^{-9}-10^{-11}$), with no further change noted during the subsequent 5 wk of the experiment.

**Occurrence of the Factor in the Blood of MS-Inoculated Mice.**—It is apparent from Fig. 1 that the decrease in PMN persists for many months after inoculation. To determine whether the factor responsible for the decrease was likewise present for long periods, we inoculated mice with serum obtained from mice that had been inoculated with MS brain homogenate. The protocol was as follows: (a) Donor mice were inoculated intracerebrally with MS brain homogenate. (b) At 12 hr, 4 and 29 wk, groups of donor mice were killed, serum collected, and differential leukocyte counts performed. (c) The sera were then inoculated into recipient mice. (d) At 3 and 5 wk, differential leukocyte counts were done on recipient mice. (e) The results were compared with similarly treated mice in which the donor mice had been inoculated with normal human brain homogenate. In addition, differential leukocyte counts were performed and serum collected from mice that had been inoculated intraperitoneally 34 wk earlier with MS brain homogenate. This serum along with that from its normal human brain-inoculated controls was inoculated intracerebrally into a group of recipient mice which were then treated as above in d. The results of the experiment (Table V) show that all of the MS-inoculated donor mice exhibited the
decreased per cent PMN except those examined 12 hr after intracerebral inoculation and that all of the recipient mice showed a decrease in per cent PMN, including those inoculated with the serum taken from donor mice only 12 hr after inoculation. Thus, the factor responsible for the decreased per cent PMN was present and detectable in the serum of donor mice from as early as 12 hr until at least as late as 8 1/2 months postinoculation. These results also show that the factor present in the MS tissue can be transmitted from mouse to mouse.

Replication of the PMN Factor in Mice.—To determine if the PMN factor can replicate in the mouse the following experiment was performed. The MS brain homogenate was diluted to $10^{-9}$ and inoculated into four mice (intracerebrally). Based on the titer noted in Table IV, mice inoculated with a $10^{-9}$ dilution of MS brain homogenate should have received 100 units of PMN factor. Two of these mice were negative for the PMN decrease at 3 wk, but the other two mice were positive at 6 wk (average PMN percentage was 6 for MS mice and 14.5 for the normal brain control mice). The brains of the 6-wk mice were homogenized and titrated, with each dilution inoculated intracerebrally into a new set of four mice. Control mice were inoculated with the mouse brain homogenate prepared from mice inoculated with normal human brain. Differential leukocyte counts were performed on mice 6 wk after inoculation. All mice inoculated with dilutions of mouse brain homogenate, which had been derived from mice inoculated with MS material, were positive for the PMN decrease up to and including the $10^{-9}$ dilution. Mice receiving dilutions beyond $10^{-9}$ and mice inoculated with material derived from normal human brain-inoculated mice had PMN percentages in the normal range. The quantitative aspects of this experiment are shown in Table VI. The results show that there was an increase of the PMN factor in mouse brain of at least $1.5 \times 10^{9}$-fold.

### TABLE V

Per Cent PMN in Mice (Recipient) Inoculated with Serum from Mice (Donor) Inoculated with MS Brain Homogenate

| Recipient inocula: % PMN in donor mice (at time of serum collection) | % PMN in recipient mice at | % PMN in recipient mice at |
|---|---|---|
| | MS Normal | MS Normal | 3 wk | 5 wk |
| 12 hr | 11† | 13 | 6‡ | 16 | 8§ | 12.5 |
| 4 wk | 8§ | 17 | 9 | 19.5 | 4 | 13 |
| 29 wk | 6 | 13 | 13.5 | 26 | 7.5 | 12.5 |
| 34 wk | 6 | 15 | 6 | 14 | 6 | 12.5 |

* Donor mice from 12 hr, 4 wk, and 29 wk had been inoculated i.c.; donor mice from 34 wk had been inoculated i.p.
† For donor mice, number = average of three mice.
§ For recipient mice, number = average of two mice.
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The mice at the end point (10^-9) had received a dilution of the original human brain material equivalent to 10^-9.

Effect of Filtration of MS Brain Material on the Per Cent PMN in Inoculated Mice.—MS brain homogenate was filtered as described in Materials and Methods. Differential leukocyte counts were performed at 2 and 4 wk post-inoculation and the pooled values are shown in Table VII. The results show that the factor causing the PMN decrease passed through all pore sizes except 25 nm.

Occurrence of the Factor Causing the Decrease in PMN Percentages in Various Tissues from Nine MS Cases.—The results of a preliminary study of the occurrence of the factor causing the PMN decrease in mice in different tissues obtained from nine MS cases are summarized in Table VIII. Samples of three brain preparations, one spleen preparation, two CSF, and three sera from nine different cases of MS were inoculated into mice. Control samples of two brains, one spleen, two CSF, and two sera from seven different normal humans were also inoculated into mice. All MS samples induced the decreased per cent PMN in 6 wk, whereas the mice inoculated with the control material all had PMN percentages in the normal range (Table VIII). The average PMN values of MS-inoculated mice when compared with the average values for mice inoculated with the corresponding normal tissue were significantly different (P of no difference ≤0.05 by Student's t test).

DISCUSSION

Three properties of the factor causing the PMN decrease in mice suggest that it is a virus: it is transmissible from mouse to mouse, it has capacity for replica-
tion, and its size is 25–50 nm. As a virus it has several unusual characteristics. The factor was present as early as 12 hr after inoculation and was still recoverable from the blood 8½ months later. The only change thus far associated with it is the decrease in PMN percentage. To this date, there are no clinical or histopathological signs of disease in any of the MS-inoculated mice. The absence of clinical disease despite the presence of virus for a long time is not unique, since similar findings have been noted, for example, with lymphocytic choriomeningitis virus (7) and lactic dehydrogenase virus (8).

TABLE VIII

| Patient Tissue Identification | Tissue Type | % PMN/mouse | Average | Statistical Significance* |
|------------------------------|------------|-------------|---------|--------------------------|
| N-1 Normal brain             | 16, 15, 14, 13, 14 | 14.1       |         |                          |
| N-2 Normal brain             | 20, 16, 17, 14, 15, 14 | 14.3      |         |                          |
| MS-1 MS brain                | 9, 8, 10, 8, 5, 8 | 8.0        |         |                          |
| MS-2 MS brain                | 5, 6, 6, 8, 8, 7  | 6.6        |         |                          |
| MS-3 MS brain                | 6, 10, 10, 5, 9, 6 | 7.6        | P < 0.001 |                          |
| N-3 Normal spleen            | 12, 14, 19, 15, 16 | 15.2       |         |                          |
| MS-4 MS spleen               | 10, 7, 6, 8, 8, 6 | 7.5        | P < 0.01 |                          |
| N-4 Normal sera              | 21, 16, 11, 13    | 15.2       |         |                          |
| N-5 Normal sera              | 22, 19, 10, 15    | 16.5       |         |                          |
| MS-5 MS sera                 | 7, 9, 7, 3        | 6.5        |         |                          |
| MS-6 MS sera                 | 6, 3, 8, 3        | 5.0        |         |                          |
| MS-7 MS sera                 | 4, 7, 9           | 6.6        | P < 0.02 |                          |
| N-6 Normal CSF               | 12, 19, 14, 10, 11 | 13.2      |         |                          |
| N-7 Normal CSF               | 12, 13, 14, 11, 12 | 12.5      |         |                          |
| MS-8 MS CSF                  | 9, 6, 13, 3, 9    | 8.0        |         |                          |
| MS-9 MS CSF                  | 7, 11, 8, 5, 7    | 7.6        | P < 0.05 |                          |

* P = probability of no difference (Student's t test). The P given for each kind of tissue is the highest value obtained after comparing each normal (N) with each MS tissue.

The "titer" of the factor in the original human brain homogenate was \(3 \times 10^{12}\) units/ml of a 10% homogenate. Such high titers are unusual among animal viruses but a number of comparable titers have been reported. The particle concentration in the blood of serum hepatitis patients can be as high as \(10^{13}/\text{ml}\) (9); plasma of chickens with avian myeloblastosis virus has been shown to contain up to \(10^{13}\) particles/ml (10). In both of these instances the concentrations are in particles per milliliter based on electron microscope observations. In addition, very high LD\(_{50}\) titers have been reported for both Semliki Forest and Bunyamwera viruses, titers of \(10^{12}\) and \(10^{11}\), respectively (11).

As yet, the question of the relationship between the factor and MS is unanswered. The cause of the PMN change in mice may very well be unrelated
to the etiology of MS in man. Changes in the proportions of leukocyte cell types have not been described in clinical studies of MS. Certain aspects of MS are consistent with the concept of a “slow infectious disease” as advanced by Sigurdsson (12). Furthermore, similarities have been described between MS and scrapie, a known “slow infection” of the CNS of lower animals (13, 14). In transmission studies a disease was induced by inoculation of MS material into sheep and mice that was very similar to scrapie (3, 4). These results are of questionable significance, since they have never been repeated. It is interesting, however, that the decrease in PMN noted in MS-inoculated mice is also observed in mice inoculated with scrapie and that the characteristics of the MS and scrapie factors that induce the decreases are similar but not identical (P. Licursi, G. S. Merz, P. A. Merz, and R. I. Carp, unpublished observations).

Regardless of whether or not the PMN factor is related to the etiology of MS, the findings in this report suggest that the decrease in PMN percentages in mice inoculated with MS material might serve as a diagnostic aid. It is important to note that every MS sample (nine samples from nine cases) caused the decrease in PMN percentages, whereas none of the normal samples (seven samples from seven individuals) affected the percentage of PMN. On the strength of these results, further testing of samples from MS patients and normals is underway to establish more firmly the correlation between the PMN decrease in mice and the presence of MS disease in man. It is of interest that material as easy to obtain as serum (three samples) caused the decrease in PMN percentages.

SUMMARY

Mice inoculated with brain homogenates from multiple sclerosis (MS) cases showed marked changes in their leukocyte differential counts, with a decrease in per cent polymorphonuclear neutrophils (PMN) and an increase in the per cent lymphocytes. These changes were based upon an absolute decrease in the number of circulating PMN. The decrease in PMN percentages was apparent at 16 hr after infection and persisted for at least 11 months. The factor responsible for the decrease in PMN was (a) recoverable from 12 hr to 8½ months after inoculation, (b) present in human brain homogenate at a concentration of \(3 \times 10^{12}\), and (c) between 25 and 50 nm in diameter. Inoculation of 100 units of factor into mice and subsequent titration showed that the factor had undergone a net increase in the mouse of at least \(10^9\)-fold. The factor causing the PMN decrease was found in all MS material thus far tested: three brains, one spleen, three sera, and two cerebrospinal fluid (CSF) from nine cases of MS. The factor was not found in normal human material that included two brains, one spleen, two sera, and two CSF.

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