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# Scrapie-Induced Changes in the Percentage of Polymorphonuclear Neutrophils in Mouse Peripheral Blood

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A decrease in the percentage of polymorphonuclear neutrophils (PMN) in the peripheral blood of mice appeared 3 days after intracerebral (IC) inoculation with scrapie mouse brain homogenate. Mice inoculated IC with normal mouse brain had PMN percentages similar to those found for uninoculated mice. This difference between normal and scrapie-inoculated mice continued throughout the preclinical phase of the disease. In the clinical phase of the disease, the percentage of PMN was either higher or lower than that found in normals. The factor causing the decrease in PMN percentages was found in the filtrates from 220-, 100-, and 50-nm filters, but not in the filtrates from a 25-nm filter. Sodium periodate treatment of the scrapie brain samples eliminated their ability to cause the decrease in PMN percentages, whereas sodium iodate had no effect. In addition to two genetically different scrapie mouse brain isolates, homogenates of mouse spleen, sheep brain, and sheep spleen from scrapie-affected animals caused a decrease in percent PMN, whereas the corresponding normal tissue homogenates did not.

Scrapie is a slow, progressive infectious disease of the central nervous system of sheep and goats (11, 19). The agent causing the disease has been passaged in mice (1), where there is an incubation period of 4 to 6 months. Thus far, all pathological changes and most of the biochemical changes have been found in the central nervous system, particularly the brain (13, 17, 18). Although the agent is found in high titer in the brain and central nervous system late in disease, it is recoverable from many other organs, including the lymphoid tissues such as spleen and lymph nodes very early after infection (5, 16). As part of a search for biochemical and physiological changes attributable to the scrapie agent outside the central nervous system, we compared differential leukocyte counts for normal and scrapie mice. The principal finding was a marked alteration in the proportion of polymorphonuclear neutrophils (PMN) in the peripheral blood.

## MATERIALS AND METHODS

**Specimen sources.** The Chandler strain of the scrapie agent was obtained from Clarence J. Gibbs of the National Institute of Health and has since been passaged in C57-BL/6J mice by intracerebral (IC) inoculation. The titer of this pool was $5 \times 10^5$ $LD_{50}$ units/ml and, unless otherwise stated, this pool was used throughout the study. Another scrapie pool (ME-7) was obtained from A. Dickinson of A.R.C. Animal Breeding Research Organization, Edinburgh, Scotland, and was tested directly, prior to passage in mice (3). Normal mouse brains were obtained from C57-BL/6J mice. Brain and spleen samples from scrapie sheep were supplied by W. W. Clark of Mission, Tex. Normal sheep material was obtained from local sources.

**Preparation of solid tissue material.** Pooled brains and spleens were minced, made up to a 20% (w/v) suspension in eagle basal medium containing penicillin (100 units/ml) and streptomycin (100 $\mu$g/ml), and homogenized in a Ten Broeck tissue grinder using 20 strokes per sample. The homogenate was then centrifuged for 15 min at 1,500 $\times$ $g$, and the supernatant fluid was removed. The remaining pellet was frozen and thawed three times, resuspended to the original volume, and then centrifuged as before. The two supernatants were pooled and stored at $-20$ C until needed. Sterility tests were performed before use.

**Mouse inoculations.** Male, weanling C57-BL/6J mice were inoculated either intraperitoneally (IP) with 0.2 ml of material or IC with 0.03 ml.

**Filtration of scrapie mouse brain material.** Extracts of scrapie mouse brain material were passed through a series of membrane filters (Millipore Corp.) of decreasing size (220, 100, 50, 25 nm). The filtrates at each stage were inoculated (IC) into groups of mice.

**Periodate treatment of mouse brain homogenates.**

Scrapie or normal mouse brain homogenates were mixed with 0.04 M sodium periodate or 0.04 M sodium iodate (as a control) in 0.2 M acetate buffer (*p*H 3.5). At 0, 90, and 360 min, samples were removed and their *p*H was brought to 7.0 with sodium thiosulfate. After dializing for 48 hr against three changes of phosphate-buffered saline (*p*H 7.2), the samples were inoculated into groups of mice.

**Total leukocyte counts.** Total leukocyte counts were done by mixing a 20-$\mu$liter sample of freshly drawn whole blood with "ZAP" (Coulter Electronics), a red blood cell lysing agent, in 10 ml of physiological saline. The number of leukocytes was determined with a Coulter electronic cell counter. Settings were 12.5 for the lower and 100 for the upper thresholds. Amplification was set at ¼ and the aperture current was ½. 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 by 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. A few drops of Giordano's Buffer (*p*H 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 (1,250 ×) for the distribution of white blood cell types.

## RESULTS

**Alterations in the leukocyte differential counts in preclinical scrapie mice.** There are four types of leukocytes found routinely in the peripheral blood of C57-BL/6J mice: PMN, lymphocytes, eosinophils, and monocytes, of which approximately 99% are either PMN or lymphocytes (4). Our results for the leukocyte distribution for uninoculated mice, aged 5 weeks to 8 months, reveal values of approximately 15% PMN, 84% lymphocytes, and less than 1% of each of the other two cell types (Table 1). The values obtained for mice inoculated with normal mouse brain (IC or IP) and analyzed from 3 days to 7 months after inoculation were essentially the same as the values shown for uninoculated mice. (Unless otherwise noted, "normal" will refer to mice inoculated with normal brain homogenate.) In sharp contrast, the average values for preclinical scrapie mice were 6% PMN and 93% lymphocytes. There was no discernable change in the percentages of eosinophils or monocytes. The shift in PMN and lymphocyte percentages following inoculation with scrapie material was independent of the inoculation route (Table 1) and, for the preclinical scrapie mice, highly statistically significant ($P$ of no difference $\leq$ 0.001 by Student's $t$ test). The average values for preclinical mice shown in Table 1 were derived in part from two experiments in which mice were inoculated by the IC or IP routes and analyzed for their leukocyte differential counts every 2 weeks throughout the preclinical phase of the disease. The percent PMN as a function of time after inoculation with normal or scrapie brain homogenate are recorded in Fig. 1. The lower level of PMN percentages in scrapie-inoculated mice became manifest as early as 2 weeks after inoculation and persisted throughout the preclinical stage of the disease.

The frequency distribution of percent PMN among normal brain-inoculated mice is plotted in Fig. 2A. The values are normally distributed about a mean of 14% with a range of 9 to 20%

TABLE 1. *Average percent leukocyte types among scrapie and normal brain-inoculated mice*

| Treatment | No. of mice | Percent leukocyte type[a] | | | |
|---|---|---|---|---|---|
| | | PMN | Lymph | Eosin | Mono |
| Uninoculated | 52 | 14.0 (3.1)[b] | 84.9 (3.3) | 0.8 (1.1) | 0.1 (0.3) |
| IP, Normal brain | 54 | 14.8 (2.8) | 83.9 (3.4) | 0.7 (1.4) | 0.3 (0.8) |
| IP, Scrapie brain preclinical | 30 | 6.1[c] (2.6) | 92.8 (2.8) | 0.4 (0.7) | 0.6 (1.1) |
| IP, Scrapie brain clinical | 82 | 13.6 (10.6) | 85.2 (10.5) | 0.9 (1.8) | 0.2 (0.4) |
| IC, Normal brain | 104 | 14.6 (2.7) | 84.4 (3.3) | 0.7 (1.0) | 0.3 (0.6) |
| IC, Scrapie brain preclinical | 133 | 6.9[c] (3.5) | 92.4 (3.7) | 0.6 (0.9) | 0.1 (0.3) |
| IC, Scrapie brain clinical | 35 | 14.5 (9.7) | 84.9 (9.6) | 0.3 (0.6) | 0.2 (0.6) |

[a] PMN, Polymorphonuclear neutrophils; Lymph, lymphocytes; Eosin, eosinophils; Mono, monocytes.
[b] Average (standard deviation).
[c] Probability of no difference from the normal control is <0.001 by Student $t$ test.

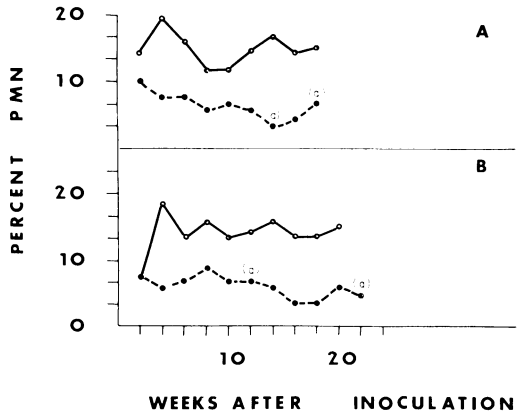


FIG. 1. *Average number of polymorphonuclear neutrophils (PMN) per 100 leukocytes at various times after inoculation with scrapie (○) and normal brain homogenate (●). A, Mice inoculated intracerebrally (IC); each point is the average for three mice, except at 14 and 18 weeks (a). B, Mice inoculated intraperitoneally (IP); each point is the average for two mice, except at 12 and 22 weeks (a). Mice began to show clinical signs at 22 weeks after IP inoculation and at 18 weeks after IC inoculation. Points shown at (a) represent mice with PMN values that were unusually high for scrapie-inoculated mice in the preclinical phase of the disease. These values were not included in calculations of the corresponding average. In B, the PMN value at (a) at 12 weeks was 68.*

independent of the inoculation route. Among the mice in the preclinical phase of scrapie, the percent PMN values were again normally distributed, but about a lower mean of 6% with a range of 1 to 9% (Fig. 2B) again independent of the inoculation route.

**Alterations in the percent PMN in clinically affected scrapie mice.** Among clinical scrapie mice, the distribution of PMN values is biomodal (Fig. 2C); the lower end of the distribution is similar in both mean and range to that of the preclinical mice. However, at the other end of the distribution, there is a clustering of percent PMN values that are, for the most part, above the upper limits of values obtained for normal brain inoculated mice. The same type distribution was observed for mice inoculated both IC and IP. This peculiar distribution accounts for the nearly identical average percent PMN seen for the control group (normal brain inoculated or uninoculated) and for clinical scrapie mice, and for the much higher standard deviation in percent PMN observed among the clinically sick mice (Table 1).

**Cell type responsible for the alteration in leukocyte differential counts.** First, from observations of the blood cell smears, it was obvious that the shift in the percent of the leukocyte cell types following scrapie inoculation could not be

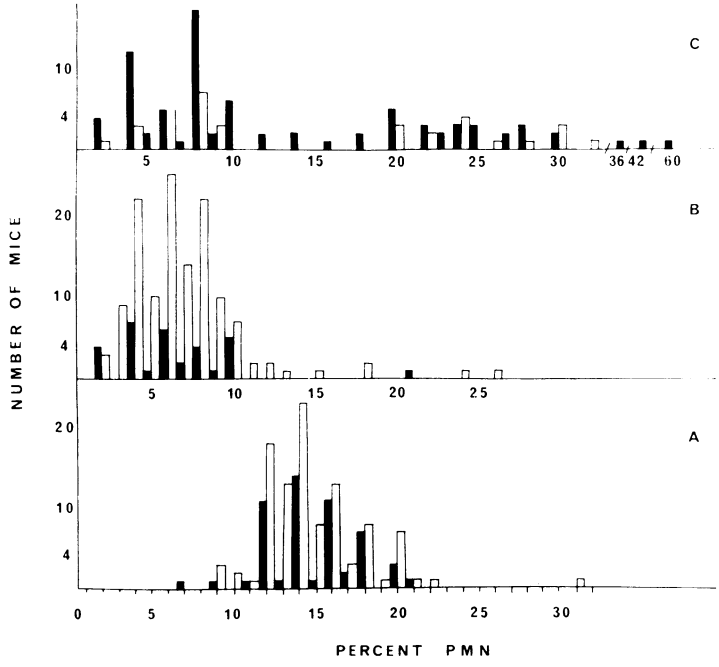


FIG. 2. *Histograms of the number of mice with given percentages of polymorphonuclear neutrophils (PMN). A, Mice inoculated with normal brain homogenate; B, mice inoculated with scrapie—preclinical phase of the disease; C, mice inoculated with scrapie—clinical phase of the disease. Solid bars represent mice inoculated intraperitoneally, open bars represent mice inoculated intracerebrally.*

accounted for by the appearance of unusual or abnormal cell types, i.e., atypical lymphocytes, juvenile PMN, etc. Rather, the change in differential counts represents a shift in the percent of normal cell types. Secondly, this shift cannot be accounted for by changes in the percentages of eosinophils or monocytes since (i) together, they account for only 1% or less of the total leukocytes, and (ii) they are not significantly altered in any of the treatment groups. The only two remaining explanations for the shift are that either there is an absolute increase in lymphocytes with a concomitant decrease in the percentage of PMN or there is an absolute decrease in the number of the PMN. These alternative explanations can be differentiated by an examination of total leukocyte counts. To obtain a decrease in the percentage of PMN, from 14.5% noted in normal mice to 6% noted in the preclinical scrapie mice, would require an increase in the absolute number of lymphocytes of 2.7-fold. Because they normally comprise such a high percentage of the total leukocytes, a 2.7-fold increase in lymphocytes would cause a 2.4-fold increase in the total leukocyte counts. In contrast, a decrease in the absolute number of PMN would decrease the total leukocyte count by only 8%.

If the change is a consequence of an increased number of lymphocytes, there should be a negative correlation between a given percent PMN value and its corresponding total leukocyte count. To test this possibility, differential leukocyte counts and total leukocyte counts were performed on normal brain-inoculated controls and scrapie-inoculated mice in the preclinical stage of the disease. These results are presented as a scatter diagram (Fig. 3). The apparent lack of association between the percent PMN and the total leukocyte counts is supported by calculation of the correlation coefficient, $r$. For the normal controls $r = -0.07$, and for the preclinical scrapie mice $r = 0.10$. Furthermore, the average total leukocyte count for normal mice (10,300 ± 3,900) was slightly higher than the average count for preclinical scrapie mice (8,100 ± 2,700). The data, used to calculate the average number of PMN per cubic millimeter (± standard deviation), were as follows: (i) Normal brain-inoculated, 1,430 PMN/mm³ (±704); (ii) preclinical scrapie mice, 540 PMN/mm³ (±250); and (iii) clinical scrapie mice, 2,650/mm³ (±1,250). It appears that the decrease in the percent PMN cannot be accounted for by an increase in the absolute number of lymphocytes and, therefore, the best explanation is that there is a decrease in the absolute number of circulating PMN.

The changes in percent PMN seen among clinically sick scrapie mice are more complex in that there are both abnormally high and low PMN percentages. The analysis for the low PMN values is the same as for preclinical mice, whereas higher PMN percentages could be obtained with either an increase in the absolute number of PMN or a reduction in lymphocytes that would produce a sharp decrease in total counts. Total leukocyte counts were plotted against PMN values (Fig. 3). When calculated separately for the high values (>15%) and the low values (<15%), the correlation coefficients were 0.05 and −0.10, respectively. Thus, it seems unlikely that either an increase or a decrease in the absolute number of lymphocytes could account for the corresponding abnormally low or abnormally high PMN percentages.

**Earliest appearance of the change in PMN percentages in scrapie-inoculated mice.** To determine

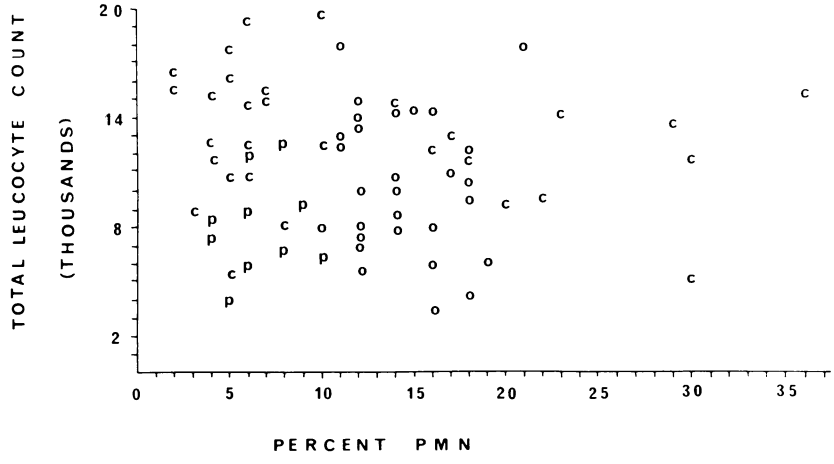


FIG. 3. *Total leukocyte counts plotted against the percentage of polymorphonuclear neutrophils (PMN). Normal mouse brain, O; scrapie preclinical, P; scrapie clinical, C.*

just how early the decrease appeared, differential leukocyte counts were done on mice at daily intervals after IC inoculation. The percent PMN among scrapie mice were clearly lower than normal by 3 days after inoculation and remained low for the remaining 21 days of the experiment (Table 2).

**Effect of periodate.** It has been reported that sodium periodate is one of the few reagents that readily inactivate(s) scrapie (12). To determine whether the factor responsible for the decrease in PMN is likewise inactivated, scrapie brain homogenate was treated with sodium periodate as described in Materials and Methods. After treatment, the material was dialyzed and inoculated into mice which were then tested for their PMN percentages at 3 and 5 weeks after inoculation. To insure that the results were due to periodate and not its reduced form, the same protocol was used except that iodate was substituted for periodate. The results along with those obtained for similarly treated normal mouse brain homogenates are shown in Table 3. The PMN factor was completely inactivated after 6 hr of exposure to sodium periodate. In contrast, sodium iodate had no effect on the ability of scrapie material to induce the PMN decrease. Since all samples were dialyzed before inoculation into mice, those results that show a decrease in PMN percentages after inoculation with scrapie material demonstrate that the PMN factor is nondialyzable.

TABLE 2. *Effect of scrapie and normal brain homogenate on percent PMN as a function of time after intracerebral inoculation*

| Time post-inoculation | Avg of PMN percentages[a] | |
|---|---|---|
| | Normal | Scrapie |
| 24 hr | 10 | 10 |
| 48 hr | 11 | 12 |
| 3 days | 10 | 6[b] |
| 4 days | 12 | 6[b] |
| 5 days | 18 | 7[b] |
| 7 days | 13 | 6[b] |
| 10 days | 13 | 7[b] |
| 11 days | 15 | 6[b] |
| 21 days | 13 | 7[b] |

[a] Average of three animals; PMN, polymorphonuclear neutrophils.

[b] Probability of no difference <0.05, (Mann-Whitney U test).

**Filtration size of the PMN factor.** Scrapie brain homogenates were prepared and filtered as described in Materials and Methods. At each stage, the filtrates were inoculated (IC) into mice. PMN percentages were analyzed at 3 and 5 weeks after inoculation, and the data for the two time periods were pooled. As seen in Table 4, the factor passed through the 220-, 100-, and 50-nm filters but not the 25-nm filter. Thus, its size lies somewhere between 50 and 25 nm. This is in good agreement with the estimate of 35 nm obtained by Gibbs et al. (10) for the size of the scrapie disease-producing agent.

**Occurrence of the PMN factor in brain and spleen from scrapie-positive mice and sheep.** The mouse-adapted Chandler strain of scrapie was used throughout these studies. Dickinson and Meikle (3) have described a series of scrapie strains with genetic characteristics that differ markedly. One of these strains (ME-7) was obtained from Dr. Dickinson and inoculated directly into mice without prior passage in mice from our colony. Mice were inoculated IC and analyzed for their PMN percentages at 3 and 5 weeks. The data for the two times of harvest were similar and were therefore pooled. The PMN values for these mice (designated scrapie brain pool 2) were compared with those values obtained for mice inoculated with the Chandler strain (scrapie brain pool 1) or with normal brain homogenate (Table 5). The results show that the ME-7 strain caused a marked reduction in the PMN percentages when compared with mice inoculated with normal brain homogenate. The values of PMN percentages obtained in mice

TABLE 3. *Effect of treatment of brain homogenate with sodium periodate or sodium iodate on the percent PMN in mice*

| Treatment | Time (min) | Normal | | Scrapie | |
|---|---|---|---|---|---|
| | | % PMN[a] | Avg | % PMN | Avg |
| Periodate | 0 | Not | Done | 5, 6, 6, 8 | 6.2[b] |
| | 90 | Not | Done | 8, 9, 7, 6 | 7.5[b] |
| | 360 | 15, 14, 14, 15 | 14.5 | 13, 13, 14, 13 | 13.2 |
| Iodate | 0 | Not | Done | 6, 9, 8, 4 | 7.0[b] |
| | 360 | 18, 18, 14, 13 | 15.6 | 8, 4, 5, 9 | 6.5[b] |

[a] PMN, Polymorphonuclear neutrophils.

[b] Probability of no difference from 360 scrapie <0.01.

TABLE 4. *Effect of filtration on the percent polymorphonuclear neutrophils (PMN) in mice inoculated with scrapie brain homogenate*

| Pore size (nm) | % PMN/mouse | Avg |
|---|---|---|
| Unfiltered | 4, 4, 5, 4, 5, 3 | 4.1 |
| 220 | 3, 6, 8, 6, 6, 5 | 5.3[a] |
| 100 | 3, 7, 5, 4, 8, 7 | 5.3[a] |
| 50 | 4, 3, 11, 6, 3, 8 | 5.3[a] |
| 25 | 12, 12, 12, 15, 13, 12 | 12.6[b] |

[a] Not significantly different from unfiltered average. $P \geqq 0.1$ (Student's $t$ test).

[b] Probability of no difference from unfiltered average <0.001 (Student's $t$ test).

TABLE 5. *Percentage of polymorphonuclear neutrophils (PMN) in mice inoculated intracerebrally with various normal and scrapie tissues*

| Tissue origin | Tissue | % PMN/mouse | Avg | Statistical significance |
|---|---|---|---|---|
| Mouse | Normal brain pool 1 | 14, 12, 12, 19, 14, 16 | 14.5 | |
| | Scrapie brain pool 1 | 7, 8, 6, 6, 3, 7 | 6.1 | $P^a < .005$ |
| | Scrapie brain pool 2 | 8, 4, 10, 8, 6, 6 | 7.0 | $P < .005$ |
| | Normal spleen 1 | 28, 20, 20, 17, 15 | 20.0 | |
| | Scrapie spleen 1 | 10, 5, 6, 7, 8, 8 | 7.3 | $P < .005$ |
| Sheep | Normal brain pool 1 | 14, 20, 12 | 15.3 | |
| | Scrapie brain pool 1 | 5, 6, 6, 8 | 6.3 | $P < .02$ |
| | Normal spleen 1 | 18, 11, 12 | 17.3 | |
| | Scrapie spleen | 6, 2, 3, 10, 9 | 6.0 | $P < .02$ |

[a] Probability of no difference from the corresponding normal control tissue by Student's $t$ test.

inoculated with the two strains of scrapie were similar.

In addition to scrapie material derived from mouse brain, homogenates of mouse spleen, sheep brain, and sheep spleen were prepared from scrapie-infected animals and tested for their ability to reduce the PMN percentages. Appropriate normal tissue homogenates were inoculated as controls. The results (Table 5) show that the PMN factor was present in spleen and brain from both scrapie-positive mice and sheep. In contrast, all mice inoculated with normal material had PMN values in the normal range (Table 1), except those inoculated with normal mouse spleen, in which the PMN values were slightly higher than normal.

## DISCUSSION

Our results indicate the following. (i) The percentages of PMN among mice inoculated with normal mouse brain homogenate is indistinguishable from that observed among uninoculated mice. (ii) Preclinical scrapie mice have a percentage of PMN that is approximately one-third that of normal mice. (iii) The decrease in the percentage of PMN in scrapie-inoculated mice is independent of IP or IC inoculation. (iv) The decrease in percentage is most likely a consequence of a drop in the absolute number of PMN, not an increase in lymphocytes. (v) Terminal scrapie mice have PMN percentage values that are generally either higher or lower than the values of the control mice. (vi) The size of the factor causing the PMN change is between 25 and 50 nm. (vii) The factor is inactivated by sodium periodate. (viii) The factor is nondialyzable. (ix) The factor is present in homogenates prepared from spleen and brain from both experimentally-induced scrapie in mice and naturally occurring scrapie in sheep.

The mechanism of the decrease in the percentage of PMN is not known. Since the total leukocyte counts in mice inoculated with scrapie and normal brain homogenates were similar, we concluded that the effect on the differential counts must be based upon changes in the absolute number of PMN in the circulating serum. The effect on PMN percentages was manifested quickly after inoculation, which suggests a toxic material. However, toxic effects on mature PMN usually result in an increase in immature forms of PMN, and certain toxins cause Heinz bodies inside of red blood cells (4). Neither phenomenon was observed. Finally, the fact that the effect was noted for both spleen and brain homogenates argues against the idea that it is a toxic material, since the responses within these two organs to scrapie infection are so markedly different (13). It appears that the factor causing the PMN change may be related to the scrapie agent itself. The estimates of the size of the PMN factor and of the infectious capability of the scrapie agent (10) are the same. Further, both the infectivity of the scrapie agent (12) and the capability of scrapie material to induce the change in PMN percentages were inactivated by periodate but not iodate. Attempts to determine the relationship between scrapie infectivity and the factor causing the PMN change are continuing.

The significance of the drop in PMN in relation to the pathogenesis of scrapie in the mouse is unclear. It is interesting that mice with scrapie

are completely normal in their immunological responsiveness to various antigens (2, 9) and that no unusual differences in their reactions to infections during the course of scrapie have been observed.

In another study (15), it was shown that the differential leukocyte counts of goats with clinical scrapie were more variable than those observed for normal goats. Of the 10 scrapie goats examined, 5 had a higher PMN percentage than any of the 10 normal animals examined. Although the number of animals examined was small, these results are, in part, similar to our findings for clinically sick scrapie mice.

The higher percentage of PMN noted in many terminal scrapie mice is difficult to interpret. There are three possible explanations. (i) The spleens of terminal scrapie mice are smaller than normal and, if this results from mechanical contraction, the process could produce an increase in circulating PMN. (ii) Secondary infections late in the disease when the mice are generally debilitated could cause the increase. (iii) The center(s) of the brain that regulates the leukocyte population could be affected by the scrapie agent at this stage of the disease.

At present, the only test for scrapie is an $LD_{50}$ in mice which is both time-consuming and a very insensitive way to detect virus. The change in leukocyte differential counts which occurred a few days after IC inoculation, in contrast to the several months required for clinical disease, may thus provide the basis for a much improved means of assaying the scrapie agent.

There are a number of slow infections of the central nervous system of man that closely resemble scrapie in clinical course, histopathological changes, and the characteristics of the causative agents (6, 7, 14). For Kuru and Jakob-Creutzfeld, the presumed causative agents have been passed in chimpanzees and smaller primates, where each causes a slow infection with spongiform degeneration, gliosis, and astrocytosis (6–8). It would be interesting to determine whether the agents of Kuru or Jakob-Creutzfeld caused changes in leukocyte differential counts in primates similar to those shown for scrapie in mice. Furthermore, if the inoculation of these agents into a small, inexpensive host such as the mouse led to rapid changes in leukocyte differential counts, it would markedly advance research in this area.

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