The Role of Mononuclear Cell Deficiency in the Production of Neutrophilic Allergic Encephalomyelitis: Parabiosis Experiments[1] (36848)

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Perivascular cuffs of mononuclear inflammatory cells are usually considered the hallmark of experimental allergic encephalomyelitis (EAE). Labeling experiments on EAE produced by passive transfer of living lymph node cells from actively immunized donors to normal recipients have shown that the perivascular cells are predominantly nonspecific and reactive in nature (1, 2). This conclusion was fortified by the ability to inhibit perivascular cuffs by X-radiation (3) or lympholytic drugs (4, 5) directed to the recipients in advance of the transfer of donor EAE cells. In these experiments, donor EAE cells were exempt from direct damage by the radiation or drugs, but might have been damaged by secondary effects of these agents (3) or prevented from transferring immunologic information to the recipients (4, 5). These possibilities were excluded when a system was devised in which a lympholytic treatment of the recipient was followed, not by elimination of the inflammatory response to subsequent transfer of EAE cells, but by a change in the *type* of response (6, 7). In this system, transfer of EAE cells caused an exudation of polymorphonuclear neutrophilic leukocytes ("neutrophilic EAE") rather than mononuclear cuffs. This was accomplished by effectuating the EAE transfer during the short period after administration of cyclophosphamide (CY) when lymphocytes were severely depressed but neutrophils were not yet reduced (relative neutrophilic leukocytosis). The appearance in the brain of neutrophils as the indicators of the autoimmune disease made it clear that the donor cells, however invisible, were unharmed and were responsible for the initial injury, and that the classical mononuclear cuffs were merely reactive nonspecific cells, not at all essential for initiation or identification of EAE.

In view of the importance of these conclusions, we have sought to substantiate the interpretation of the role of CY in production of neutrophilic EAE by reversing the effects of the drug. For this purpose, drug-treated recipients in parabiosis with normal animals were used.

Methods. Inbred Lewis rats (Microbiological Associates, Inc., Bethesda, MD) were used because histocompatibility was essential for parabiosis and for passive transfer of EAE. They were maintained in suspended wire cages on Purina Laboratory Chow and tap water. Pairs of male rats, 6–14 wk old, were matched in weight to within 10 g and united in parabiosis. Skin incisions were made from ear to base of tail on the apposing sides of the prospective partners, under ether anesthesia. Adjacent scapulas were sutured together. The wounds were dusted with neosporin antibiotic powder and penicillin was injected into the gluteal muscles. Ventral and then dorsal skin flaps were united with surgical clips. Tetracycline was added to the drinking water. Wound clips were removed after 2 wk. The parabionts were used 2–3.5 wk after surgery, when the cross-circulation was well established.

Neutrophilic EAE (7) was produced in the right members of each of 53 parabiotic pairs divided among nine experiments. First, a thermal brain injury was produced in order to localize and accelerate the subsequent EAE transfer. Two days later, when the brain

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reaction to thermal injury was well established, CY (125 mg/kg) (except half this dose in one experiment) was injected in the dorsal penile vein, the peritoneal cavity, or the subcutis. Regardless of route, the drug caused marked depletion of the hemopoietic and lymphoid tissues of the right partners. (The left partners remained untreated, inasmuch as it was our purpose to have them replenish the depleted right partners. Fortunately, CY did not traverse the parabiotic cross-circulation, probably because it disappeared from the circulation rapidly.) One day later, the right partners were given a passive transfer of living lymph node cells draining inoculation sites of a set of donor rats with early EAE. The donor:recipient ratio varied from 1:1 to 3:1. The donors had been prepared 7 days in advance by immunization in the right hind foot with guinea pig spinal cord emulsified in Freund's complete adjuvant, plus the ancillary adjuvant, pertussis vaccine. EAE lesions were fully developed 24 hr after transfer when the recipient parabiotic pairs were sacrificed.

Controls included single and parabiotic recipients given EAE cells but no drug (ordinary EAE), single recipients given CY and EAE cells (neutrophilic EAE), and most important, 53 parabiotic pairs *both* members of which were treated with CY before the right partner was given EAE cells. The latter arrangement, incorporated into every experiment, provided a control for nonspecific effects of parabiosis while it prevented any replenishing effect inasmuch as both partners were equally depleted of leukocytes.

Histology. Forebrains, spleens and vertebrae were fixed in Bouin's fluid. The entire thermal injury was included in four or five coronal slices, embedded in paraffin and stained with hematoxylin-eosin. Slides were randomized and scored after each of the nine experiments, without knowledge of group of origin. Later, slides of all nine experiments were pooled, randomized, and scored again. The two readings did not differ significantly, and only the latter are reported. Neutrophils and mononuclear cells were scored separately and at different times. Neutrophils were graded at 100× magnification as follows: 1, few, in one or several areas. 2, innumerable in one of the 4 or 5 slices on the slide; small numbers elsewhere. 4, innumerable in two or more of the slices. Mononuclear cells were graded at 40× magnification as follows: 1, up to five perivascular cuffs, usually very mild, only a single layer of cells, often incompletely circumferential. 2, 5 to 10 cuffs, usually completely circumferential, a few multilayered cuffs. 4, many cuffs usually multilayered, at least a few in each slice, similar in number and intensity to untreated controls with ordinary localized EAE. Grade 3 was intermediate between grades 2 and 4. Neutrophils and mononuclear cells could not be differentiated at 40× magnification, but neutrophils were not present in the perivascular cuffs in sufficient numbers to impair the validity of cuffs as a measure of mononuclear cell content of the EAE lesions.

Results. Passive transfer of EAE cells produced lesions in a narrow zone adjacent to the necrotic area caused by the thermal injury. In recipients that were not pretreated with CY, the vessels were surrounded by cuffs of mononuclear cells, predominantly small lymphocytes, and similar cells infiltrated the parenchyma (ordinary EAE). There were few neutrophils. In control (single) recipients that had been pretreated with CY, the mononuclear cells were greatly decreased while neutrophils were greatly increased in vessel walls and especially in the neutrophil, as has been described (neutrophilic EAE) (7). Identical lesions were observed in control parabiotic pairs after both members had been given CY (Tables I, II). Therefore, parabiosis, *per se*, did not interfere with development of neutrophilic EAE.

The findings were different when the right parabiotic partner had been pretreated with CY but the left partner remained untreated. The right partner had a considerable increase in the mononuclear content of the EAE lesions as a result of parabiosis with the normal animal (Table I, last column). In a few rats, the number of mononuclear cuffs reached the range commonly observed in rats with ordinary EAE. In most of the right partners, however, the mononuclear component remained less than normal (av score,

TABLE I. Mononuclear Cell Infiltrates in Neutrophilic EAE Lesions in the Right Partners of 106 Parabiotic Pairs.

Histologic grade	Cyclophosphamide treatment	
	Both partners[a]	Right partner[b]
4+	0[c]	5[c]
3+	1	4
2+	13	28
1+	32	16
0	7	0
Totals	53	53
Av scores	1.2+	2.0+

[a] These are the controls inasmuch as cyclophosphamide treatment of *both* partners prevented reversal of the drug effects.

[b] These are the experimentals inasmuch as the untreated left partner was able to replenish the right partner and thereby reverse the drug effects.

[c] Number of rats (right partners) with infiltrates of indicated severity. Combined results of nine experiments.

2.0), but distinctly more abundant than in the controls (av score, 1.2). The standard error of the difference of these means was 0.148, statistically significant at the 1% level.

Corresponding to this finding, but in the opposite direction, were the observations on neutrophils. Parabiosis to a normal animal did not eliminate neutrophils from the EAE lesions of the right partners, but there was a definite decrease in the scores (Table II, last column). The average score was 2.1 compared to the control average of 2.8. The standard error of the difference of these means was 0.66, statistically significant at the 5% level.

One further experiment differed from the foregoing only in that the 10 recipient pairs were sacrificed 2 days rather than 1 day after the EAE transfer. The effects of parabiosis were the same as described above.

An additional experiment was done in which triethylene melamine was substituted for CY. Administration of 1 mg/kg of this drug iv to both members of four parabiotic pairs produced the usual features of neutrophilic EAE, as reported for single animals (7). As above, parabiosis of the drug-treated rat to a normal rat (4 pairs) increased the mononuclear cells and decreased the neutrophils in the EAE lesions.

Other tissues. Sections of spleen and bone marrow of untreated left partners were normal, proving that CY did not cross the parabiotic union to a detectable degree. Marrow of CY-treated right parabionts was devastated and spleens were markedly depleted of lymphocytes. The depletion of splenic lymphocytes was less profound in animals united with a normal rat than in pairs both members of which were given CY. This was another indication of reversal of CY effects by parabiosis with a normal animal. No such reversal was detected in marrow.

Evaluation of cross-circulation. In some experiments, thermal injuries of the brain were inflicted on the left as well as right parabiotic partners. EAE lesions were found in the left partner's brain 1 day after transfer of EAE cells into the right partner. This was proof of the patency of the cross-circulation for leukocytes. This experience confirms the conclusion of Lipton and Freund (8) that EAE in one parabiont following active immunization of the other parabiont was due to transfer of EAE cells rather than transfer of antigenic material from the inoculum.

EAE lesions were less severe in the left parabiont than in the right, as might be expected. Neutrophilic lesions were found in a few left partners when both parabionts had been pretreated with CY.

TABLE II. Polymorphonuclear Neutrophil Infiltrates in Neutrophilic EAE Lesions in the Right Partners of 106 Parabiotic Pairs.

Histologic grade	Cyclophosphamide treatment	
	Both partners[a]	Right partner[b]
4+	22[c]	11[c]
3+	11	9
2+	10	14
1+	8	15
0	2	4
Totals	53	53
Av scores	2.8+	2.1+

[abc] See Table I.

Discussion. The following evidence indicates that neutrophilic EAE in single rats is caused by a CY-induced deficiency of nonspecific mononuclear cells (7): (a) the brief period after CY administration during which EAE cells can produce neutrophilic infiltrates in recipients corresponds with the duration of drug-induced lymphopenia and relative neutrocytosis; (b) the increase of neutrophils in the lesions is accompanied by a decrease of mononuclear cells; (c) neutrophilic infiltrates can be produced by related cytotoxic drugs or X-radiation; (d) neutrophilic infiltrates are prevented if conventional mononuclear EAE lesions are produced in the brain just before the treatments designed to yield neutrophilic EAE at the same site. However, final proof of the nature of any deficiency disease requires reversal of the process by replacement of the deficient constituent. Replacement of the deficient mononuclear cells by injection of normal spleen or lymph node cells was not practical because the design of these experiments required that the deficiency be corrected within 24 hr. We have already reported inability of transferred cells to reverse the inhibitory effects of X-radiation on conventional passive EAE in 24 hr (3), while Werdelin, doing more protracted experiments, was able to reverse radiation effects in 5 days (2); the additional time may have been needed to overcome mechanical damage during cell preparations or initial sequestration of transfused cells. Therefore, we resorted to cross-circulation established in advance by parabiosis as a source of normal blood leukocytes.

Parabiosis with a normal animal increased the mononuclear cells and inhibited the neutrophils in the infiltrates of CY-treated parabionts. That the changes were not more dramatic was due to the slow and limited blood exchange between parabiotic partners which means that relatively few normal mononuclear cells from the left parabiont were available at any one moment to enter EAE lesions in the right parabiont. On the other hand, neutrophils were available in almost undiminished numbers in the right parabiont because CY had not yet eliminated its blood neutrophils or destroyed the reserve supply of mature granulocytes in its own bone marrow (9). In view of these facts, the relatively modest reversal of CY effects on EAE lesions by parabiosis to a normal animal is sufficient to confirm the hypothesis that neutrophilic EAE is caused by a deficiency of mononuclear cells.

Alternative explanations for the diminution of neutrophils following parabiosis with a normal animal and the reasons for excluding them are as follows:

1. Loss of CY from treated to normal partner: no CY effects were detected in the normal partner's spleen or bone marrow; a dose of 62.5 mg/kg (the amount remaining in the treated partner even if one made the unlikely assumption of a uniform distribution between the two partners) was ample to produce neutrophilic EAE.

2. Loss of EAE cells from treated to normal partner: in one of the experiments, equal numbers of EAE cells were given to *both* partners, so that no net loss or gain could occur, and the results were similar to the other experiments.

3. An inhibitory factor or effect in serum erythrocytes or neutrophils transferred from normal to treated partner: there is no reason to expect such an unlikely event; in a separate experiment on six nonparabiotic rats, IV injection of 10 or 20 ml normal rat serum did not interfere with development of neutrophilic EAE.

4. Inhibition by nonspecific stress (10): neutrophilic EAE was not at all inhibited in the control series of parabionts that were submitted to identical stresses (Tables I, II).

Furthermore, alternates 2, 3 and 4 could not account for the *increase* of mononuclear cells concomitant with the *decrease* of neutrophils, or the increase of lymphocytes in the spleen.

Summary. Experimental allergic encephalomyelitis (EAE) produced during a transient period of mononuclear cell deficiency and relative neutrophilic leukocytosis induced by cyclophosphamide is characterized by neutrophilic rather than mononuclear cell infiltrates. Parabiosis to a normal animal increased the mononuclear content and inhibited the neutrophils in the infiltrates, presumably because the normal parabiont replenished the nonspecific mononuclear cells

made deficient by cyclophosphamide. This evidence supports the hypothesis that neutrophilic infiltrates in EAE are caused by a deficiency of nonspecific mononuclear cells, and that mononuclear infiltrates in ordinary forms of EAE are reactive, nonspecific cells.

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