Reduction of Allergic Encephalomyelitis Incubation Period to Five Days

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Experimental allergic encephalomyelitis (EAE) is thought to belong to the delayed hypersensitivity class of immunologic reactions. There are many reports of delayed hypersensitivity with incubation periods of only 4 or 5 days. Indeed, guinea pigs develop skin reactivity of the delayed type as early as 4 days after immunization with neural antigen and adjuvants, many days before they develop clinical signs of EAE. Histologic lesions of EAE—perivascular infiltrates of mononuclear inflammatory cells—have been observed in the nervous system as early as 6, 7, or 8 days after immunization in a few rats, guinea pigs, and rabbits. Most animals, however, do not develop lesions of EAE until a few or many days later. Only the hyperacute form of EAE, produced with the aid of pertussis vaccine, regularly causes lesions and clinical signs after 7 or 8 days in most or all rats.

The present work has resolved the apparent discrepancy in incubation periods between EAE and other agents causing delayed hypersensitivity. We were not content to await the natural evolution of EAE. We established the presence in lymph nodes of cells capable of causing EAE infiltrates by the techniques of passive transfer and "induced localization." It is well known that physical, chemical, or anoxic injuries to the brain induce the localization of EAE on the periphery of the damaged area. Not only is the threshold of disease lowered by this procedure, but the lesions are spatially concentrated and easy to find. In an earlier work, the lesions of hyperacute EAE were found clustered around intracerebral implants of graphite as early as 4 or 5 days after immunization. The present work confirms this observation with more advanced techniques. It proves that encephalitogenic cells are regularly present in lymph nodes and can cause perivascular infiltrates in the brain 5 or even 4 days after immunization in both the ordinary and hyperacute forms of EAE.

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Supported wholly by Grant 536A10 from the National Multiple Sclerosis Society.

Accepted for publication April 4, 1969.

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Materials and Methods

Female Lewis rats, 8–12 weeks old (150–200 g), from Microbiological Associates, Inc., were immunized with 0.05 ml of a water-in-oil emulsion made with equal parts of guinea pig spinal cord homogenate and Freund's complete adjuvant. The homogenate consisted of 4 parts of strained, previously frozen cord and 6 parts of saline. The adjuvant consisted of 8.5 parts mineral oil (Bayol F), 1.5 parts emulsifying agent (Arlace A), and killed tubercle bacilli (4 mg/ml). No preservatives were added, but the emulsion was heated to 60°C for 1 hr immediately before use. Injection of this material into one of the pads of the sole of the right hind foot produced the usual form of EAE, hereinafter designated "ordinary EAE." When, in addition, 0.1 ml of a concentrated pertussis vaccine (about 20 billion organisms) was injected intradermally into the dorsum of the same foot at the same time, the rats developed the hyperacute form of EAE. Hyperacute EAE is characterized by early onset of clinical signs, great severity of lesions, and high mortality. These designations will be applied in this paper even though the rats were killed before appearance of the characteristic signs and lesions.

In one experiment (Table 2), ordinary EAE was induced by injection of 0.25 ml of 80% guinea pig cord homogenate, without adjuvant, as previously described. Hyperacute EAE was produced in this experiment by injection of pertussis vaccine in the dorsum of the foot at the same time that the aqueous homogenate was given in the footpads.

The lesions of EAE were induced to localize in the right cerebral hemisphere by creating a zone of coagulation necrosis. This was accomplished by applying one of the flat surfaces at the end of a 371/2-in pyramid-tip preheated electric soldering iron for 7 sec to the exposed but intact calvaria. Thermal injury of the dorsal part of the spinal cord was produced by applying the point of the soldering iron to the exposed spinous processes of upper lumbar vertebrae for 15 or 20 sec. These rats often were paralyzed, whereas thermal injuries of the brain caused no clinical manifestations. Skin incisions were closed with metal clips. Surgical procedure and foot injections were done with the aid of ether anesthesia and sterile precautions. The timing of the thermal injury was important because the age of the injury determines its ability to induce localization of EAE. In one experiment, thermal injuries were inflicted 3 days before the rats were sacrificed; in other experiments, all were done immediately after the inoculation of the feet, regardless of the planned day of sacrifice. For the range of times under investigation, both plans gave thermal injuries capable of inducing localization of EAE; and the results were similar.

Passive transfer of EAE was accomplished with lymph node cells from donor rats immunized as described above. The right popliteal, inguinal, sacral, lumbar, renal, and axillary nodes draining the sites of inoculation were excised, cleaned, and dissociated into a cell suspension. The cells were washed twice with saline and injected into the dorsal penile vein of anesthetized isogenic Lewis recipient rats. The recipients, but not the donors, had thermal injuries of the brain.

Tissues were fixed in Bouin's solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin and with phosphotungstic acid-hematoxylin (for fibrin). The brain was cut in coronal sections. The portion of spinal cord with a thermal injury and the enveloping vertebral column (decalcified) were cut in cross section. The remainder of the cord, or the entire cord in the absence of a thermal injury, was removed from the column by the "segmented sleeve" technique and embedded longitudinally.

Results

As a base line for data which follow, we present our observations on the earliest perivascular infiltrates observed in the natural evolution of
EAE (Table 1). In ordinary EAE, only 2 rats among 60 killed after 5 days, and 3 among 28 killed after 7 days, had lesions. In each instance, only one perivascular infiltrate was found on the slide of the entire cord and hindbrain (the areas of predilection). A few additional rats had minor mononuclear infiltrates in the leptomeninges. All rats killed 8, 9, or 10 days after immunization had lesions. The number of lesions varied from few to many on each of these days.

In hyperacute EAE, mild lesions were detected after 6 days, but not after 5 days, confirming a previous report. After 7 or 8 days, most or all rats have clinical signs and innumerable lesions, so those relatively late times were not investigated further. There is no doubt that the appearance of signs and lesions is accelerated in hyperacute EAE.

**Induced Localization**

To what extent can the appearance of perivascular infiltrates be accelerated by a thermally induced zone of cerebral coagulation necrosis? Perivascular infiltrates of EAE were found adjacent to the thermal injuries in almost all rats killed 5, 6, or 7 days after inoculation, except when no adjuvants were employed (Table 2). A few rats killed 4 days after inoculation had lesions, but these were few and mild. By 5 days after inoculation, almost every rat had perivascular infiltrates (Fig 1 and 2). Usually about a dozen (range 6–24) were detected by scanning the entire slide at a magnification of 40×. Additional inflammatory cells, around small vessels and in the intervening parenchyma, could be detected at higher magnifications. There was progression in number and severity in the ensuing days. There was no significant difference in incidence or severity of early lesions between hyperacute EAE and ordinary EAE except for the group without adjuvants. However, some of the rats killed 6 or 7 days after induction of hyperacute EAE had

* Fewer positive results were obtained when rat cord, a weaker antigen, was substituted for guinea pig cord in the inoculum.

<table>
<thead>
<tr>
<th>After Immunization (days)</th>
<th>Perivascular infiltrates*</th>
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<tbody>
<tr>
<td></td>
<td>Ordinary EAE</td>
</tr>
<tr>
<td>5</td>
<td>2/60</td>
</tr>
<tr>
<td>6</td>
<td>0/4</td>
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<tr>
<td>7</td>
<td>3/28</td>
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<td>8</td>
<td>4/4</td>
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<tr>
<td>9</td>
<td>4/4</td>
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<tr>
<td>10</td>
<td>4/4</td>
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* Numerator: number of rats with EAE lesions in spinal cord or hindbrain; denominator: total number of rats.
† Later times were not investigated because most rats have clinical signs and all or almost all have histologic lesions by 7 days after immunization.
Table 2. Occurrence of Perivascular Infiltrates Adjacent to Thermal Injuries of Brain or Cord

<table>
<thead>
<tr>
<th>After Immunization (days)</th>
<th>After Thermal Injury (days)</th>
<th>Perivascular Infiltrates*</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Brain Injury Only</td>
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<tr>
<td></td>
<td></td>
<td>Brain Injury Only</td>
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<tr>
<td>4</td>
<td>3</td>
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<td>5</td>
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<td>6</td>
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<td>5</td>
<td>5</td>
<td>4/4</td>
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<tr>
<td>6</td>
<td>6</td>
<td>4/4</td>
</tr>
</tbody>
</table>

* Data from four experiments represent incidence of EAE lesions adjacent to the thermal injuries; severity of lesions and observations on controls are described in text. Guinea pig spinal cord was the antigen in all experiments.
† Freund's adjuvant only was used in all cases except those marked §, in which case no adjuvant was used.
‡ Freund's adjuvant and pertussis vaccine were used in all cases except in those marked †, in which case only pertussis vaccine was used.
§ Data from fourth experiment represent incidence of EAE lesions adjacent to the thermal injuries; severity of lesions and observations on controls are described in text. Guinea pig spinal cord was the antigen in all experiments.
|| Freeman's adjuvant only was used in all cases except those marked §, in which case no adjuvant was used.

sufficient fibrin in the lesions to be demonstrable in the phosphotungstic acid-hematoxylin stain. Clinical signs were present at 7 days in these rats.

In the preceding experiments, EAE lesions were induced to localize in the forebrain. However, EAE lesions are much more numerous in the spinal cord than in the forebrain when the disease is allowed to evolve naturally, uninfluenced by thermal injuries. Therefore, an experiment was done in which immunized rats were killed at intervals after thermal injuries had been induced in both forebrain and spinal cord. The perivascular infiltrates of EAE were observed in cord and brain 5, 6, or 7 days, but not 4 days, after immunization in both the ordinary EAE and hyperacute EAE groups (Table 2; Fig 3). There was no difference in induced localization between brain and cord, except that fibrin-containing lesions were somewhat more common in the cord than in the brain of rats with hyperacute EAE. The lesions in rats with ordinary EAE were much milder in this experiment than in
the first two. Probably the paraplegia induced by the thermal lesion of the cord was stressful to the animals, and it is known that stress inhibits EAE. The relatively stronger antigenic stimulus in rats with hyperacute EAE appeared to be unaffected by the stress, and their EAE lesions were as numerous as in the previous experiments.

The observation of mild EAE in a few rats as early as 4 days after immunization prompted two experiments in which guinea pig cord in Freund’s adjuvant was inoculated into the right foot of rats that had been splenectomized or adrenalectomized 3 days earlier. In addition, groups of intact rats were given the inoculum into the right foot, into all four feet, or directly into surgically exposed inguinal or cervical lymph nodes. Of 29 rats killed 4 days after inoculation, definite EAE lesions were observed in 18. However, none of these ancillary procedures consistently increased the intensity of lesions to the level observed after 5 days.

It has been established by control material in previous studies that perivascular infiltrates did not occur adjacent to thermal injuries of the brain even if the rats were immunized with Freund’s adjuvant, pertussis vaccine, and a non-neutral (adrenal) tissue. Nor did inflammatory lesions occur after passive transfer of lymph node cells or blood from donor rats immunized with adjuvants with or without non-neutral tissue. Additional controls done in the present series of experiments included 16 rats killed 3–6 days after immunization with isologous testis tissue and adjuvants, and simultaneous induction of a thermal injury of the brain. In addition, 4 rats treated with pertussis vaccine only, and 13 nonimmunized rats were given thermal injuries of both brain and cord or brain only. As might be expected, the neural tissue adjacent to the zones of coagulation necrosis exhibited edema, macrophages, and prominent vessels (Fig 4). Nevertheless, there were no perivascular lymphocytic infiltrates or any sign of inflammation in these control animals. When read “blind” in randomized slides of each experiment, every control was scored as zero.

**Passive Transfer**

The relationship between the immunizing events in lymph nodes and the development of EAE has been proved by passive transfer of the disease with lymph node cell suspensions. It is our contention that EAE produced adjacent to thermal injuries after 5 days of active immunization is not different from EAE occurring a few days later in conventional experiments. If this is true, it should be possible to transfer the disease with cell suspensions prepared from lymph nodes taken 5
days after immunization. This was accomplished in four separate transfer experiments, two with ordinary EAE donors and two with hyperacute EAE donors (Table 3). In these experiments, thermal injuries were produced in the recipients 1–3 days before the transfer. The recipients were killed 1 day after the transfer. EAE lesions in recipients approximated in severity those observed after 5 days of active immunization in the experiments recorded in Table 2. In order to accomplish this result with ordinary EAE donors, however, it was necessary to use relatively high donor to recipient ratios (4:1, 8:1). Other recipient rats not included in Table 3 were allowed to survive for longer periods, and some of these also developed EAE lesions despite a donor to recipient ratio of only 1:1. In fact, some rats that survived 7 days had clinical signs of EAE, and perivascular infiltrates throughout spinal cord and hindbrain, as well as adjacent to the thermal injury of forebrain.

On the other hand, large volumes of fresh serum from the same donors injected into recipients prepared with thermal injuries failed to transfer EAE (Table 3). Nor was a synergistic effect observed when serum and cells were given together (Table 3).

Passive transfer of EAE after 5 days' immunization of donors has been reported previously in guinea pigs by Stone and by Falk, Kies, and Alvord. In their experiments, however, the recipients were allowed to survive many days, which may have permitted further maturation of the donor cells. We are not aware of any previous cellular passive transfer of EAE in which the total elapsed time for donor plus recipient was only 6 days.

Table 3. Passive Transfer of EAE with Lymph Nodes' Cells Harvested 5 Days after Immunization of Donors

<table>
<thead>
<tr>
<th>Donor:Recipient ratio</th>
<th>EAE in recipients*</th>
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<tr>
<td><strong>Donors with ordinary EAE</strong></td>
<td></td>
</tr>
<tr>
<td>8:1</td>
<td>2/2</td>
</tr>
<tr>
<td>4:1</td>
<td>5/7</td>
</tr>
<tr>
<td>1:1</td>
<td>0/8</td>
</tr>
<tr>
<td>1:1 + Serum†</td>
<td>0/2</td>
</tr>
<tr>
<td>Serum only†</td>
<td>0/2</td>
</tr>
<tr>
<td>Saline only</td>
<td>0/4</td>
</tr>
<tr>
<td><strong>Donors with hyperacute EAE</strong></td>
<td></td>
</tr>
<tr>
<td>1:1</td>
<td>7/7</td>
</tr>
</tbody>
</table>

* Combined results of two experiments with ordinary EAE and two experiments with hyperacute EAE.
† Incidence of perivascular infiltrates adjacent to thermal injury of brain; recipients killed 1 day after passive transfer.
† Serum obtained from donors of lymph nodes; 18 ml injected IV into each recipient within 3 hr of bleeding.
Discussion

The present results and those of other investigators\textsuperscript{3,4} on the natural evolution of ordinary EAE in rats showed that perivascular infiltrates were rare 5, 6, or 7 days after immunization, but they did occur. The addition of pertussis vaccine to the immunizing materials caused a definite acceleration of the temporal sequence, in that EAE lesions occurred very frequently 6 or 7 days after immunization.\textsuperscript{8} This hyperacute form of EAE is a very intense stimulus, as evidenced by the high incidence of clinical signs of disease 7 or 8 days after immunization,\textsuperscript{8} the severe mortality, the massive exudation of fibrin into the lesions, and the effectiveness of passive transfer with relatively low doses of cells (Table 3). In the present context, however, it is worth emphasizing that the features which are seen \textit{regularly} in hyperacute EAE are no different from those which occur \textit{occasionally} in ordinary EAE.

A similar comparison can be made between the natural evolution of EAE and its induced localization. Perivascular infiltrates occurred \textit{regularly} adjacent to thermal injuries after immunizing periods so brief that they permitted lesion production in the naturally evolving disease only \textit{occasionally}. Passive transfer experiments have demonstrated that the thermal injury lowers the threshold of the brain for EAE lesions.\textsuperscript{12,19} It may be concluded that the release of an encephalitogenic agent from lymph nodes into the circulation occurs regularly after 5 days of immunization, but the amount is usually (not always) insufficient to cause EAE lesions unless the threshold is reduced by a thermal injury. Additional evidence for the early development of immunocompetence in lymph node cells is provided by tissue culture experiments. Koprowski and Fernandes observed contactual agglutination around glial cells with EAE lymph node cells harvested as early as 4 days after immunization.\textsuperscript{23} Winkler first observed demyelinating activity of lymph node cells in trigeminal ganglion cultures 4 days after injection of peripheral nerve antigen and adjuvants.\textsuperscript{24} Dowling and Cook found that peripheral blood leukocytes taken 5 days after EAE immunization elaborated a demyelinating agent in tissue culture.\textsuperscript{25} Hughes and Newman noted inhibition of exudate cells by an encephalitogenic antigen as early as 5 days after immunization.\textsuperscript{26}

It is of interest that addition of pertussis vaccine to Freund's adjuvant did not reduce the minimum period needed for the release of the encephalitogenic agent from lymph nodes (Table 2). Why, then, does addition of pertussis vaccine to Freund's adjuvant reduce the incubation period for the natural evolution of EAE? Perhaps pertussis vaccine af-
fects the nervous system by increasing permeability, as has been reported for other tissues. However, our previous investigations have suggested that pertussis operates in the draining lymph nodes. It may synchronize and/or intensify the immunizing events and proliferation in lymphoid cells, or it may facilitate the discharge of the immunized cells from the nodes, but it does not make the minimum immunizing period shorter than that which is required for neural tissue in Freund's adjuvant.

What is the nature of the encephalitogenic agent released from lymph nodes into the circulation 5 days after immunization? Several authors reported permeability changes in the brain in the absence of inflammatory cells at the same site, but those observations were made in animals with fully developed EAE and might have been caused by undetected cellular infiltrations in nearby areas; no permeability changes were found by these authors before the onset of conventional inflammatory disease. On the other hand, immunofluorescence studies by Pette et al and by Oldstone and Dixon revealed perivascular exudation of complement or of fibrinogen as early as 5 or 6 days following immunization of monkeys, rabbits, or rats, whereas perivascular cellular infiltrates were not detected until a few days later. The precocious appearance of increased permeability to complement or fibrinogen suggested that a humoral factor might initiate lesions, rather than the inflammatory cells that appeared later; but the present investigation has revealed that perivascular cellular infiltrates can be induced to appear (by thermal injury) at the very time (5 days) that Pette et al and Oldstone and Dixon began to find permeability changes without cellular infiltrations. Our results suggest that a primary pathogenetic role of immunized lymphoid cells cannot yet be excluded, for the following reasons:

1. Lymphoid cell infiltrates may occur regularly in the intact nervous system 5 days after immunization, but the cells may be too few to be detected by routine histologic methods. Comparison of results of the exquisitely sensitive immunofluorescence technique with the relatively insensitive histologic technique for detection of small numbers of cells in a large organ might be misleading. In allergic adrenalitis, a related autoimmune disease, escape of inflammatory cells through vessel walls has been detected at least 2 days earlier by electron microscopy than by routine histologic study.

2. Immunized lymphoid cells might reach the vessels of the nervous system and alter their permeability without traversing the endothelial barrier. In this connection, demonstrations of attachment of lymphoid
cells to endothelium in EAE and in allergic neuritis are of interest.

3. Immunized lymphoid cells might reach the vessels of the nervous system, traverse the endothelial barrier, alter the permeability, but quickly disappear by reverse migration or dissolution.

The passive transfer experiments reported here prove that washed lymph node cells after 5 days of immunization are capable of causing perivascular infiltrates. We cannot exclude the possibility that the transferred cells produced a soluble encephalitogenic agent in the recipient rat. Nor does failure of passive transfer with serum exclude the possibility of a humoral agent with a primary effect on permeability. However, the present investigation has revealed a previously unrecognized rapidity in the development of encephalitogenicity in lymph node cells which should be considered before discarding a direct role for lymphoid cells in initiation of perivascular inflammation in EAE.

Summary

The phenomenon of induced localization around thermal injuries in the brain permitted the demonstration of typical perivascular infiltrates of allergic encephalomyelitis (EAE) as early as 4 days after active immunization of rats with neural tissue and adjuvants. Five days after immunization, almost every animal had the specific perivascular inflammation of EAE in brain or spinal cord adjacent to zones of thermal coagulation necrosis. The heat injuries lowered the threshold for development of EAE in the adjacent parenchyma and facilitated the detection of the perivascular infiltrates. Furthermore, washed lymph node cell suspensions, but not serum, prepared from donors after only 5 days of immunization, transferred the disease passively and rapidly (1 day) to nonimmunized recipients. The speed with which lymph node cells assumed competence for production of EAE is similar to that reported in other forms of delayed hypersensitivity and should be considered in the assessment of humoral versus cellular theories of pathogenesis.

EAE lesions were produced by neural antigen in 5 days when the adjuvant was either pertussis vaccine or killed mycobacteria in oil. The combination of both adjuvants did not effect any further reduction in incubation period, although pertussis vaccine caused a change in intensity and character of lesions and clinical signs of EAE.

References


We are indebted to B. H. Brown for technical assistance; to Dr. H. B. Devlin of Parke, Davis and Company for generous gifts of pertussis vaccine; and to Morris Moritz for the photomicrographs.
Legends for Figures

All photomicrographs are derived from the margins of thermal injuries of brain or cord in rats that were killed 5 days after immunization. All sections were stained with hematoxylin and eosin.

Fig 1. Longitudinally cut cerebral vein and several venules have perivascular lymphocytic infiltrates, 5 days after immunization with spinal cord tissue and Freund's adjuvant. Zone of coagulation necrosis in right upper corner is the result of thermal injury produced 3 days before sacrifice. Eighteen EAE lesions were detected in this slide by scanning five cross sections of thermal injury at a magnification of 40×. × 135.

Fig 2. Zone of cerebral coagulation necrosis at top is 5 days old and has early cavitation and many phagocytes. Two vessels with lymphocytic infiltrates extend into necrotic zone (arrows). A third vessel, in lower left corner, is in adjacent viable parenchyma. Thirteen vessels were detected in this slide by scanning four cross sections of thermal injury. Immunization as for Fig 1. × 135.

Fig 3. The 5-day-old zone of coagulation necrosis on right is in posterior column of spinal cord. There are many lymphocytes (arrows) in arachnoid and pia and around two small parenchymal veins (5 days after immunization with spinal cord tissue, Freund's adjuvant, and pertussis vaccine). Twenty-one perivascular infiltrates were found in four cross sections of spinal thermal injury, and 14 in four cross sections of cerebral thermal injury. × 200.

Fig 4. The 5-day-old zone of cerebral coagulation necrosis at top has early cavitation, as in Fig 2. This control animal was immunized with testis tissue, Freund's adjuvant, and pertussis vaccine 5 days before sacrifice. There were no perivascular infiltrates in any cross section of this or other control rats. × 135.