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# Improvements for Consistently Inducing Experimental Allergic Encephalomyelitis (EAE) in Rats: I. Without Using Mycobacterium. II. Inoculating Encephalitogen into the Ear (39272)

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The animal model disease experimental allergic encephalomyelitis (EAE), has been used fairly extensively to evaluate possible drugs and natural immunoregulators that may be useful in treating multiple sclerosis (MS.) (1) and some immunological disorders (2). There are, perhaps, as many differences as similarities between these two paralytic CNS diseases, EAE and M.S. (3, 4). EAE in the laboratory animal will probably continue to be used not only (1) to investigate problems relating to the establishment and control of multiple sclerosis allergic encephalomyelitis or allergic neuritis in man (4) and other diseases that may involve cell-mediated immunity, but also (b) for future evaluation of potential immunoregulatory agents (2, 5).

EAE is normally induced in laboratory animals such as rabbits, guinea pigs, and rats by the injection of an encephalitogen, emulsified with Freund's complete adjuvant, i.e., a finely dispersed suspension of a heat-killed *Mycobacterium* in a mineral oil. The emulsion may be injected either intracutaneously (6), or into a footpad (7, 19), or into inguinal lymph nodes (5, 8). When the Newbould technique of injecting small quantities, 10–20 $\mu$l of the conventional encephalitogen/adjuvant-emulsion directly into inguinal lymph nodes is employed, the incidence of EAE in Lewis rats is 100% and paralysis, which characterizes severe EAE, usually becomes evident within 8 days after inoculation (5, 7, 8).

Use of these methods does, however, raise some problems particularly in evaluating possible inhibitors of cell-mediated immunity:

(i) What part does the mycobacterial component of the adjuvant play in the production of this EAE? We have observed that rats frequently developed polyarthritis around 16 to 22 days after the injection of an encephalitogen admixed with Freund's complete adjuvant containing quantities of *M. tuberculosis* (less than 10 $\mu$g/animal) that were nearly always insufficient to cause adjuvant arthritis per se (8).

(ii) When the development of EAE is successfully prevented with drugs, it is uncertain whether the drug action is directed primarily against the recognition of the mycobacterial component (or development of a pre-arthritic phase) rather than against the more specific process of auto-allergic sensitization to the encephalitogen.

(iii) The Newbould technique requires surgery to expose the inguinal nodes for inoculation of the encephalitogen, with concomitant stress (reflected in weight loss) even when the *strictest* aseptic technique was employed. This surgical stress could affect the general health of an animal or even enhance the disease, as has been discussed elsewhere (9).

(iv) As an alternative to surgery, even relatively small amounts of encephalitogen and *M.tb.* (0.02-ml total vol) inoculated into a footpad nearly always causes local inflammation (e.g., paw swelling), principally due to the adjuvant present and may sometimes drastically alter the half-life (toxicity) of applied drugs (10, 11).

EAE can be initiated in Lewis rats by omitting the mycobacterial component from Freund's adjuvant and is histologically identical with EAE induced with a mycobacterial adjuvant (12, 13, 14). Varying the oily component of the encephalitogenic emulsion permits use of alternate adjuvants for EAE induction (5, 15). This, in essence, largely circumvents problems (i) and (ii) cited above. By using the ear as a site of

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inoculation, problems (iii) and (iv) can largely be circumvented as well.

In this report, we compare some of these and other methods for inducing EAE in a susceptible strain of rat (inbred Lewis). EAE induced in the inbred Fisher strain, outbred Wistar strain, and Wistar–Fisher hybrid offspring ($F_1$ hybrid) is mentioned as well.

*Materials and Method.* Dried *Mycobact. tuberculosis (M.tb.)* (Central Veterinary Laboratory, Weybridge, England) was extracted with ether and ethanol (1:1 v/v), then finely ground in a mortar and pestle before despersion in the oily vehicle.

Highly inbred, male Lewis rats, 200 to 250 g (Microbiological Assoc., Walkerville, Maryland), were used for most of these studies. Two-hundred-gram inbred Fisher rats, 150- to 200-g outbred Wistar rats, and 125-g Wistar–Fisher ($F_2$ hybrid) offspring (Hilltop Laboratories, Chatsworth, California) were also used for these experiments. Spinal cord obtained from white Hartley female guinea pigs (Lab Associates, Kirkland, Washington) blended with four vol 0.25% aqueous phenol solution and frozen at −20° was used as the encephalitogen (GPSC). The preparation of the encephalitogenic emulsions, Freund's type adjuvants, and adjuvants containing no *M.tb.* are described fully elsewhere (5, 15).

These emulsions were injected either intranodally through a 30-gauge stainless steel needle (5, 8) or intradermally through a 26-guage needle into the pinna of the ear (5) or into the plantar region of a rear paw (19). Injections into the pinna of the ear were made into the middorsal side from the outer edge, angling toward the head and avoiding areas of blood vessels. These injections were made closer to the head than those made using the Kulka method (16). In this way a necrotic scab is not torn off by the rat displaying normal activity. Each injection totalling 20 $\mu$l in volume (containing 2.5 mg of GPSC and 10 $\mu$l of oil with or without 10 $\mu$g of *M.tb.*) was used throughout the studies.

Groups of three to four animals were placed on sawdust in plastic cages and permitted food and water *ad libitum*. Rats were weighed and scored on the day of injection (Day 0), Day 3, and daily thereafter. Each rat was observed and scored 0 to 10 points for signs of clinical EAE according to weight loss (assigning 1 point for each 20 g lost) throughout the period during which the disease occurred, totalling 5 points; tail flop, 1 point; posterior paralysis, 3 points; whole body paralysis or death, 5 points (5). Group averages were computed by using the maximum disability of each rat during the course of its disease. Each experiment used at least three, but not more than nine rats per encephalitogenic emulsion tested. Tabular data are based on the observations from 46 separate experiments conducted over 14 months. Rats were allowed to develop EAE, recover from clinical symptoms, and then were observed up to Day 30 to ensure that no recrudescence occurred, at which time they were sacrificed. We did not observe recrudescence in any of our undrugged rats. For studies in which organs were removed and blood analyzed, rats were allowed to begin to recover and then sacrificed 1–3 days after the maximum clinical symptoms of EAE were apparent, when a body weight gain was observed.

For the leukocyte attraction studies, 0.5 ml of the various oils was injected ip into Swiss/Webster male mice (Hilltop Laboratories, Chatsworth, Calif.). After 24 or 48 hr these mice were sacrificed by cervical dislocation, the peritoneal exudate was removed, washed once, and resuspended in 1 ml of saline. The resuspended cells were then counted as "Zaponin" resistent cells on a Model B coulter counter.

For skin test studies, 0.1 ml of the oil or emulsion to be tested was injected subdermally into shaved backs of Lewis rats. Skin lesion diameters (millimeters) were read at 24 and 48 hr after inoculation.

Blood for smears and albumin determinations was obtained by cardiac puncture and placed in centrifuge tubes containing small amounts of heparin. Blood smears were stained with Wright's stain and total WBC counts were obtained with a Model B Coulter Counter.

Sleep times were determined after injecting sodium hexobarbital (150 mg/kg, ip) (11).

*Results. EAE induction with and without*

*M.tb.* The data in Table I-B show that when *M.tb.* is added to the encephalitogenic emulsions containing oily vehicles other than mineral oil (15), the EAE which develops is as severe or even more so than that engendered by the conventional Freund's complete adjuvant-GPSC emulsion. Weight losses are similar as are onset days of clinical symptoms and total disability scores.

The data in Table I-C show than when *M.tb.* is omitted from encephalitogenic emulsions containing mineral oil or squalane or butyl palmitate or butyl stearate, onset of paralysis is several days later, and the disease itself is less severe than the disease that develops when *M.tb.* is added to any of these emulsions. Using only the oily vehicles as adjuvants, the incidence of EAE is not always 100%. However, by using some alternate oily vehicles, e.g. squalene or hexadecane, admixed with equal volumes of encephalitogen, an EAE is induced that is quite similar in all respects to that developing in the conventional model (with F.C.A. as the adjuvant), i.e., total disability score, weight loss, and onset of clinical symptoms. Note that here, the disease is induced with 100% incidence *without* needing a mycobacterial component in the adjuvant.

*Effects of varying the inoculation site.* Data in Table II show that 20 μl of the encephalitogenic emulsions prepared with squalene or hexadecane (containing 2.5 mg of GPSC but no *M.tb.*) was quite sufficient to induce EAE in Lewis rats when injected into any one of three different sites. Intranodal injections proved to be the most effective way by which EAE can be induced in the shortest period of time.

Rats which were injected in the footpad manifested a slight inflammation as swelling occurring at the site of inoculation. The onset of clinical symptoms was slightly later than in the node-injected animals. Injection into the ear delayed the time of onset even more. However, in each of these experiments weight losses and disability scores were quite comparable for each encephalitogenic emulsion used.

*Systemic effects of EAE induced with no M.tb.* Splenic and thymic atrophy may also indicate depletion of T cells associated with autoimmune diseases (17, 19). Thymic atrophy is evident in each group of rats exhibiting symptoms of EAE but most severe in the rats with EAE induced with GPSC and FCA. This evidence of further stress with FCA parallels the finding of concomitant

TABLE I. COMPARISON OF EAE INDUCED IN LEWIS RATS BY VARIOUS OILS ADMIXED WITH EQUAL VOLUMES OF ENCEPHALITOGEN AND INJECTED INTO INGUINAL LYMPH NODES.

| Encephalitogen with | Weight loss | Disability score[a] | Onset of EAE[b] | Incidence[c] |
|---|---|---|---|---|
| A. Controls | | | | |
| (Nondiseased) | (gain) | | | |
| Normal Lewis | 90.6 ± 3.9[d] | 0 | 0 | 0/31 |
| B. *M.tb.* and: | | | | |
| Mineral oil | 57.9 ± 3.8 | 6.5 ± 0.3 | 7.2 ± 0.3 | 34/34 |
| (F.C.A.) | 65.3 ± 5.4 | 8.3 ± 0.2 | 5.5 ± 0.7 | 8/8 |
| Hexadecane | 43.3 ± 3.4 | 6.9 ± 0.2 | 7.3 ± 0.2 | 13/13 |
| Squalene | 56.1 ± 7.8 | 6.7 ± 0.5 | 7.7 ± 0.5 | 13/13 |
| Squalane | 55.7 ± 7.9 | 5.8 ± 0.8 | 8.3 ± 0.3 | 6/6 |
| Butyl palmitate | 34.7 ± 4.7 | 5.6 ± 0.7 | 6.5 ± 0.3 | 7/7 |
| Butyl stearate | | | | |
| C. No *M.tb.*: | | | | |
| Hexadecane | 46.6 ± 4.0 | 5.8 ± 1.4 | 8.0 ± 0.2 | 23/23 |
| Squalene | 47.1 ± 2.7 | 5.8 ± 0.2 | 7.6 ± 0.2 | 30/30 |
| Squalane | 36.0 ± 4.0 | 3.5 ± 0.4 | 8.0 ± 0.2 | 24/28 |
| Mineral oil | 44.0 ± 3.4 | 4.0 ± 0.3 | 9.2 ± 0.2 | 19/21 |
| Butyl palmitate | 32.2 ± 6.6 | 3.3 ± 0.6 | 11.0 ± 1.2 | 8/13 |
| Butyl stearate | 5.2 ± 2.0 | 1.0 ± 0.3 | 9.7 ± 0.6 | 1/6 |

[a] Total disability score includes maximum weight loss and maximum clinical symptoms of EAE. Number expressed = average of all rats studied.

[b] Days after antigen inoculation.

[c] Number of rats exhibiting outward clinical symptoms of EAE/number of rats injected.

[d] Mean ± SEM

arthritis when *M.tb.* is used in the adjuvant initiate EAE. Hypoalbuminaemia, another parameter of adjuvant arthritis (18), is evident when EAE is initiated with *M.tb.* and when the antigen is inoculated into the footpad or ear of rats.

The total blood leukocyte count from rats inoculated in the inguinal nodes, although higher than normal ($19 \times 10^6$ cells/ml) does not differ significantly from that of the control, except in the group with squalene as sole adjuvant. However, as reported by Rosenthale (19), a partial reversal of the polymorphonuclear leukocytes (PMN):lymphocyte ratio occurred. This was less severe for squalene-induced EAE. Although the outward clinical symptoms of this latter EAE are as severe as the EAE induced with hexadecane or FCA, the total disease perhaps may not be as severe *or even more* drug sensitive than the others mentioned.

An increased sleep time, as indicating slower metabolism of barbiturate hypnotics is a measure of systemic response to acute inflammation leading to liver dysfunction that occurs with adjuvant arthritis (11). Slight increases in sleep times were found when rats were recovering from clinical EAE. The greatest increases were observed in rats with EAE initiated by encephalitogenic emulsions containing the mycobacterial component, particularly when the inoculation sites were the footpad or the ear.

*Enhancement of "marginal" adjuvants by M.tb.* There are many oily vehicles which, when admixed with *M.tb.* or other arthritogenic organisms, will not induce a polyarthritis in Lewis rats. We have assayed a few of these "marginal" oils, with and without *M.tb.*, admixed with GPSC to see if these oils might still induce EAE. The data are summarized in Table III. The adjuvanticity of all three oils was enhanced by the addition of *M.tb.* when assayed by EAE induction. In the case of oleyl alcohol or methyl oleate, adjuvant arthritis did not develop

TABLE II. COMPARISON OF EAE INDUCED IN LEWIS RATS AFTER INOCULATION OF THE ENCEPHALITOGEN AT VARIOUS SITES.

| Encephalitogen admixed with: | Amount injected | Weight Loss | Disability score[a] | Onset of disease[b] | Incidence |
|---|---|---|---|---|---|
| A. Freund complete adjuvant (F.C.A.) | | | | | |
| Node | 10–20 μl | 57.9 ± 3.8[c] | 6.5 ± 0.3 | 7.2 ± 0.3 | 34/34 |
| Footpad | 20 μl | 70.1 ± 6.9 | 7.3 ± 0.5 | 10.0 ± 0.5 | 7/7 |
| Ear | 20 μl | 58.5 ± 4.3 | 6.7 ± 0.4 | 12.0 ± 0.4 | 14/14 |
| B. Hexadecane | | | | | |
| Node | 10–20 μl | 46.6 ± 4.0 | 5.8 ± 0.3 | 8.0 ± 0.2 | 23/23 |
| Footpad | 20 μl | 28.8 ± 3.0 | 4.8 ± 0.5 | 9.5 ± 0.2 | 7/7 |
| Ear | 20 μl | 37.8 ± 4.4 | 4.4 ± 0.4 | 9.6 ± 0.3 | 19/20 |
| Squalene | | | | | |
| Node | 10–20 μl | 47.1 ± 2.7 | 5.8 ± 0.2 | 7.6 ± 0.2 | 30/30 |
| Footpad | 20 μl | 46.5 ± 2.9 | 5.8 ± 0.6 | 9.6 ± 0.3 | 6/6 |
| Ear | 20 μl | 45.9 ± 2.9 | 5.4 ± 0.4 | 12.8 ± 0.3 | 20/20 |

[a] See footnote to Table I.
[b] See footnote to Table I.
[c] Mean ± SEM.

TABLE III. EFFECT OF MARGINAL ADJUVANTS WITH AND WITHOUT *M.tb.* INITIATING EAE IN LEWIS RATS.

| Encephalitogen admixed with: | Average weight loss (g) | Total disability[a] score | Incidence[b] |
|---|---|---|---|
| A. *M.tb.* and Vehicle | | | |
| Oleyl alcohol | 0.8 ± 0.8 | 0.4 ± 0.4 | 1/5 |
| Dodecane | 16.8 ± 2.9 | 1.7 ± 0.4 | 2/6 |
| Methyl oleate | 28.9 ± 6.3 | 3.5 ± 0.5 | 14/15 |
| B. Vehicle only (No *M.tb.*): | | | |
| Oleyl alcohol | 5.5 ± 1.7 | 0.7 ± 0.2 | 1/8 |
| Dodecane | 7.9 ± 5.1 | 0.9 ± 0.3 | 3/9 |
| Methyl oleate | 12.2 ± 3.5 | 2.0 ± 0.4 | 12/17 |

[a] See Table I.
[b] No rats with clinical symptoms/number of rats injected.

(incidence <10%) when these oils were emulsified with *M.tb.* and injected into Lewis rats (15).

*"Modified" EAE in various rat strains.* Since EAE has been shown in these experiments to be initiated in Lewis rats without using a mycobacterial adjuvant with the oily vehicle, a further step was to assay these oils for adjuvant sufficiency in inducing EAE in other rat strains. Outbred Wistar and inbred Fisher rats were compared with the inbred Lewis along with a Wistar/Fisher $F_1$ hybrid (Table IV). The inbred Fisher strain is as sensitive as the inbred Lewis, to EAE induced with the oily (no *M.tb.*) encephalitogenic emulsion. However, the $F_1$ hybrid and the outbred Wistar are only moderately sensitive to EAE induced without mycobacterium.

These observations do not disagree with Paterson's reports (12, 13, 20) or that of Hughes *et al.* (21) when the disease is scored by outward clinical symptoms. Different sources and quantities of GPSC used may determine the incidence of EAE. In our experiments the amount of encephalitogen is much less (2.5 mg of GPSC) than that generally used by other investigators.

In 1961, Levine and Wenk reported a correlation between incidence of EAE and the degree of inflammation at the inoculation site. We repeatedly observed that the ear and the footpad become rather more inflamed in Lewis rats than in Wistar rats, and this correlated well with the EAE incidence in the susceptible (Lewis) vs nonsusceptible (Wistar) rat strain.

Changing the oily component of the encephalitogen may have altered its "inflammagenicity" as well. The presence or absence of even the small amounts of *M.tb.* (10 μg/inoculum) may influence the total inflammatory effect of the encephalitogen inoculum. Some experiments were carried out to find if the oil itself was inflammatory (Table V).

McMasters (23) has shown that there is a slight gradient of pressure from the tissue to lymph which is increased during the development of inflammatory edema and is important in lymph function. These facts could explain why the symptoms of EAE, induced via lymph node inocula, can be observed earlier than when the EAE was induced via footpad inoculation; in turn in less time than the EAE induced by ear injections of the encephalitogen. Since the ear is a small thin tissue, there is less swelling and restricted inflammation. The skin of the footpad when swollen becomes tense, causing pressure. Injection directly into the node eliminates the additional time component for the antigen to be translocated to the lymph node itself and therefore decreases the overall time before clinical symptoms appear.

Table V shows the numbers of leukocytes attracted in 24 to 48 hr by some oils injected into the peritoneal cavity of mice. Of special note are squalane and butyl palmitate. Squalane or butyl palmitate together with GPSC, induce a poor-to-fair EAE. When *M.tb.* is added to the emulsion, the EAE is slightly enhanced, but adjuvant arthritis develops several days later. Squalane and butyl palmitate when emulsified with *M.tb.* are good arthritogens, but only moderate

TABLE IV. COMPARISON OF EAE INDUCED IN SEVERAL RAT STRAINS (FOLLOWING ENCEPHALITOGEN INOCULATION INTO INGUINAL NODES OR EAR).

| | | Rat strain | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Encephalitogen admixed with: | Route | Incidence (%) | Wistar | Incidence (%) | Fisher | Incidence (%) | Wistar × Fisher $F_1$ hybrid | Incidence (%) | Lewis |
| Freund's complete adjuvant | Node | 87[a] | 2.4 ± 0.6[b] | 100 | 7.5 ± 0.8 | – | ND | 100 | 6.5 ± 0.3 |
| | Ear | 66 | 3.6 ± 1.5 | 33 | 1.3 ± 0.3 | 33 | 1.0 ± 1.0 | 100 | 6.7 ± 0.3 |
| Hexadecane | Node | 66 | 2.2 ± 0.7 | 100 | 6.7 ± 0.4 | 100 | 2.5 ± 0.5 | 100 | 5.8 ± 0.3 |
| | Ear | 16 | 0.3 ± 0.3 | 100 | 5.3 ± 0.3 | 100 | 5.7 ± 1.3 | 95 | 4.4 ± 0.4 |
| Squalene | Node | 0 | 1.0 ± 0.0 | 100 | 5.7 ± 0.4 | – | ND | 100 | 5.8 ± 0.2 |
| | Ear | 50 | 0.8 ± 0.3 | 100 | 6.0 ± 0.4 | 100 | 3.0 ± 1.0 | 100 | 5.4 ± 0.4 |

[a] Incidence = number of rats with clinical symptoms/number of rats inoculated (%)
[b] Mean ± SEM.

TABLE V. MOUSE PERITONEAL LEUCOTAXIS BY VARIOUS ADJUVANT OILS CORRELATED WITH THEIR IRRITANCY AND ADJUVANTICITY IN RATS.[a]

| Oil | Inflammation score: Peritoneal leucocyte count ($N \times 10^{6}$)[e] 24 hr | Peritoneal leucocyte count ($N \times 10^{6}$)[e] 48 hr | Inflammation score: Skin lesion diameter 24 hr | Skin lesion diameter 48 hr | Adjuvanticity for inducing: Arthritis[c] | Adjuvanticity for inducing: EAE[d] |
|---|---|---|---|---|---|---|
| None[b] | 2.9 ± 0.82 | 3.0 ± 0.3 | 0 | 0 | 0 | 0 |
| Hexane | – | 2.1 ± 0.0 | – | – | 0 | 0 |
| Oleyl alcohol | 5.8 ± 1.2 | – | 21.1 ± 0.6 | 23.4 ± 0.4 | 0 | <1 |
| Mineral oil | 2.7 ± 0.5 | 9.7± 2.6 | 10.6 ± 0.4 | 5.1 ± 1.9 | +++ | 4 |
| Squalane | 3.4 ± 0.2 | 9.0± 1.9 | 11.2 ± 0.2 | <5.0 | ++++ | >3 |
| Butyl palmitate | 5.5 ± 1.0 | 19.5± 2.6 | 10.7 ± 0.3 | <5.0 | ++++ | >3 |
| Hexadecane | 8.0 ± 0.3 | 24.0± 3.5 | 15.9 ± 0.4 | 16.3 ± 0.4 | ++++ | >5 |
| Squalene | 12.9 ± 1.1 | 34.3± 2.3 | 13.1 ± 0.8 | <5.0 | ++++ | >5 |

[a] Irritancy assayed by the skin lesion when injected subdermally into shaved backs of Lewis rats.
[b] Saline control.
[c] Adjuvant arthritis induced in Wistar and/or Lewis Rats. Reference 15, score 0 to ++++.
[d] EAE score = average for total disability score. Average based on EAE initiated without *M.Tb.* See Table I.
[e] $N$ = average total peritoneal leucocyte count per cavity. Data = mean ± SEM.

encephalitogenic adjuvants when used without *M.tb.* The correlation between the chemotactic activity of these particular oils in the peritoneal cavity of mice and with the severity of the EAE they induce in rats is a curious but interesting one.

*Discussion.* If EAE is to be used successfully as an experimental assay for the development of new immunoregulatory agents (vis a vis antiinflammatory drugs), the potential interference from an adjuvant arthritis might be eliminated. The conventional procedure is to use frozen guinea pig spinal cord suspended in a phenol solution and emulsified with Freund's complete adjuvant. It is a reasonable conjecture that the animals' immune system then has to contend with two immunogenic insults: (i) one which results in "true" EAE, and (ii) another one which results first in a suppressed and then later with manifestation of a response to some component in a complete adjuvant leading to the adjuvant arthritis rats. EAE may develop in rats even when the normal mycobacterial component of a conventional "complete" adjuvant is omitted. The weight changes, which provide a quantifiable index for progressively monitoring the EAE, then reflect the true course of the disease. They are not confounded by the further weight loss induced by inflammation in response to the mycobacterial component. Studies underway in this laboratory have indicated that drugs such as cyclophosphamide may sometimes have a different effect on the EAE induced using a mycobacterial adjuvant from that on the EAE induced with non-bacterial adjuvants (i.e., oily vehicle only) (5). Weight loss, stiff rear limbs, or stiff tail, which result from the onset of adjuvant arthritis, could be misinterpreted as indications of paralysis caused by EAE. It can be argued that the onset of EAE is earlier than that of adjuvant arthritis (when the two diseases develop in the same animal), and this seems to be true when nondrugged controls are considered. However, there is a risk of scoring a "false EAE" which may in fact be a low-level arthritis, if a drug delays the onset of EAE beyond the 15th day after an encephalitogen-oil-mycobacterium emulsion is injected (24).

The use of an encephalitogenic emulsion prepared with a pure, chemically defined, oil is preferable on theoretical and logical grounds to using emulsions prepared with mineral oils, because of the variability in composition and sterility of mineral oils from different sources.

The special techniques and time required for intranodal injections need not be elaborated here. It is sufficient to say that it requires much practice even though it proves to be the most effective method of those

discussed to induce 100% incidence of EAE. The temporary stress of surgery does not appear to enhance the disease.

Footpad injections although easier to administer without practice, do place additional stress on rats by allowing a large area of inflammation to develope and for longer periods of time. Hence, at the onset of EAE, the animals may be contending with an inflammatory insult as well as with an immunological insult, as seen by increased sleep time.

The ear route of inoculation thus offers two decided advantages to those investigators who may have to predose the animals with a drug. Such lympholytic drug as cyclophosphamide, dexamethasone, or paramethasone, given before the time of initiating disease, will shrink the nodes to sizes which are often very difficult to see. It is then virtually impossible to confidently inject the residual node mass with sufficient encephalitogenic emulsion to initiate EAE. Drug evaluation based on observations of EAE in animals under the latter circumstances is then particularly uncertain; i.e., was the drug truly ameliorating the EAE, or was insufficient encephalitogen introduced into the animal to initiate florid disease?

Using a combination of these methods does allow more opportunities for investigators to study EAE. Injection of an encephalitogenic "chemically defined oil" emulsion into the ear is easily carried out, and measured inocula are assured. We have repeatedly observed that 20 $\mu$l of this emulsion, when injected into one ear or one footpad, induced all the clinical signs of EAE. Less than 20 $\mu$l will result in variable responses, which seem to reflect the susceptibility of the particular animal to EAE.

Whether one chooses to use an intranodal, footpad, or ear route of inoculation, the omission of *M.tb.* is important in inducing a "refined" EAE, i.e., one not complicated with concomitant adjuvant arthritis.

*Summary.* Several methods of inducing experimental allergic encephalomyelitis (EAE) in rats were examined using different (i) rat strains, (ii) combinations of encephalitogen with different adjuvants, and (iii) sites of encephalitogen inoculation. The time course and severity of the ensuing diseases were determined and methods delineated for inducing a disease with limited variability and high incidence.

Omitting the mycobacterial component from the adjuvant eliminated the complication of adjuvant arthritis, which may develop after the appearance of EAE. Encephalitogenic emulsions prepared with an equal volume of frozen guinea pig spinal cord (GPSC) and hexadecane or squalene, injected into two inguinal nodes or one foot pad of Lewis rats, provided two quick and easy ways to induce EAE. Emulsions of encephalitogen with Freund's complete adjuvant or hexadecane, injected into the ear, also induced EAE but lengthened the time between the antigen inoculation and clinical symptoms which accompany the onset of EAE disease. However, injection into the ear offers an advantage over the Newbould technique (direct instillation of encephalitogen in pre-exposed lymph nodes), since the animals can be confidently predosed with drugs which may reduce lymphoid mass. Effects of local inflammation on systemic drug metabolism are also minimized when using the ear route.

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