HYPERACUTE ALLERGIC ENCEPHALOMYELITIS

Lymphatic System as Site of Adjuvant Effect of Pertussis Vaccine

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Experimental allergic encephalomyelitis (EAE) is characterized by disseminated perivascular infiltrates of mononuclear, hematogenous inflammatory cells in the central nervous system (CNS) of animals sensitized with CNS antigen. The hyperacute form of EAE is distinguished by an abundance of fibrin, edema and polymorphonuclear leukocytes in the exudate, by its clinical severity, its close resemblance to the human disease known as acute hemorrhagic necrotizing encephalopathy, and the special circumstances surrounding its induction.[1] Among the latter, the role of aqueous pertussis vaccine as an immunologic adjuvant is of particular interest. The mechanism through which this vaccine, universally employed in childhood prophylactic medicine, converts EAE to the accelerated, highly lethal, hyperacute form, is the subject of this study.

Previous studies revealed that an aqueous mixture of pertussis vaccine and CNS antigen produced the hyperacute form of EAE in rat strains of high susceptibility.[1] Transfer of living lymph node or spleen cells from donor rats with hyperacute EAE produced ordinary EAE in normal recipients.[1,2] The component of *B. pertussis* responsible for its adjuvant effect was not its endotoxin or heat-labile toxin, but was the histamine-sensitizing factor or a closely associated substance.[3] These studies have established the structural features of hyperacute EAE and its relation to ordinary EAE, but not the mechanism or location of pertussis action. The experiments described here indicate that this adjuvant effect does not occur at the original site of inoculation or in the target neural tissues, but rather in the regional lymph nodes which drain the site of CNS antigen inoculation.

Methods

Male and female Lewis rats (Microbiological Associates, Inc., Bethesda, Md.), usually weighing 250 to 350 gm, were maintained on Purina® Laboratory Chow

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and tap water *ad libitum*. EAE was induced by injections of previously frozen guinea pig, or occasionally rat, spinal cord tissue. The tissue was heated to 60° C for 45 minutes immediately before use to reduce bacterial contamination. In each case, it was injected into the right hind foot in one of two ways. Aqueous suspensions were prepared by homogenizing 8 parts of cord tissue with 2 parts of saline; the mixture was injected intradermally in a dose of 0.25 ml (200 mg wet weight of tissue) distributed equally among 5 of the 6 cutaneous pads on the sole of the right hind foot. Water-in-oil emulsions were prepared by first homogenizing 4 parts of cord tissue with 6 parts of saline and then emulsifying the homogenate in an equal volume of Freund's complete or incomplete adjuvant. Emulsification was aided by cycling between 2 syringes through a double-hubbed needle. The emulsion was injected intradermally in a dose of 0.05 ml (10 mg wet weight of tissue) in a single footpad. Freund's complete adjuvant consisted of 8.5 parts mineral oil (Bayol F), 1.5 parts emulsifying agent (Arlacel A), and killed tubercle bacilli (4 mg per ml); incomplete adjuvant lacked the tubercle bacilli.

Pertussis organisms were available as a concentrated vaccine (hereinafter referred to as vaccine). It contained approximately 200 billion organisms or 4 mg solids per ml. The vaccine was mixed with the aqueous antigen in a dose of 0.05 ml, replacing the saline, or 0.10 ml, in which case the total volume of inoculum was increased by 0.05 ml. When the vaccine was given separately, a dose of 0.05 ml was injected intradermally, on the dorsum of the right hind foot or elsewhere as indicated in the text, or a dose of 0.20 ml was injected intravenously (dorsal penile vein). All injections were performed with sterile precautions and the rats were anesthetized with ether. Splenectomy and carotid artery injections required the usual surgical procedures. Wounds were closed with metal clips. Techniques of passive transfer and some other special procedures are described in the text.

The earliest indications of EAE were paralysis and loss of tonus of the entire or distal end of the tail. Advanced EAE was manifested as weakness, ataxia, paralysis and urinary retention. Usually, the rats were not sacrificed until they were severely paralyzed. Rats that did not become paralyzed were sacrificed as soon as an improvement in their signs indicated that they had passed the acme of their disease. The CNS was fixed in Bouin's solution, other tissues in acetate-buffered formalin. The entire hindbrain in transverse sections and the entire spinal cord in longitudinal sections were embedded in paraffin, and were sectioned and stained by hematoxylin and eosin or phosphotungstic acid-hematoxylin (with the specificity for fibrin increased by differentiation in 30 per cent ferric chloride in 95 per cent ethanol[4]). Lymph nodes, adrenals and thymuses were weighed after fixation.

The efficacy of each experimental procedure was evaluated by its ability to convert EAE to the hyperacute form. This was done by counting the total number of perivascular exudates in the spinal cord that contained sufficient fibrin to be visible at 40 × magnification.[3] For convenience in presentation, these were scored as follows: 1+, 1 to 10 vessels; 2+, 11 to 20 vessels; 3+, 21 to 50 vessels; and 4+, more than 50 vessels.

Results

Topographic Dissociation of Pertussis Vaccine and CNS Antigen (Table I). Previous experiments demonstrated that pertussis vaccine acted as an adjuvant in the production of hyperacute EAE when mixed with aqueous CNS antigen.[1,3] In the present work pertussis vaccine retained its adjuvant property when injected separately into footpad sites of antigen deposition, into adjacent sites on the sole of the foot, or into the dorsum of the same foot. The onset of disease was rapid in

each instance; it progressed quickly to severe paralysis, and histologic sections revealed more than 50, and sometimes 100 to 200 vessels surrounded by fibrinous exudate. The administration of pertussis vaccine at more remote sites (opposite foot, neck, peritoneal cavity) was less effective, but not devoid of activity; all the animals developed clinical signs of EAE but the onset was not as rapid, nor was fibrin as abundant in the lesions as in rats with hyperacute EAE. Presumably, remote injections decreased, but did not eliminate, the possibility of a common lymphatic drainage for CNS antigen and pertussis. On the other hand, the intravenous injection of pertussis vaccine (a 4-fold increase of dosage over that used for local injections) was fully effective as an adjuvant in the production of hyperacute EAE. These rats exhibited severe paralysis and abundant fibrin was evident in the lesions, but, interestingly, the onset of clinical signs was not accelerated. Control animals that received aqueous CNS antigen but no pertussis vaccine usually had clinical signs of EAE. Their signs were less severe and less progressive. All these control rats showed histologic lesions of EAE (Lewis rats are susceptible to CNS antigen even without any adjuvant [5]), but the lesions contained little or no fibrin.

TABLE I

PRODUCTION OF HYPERACUTE EAE: DEPENDENCE ON TOPOGRAPHIC RELATION BETWEEN SITES OF ANTIGEN AND PERTUSSIS VACCINE INJECTIONS

Antigen in rt. footpads	Site of pertussis vaccine	No. of rats	EAE	
			Onset	Fibrin *
200 mg cord	Rt. footpads, mixed	10	7.8 days	3.7
" "	" " , same sites	5	8.0	2.6
" "	" " , adjacent	12	7.3	2.6
" "	Rt. foot dorsum	32	7.1	3.6
" "	Left " "	6	10.8	.8
" "	Neck	11	11.5	1.7
" "	IP	12	11.0	1.8
" "	IP †	4	12.3	2.3
" "	IV †	18	11.9	3.6
" "	None	32	12.5 ‡	.1
10 mg cord in FA §	Rt. footpad, mixed ¶	12	7.3	3.4
" " " "	Rt. foot dorsum	11	7.5	3.2
" " " "	IV †	5	9.8	3.8
" " " "	None	17	12.9 ‡	.1

Antigen and pertussis vaccine administered at same time. Tables I and II represent composite results of 8 experiments.

* Group averages of scores based on number of vessels surrounded by fibrin-rich exudate; scale 0 to 4+ as described in text.

† 0.2 ml; all others received 0.05 ml.

‡ In these groups only, there were some rats that exhibited no clinical signs; they were not included in the calculation of the average day of onset.

§ FA, Freund's adjuvant, incomplete. Similar results were obtained with complete Freund's adjuvant.

¶ Dried pertussis organisms incorporated in either aqueous or oily phase before emulsification; results identical, therefore combined.

In a previous study[1] we produced hyperacute EAE only when pertussis vaccine was mixed with CNS antigen in a completely aqueous system. In the present work, however, with variation in dose and technique, pertussis vaccine has exhibited equal adjuvant property when incorporated in water-in-oil emulsion. Dried pertussis organisms (0.4 mg per dose) were incorporated into Freund's incomplete or complete adjuvant, or into the aqueous antigen, before emulsification. Rats that received these preparations in the footpad exhibited the onset of clinical signs of EAE as early as 7 or 8 days after inoculation; all became paralyzed after an additional 1 or 2 days, and the histologic lesions were replete with fibrin. In contrast, rats that received the same dose of CNS antigen in Freund's complete or incomplete adjuvant but without any pertussis vaccine, had ordinary EAE (less severe signs, later onset, little or no fibrin in the lesions). The adjuvant property of pertussis organisms in replacing mycobacteria in Freund's adjuvant has been demonstrated in guinea pigs by Wiener, Tinker and Bradford[6] and by Shaw, Alvord, Fahlberg, and Kies.[7] Contrary to our results in Lewis rats, these authors found that pertussis organisms were only moderately effective and their guinea pigs developed the ordinary form of EAE.

With injections into the footpad of CNS antigen in water-in-oil emulsion, as with aqueous antigen, direct incorporation of pertussis vaccine was not required for activity. Hyperacute EAE was produced with equal facility by separate injections of pertussis either in the dorsum of the foot or intravenously. The incubation period was 1 to 3 days longer after the intravenous injection of the vaccine than after an injection by other routes, but exudation of fibrin was equally exuberant. In an experiment not recorded in Table I, an intravenous dose of 0.05 ml was as effective as the 0.2 ml dose usually used by this route.

Temporal Dissociation of Pertussis Vaccine and CNS Antigen (Table II). Within certain limits hyperacute EAE could be produced when CNS antigen and pertussis vaccine were given at different times. Pertussis vaccine introduced into the dorsum of the foot 4 days before the aqueous antigen was injected into the footpads had little adjuvant effect, but full effect was obtained when the pertussis vaccine followed antigen by 2, 4 or 7 days. The incubation period before the onset of signs of EAE lengthened progressively, however, as pertussis administration was delayed. Intravenously introduced pertussis vaccine converted EAE to the hyperacute form whether given 5 days before or 7 days after the aqueous CNS antigen. It was interesting that even the prior administration of pertussis vaccine by the intravenous route failed to reduce the incubation period to the 7- to 8-day interval character-

istic of the hyperacute EAE produced by the local injection of pertussis vaccine.

Delayed administration of pertussis vaccine into the dorsum of the foot was effective even when the antigen injected into the footpad was

TABLE II

PRODUCTION OF HYPERACUTE EAE: DEPENDENCE ON TEMPORAL RELATION BETWEEN ANTIGEN AND PERTUSSIS VACCINE INJECTIONS

Antigen in rt. footpad	Site of vaccine	Time of vaccine *	No. of rats	EAE	
				Onset	Fibrin
200 mg cord	Rt. foot dorsum	Day −4	6	10.0 days	.8
" "	" " "	+2	11	7.5	3.5
" "	" " "	+4	5	10.4	3.6
" "	" " "	+7	14	11.3	3.7
" "	IV	−5	5	9.8	3.8
" "	"	+7	11	14.6	3.0
10 mg cord in FA	Rt. foot dorsum	+7	6	11.3	4.0

* Pertussis vaccine administered at indicated number of days before or after antigen injection. Dose, 0.05 ml in foot and 0.2 ml intravenously.

incorporated in Freund's adjuvant, but prior administration has not been tested.

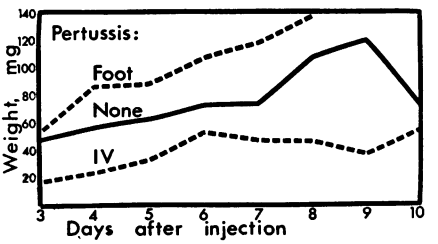
The Regional Lymph Node in Hyperacute EAE

The adjuvant effect of pertussis vaccine when given in a different but nearby location or at a different time than the antigen, and the lesser effectiveness when given in remote sites, suggested that the regional lymph node was the main locus of its action. To determine if this hypothesis could account for the effectiveness of the intravenous route, or for the difference in incubation periods between local and intravenous routes, the following experiments were performed. The right popliteal and lumbar lymph nodes were examined in rats sacrificed at various times after the inoculation of guinea pig cord antigen in Freund's incomplete adjuvant. On the day of inoculation, the animals were given an injection of pertussis vaccine (0.05) ml into the dorsum of the right foot, 0.2 ml vaccine intravenously, or no vaccine at all.

No Pertussis Vaccine Administered. The draining nodes were hyperplastic and contained clear vacuoles of oily inoculum after 3 days. During succeeding days the enlargement progressed (Text-fig. 1). After 7 days, the sinusoids contained a few polymorphonuclear leukocytes, some eosinophilic plasmatic exudate and small amounts of fibrin but no thrombi. Epithelioid cell granulomas appeared at 10 days.

Pertussis Vaccine Injected into the Dorsum of the Foot. The draining nodes were similar to those described above after 3 days (Fig. 1).

Subsequently, they enlarged more rapidly (Text-fig. 1). After 5 days, there was a marked change in appearance. The subcapsular sinusoids were dilated and contained polymorphonuclear leukocytes and plasmatic exudate. Six days after inoculation, the sinusoids contained fibrin



TEXT-FIG. 1. Weights of popliteal lymph nodes from rats sacrificed at various intervals after the injection of spinal cord antigen in Freund's incomplete adjuvant into a footpad. Groups of rats were given no additional treatment (solid line) or pertussis vaccine (interrupted lines) either into the dorsum of the same foot or intravenously. Each point on each curve represents the average of 3 animals.

thrombi and leukocytes. The thrombosis progressed during succeeding days and was noted in deeper sinusoids and in afferent lymphatic vessels as well.

Pertussis Vaccine Administered Intravenously. The draining nodes were smaller and less cellular than in either of the above groups, although they appeared larger than normal (Text-fig. 1 and Fig. 2). There was only slight enlargement subsequently. Despite these remarkable differences in size and cellular composition, the sinusoids revealed an accumulation of leukocytes and progressive thrombosis after 5 days, as severe as the changes which followed local pertussis inoculation (Figs. 3 to 5). After both routes of pertussis vaccine administration, thrombosis was restricted to nodes draining the site of CNS antigen inoculation, and was absent from distant nodes (mesenteric, left popliteal, axillary).

Thrombosis in the sinusoids of hyperplastic lymph nodes draining local sites of pertussis vaccine inoculation has been described before.[1,8] It was unexpected to find the same change in the much smaller lymph nodes draining the antigen emulsion after intravenous injection of pertussis vaccine. It is unlikely that the two effective methods for producing hyperacute EAE also produced the same lesion in the draining lymph nodes as a coincidence. On the other hand, no claim is made that sinusoidal thrombosis *per se* is important in the pathogenesis of hyperacute EAE. It is more likely an easily detected signpost of an underlying change caused by pertussis vaccine.

Certain differences were noted in the sites of inoculation of CNS

antigen in the footpads. Rats that received pertussis vaccine in the dorsum showed slightly more inflammation than those without such treatment. The lesions in rats that received pertussis vaccine intravenously exhibited fewer inflammatory cells but more edema at all stages.

Stress. Leukocytosis. It was important that thrombosis of lymph node sinusoids occurred at the same time after the local as after the intravenous introduction of pertussis vaccine. Therefore, the delay in the onset of hyperacute EAE after the intravenous administration of pertussis vaccine compared to its local introduction could not be attributed to a delay in the pertussis organisms (or their active fraction) reaching the nodes. The relative depletion of the lymph nodes after the intravenous injection of pertussis vaccine suggested an alternative explanation: nonspecific stress.[9] Following pertussis vaccination, Chedid and Boyer described adrenal hypertrophy which they attributed to stress,[10] and Schayer and Ganley found an increased content of blood corticosteroids.[11] The following experiment explored this possibility (Table III and Text-fig. 2).

TABLE III

STRESS CAUSED BY PERTUSSIS VACCINE

	Thymus weight			*Adrenal weight*		
Day	No vaccine	Dorsum vaccine	IV vaccine	No vaccine	Dorsum vaccine	IV vaccine
0	489 mg	489 mg	489 mg	16 mg	16 mg	16 mg
3	403	380	344	18	16	20
4	490	557	350	20	18	22
5	417	403	343	22		23
6	390	413	330	22	20	24
7	397	437	312	20	18	22
8			353			26
9	350	417	268	20	20	27
11	417	395	379	22	20	27
17			263			28

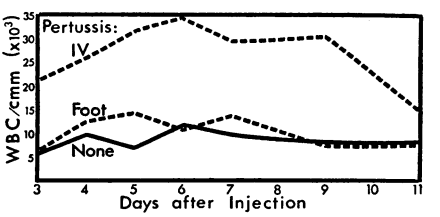
Each value is an average for a group of 3 male Lewis rats sacrificed on the day indicated, except values at zero time are based on 9 normal untreated rats from the same shipment as the experimental animals. All rats except normals received Freund's incomplete adjuvant into the right footpad on day 0 to mimic the effect of our usual injection without actually producing EAE. Pertussis vaccine is injected at the same time, as indicated. Body weights on day 0 were approximately 300 gm. Blood counts in these animals are presented in Text-figure 2.

All rats except normal controls were given 0.05 ml of an emulsion made of equal parts of saline and Freund's incomplete adjuvant in order to mimic the usual injection. CNS antigen was omitted because EAE itself is a stressor and causes increased serum corticoid levels[12] that would confuse the results. Some rats were given no further treat-

ment. Others, at the same time, were given 0.05 ml pertussis vaccine in the dorsum of the right foot or 0.2 ml intravenously. Three rats from each group were sacrificed at intervals (Table III).

The injection of Freund's incomplete adjuvant alone or with pertussis vaccine into the dorsum of the same foot caused an insignificant loss of body weight, only small and irregular changes in the thymus and adrenal weights and in the histologic appearance of the spleen, and only minor and transitory leukocytosis.

In contrast, the intravenous administration of pertussis vaccine caused an obvious indisposition and an average loss in weight of 21 gm in only 1 day. This loss was not made up later. The thymuses underwent rapid and moderately severe atrophy while the adrenals exhibited slower but progressive hypertrophy. There was less local inflammatory response to Freund's incomplete adjuvant, smaller draining (popliteal and lumbar) lymph nodes and depleted non-draining (mesenteric) nodes compared to the other groups. These changes could be caused, at least in part, by a nonspecific stressful effect of intravenous pertussis. Only the changes in spleen and blood count could not be related to stress. The spleen was enlarged due to an increase of red pulp. Germinal centers were prominent and demarcations among corpuscles, perifollicular sheaths and red pulp were blurred. The splenic changes were probably caused by antigenic stimulation. Lymphocytes in spleen were reduced in number, as they were in lymph nodes. The white cell counts were markedly elevated, a well known result of intravenous pertussis vaccination.[13,14] Right atrial blood counts are charted in Text-fig. 2; left



TEXT-FIG. 2. White blood cell counts from the right atrium (same animals described in Table III). All rats received Freund's incomplete adjuvant in a footpad to mimic the effect of our usual injection without actually producing EAE. Groups of 3 rats were given no additional treatment (solid line), or injections of pertussis vaccine (interrupted lines) either into the dorsum of the same foot or intravenously. The average white cell count of 9 normal, completely untreated rats was 7,100 per mm³. Intravenously administered pertussis vaccine caused severe, persistent leukocytosis; the count was still elevated to 12,150 per mm³ after 17 days.

atrial counts were usually 30 to 60 per cent of the right atrial values, but followed a similar pattern.

Severe leukocytosis was produced by the intravenous but not by the

local injections of pertussis vaccine. But both routes were effective in converting EAE to the hyperacute form. Therefore leukocytosis, *per se,* is not likely to be important in the adjuvant effect of pertussis vaccine.

Inasmuch as the intravenous administration of pertussis vaccine causes stress, and stress inhibits EAE,[15] it is reasonable to ascribe the slight delay in the onset of hyperacute EAE after the intravenous injection of pertussis vaccine to this nonspecific cause.

The Spleen. It has been noted above that the intravenous injection, but not the local introduction of pertussis vaccine caused changes in the spleen attributable to antigenic stimulation. The fact that the intravenous and local administration of pertussis vaccine were both effective in converting EAE to the hyperacute form indicated that the splenic changes were not essential factors; this has already been argued in connection with the leukocytosis. To confirm this contention, 4 groups of 8 to 10 rats each were given inoculation with guinea pig cord antigen in Freund's incomplete adjuvant. One day later, all rats were given 0.2 ml pertussis vaccine by intravenous route. The first group consisted of intact animals. The other groups underwent splenectomy 8 days before, 1 day after, or 4 days after the pertussis vaccine injection. All the rats developed severe paralysis and the lesions contained abundant fibrin. The average incubation periods were 8.1 days for the controls and 8.2, 8.2 and 8.9 days, respectively, for the groups undergoing splenectomy. Clearly, the spleen was not essential in the conversion of EAE to the hyperacute form by the intravenous administration of pertussis vaccine.

Effect of Pertussis Vaccine on Passively Transferred EAE. Some of the evidence indicating a crucial role of the regional lymph node in the adjuvant effect of pertussis vaccine could apply with equal force to the site of inoculation. In fact, the regional lymph node often mirrors the primary focus of inflammation. Such a parallel has been noted above, inasmuch as the intravenous injection of pertussis vaccine caused a decrease of inflammation in the site of inoculation and a decrease of hyperplasia in the regional lymph node following injections of Freund's incomplete adjuvant with or without CNS antigen. Therefore, it was important to determine whether pertussis vaccine had an adjuvant effect on EAE acquired by the passive transfer of living lymphoid cells, a form of EAE in which there is no site of inoculation of antigen. Passive transfer of EAE has been accomplished by intravenous injection into normal recipients of actively immunized lymph node[16] or spleen[2] cells from donors that had developed or were about to develop EAE. It is possible that some CNS antigen contained within the donor cells is transferred to the recipient. The amount of antigen carried over to the recipient is not likely to be large enough to create multiple "sites of inoculation"

and subsequent active sensitization of the recipient, especially by the relatively inefficient intravenous route. Other evidence against the possibility of active sensitization of recipients during the course of passive transfer includes the short incubation period (as little as 24 hours in some unpublished experiments), and the requirements that the donor cells be living and that the recipient be histocompatible or tolerant of the cells' histocompatibility antigens.[16]

Donor rats received guinea pig cord antigen in Freund's complete adjuvant into one of the right footpads. As soon as EAE developed, the animals were anesthetized, and their right popliteal, inguinal, axillary, lumbar, sacral and renal lymph nodes were excised aseptically. The nodes were cleaned of fat, rinsed in saline, minced and a cell suspension prepared by pressing through an 80-gauge stainless steel screen. The suspension was centrifuged for 15 minutes at 200 g and 1 to 8° C. The sedimented cells were suspended in saline, re-sieved, and injected intravenously into isologous rats. The recipients were normal or were pre- or post-treated with intravenous pertussis vaccine. In 3 experiments compiled in Table IV, the donor: recipient ratio varied from 1:1 to 4:1 and the dose of lymph node cells varied from 1.8×10^8 to 2.0×10^9.

TABLE IV

PRODUCTION OF HYPERACUTE EAE BY PASSIVE TRANSFER OF LYMPHOID CELLS AND INTRAVENOUS INJECTION OF PERTUSSIS VACCINE

Vaccine	No. of rats	EAE	
		Onset	Fibrin
Day—1	2	4.0 days	3.0
Day 0	8	6.4	3.9
Day+1	5	4.4	3.2
None	11	3.6	0.3

On day zero all rats received living lymph node cells from isologous donors that had just developed EAE. Pertussis vaccine, 0.2 ml, administered intravenously to recipients as indicated above. Composite results of 3 experiments, each of which included controls (no pertussis vaccine).

All recipients developed EAE (Table IV). The normal recipients had onset 2 to 4 days after the passive transfer and some were severely paralyzed. They had ordinary EAE with little or no fibrin around the vessels. The pertussis vaccine-treated recipients had onset 3 to 10 days after passive transfer, with equal or more severe signs. Their EAE lesions contained plentiful fibrin. In two other experiments, similar but less regular results were obtained with passive transfer of spleen cells from appropriately immunized donors (see reference 2 for method of immunization) into pertussis treated recipients.

This is the first time that the hyperacute form of EAE has been produced by passive transfer. All previous transfers, whether from donors with ordinary EAE[16] or with hyperacute EAE,[1,2] have produced ordinary EAE in the recipients. In the present context, these experiments indicated that the intravenous administration of pertussis vaccine had an adjuvant effect in the induction of hyperacute EAE even in a system in which there was no local site of CNS antigen inoculation.

Role of Recipient's Lymphatic System in Passive Transfer. These results with passively transferred EAE permitted the inference that the site of inoculation of antigen was not the crucial locus of pertussis vaccine effect in actively produced hyperacute EAE. These findings, and our other data, focussed attention on the regional lymph node. But, in actuality, if there is no local inoculation site in the passive transfer system, neither is there any draining lymph node! If pertussis vaccine influences both the active and passive forms of EAE through identical or related mechanisms, then the lymphatic system of the recipient must have some role in the passively transferred disease. This role was demonstrated in the following experiment.

Sixteen donor Lewis rats were subjected to active sensitization with guinea pig cord antigen in Freund's complete adjuvant which was introduced into the right footpad; pertussis vaccine was injected into the dorsum of the foot. As soon as EAE developed, the draining lymph nodes were removed, processed as described above and injected intravenously into 4 primary recipients (donor:recipient = 4:1; 9.6×10^8 cells per recipient). Three days later, the primary recipients were sacrificed and almost all of their lymph nodes were removed, processed and injected into a single secondary recipient (6×10^8 cells). Their spleens were pooled, processed and injected into another secondary recipient (2.1×10^9 cells). The secondary recipient of lymph node cells exhibited clinical signs of EAE and both secondary recipients showed lesions of ordinary EAE when sacrificed 3 days after the secondary passive transfer.

This experiment proved that cells with the ability to produce EAE reside in the recipient's lymph nodes and spleen during the incubation period after passive transfer, just as similar cells reside in the draining lymph nodes during the incubation period after active sensitization. This finding is an essential basis for the hypothesis that pertussis vaccine exerts its adjuvant effect in the lymphatic system in both the active and passive forms of EAE.

Does Pertussis Vaccine have a Direct Effect on the CNS? Pertussis organisms contain endotoxin,[3] and certain endotoxins have been reported to cause transient damage to the blood–brain barrier in rabbits

after intravenous or intracarotid injection.[17,18] Therefore, it is possible that pertussis vaccine may convert EAE to the hyperacute form by a direct effect on the CNS. Some of the data presented above do not support this possibility: (1) pertussis vaccine was much more effective in converting EAE to the hyperacute form when injected into the right foot (previously given injections of CNS antigen) than in other peripheral sites (left foot, neck, peritoneal cavity); (2) pertussis vaccine was equally efficacious in converting EAE to the hyperacute form after injection into the right foot or intravenously, although the latter route should provide more ready access to the CNS; (3) the incubation period was longer after intravenous than after foot injections.

In addition, no clinical or histologic evidences of a direct effect on the CNS were detected in rats after 10 days if they had received no treatment except the intravenous or intracarotid injection of 0.2 ml pertussis vaccine (4 and 6 animals, respectively).

Finally, the effects of intracarotid injection of pertussis vaccine on both the passive and active forms of EAE were studied. This route ought to provide the best opportunity to observe a direct effect of pertussis vaccine on the CNS, particularly the forebrain. Three rats were given an intravenous passive transfer of 2.6×10^8 lymph node cells from donors with EAE (donor: recipient = 4:1), and 5 rats were sensitized by the injection of guinea pig cord antigen emulsified in Freund's complete adjuvant. Shortly thereafter these were given 0.2 ml pertussis vaccine into the left common carotid artery. The artery was doubly ligated to prevent hemorrhage (unilateral ligation is innocuous in Lewis rats[19]).

All recipients of the passive transfer developed hyperacute EAE after 10 days. All 5 actively sensitized rats developed hyperacute EAE after 9 days; popliteal and lumbar nodes draining the sites of antigen–adjuvant emulsion exhibited thrombosis of sinusoids similar to that observed after the intravenous injection of pertussis vaccine. In all 8 rats with hyperacute EAE produced by both passive and active methods, the lesions were found in the usual sites of predilection, spinal cord and hindbrain, and only to a minor degree in the forebrain areas that received the first effects of intracarotid pertussis vaccine injection. The incidence and distribution of lesions did not differ from those in the control groups in which pertussis vaccine was administered into the foot or intravenously. Although all experimental and control rats showed abundant fibrinous exudates in the spinal cord, only one rat exhibited a single fibrin-containing lesion in the forebrain, and this was a control animal (vaccine in the right foot).

The failure of the intracarotid introduction of pertussis vaccine to

cause brain lesions or to enhance or localize EAE lesions in the forebrain, and the relative activities of pertussis vaccine injections in various parts of the body, indicated that the adjuvant effect was not a direct action on the CNS.

Discussion

Alvord, Shaw, Fahlberg and Kies [20] found that the separate injections of CNS antigen and Freund's complete adjuvant produced EAE provided they were given into the same site.[20] Aspermatogenesis [21] and hypersensitive reactions to ovalbumin [22] have been produced by the injection of antigen and Freund's adjuvant into separate sites provided that both were on the same side of the midline and presumably had common lymphatic drainage. The present work has also focussed attention on the draining lymph node, but has revealed a much greater latitude in the permissible dissociation, topographically and temporally, between antigen and pertussis vaccine adjuvant. In fact, pertussis vaccine exhibited an adjuvant effect even after intravenous injection, and even when given so late after the injection of CNS antigen that signs of EAE had developed.[23] In mice, pertussis vaccine introduced into the peritoneal cavity caused an increase of EAE following the intradermal injection of CNS antigen [24] and anaphylactic sensitization following the subcutaneous injection of heterologous erythrocytes.[25] These properties of pertussis vaccine may be due to its aqueous nature which might facilitate entry into antigen deposits in draining lymph nodes. But the fact that pertussis vaccine had an adjuvant effect on EAE produced by passive transfer suggests the existence of additional modes of action.

Morse has attributed the lymphocytosis that follows pertussis vaccine inoculation in mice to mobilization of lymphocytes from the nodes into the blood stream.[14] Our results indicate that the degree of leukocytosis *per se* is not a crucial factor. But measurements on the peripheral blood reflect the output of the entire lymph node system. It is possible that local pertussis inoculation induces a discharge of immunopotent cells from draining nodes into the blood without causing a detectable effect on the total white cell count. Therefore, pertussis vaccine, whether administered locally or intravenously, whether given to rats with actively or passively induced EAE, may act by a mechanism closely related to that which causes leukocytosis. In broader terms, pertussis vaccine probably influences the kinetics of sensitization, multiplication or release of cells with encephalitogenic potency in lymphatic tissue. This effect may be related to the increased DNA and protein production and turnover in lymph node cells caused by pertussis vaccine.[26,27]

This hypothesis is supported by the evidence of a topographic rela-

tionship between sites of antigen and pertussis vaccine inoculations, and by the exclusion of the local inoculation site, spleen and CNS as primary targets. Furthermore, it is reinforced by the fact that the adjuvant effect of pertussis vaccine, whether administered locally or systemically, is accompanied by a specific morphologic change in the regional node draining the site of CNS antigen inoculation. Finally, it is based on the assumption that the similar effects of the local and intravenous administration of pertussis vaccine on the active production of EAE, and the intravenous administration of pertussis vaccine on the passive production of EAE, are due to operation of a common mechanism. Our theory may fail if this unitarian assumption is incorrect. For example, the relative lymph node depletion and other changes that followed intravenous pertussis alone are attributed to a secondary action, nonspecific stress, and we discount their importance in development of hyperacute EAE. It is conceivable that the relatively depleted lymph nodes observed after the intravenous administration of pertussis vaccine favor development of hyperacute EAE by providing more room for the development of cells specifically immunized against CNS antigen. Provisionally, we reject this alternate hypothesis because it cannot apply to the swollen, hyperplastic nodes found after local pertussis inoculation.

Summary

The hyperacute form of experimental allergic encephalomyelitis (EAE) was produced by inoculation of CNS antigen into the footpads of Lewis rats in conjunction with the injection of pertussis vaccine. Pertussis vaccine was effective when mixed with antigen, injected separately into or adjacent to the site of antigen inoculation, injected into the dorsum of the same foot, or administered intravenously. Inoculations in the opposite foot, neck or peritoneal cavity were much less effective. Similar results were obtained with CNS antigen in aqueous suspension or in water-in-oil emulsion in Freund's complete or incomplete adjuvant. Pertussis vaccine was effective when given after the antigen, or under some conditions, before the antigen. The fact that an intimate association between CNS antigen and pertussis vaccine was not required, and the topographic relationships, suggested that the locus of adjuvant activity of pertussis vaccine was in the regional lymph node.

In the presence of local inflammation caused by CNS antigen and Freund's adjuvant, pertussis vaccine caused thrombosis of sinusoids in the regional lymph nodes. Thrombosis was found after both the local and the intravenous injections of pertussis vaccine, although other characteristics of the nodes differed markedly according to the route. Both

routes were effective in the conversion of EAE to the hyperacute form. The parallel between EAE and lymph node sinusoid thrombosis provided morphologic support for a pathogenetic role hypothesized for the regional lymph node. Certain differences between the effects induced by the local and intravenous administration of pertussis vaccine were caused by nonspecific stress that followed the intravenous administration only.

Neither splenic hyperplasia nor the degree of blood leukocytosis were crucial factors in the action of pertussis vaccine, inasmuch as they did not parallel the production of hyperacute EAE. Nor did splenectomy interfere with the adjuvant effect of pertussis vaccine introduced intravenously. There was no evidence of a significant direct effect on the CNS of pertussis vaccine even after intravenous or intracarotid inoculations.

Pertussis vaccine retained its adjuvant activity in that form of EAE produced by the passive transfer of living lymphoid cells from donors sensitized against CNS antigen. This is the first time that hyperacute EAE has been produced by passive transfer. In this type of EAE there is no local site of antigen inoculation. Assuming that pertussis vaccine acted by the same mechanism in both the passive and active diseases, this observation permits the inference that the local inoculation site was not a crucial place for adjuvant activity. Neither is there a regional draining lymph node in the passive form of EAE. The lymphatic system as a whole was involved, however, in the development of EAE by passive transfer. This was proved by serial transfer, in which lymph node and spleen cells taken from primary recipients of a passive transfer produced EAE in secondary recipients. This is the first report of serial transfer of EAE and it proves that cells with encephalitogenic potency reside in the lymphoid tissue of the recipient during the incubation period of passively acquired EAE.

All of these results indicate, or are compatible with, the lymphoid system as the site of adjuvant action of pertussis vaccine in both the active and passive forms of EAE.

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[*Illustrations follow*]

Legends for Figures

All photomicrographs were prepared from sections of popliteal or lumbar lymph nodes draining the site of inoculation of CNS antigen–incomplete adjuvant emulsion in right footpad. Pertussis vaccine was injected at the same time either into the same area of lymphatic drainage (dorsum of the right foot) or intravenously, as specified. Optically empty, rounded spaces in all the photographs represent droplets of the antigen–adjuvant emulsion.

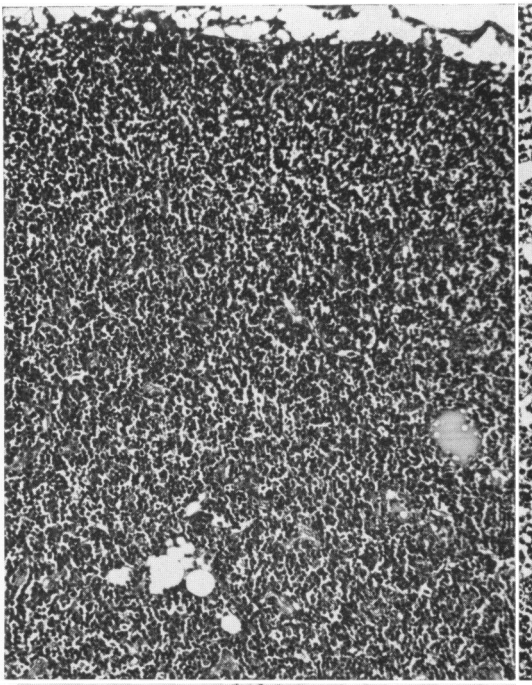
Fig. 1. Pertussis vaccine in dorsum of the foot. Four days after inoculation, there are droplets of antigen–adjuvant emulsion in a subcapsular (top) and a deep sinusoid (bottom left). There is intense hyperplasia of the lymph node tissue. Hematoxylin and eosin stain. × 115.

Fig. 2. Pertussis vaccine injected intravenously. Four days after inoculation, droplets of antigen–adjuvant emulsion appear in the subcapsular sinusoid (top) but there is much less hyperplasia than in Figure 1. Parenchymal sinusoids and vessels are relatively conspicuous because of the paucity of lymphocytes. See Text-figure 1 for weights of nodes. Hematoxylin and eosin stain. × 115.

Fig. 3. Despite differences in the response of the lymph node parenchyma, sinusoidal thrombosis commenced after 5 days following injection of pertussis vaccine either in the foot or intravenously (latter depicted here, 10 days after inoculation). There are darkly stained fibrin thrombi in the subcapsular sinusoid around the entire node and in 2 afferent lymphatic vessels (arrows). Phosphotungstic acid–hematoxylin stain. × 18.

Fig. 4. A higher magnification of the fibrin thrombus shown in the upper lymph vessel in Figure 3 and in the subjacent subcapsular sinusoid. Phosphotungstic acid–hematoxylin stain. × 115.

Fig. 5. Another lymph node from the same rat. There is a thrombus in an afferent lymphatic (above) with direct extension into the subcapsular sinusoid (below). The thrombus is undergoing organization (upper right corner). There is pericapsular inflammation. Hematoxylin and eosin stain. × 115.



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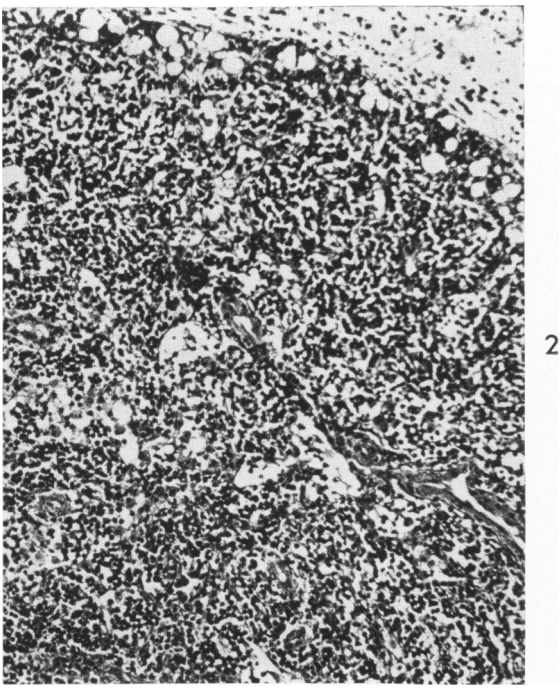

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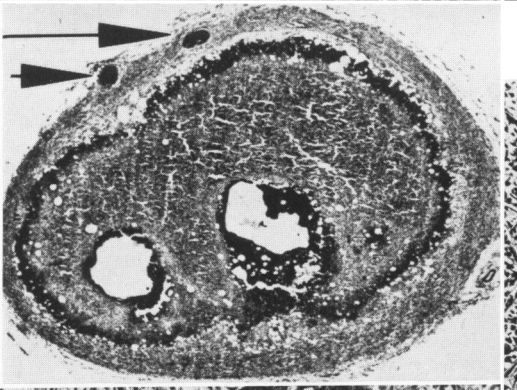

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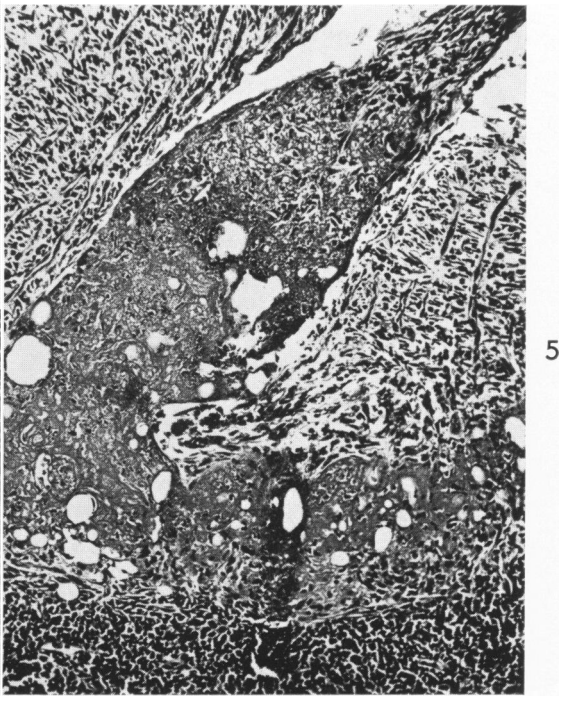

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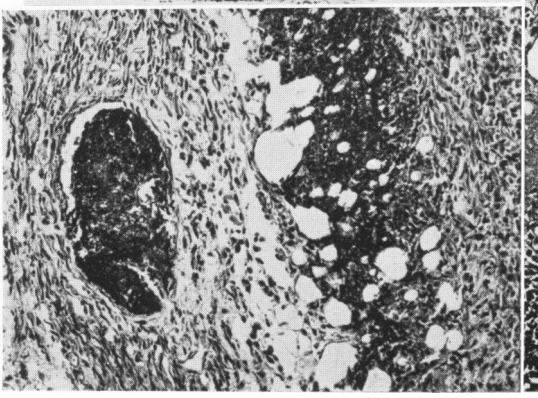

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