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SWELLING OF THE BRAIN IN MICE CAUSED BY PERTUSSIS VACCINE - ITS QUANTITATIVE DETERMINATION AND THE RESPONSIBLE FACTORS IN THE VACCINE

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SUMMARY: Intracerebral injection of vaccine into the mouse induced swelling of the brain. The swelling reached the maximum in the intensity by day 1 and persisted for several days. A method for quantitative determination of the brain-swelling activity of the vaccine was developed. A positive regression coefficient was found only between the brain-swelling and the lymphocytosis-promoting activities. Such activity was no longer shown with the vaccine heat-treated for 30 min at 80 C, but it was restored upon addition of the lymphocytosis-promoting factor (LPF) that caused no brain swelling by itself. The activity, therefore, was ascribed to cooperation of LPF and a certain heat-stable component other than endotoxin contained by pertussis vaccine.

INTRODUCTION

Administration of pertussis vaccine causes a variety of untoward reactions in children. The most distressing reactions are encephalopathy and neurological disturbances (1-6), which have occasionally been observed and in rare cases produced severe brain damage and even death (4,7,8). Such unfortunate incidents have frequently lowered the acceptance rate of the vaccines and have even brought on vaccination boycotts (4,5,6,9,10).

Although there have been numerous reports on the biological activities and toxic substances of *B. pertussis* cultures and of pertussis vaccine, there is little information concerning the possible factor(s) responsible for postvaccination encephalopathy or neurological disturbances. A major reason why such an unfavorable situation has lasted for a long time may be the total lack of an experimental model for studying the postvaccination neurological complications.

In this paper, we report that the intracerebral injection of pertussis vaccine induces the brain swelling in mice, and that such brain-swelling activity of the vaccine can be quantified by using the corrected weight of the brain as a response value. Furthermore, we show that at least two distinct and separable components contained in the vaccine may be involved in the activity.

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MATERIALS AND METHODS

Mice: Three-week-old female mice of JCI:NIH(s) strain were used unless otherwise is described. The NIH(s) strain (11,12) was introduced into Japan Clea Co. from the National Center for Drugs and Biologics, USA, by courtesy of Dr. C. R. Manclark (Office of Biologics) through Dr. Y. Nakase (Kitasato University, Tokyo, Japan) in 1979 and is being bred there as a closed colony named JCI:NIH(s). The mice were weighed every morning for 2 or 3 days after purchased from this company. The mice gaining body weight were randomly divided into groups of five each. Suckling mice of this strain were produced in this department. Baby mice born on the same date were randomly divided into groups of five to seven before used. Each group was assigned to a nursing mother mouse.

Reference pertussis vaccine: The National Reference Pertussis Vaccine (for toxicity tests) (13) was used and hereafter is referred to as R1. This is a dried product containing inactivated pertussis bacilli corresponding to 60 histamine-sensitizing units (HSU), 60 lymphocytosis-promoting units (LPU) and 1.380 body weight decreasing units (BWDU) per ampule. The unitage assignment was detailed in previous papers (14,15).

Diphtheria-pertussis-tetanus combined (DPT) vaccine: DPT vaccine produced by six different manufacturers in Japan were used. The pertussis component in these DPT vaccines produced before 1981 was a whole cell vaccine. Acellular pertussis vaccine or the precipitated purified pertussis vaccine was prescribed in the Japanese Minimum Requirements for Biological Products in 1981 (13).

Endotoxin, histamine-sensitizing factor (HSF) and lymphocytosis-promoting factor (LPF) activities of these vaccines were assayed according to Kurokawa et al. (13,14) and expressed in BWDU, HSU and LPU, respectively.

LPF preparation: A partially-purified LPF preparation, L21, was used. The method of purification was described in a previous paper (16). The preparation is being used as a laboratory reference instead of R1 vaccine for assaying LP activity of preparations containing little endotoxin, because both the coefficient and the intercept of regression of the leukocytosis response on dose of LPF are modified by endotoxin (17).

Experimental procedures: DPT and R1 vaccines were each diluted in saline and 0.02-ml portions were injected intracerebrally into mice. Ten mice were used for each dose, unless otherwise is stated. The mice injected with saline served as a control. One day after the injection, the mice were weighed and killed by exsanguinating from both the carotid and the jugular cut with a pair of scissors.

The brains of the killed mice were removed. Each brain was wrapped in a previously weighed piece of aluminium foil (3 x 10 cm) and stored in a small container surrounded with crushed ice. After all the brains were harvested, each was weighed, and the net weight was calculated by subtracting the weight of the aluminium foil from that of the wrapped brain.

Statistical analyses: Since there was a significant positive correlation between the brain weight and the body weight (see Results), the brain weight was corrected to its body weight equivalence for eliminating possible bias due to the differences in the body weight. Covariance analysis (18-20) was used for correcting the weight of the brain to its body-weight equivalence, and the corrected brain weight was used as the swelling response. Parallel line assay (18) was used to test the validity of assay results and to estimate the relative brain-swelling activity.

RESULTS

Correlation between the Brain and the Body Weights in Mice

The correlation between the brain and the body weights was tested in groups of mice of ages 1, 3, 5, 7, 15 and 21 days. Thirty to 40 mice of each age were used

for the test. There was a significant positive correlation coefficient between the brain and body weights in each age group. Figure one shows the correlation in the group of 21-day-old mice. The correlation coefficient calculated was 0.617. The finding indicates that the weight of the brain varied with its body weight even in mice of the same age until 21 days. Hereafter, the brain weight was used as the response after correction to its body-weight equivalence by covariance analysis.

The Brain Swelling due to Pertussis Vaccine

Groups of mice of various ages were intracerebrally injected with R1 vaccine. Fourteen to 16 mice of each age were used. One day after injection, the brains were harvested and weighed. The mean brain weight of each age group was compared with that of the corresponding control age group (Fig. 2).

A significant increase in the mean brain weight of the mice inoculated with the vaccine was demonstrated in all age groups, except in the 5 day-old mice whose increase in the mean brain weight was not significantly different from that of the control mice. A 6 to 12% increase in the mean brain weight was induced by injection of the vaccine irrespective of the age of mice, and almost the same variances in the brain weight were found in all age groups. Hereafter, 21-day-old female mice were used for convenience.

Fig. 1. Correlation between the brain and body weights in mice.

Thirty 21-day-old mice were used for the test. The correlation coefficient calculated was 0.617 ($F_{0.01} = 17.86 > F_{28}^1 = 4.196$). Each square represents an individual mouse.

Fig. 2. The brain swelling caused by pertussis vaccine.

Groups of mice of ages 2, 5, 7, 15 and 21 days were intracerebrally inoculated with R1 vaccine (●) or saline (○). The brains of the mice were weighed the next day.

Each point represents the mean brain weight of 14 to 16 mice, and each vertical bar the confidence interval of the mean.

*The difference between the vaccine- and saline-treated mice is significant at $p=0.05$. **The difference is significant at $p=0.01$.

Time Course of the Brain Swelling

Fifty mice were injected with a threefold diluted R1 and divided into five groups of 10 mice each. Each group was killed on days 1, 2, 3, 5 and 7 after injection, and the mean brain weights on each day were compared with those of the respective control groups.

As shown in Fig. 3, the brain swelling reached the maximum on day 1 and persisted for at least 5 days. The longer the period from injection to the brain harvest, the smaller the difference in the mean brain weight between the experimental and the control mice; no detectable difference was observed on day 7. In addition, the variance of the brain weight did not vary greatly with the day of the harvest. It is, therefore, preferable to determine the brain-swelling activity of pertussis vaccine on day 1 after injection.

Fig. 3. Time course of the brain swelling.

Mice were intracerebrally injected with 3-fold diluted R1 (●) or saline (○). Each point represents the mean brain weight of 10 mice and each vertical bar the confidence interval of the mean. **See the legend to Fig. 2.

Fig. 4. Log dose-response regression of the brain-swelling reaction to R1.

Each open circle represents the mean brain weight of 10 mice and each vertical bar the confidential interval of the mean.

Log Dose-response Regression of the Brain Swelling

Log dose-response regression of the brain-swelling reaction for R1 vaccine is shown in Fig. 4. The regression line did not significantly deviate from linearity over a range of doses of 0.02 to 0.0002 ml of R1, and the regression coefficient was estimated at 0.0212. A similar log dose-response regression was obtained with other DPT vaccines. In Fig. 5 depicted are the two regression lines of R1 and a DPT vaccine. Parallelism between the two lines is not denied, indicating that the brain-swelling activities of DPT vaccines can be estimated as relative values to that of R1 by parallel line assay.

Correlations between the Relative Brain-swelling Activity and the Endotoxin, HSF and LPF Activities of DPT Vaccine

Injection of pertussis vaccine into the mouse paw induces marked paw swelling. Our previous report (21) showed that the paw-swelling activity of pertussis vaccine is due to the combined action of endotoxin and HSF. Hence, we tried to determine whether its brain-swelling activity is also due to the action of any one or any combination of endotoxin, HSF and LPF.

Nine lots of DPT vaccine were quantified for the relative brain-swelling activity (RBSA), in addition to endotoxin, HSF and LPF activities, and partial regression coefficients between RBSA and the respective toxic activities were calculated by multiple regression analysis (19,20,22) (Table I). The partial regression coefficients between RBSA and endotoxin and between RBSA and LPF were positive, but only the latter was significant. The coefficient between RBSA and HSF was negative and not significant.

Fig. 5. Parallelism between the regression lines of R1 and a test vaccine. -o-; R1, -●-; the test vaccine. See the legend to Fig. 4.

Table I. *Partial regression coefficients between the relative brain swelling activity and the respective toxic activities of endotoxin, LPF and HSF*

Toxic component	Partial regression coefficient
Endotoxin	0.385
LPF	2.860*
HSF	-0.140

* probability, $P < 0.05$

Effect of Heating on the Brain-swelling Activity of Pertussis Vaccine

It is known that LPF is relatively heat labile; the LP activity is abolished upon incubation at 80 C for 30 min. The R1 vaccine heated at 80 C for 30 min was quantified for the brain-swelling activity. Such heating not only inactivated the LP activity but also greatly reduced the brain-swelling activity, to one-tenth of the initial activity (Fig. 6).

Restoration of the Brain-swelling activity of the Heat-inactivated Reference Vaccine by Addition of LPF

The heat-inactivated R1 vaccine was mixed with an equal amount of LPF of 10 LPU* per ml; the LP activity of the mixture was almost twice that of the unheated R1. The mixture was assayed for the brain-swelling activity (Fig. 6). The heated vaccine, whose RBSA was estimated at 0.1, completely restored its activity when mixed with LPF; the activity of the mixture, RBSA 6.8, was even higher than that of the unheated R1 vaccine. The LPF preparation itself may have had no brain-swelling activity, as no activity was detectable even by intracerebral injection of LPF in an amount 1.4 times that in the mixture used in the above experiment (Fig. 7).

* The unitage assigned for the laboratory LPF reference, L21 (17).

Fig. 6. Effect of heating on the brain swelling activity of R1 vaccine.

R1 vaccine was heated at 80 C for 30 min. R1, heated R1 and a mixture of the heated R1 and LPF (5 μ g of protein per ml) were each assayed for the brain-swelling activity.

◐; the mixture of heated R1 and LPF, ○; R1, ●; heated R1. See the legend to Fig. 4.

These results clearly indicate that the brain-swelling activity of pertussis vaccine is elicited by the cooperation of at least two factors; one is relatively heat labile, possibly LPF, and the other is heat stable.

Endotoxin or lipopolysaccharide is known to be heat stable. It is possible that the heat-stable factor involved in the brain-swelling activity is endotoxin itself which remained in the heated vaccine. Hence, we attempted to determine whether endotoxin can substitute the heat-stable factor that plays a role in the brain-swelling activity.

An endotoxin preparation, WE-1*, extracted from a culture of *Escherichia coli* 0-111 by the phenol-water method (23) was tested for the brain-swelling activity (Fig. 8). No activity was observed after injection of the endotoxin alone even at a concentration of 200 μ g per ml, which roughly corresponded to the endotoxic activity of the reference vaccine when assayed as the body-weight decreasing activity in mice (24). A mixture of equal amounts of LPF at 10 LPU per ml and endotoxin at 800 μ g per ml was determined for the brain-swelling activity (Fig. 9). It was only 0.1, although the brain-swelling activity of the heat-inactivated R1 was completely re-

* The biological activity of the preparation was described in a previous paper (25).

Fig. 7. The brain-swelling activity of LPF.
 -●-; R1, -o-; LPF. See the legend to Fig. 4.

Fig. 8. The brain-swelling activity of endotoxin.
 -●-; R1, -o-; endotoxin. See the legend to Fig. 4.

Fig. 9. The brain-swelling activity of a mixture of endotoxin and LPF. The mixture consisted of equal amounts of LPF at 10 μ g of protein per ml and endotoxin at 800 μ g dry weight per ml.

—●—; R1, —○—; the mixture of LPF and endotoxin. See the legend to Fig. 4.

stored by the addition of LPF at the same concentration (see Fig. 7). The result ruled out the possibility that endotoxin is the heat-stable factor remaining in the heated-R1 vaccine.

DISCUSSION

In the present study, the weight of the brain was used as a response for estimating the brain-swelling activity of pertussis vaccine in mice. The brain weight was corrected to its body weight equivalence for eliminating the effect of the body weight on the brain weight, and it was disclosed that pertussis vaccine induces the brain swelling in mice when injected intracerebrally. Injection of pertussis vaccine decreases the body weight of mice, reaching the maximum on day 1, due mainly to the endotoxic activity of the vaccine (24). It is therefore likely that the increase in the corrected weight of the brain in mice given the vaccine may result from the decrease in the body weight. Nevertheless, the increase in the brain weight was observed even when the body weight on the day of injection was used for correcting the brain weight instead of that on the day of harvest of the brain.

The swelling reached the maximum on day 1 after injection and persisted for several days. The intracerebral injection of *B. pertussis* into mice causes expansion of the ventricles with a large amount of exudate as a consequence of proliferation of the

bacteria on the ventricle wall (26), and the swelling of the head, therefore, is observed clinically. The brain swelling after administration of pertussis vaccine may be caused also by accumulation of exudate, since the swollen brain harvested on day 1 after injection showed histologically perivascular edema with some polymorphonuclear cell infiltration in the chorioid plexus, the cortex and the white matter, in addition to meningitis probably caused by the injury from the injection; no edematous was found in the brain of the mice injected with heat-inactivated vaccine.

Statistical analyses of the data on the endotoxin, HSF and LPF contents and the brain-swelling activities of DPT vaccines disclosed that the brain-swelling activity is correlated only with the LPF content. This finding is contrary to the observation that the mouse paw-swelling activity of pertussis vaccine is correlated with both the endotoxin and HSF contents (21). Furthermore, the latter activity was reproduced with a mixture of endotoxin and HSF preparations (21), while the former was not with LPF alone.

The brain-swelling activity of pertussis vaccine was relatively heat labile since it was abolished by heating at 80 C for 30 min. The activity of the heat-inactivated pertussis vaccine, however, was completely restored by adding an appropriate amount of LPF. This finding indicates that in addition to the heat-labile factor, possibly LPF, a heat-stable factor remaining in the heated vaccine is indispensable for inducing the swelling.

A mixture of *E. coli* endotoxin and LPF did not induce any brain swelling in mice, indicating that endotoxin could not substitute the heat-stable factor. A similar result was obtained with an endotoxin preparation of *B. pertussis* (data not shown). There was, however, another possibility that intrinsic integrate(s) in the heated pertussis cells such as endotoxin may play the role of the heat-stable factor. The possibility seems unlikely, however, from the circumstantial evidence that heated cholera vaccine, whose endotoxin activity was comparable to that of heated-R1 vaccine, did not induce any brain-swelling activity when injected together with LPF. Hence, the heat-stable factor may be some other toxic substance than endotoxin, LPF or HSF contained by *B. pertussis* cells. More work is necessary to identify the factor and to characterize its action. It should be noted that a kind of neurotoxin found in *B. pertussis* having a unique toxicity in guinea pigs was reported as being heat stable (27).

Intraperitoneal injection of the vaccine did not induce any detectable change in the brain weight in suckling or young adult mice for at least 3 days (data not shown). Some investigators, however, have reported an increase in cerebellar vascular permeability in the mice and rats injected intraperitoneally or intravenously with an HSF preparation or killed pertussis cells (28-30). Recently, there have been interesting reports stating that a 100% increase in the cerebellar cyclic GMP (cGMP) level in rats was found between 3 and 5 days after ip injection of pertussis vaccine

(31), and that such an effect was reproduced by injection of LPF (32). Therefore, we thought that the brain swelling would be produced by injecting LPF and the heat-inactivated pertussis vaccine separately at different sites; the former was injected intravenously and the latter intracerebrally. However, the intracerebral injection of heat-inactivated vaccine induced little or no brain swelling in the mice having been given intravenously 0.2 ml of 10 LPU per ml 2 days before (data not shown).

It is generally accepted that most cerebrospinal fluid (CSF) is formed in the ventricular system; the chorioid plexus contributes almost 80 to 90% of the CSF secretion (33,34). It is certain that the injection of pertussis vaccine into the brain temporally disturbs not only the blood-brain barrier but also the blood-CSF barrier. Such temporal disturbances of these barriers may also contribute to the brain-swelling activity of pertussis vaccine, though the weights of the brains of the mice given saline were not different from those of mice given no injection.

It is important to develop a useful animal model for evaluating neurological complications following vaccination with pertussis vaccine. Steinman et al. (35) described a murine model for pertussis vaccine encephalopathy; the mice that had an appropriate major histocompatibility genotype died with shock-like symptoms within 2 hr after immunization with bovine serum albumin and pertussis vaccine. Although the time course of this response suggests an anaphylactic reaction, the postmortem examination of the brain revealed diffuse vascular congestion and parenchymal hemorrhage in both the cortex and white matter. The brain-swelling activity of pertussis vaccine described in the present report may also be useful as an experimental model for studies on a factor(s) responsible for the postvaccination neurological complications. In contrast to the former, this model may be characteristic of involvement of little or no immunological reaction in inducing the brain swelling.

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