Effect of Lymphocytosis-Promoting Factor from Bordetella pertussis on Cerebellar Cyclic GMP Levels

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Dermonecrotic toxin (DNT), lipopolysaccharide (LPS), and lymphocytosis-promoting factor (LPF) were isolated from Bordetella pertussis and tested for neuroactivity. When injected intraperitoneally into rats, a dose of 0.13 mg of LPF per kg elevated the cyclic GMP level in cerebellum by approximately 70%, whereas DNT (0.5 mg/kg) and LPS (1.5 mg/kg) were without effect. This action of LPF on the central nervous system was dose dependent and did not require the administration of any additional agent, such as histamine.

Pertussis vaccination may affect the central nervous system and produce complications such as persistent screaming, shock, convulsions, and encephalopathy (14). Some of the effects are similar to the neurological complications following pertussis infection. The frequency of serious adverse reactions after injection with pertussis vaccine is about 1/3,000 vaccinated children (17, 32). A possible association between serious neurological illness and immunization with pertussis vaccine has been reported (22), but the mechanism of such adverse reactions is not known. There are as yet no experimental models for studying the effects of pertussis vaccine on the central nervous system (35). Most studies concern effects on the periphery and include an examination of the hypersensitization to histamine, serotonin, and bradykinin, active and passive anaphylaxis, and sensitization to stress and cold (23).

One would expect that a neurotoxin from Bordetella pertussis would affect the intracellular cyclic nucleotide concentrations in nervous tissue, and a preliminary study examined three brain regions: (i) hypothalamus, which is especially rich in histaminergic nervous tissue (18, 29); (ii) cerebral cortex, where electrophysiological effects (electroencephalogram) have been described after pertussis vaccination (19); and (iii) cerebellum, since pathological studies have shown changes in this part of the brain in mice after intracerebral pertussis infection (8). When a killed whole-cell vaccine was injected intraperitoneally into rats, a 100% increase was found in cerebellar cyclic GMP (cGMP) levels between 3 and 5 days after the injection (2). This effect was used to assay the neuroactivity of three purified toxins from B. pertussis (27): (i) dermonecrotic toxin (DNT), an intracellular protein toxin; (ii) lipopolysaccharide (LPS), a structural component of the outer membrane; and (iii) lymphocytosis-promoting factor (LPF), a membrane-associated protein toxin which is alternatively described as histamine-sensitizing factor or islet activating protein. The results showed that only LPF produced a significant increase in cerebellar cGMP levels and therefore may be responsible for the effects observed with killed whole-cell vaccine (2).

MATERIALS AND METHODS

Toxin preparations. The toxins were prepared as described elsewhere (3). In brief, DNT was prepared by extracting B. pertussis strain 18323 (National Bacteriological Laboratory, Stockholm, Sweden) with 1.0 M sodium acetate for 3 days at room temperature. After centrifugation, 10-ml volumes of the supernatant were chromatographed on a Sephacryl S-300 column (28 by 360 mm). Fractions of 5 ml were collected; 0.1 ml of the fractions containing DNT produced hemorrhagic necrosis (15 by 15 mm) when injected intradermally into rabbits. The 50% lethal dose in mice (18 to 20 g; NMRI, Eklund, Sweden) by the intraperitoneal route was between 0.25 and 0.5 ml. The protein content of the fractions, as determined by the method of Bradford (5), using bovine serum albumin as the standard, varied between 50 and 100 μg/ml.

LPS was extracted from bacteria by the method of Westphal et al. (34). The final preparation did not contain any detectable protein (determined as above). In the limulus assay (Pyrostat reagent kit, Millipore Corp., Freehold, N.J.), the final dilution producing a positive reaction was 0.1 ng/ml (wt/vol).

LPF, which binds to sialic acid-containing proteins, was prepared by affinity chromatography according to the method of Irons and MacLennan (20). However, in this study the method was modified by using fetuin instead of hapten in the ligand coupled to the Sepharose 4B matrix. Bacteria were grown in a liquid medium (6), and the culture filtrates were acid precipitated at pH 4.0 overnight at 4°C. The pelleted material was suspended in and dialyzed against phosphate-buffered saline, pH 7.2, containing 0.5 M NaCl and applied to a fetuin-Sepharose column equilibrated with the same buffer. After the column was washed, LPF...
TABLE 1. Effect of *B. pertussis* toxins on cerebellar cGMP levels

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cGMP (pmol/mg protein)</th>
<th>SEM</th>
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<tbody>
<tr>
<td>DNT (100 μg)</td>
<td>114 ± 18</td>
<td></td>
</tr>
<tr>
<td>LPS (300 μg)</td>
<td>89 ± 25</td>
<td></td>
</tr>
<tr>
<td>LPF (50 μg)</td>
<td>184 ± 40</td>
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</tbody>
</table>

The cerebellar cGMP concentration of the control rats was 3.6 ± 0.8 pmol/mg of protein for DNT and 2.9 ± 1.0 pmol/mg for LPS and LPF.

was eluted by raising the pH to 11.5 with 0.1 M glycine-NaOH buffer. The eluted LPF was then dialyzed against the starting buffer. The purified LPF did not contain any detectable sugar as measured by the phenol-sulfuric acid method (10). The hemagglutinating activity in the hemagglutinating test (25) was between 5,120 and 10,240 hemagglutinating units per mg of protein. This compares well with the activities of the highly purified preparations of Irons and MacLennan (20), who reported 6,000 to 8,000 hemagglutinating units per mg of protein, and with the crystalline preparation of Munoz et al. (24), who reported 5,000 hemagglutinating units per mg of protein.

**Assay of neuroactivity.** One-milliliter volumes of the toxin preparations were injected intraperitoneally into male Sprague-Dawley rats (180 to 200 g). Control rats were injected in the same way with phosphate-buffered saline containing 0.5 M NaCl. The animals were sacrificed 3 days later. Injection and decapitation were carried out between 8 and 10 a.m. to exclude effects of diurnal variations. After decapitation, the cerebellum was rapidly dissected on dry ice within 1 min. The cerebella were homogenized in 1.0 ml of ice-cold perchloric acid (7%, wt/vol), and the cyclic nucleotides were extracted according to Folbergrova (13). cGMP was measured by radio-immunoassay according to Steiner et al. (30), with standards containing tissue extracts in which cyclic nucleotides were hydrolyzed by incubation with bovine heart 3',5'-cyclic nucleotide phosphodiesterase (Sigma Chemical Co., St. Louis, Mo.) before addition to the radioimmunoassay. Protein was determined by the biuret method (16). Values for cGMP represent the mean ± standard error obtained from four cerebella, each assayed in triplicate.

**RESULTS AND DISCUSSION**

The DNT preparation was slightly contaminated with LPS (about 10 μg/ml), but did not contain any detectable LPF. No contaminating LPF or DNT activities could be detected in the LPS preparation, and no contaminating LPS or DNT could be detected in the LPF preparation. Table 1 summarizes the effects of intraperitoneal injection of the three toxins on cerebellar cGMP levels. It can be seen that DNT and LPS were without effect, whereas LPF gave about a twofold increase in cGMP concentration. The highest amount of DNT tested (0.5 mg/kg) corresponded to two to four times the 50% lethal dose in mice, and higher doses could not be used due to the lethality of DNT. It was expected that DNT would be without effect, since the whole-cell vaccine used in the previous report (2) did not contain any detectable amounts of DNT. The amount of LPS used (1.5 mg/kg) was 5 to 10 times more than the amount present in the dose of the whole-cell vaccine that gave the maximal increase in cGMP concentration. The lack of effect of LPS was also expected since the neurotoxicity is supposedly specific for *B. pertussis*, and the biological activities of LPS prepared from different species of gram-negative bacteria are very similar. If the neurotoxicity was associated with LPS, it would have been found in connection with other gram-negative bacteria.

LPF was tested at concentrations up to 50 μg of protein per rat, but a significant rise in cGMP concentration was seen at 25 μg/rat (= 0.13 mg/kg of body weight) (Fig. 1A). The observed differences, i.e., an increase from 2.9 ± 1.0 to 5.0 ± 0.7 (25 μg of LPF) and to 5.3 ± 1.1 pmol of cGMP per mg of protein (50 μg of LPF), were both statistically significant (*P < 0.05*). For comparison with the effects of LPF, the dose-response curve of a whole-cell vaccine is also included in Fig. 1 (note the different spacing of doses). Here significant increases in cGMP were found for all doses of vaccine tested (*P < 0.05*) (Fig. 1B).

The time course of the increases in cGMP levels (2) coincides with other physiological changes induced by LPF, including lymphocytosis, histamine sensitization, and passive anaphylaxis (25). When leukocytes were counted from the rats injected with LPF, a three- to fourfold increase was obtained for all doses of LPF (6.25 to 50 μg/rat), whereas significant increases in cGMP levels occurred first at 25 μg/rat. Indeed, the correlation coefficient calculated between cGMP concentrations and leukocyte counts for individual rats (data not shown) was as low as 0.33. The body temperature of the rats was also measured and was found to be normal during the course of the experiments, except that the rats injected with 50 μg of LPF showed an immediate decline in body temperature (about 2°C) and a reduction of the heart rate to half of the normal value. However, these values were restored to normal levels within 24 h, and hence the reduction in body temperature did not correlate with the lymphocytosis, which is in contrast to the correlation found in lethally infected mice (28).

We have shown that the LPF of *B. pertussis* affects the cGMP levels in rat cerebellum. The effect is dose dependent and represents an additional action of LPF on a parameter in the central nervous system. Whether LPF is present in the brain during human infection or injection of humans or animals with pertussis vaccine is not known. However, it has been shown by...
FIG. 1. Dose dependence of elevation of cGMP levels in rat cerebellum, using purified LPF (A) and a whole-cell vaccine (B). The whole-cell vaccine was prepared from strain 44122 (National Bacteriological Laboratory, Stockholm, Sweden) inactivated with thimerosal in a manner different from that used for other vaccine preparations (2). The cerebellar cGMP concentration of the control rats was 2.9 ± 1.0 pmol/mg of protein for LPF and 3.8 ± 0.5 pmol/mg for the whole-cell vaccine. * Significantly different from control (P < 0.05).

using $^{131}$I-labeled human serum albumin (1) that B. pertussis vaccine causes a measurable increase in cerebral vascular permeability in mice. Protein may also reach the brain via active transport (33). Thus, it is possible that LPF, a component of the vaccine, is present in the brain. The rise in cGMP concentration can be explained in various ways: (i) LPF acts on the α-adrenergic or the H$_2$-histaminergic receptors; (ii) LPF stimulates guanylate cyclase, in analogy with the action of cholera toxin (12, 26) and Escherichia coli heat-labile toxin (9) on adenylate cyclase, and of E. coli heat-stable toxin on guanylate cyclase (11); (iii) LPF affects the coupling between the above-mentioned receptors and guanylate cyclase; or (iv) LPF inhibits 3',5'-cyclic nucleotide phosphodiesterase which degrades cGMP.

The doses of LPF required to enhance cerebellar cGMP levels (0.13 mg/kg) are not comparable to those reported for LPF needed to induce other effects involving the nervous system, e.g., histamine sensitization (0.5 ng/mouse) and experimental allergic encephalomyelitis (20 ng/rat), since these latter effects both require the administration of a second agent, 0.5 mg of histamine per mouse and 200 mg of spinal cord cells per rat, respectively (24). There are, on the other hand, some reports where drugs have been injected intraperitoneally and their effects on cerebellar cGMP levels have been evaluated: 0.5 mg of apomorphine per kg increases cGMP levels by 25%, and 0.5 mg of haloperidol per kg decreases cGMP by 80% (4). It has also been shown that intraperitoneal injection of 0.05 mg of a highly toxic convulsive bicyclic phosphorus ester (4-isopropyl-1-phospha-2,6,7-trioxabicyclo[2.2.2]octane-1-oxide) (21) per kg and of other convulsive agents (7, 15) increases cerebellar cGMP levels. This is in line with the possibility that the increase in cerebellar cGMP levels induced by LPF might be related to the neurological complications, such as convulsions, observed after vaccination.

The side effects of the vaccine taken together with its disputed efficacy have lead to low acceptance of the vaccine in the United Kingdom (31) and other areas of the world. Although LPF is toxic, it may be an important immunogen in pertussis vaccine. It is also probable that LPF will be included in all effective vaccines, so it is equally important that the toxicity of LPF (and its effects on the brain) is controlled in order to make the vaccine safe and acceptable.

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LITERATURE CITED


