Biphasic Effect of Pertussis Vaccine on Serum Insulin in Mice

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Administration of pertussis vaccine, consisting of whole, killed *Bordetella pertussis* organisms, causes hyperinsulinemia and enhanced secretion of insulin in response to a variety of secretagogues in rats and mice. In examining the time course and properties of this phenomenon, we discovered two distinct and separate effects of the bacteria on glucose and insulin levels in mice. First, a heat-stable (80°C for 30 min) component causes a brief hyperinsulinemia which is measureable by 1 h, maximal at 8 h, and ends in less than 48 h. This effect appears to be due to *B. pertussis* endotoxin, is mimicked by *Escherichia coli* endotoxin, and is associated with a transient, mild hypoglycemia. Second, there is a heat-labile component of the *B. pertussis* organism which induces a sustained (>14 days), dose-dependent hyperinsulinemia which reaches a maximum at 5 to 7 days and has no associated hypoglycemia. The two effects are further distinguishable in that the early, endotoxin-induced hyperinsulinemia exhibits the normal suppressibility by exogenous epinephrine, whereas epinephrine markedly enhances the hyperinsulinemia occurring at 7 days. These two effects of *B. pertussis* appear to be mediated by different mechanisms and may be important in the well-recognized reactogenicity of pertussis vaccine in humans.

Components of *Bordetella pertussis* have a number of characteristic biological effects in experimental animals, including decrease or loss of epinephrine-induced hyperglycemia and increase in sensitivity to the lethal effects of histamine (24, 30, 33). It was previously thought that the decreased response to epinephrine was mediated by β-adrenergic blockade induced by the bacteria (33). More recently, however, it has been shown by direct binding studies that *B. pertussis* administration does not cause blockade of the β-adrenergic receptor (19). It is now known that a factor from the organism causes fasting hyperinsulinemia or enhanced insulin secretion in response to insulin secretagogues (or both) (16, 32, 36). In addition, several studies suggest that the histamine-sensitizing and the insulin-secretory activities reside on the same molecule (28, 36).

A line of mice (HSFS/N) has been developed by selectively breeding for susceptibility to the histamine-sensitizing effects of pertussis vaccine (26). In the present study, the HSFS/N mouse was used to examine the time course and properties of the alteration in insulin secretion induced by pertussis vaccine. We demonstrated that the effects of pertussis vaccine are clearly separable into two previously unrecognized phases produced by different components of *B. pertussis*.

First, there is an endotoxin-induced, hyperinsulinemia lasting less than 2 days. After administration of intact organisms, this effect overlaps with a hyperinsulinemia which is maximal at 5 to 7 days and lasts several weeks. The two responses appear to be mediated by different mechanisms. These observations have implications for the understanding of the control of insulin secretion in general and of mechanisms involved in other biological effects of *B. pertussis*, including reactions to pertussis vaccine.

MATERIALS AND METHODS

Vaccine. Pertussis vaccine lot 7b is a whole-cell preparation of killed *B. pertussis* 27 preserved with 0.01% thimerosal and freeze-dried. Immediately before use, vaccine was reconstituted with 0.01% thimerosal-saline to the appropriate opacity unitage (opu; 1 opu represents approximately 6×10^8 organisms). Heat inactivation of vaccine was accomplished by preparing the vaccine as described above and then heating in a controlled-temperature water bath at 80°C for 30 min immediately before use. *Escherichia coli* endotoxin extracted from *E. coli* O127:B8 was obtained from Difco Laboratories, Detroit, Mich. (3123-35 Bacto lipopolysaccharide W). *B. pertussis* endotoxin was prepared by the phenol method described by Kabat (21).

Animals. A line of mice, HSFS/N, derived from the N:NIH(S) strain has been selectively bred for suscep-

tibility to sensitization by histamine-sensitizing factor of pertussis vaccine (26). Because there appears to be a relationship between histamine-sensitizing activity and altered insulin metabolism (28, 36), it was felt that this inbred mouse line might be useful in further studies of glucose/insulin homeostasis after administration of pertussis vaccine. Initially, we compared males and females of the HSFS/N and N:NIH(S) lines in the response of serum glucose and insulin to pertussis vaccine. The maximum insulin response occurred at 7 days after injection and, as shown in Table 1, each group of mice demonstrated a significant hyperinsulinemia with normal glucose. The most striking response was that of the HSFS/N female with a serum insulin level of $1,420 \pm 210$ μU/ml (mean $\pm$ standard error of the mean) and a serum glucose level of 120 ± 5 mg/dl. On the basis of these initial data, the HSFS/N female was selected for use in all subsequent studies.

The animals were fed a diet of National Institutes of Health open formula rat and mouse ration. Food was removed 16 h before initial injection of vaccine or saline, and the fast was continued for animals to be sacrificed within 8 h. Other animals had food returned but were again fasted for 16 h before sacrifice. Females were separated from males at the time of weaning to prevent the possibility of pregnant animals being used. At the time of injection, the animals were 4 to 5 weeks old and weighed 16 to 20 g.

Experimental protocol. All animals were injected intravenously with either sterile saline solution (controls), vaccine, or endotoxin in a volume of 0.5 ml after a 16-h fast. For dose-response and time course studies, the animals were bled on the day indicated. For challenge experiments, glucose (100 mg) or epinephrine (2 mg of L-epinephrine bitartrate [Sigma Chemical Co., St. Louis, Mo.] per kg) was administered intraperitoneally or subcutaneously, respectively, in 0.5 ml of sterile saline 30 min before bleeding. Control animals received sterile saline solution intraperitoneally. For bleeding, animals received light ether anesthesia. The brachial artery was severed, and blood was collected in a Pasteur pipette. A 1- to 1.5-ml volume of blood was placed in plastic tubes and allowed to clot. After clot retraction, serum was separated by centrifugation, removed, and stored at $-20°C$ until assay. The total time from bleeding to freezing was less than 2.5 h.

Insulin and glucose assay. Serum insulin was measured by radioimmunoassay, using porcine ^{125}I-labeled insulin (New England Nuclear Corp., Boston, Mass.) as tracer, guinea pig anti-insulin serum (Burroughs Wellcome Co., Research Triangle Park, N.C.)

as antibody, and mouse insulin (Novo Laboratory, Mamaroneck, N.Y.) as standard. The ^{125}I-labeled insulin had a specific activity of 100 μCi/μg of protein and was added to the assay at a final concentration of 60 pg/ml. The assay mix consisted of insulin standard (final concentration, 5 to 500 μU/ml) in 100 μl of 40 mM phosphate buffer (pH 7.0) or sample serum, and 100 μl of anti-insulin serum (1:400,000 dilution). After a 6-h incubation at 30°C, ^{125}I-labeled insulin tracer was added in 200 μl. This mixture was incubated for 20 h at 37°C. The insulin-antibody complexes were precipitated by the addition of 1.0 ml of 20% (wt/vol) polyethylene glycol 6000 (Serono Laboratories, Inc., Braintree, Mass.) and 0.1 ml of hormone-free carrier serum (Serono) as described by Desbuqois and Aurbach (10). The tubes were mixed thoroughly, and the precipitate was sedimented by centrifugation (3,000 $\times$ g for 15 min). The supernatant liquid was carefully decanted, and the pellet was counted in an LKB gamma counter. Total counts added was approximately 10,000 cpm with maximal binding ranging from 60 to 70%. A standard curve was plotted as percent binding versus insulin concentration. All samples were assayed in duplicate, and the results agreed within $\pm 5\%$ or were repeated. Many insulin values clearly exceeded the standard curve, and those specimens were assayed in a minimum of two, and generally three, dilutions, each of which was calculated to the same final value $\pm 5\%$. Nonspecific binding of ^{125}I-labeled insulin tracer to mouse serum was evaluated by omitting anti-insulin serum from the assay and was determined not to exceed 2% of the total counts.

Glucose was assayed in nonhemolyzed serum samples by using the hexokinase method described by Bondar and Mead (7). Statzyme reagents were obtained from Worthington Diagnostics, Freehold, N.J. The glucose and insulin assays were carried out under contract, with close cooperation, by Bionetics Medical Laboratories, Kensington, Md.

Differences between the control and treated groups and among the treatment groups were compared by using the two-tailed Student's t test.

RESULTS

Dose response. After selection of the HSFS/N female as the experimental animal based on the preliminary data shown in Table 1, we evaluated the effect of vaccine dose on insulin levels 3 and 7 days after injection. As shown in Fig. 1, there was a dose-dependent response of the serum

TABLE 1. Serum glucose and insulin values 7 days postinjection[a]

Mouse strain	Sex	Control[b]		Vaccinated[c]	
		Glucose (mg/dl)	Insulin (μU/ml)	Glucose (mg/dl)	Insulin (μU/ml)
N:NIH (S)	Male	129 ± 12	46 ± 15	137 ± 4	520 ± 43
N:NIH (S)	Female	153 ± 12	41 ± 11	116 ± 4	398 ± 16
HSFS/N	Male	102 ± 7	59 ± 12	139 ± 9	588 ± 58
HSFS/N	Female	135 ± 11	45 ± 8	120 ± 5	$1,420 \pm 210$

[a] These values represent mean $\pm$ standard error.
[b] Control animals received 0.5 ml of sterile saline intravenously.
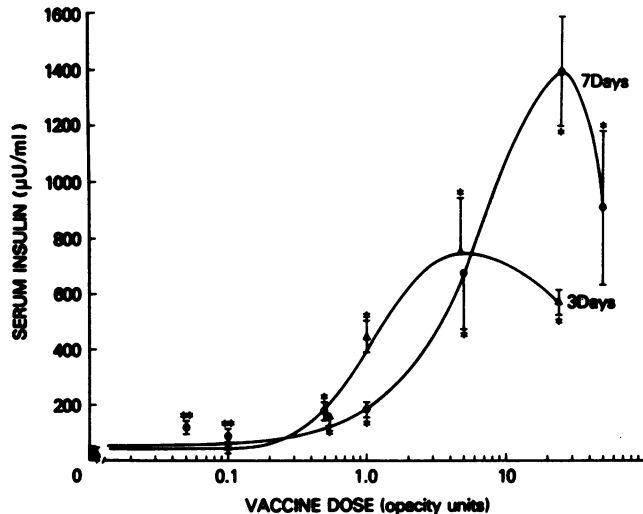[c] Vaccinated animals received 25 opu of pertussis vaccine.

FIG. 1. Effect of pertussis vaccine dose on serum insulin in mice. Female HSFS/N mice were given a single intravenous injection of pertussis vaccine (lot 7b) in 0.5 ml. Vaccine dose ranged from 0.4 to 25 opu as indicated on the abscissa. Mice were bled either 3 (▲) or 7 (●) days later, and the sera were assayed for insulin as described. Each point represents five to nine animals and insulin values are given as mean ± standard error. Asterisks indicate differences from control animals (given sterile saline) at $P < 0.05$ (**) or $P < 0.01$ (*).

insulin at both time points. The maximal insulin level achieved was at 7 days after administration of 25 opu of vaccine.

Time course. Having established an optimal dose of vaccine for these experiments, we studied the time course of vaccine effects. After 25 opu of pertussis vaccine, there was a significant ($P < 0.01$) elevation of the serum insulin, beginning at 4 h and persisting for more than 14 days (Fig. 2A). There appeared to be an early phase of moderate hyperinsulinemia (350 to 450 μU/ml) during the first 24 h and a second peak of marked hyperinsulinemia (1,000 to 1,450 μU/ml) at 5 to 7 days. The serum insulin values at 5 and 7 days, although significantly ($P < 0.01$) increased over days 1 and 3, did not differ from each other. Mean serum glucose levels (Fig. 2B) of pertussis-treated mice differed from those of controls only at 8 h ($P < 0.01$) and 24 h ($P < 0.05$), coinciding with the early phase of hyperinsulinemia. During the later phase of marked insulin elevation, the vaccinated mice had normal glucose levels. These data suggested that components of *B. pertussis* might have two separate effects on the glucose/insulin homeostasis of the vaccinated animal.

Effect of heat-inactivated pertussis vaccine and *E. coli* endotoxin. Bacterial endotoxins have profound effects on carbohydrate metabolism and have been reported to cause hyperinsulinemia in rats and hypersecretion of insulin in response to glucose in rats and dogs (5, 6, 8). These effects are of short duration, occurring

over a course of 1 to 6 h. Because *B. pertussis* contains substantial amounts of endotoxin (25), we examined the effects of heat-inactivated pertussis vaccine and *E. coli* endotoxin on the glucose and insulin levels of the HSFS/N female mouse.

Both the heat-treated pertussis vaccine and the purified *E. coli* endotoxin elicited a prompt, limited hyperinsulinemia (Fig. 3A). The serum insulin was significantly elevated by 1 h and maximal by 8 h, and had returned to control values by 48 h. As suggested by the earlier experiment with unheated pertussis vaccine, this acute hyperinsulinemia was associated with a significant hypoglycemia in animals receiving either the endotoxin or heat-treated vaccine (Fig. 3B). As has been noted by other investigators (4, 8, 12, 27), there was a transient hyperglycemia observed at 1 h after injection of endotoxin or heated vaccine. In addition, animals given heat-inactivated pertussis vaccine developed an unexplained hyperglycemia at 48 h. Mean serum glucose values for both treatment groups had returned to control levels by 72 h. *B. pertussis* endotoxin, prepared by the method of Kabat (21), elicited a similar hyperinsulinemia at 8 h postinjection (Table 2).

Effect of exogenous glucose and epinephrine. The data presented thus far confirmed our initial impression that heat-stable and heat-labile components of the pertussis vaccine might be mediating two separate effects. To evaluate this concept further, we made use of the observation
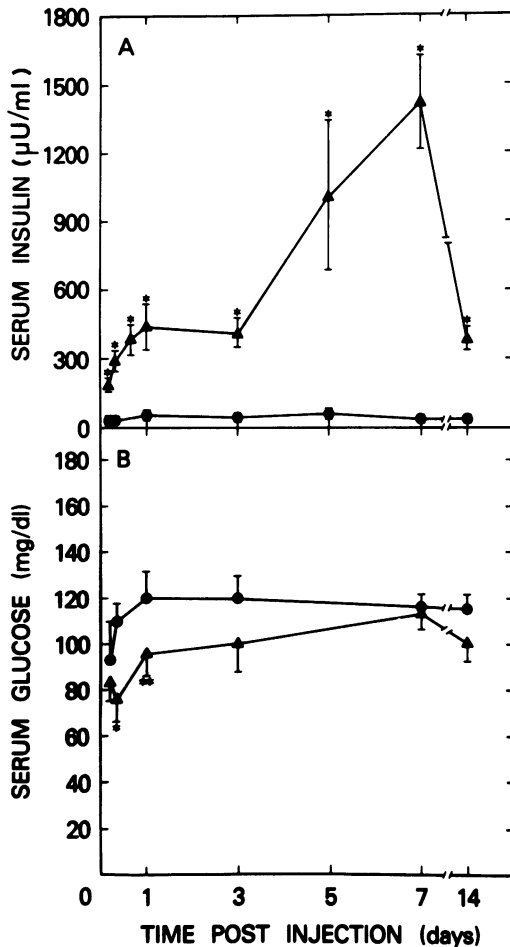
FIG. 2. Time course of pertussis vaccine effects on serum insulin (A) and glucose (B). Mice were given 25 opu of pertussis vaccine (▲) or saline (●) intravenously and bled at the designated times after injection (first point at 4 h). Ten to 16 animals were used per point, except at days 5 and 14 where $n = 5$. Values represent mean ± standard error. Asterisks indicate differences from control animals at $P < 0.05$ (**) or $P < 0.01$ (*).

yielded equivalent increases in insulin at 8 h, which were significantly reduced by both glucose and epinephrine (Fig. 4A). The paradoxical reduction in serum insulin after glucose administration was felt to be a result of dilution due to acute, osmotic plasma volume expansion. At 8 h
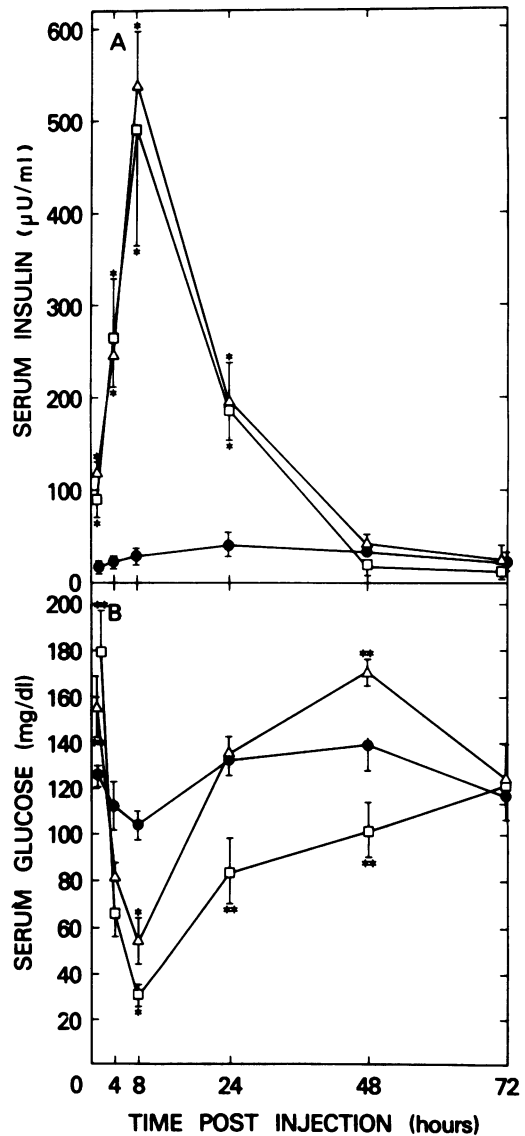
that after pertussis vaccine administration epinephrine stimulates rather than inhibits insulin secretion (16). Eight hours and 7 days were chosen as the times of maximal effect of the heat-stable and heat-labile components, respectively. Unheated and heat-inactivated pertussis vaccines were compared in terms of their effects on the response of mice to exogenous glucose (100 mg intraperitoneally) or epinephrine (2 mg/kg subcutaneously). At 30 min after glucose administration to control animals, serum insulin was increased more than twofold (Fig. 4A). Subcutaneous epinephrine, on the other hand, resulted in a halving of the basal serum insulin level. The unheated and heat-treated vaccines

FIG. 3. Time course of heat-inactivated pertussis vaccine and E. coli endotoxin effects on serum insulin (A) and glucose (B). Mice were given 25 opu of heat-inactivated (80°C for 30 min) pertussis vaccine (△), 0.2 mg of E. coli endotoxin (□), or saline (●) intravenously and sacrificed at the times indicated. Each point represents 5 to 10 animals. Values are given as mean ± standard error. Asterisks indicate differences from control animals at $P < 0.05$ (**) or $P < 0.01$ (*).
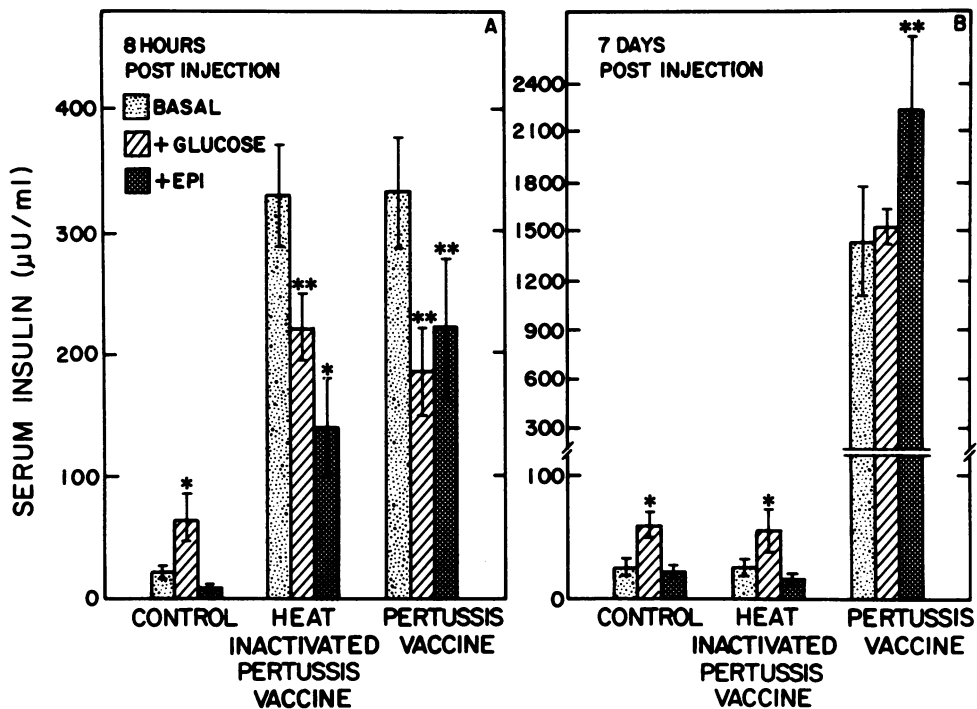
FIG. 4. Effect of exogenous glucose and epinephrine on serum insulin at 8 h (A) and 7 days (B) after administration of unheated or heat-inactivated pertussis vaccines. Mice were given 25 opu of unheated or heat-inactivated pertussis vaccine intravenously. At 8 h or 7 days after vaccine administration, animals were challenged with 0.5 ml of saline intraperitoneally (basal), 100 mg of glucose intraperitoneally, or 2.0 mg of epinephrine per kg subcutaneously. All animals were bled 30 min after challenge. Each group represents five to nine animals. Values are given as mean ± standard error. Asterisks indicate differences from the basal value for the same treatment group with $P < 0.05$ (**) or $P < 0.01$ (*). Note change of scale in part B. The glucose values (mg/dl) ± standard error corresponding to the insulin values depicted in (A) (8 h) are as follows. Control: basal, 110 ± 11; + glucose, 318 ± 88; + epinephrine, 194 ± 14. Heat-inactivated pertussis vaccine: basal, 65 ± 4; + glucose, 213 ± 4; + epinephrine, 176 ± 5. Untreated pertussis vaccine: basal, 69 ± 9; + glucose, 226 ± 22; + epinephrine, 152 ± 5. The glucose values (mg/dl) ± standard error corresponding to the insulin values depicted in (B) (7 days) are as follows. Control: basal, 106 ± 13; + glucose, 183 ± 5; + epinephrine, 216 ± 25. Heat-inactivated pertussis vaccine: basal, 117 ± 5; + glucose, 211 ± 21; + epinephrine, 178 ± 18. Untreated pertussis vaccine: basal, 110 ± 8; + glucose, 109 ± 3; + epinephrine, 106 ± 15.

the effects of the unheated and heat-treated vaccines were essentially identical. By 7 days, however, the basal response to the heat-inactivated vaccine as well as the responses to exogenous glucose and epinephrine were the same as in controls (Fig. 4B; note change of scale). With

TABLE 2. Serum insulin values 8 h postinjection[a]

Test material	Dose	Insulin level (μU/ml)
Saline (control)		22 ± 5
E. coli endotoxin	200 μg	480 ± 123[b]
B. pertussis endotoxin	10 μg	235 ± 46[b]
Heat-inactivated pertussis vaccine	25 opu	540 ± 56[b]

[a] These values represent mean ± standard error.
[b] Significantly different from control ($P < 0.01$).

unheated vaccine, on the other hand, the basal hyperinsulinemia had increased to 1,440 ± 350 U/ml, and the administration of epinephrine further increased the insulin level to 2,240 ± 440 μU/ml ($P < 0.05$). In addition, the normal hyperglycemic responses to glucose and epinephrine were abolished (basal, 110 ± 8 mg/dl, versus postglucose, 109 ± 3 mg/dl, and postepinephrine, 106 ± 15 mg/dl) in the unheated-vaccine-treated animals.

DISCUSSION

Although it has been known for a decade that administration of pertussis vaccine induces hyperinsulinemia in rats and mice (16), the mechanism of this dramatic response and its possible role in the biological effects of the organism have been unclear. In the present study, we demon-

strated that whole-cell pertussis vaccine has a biphasic effect on serum insulin in HSFS/N mice and that the two parts of the response appear to be mediated by separate components of the bacterium. In this animal system, the acute response is mediated by a heat-stable element of the organism and is reproduced by endotoxin administration. As has been noted in endotoxin-treated rats, rabbits, and dogs (4, 8, 12, 27), there is a transient hyperglycemia at 1 h, which is felt to be a result of acute glycogenolysis and decreased peripheral utilization of glucose. Thereafter, animals treated with heat-inactivated vaccine or endotoxin develop a significant hyperinsulinemia and associated hypoglycemia which resolves in less than 48 h (Fig. 3). This is in contrast with earlier observations in dogs and rats which showed endotoxin-induced hypoglycemia to be a hypoinsulinemic, generally preterminal state associated with depletion of glycogen stores (15, 20). In humans, low doses of endotoxin elicit increases in levels of glucose, basal and glucose-stimulated insulin, glucagon, growth hormone, adrenocorticotropin, and cortisol (31). It is of note that unheated pertussis vaccine has the same effect at 8 h as heat-inactivated vaccine and endotoxin, although the insulin levels were often not as high and glucose levels were only slightly decreased (Fig. 2).

The second phase of the response to pertussis vaccine in the HSFS/N mouse consists of marked hyperinsulinemia which is maximal at 5 to 7 days, is mediated by a heat-labile component of the organism, does not result in hypoglycemia, and is enhanced by exogenous epinephrine (Fig. 2 and 4). This paradoxical catecholamine effect explains the loss of epinephrine-induced hyperglycemia in pertussis vaccine-treated rats and mice (16, 19, 24, 33), the observation which originally prompted Gulbenkian et al. to examine insulin levels in those animals (16). These workers found hyperinsulinemia as early as 1 day after vaccine administration and demonstrated that the observed levels represented biologically active insulin (16). In that study, blood glucose was not measured at the early (1 day) time point, and no distinction was made between heat-stable and heat-labile elements of the vaccine. Although Gulbenkian hypothesized that the hyperinsulinemia in conjunction with normal serum glucose might reflect peripheral insulin resistance, recent studies by Furman et al. have provided a more reasonable explanation (14). The hyperinsulinemia associated with administration of pertussis vaccine or infection with B. pertussis appears to be at least partly a result of catecholamine release at the time of anesthesia before sacrifice. Elimination of the use of anesthesia or pretreatment of the mice with the β-adrenergic blocking agent

propranolol markedly reduced the hyperinsulinemia. In their studies, however, only the component corresponding to the late (second) phase of hyperinsulinemia was investigated. It is likely from the different characteristics of the response at 8 h that it is mediated by a different mechanism and is not a function of catecholamine release with anesthesia. The normal response to exogenous epinephrine at 8 h (Fig. 4A) supports this contention.

The data of Furman et al. (14) are consistent with the data of Ui and co-workers, who have demonstrated that administration of B. pertussis cells, fractions of culture medium, or purified islet-activating protein to Wistar rats results in enhanced insulin secretion in response to glucose and other secretagogues, but little or no effect on basal insulin levels (35). In these experiments, blood was collected from unanesthetized rats. Treatment of the animals 3 days before sacrifice did not affect islet morphology or insulin content (22), but did markedly potentiate glucose-stimulated insulin release. In keeping with the data presented here, islet-activating protein-induced alteration of insulin secretion in vivo exhibited a lag of 24 h and peaked at 7 to 10 days (34). It is of note that islet-activating protein, also known as pertussis toxin, lymphocytosis-promoting factor, histamine-sensitizing factor, and pertussigen, is now known to act on a number of other tissues both in vivo and in vitro and that its biological effects are associated with the ADP-ribosylation of a host target cell membrane protein of $M_r = 41,000$ (9, 23).

An additional question of importance is whether this altered insulin metabolism is involved in or is responsible for any of the other biological activities of B. pertussis, such as sensitization to the lethal effects of histamine (24, 30, 33), induction of leuko-lymphocytosis (28), and modulation of delayed hypersensitivity (1, 18, 29). It has been shown previously that exogenous insulin sensitized mice to histamine death and the pertussis-induced sensitization is reduced by prior treatment with alloxan (13). Bergman and Munoz (3) have recently reported that CFW mice given endotoxin from B. pertussis, E. coli, or Salmonella enteritidis are transiently sensitized to histamine over a time course corresponding to the early endotoxin-induced hyperinsulinemia demonstrated here. Ui et al. (34) and Yajima et al. (36), and Morse and Morse (28), have found that their preparations of islet-activating protein and lymphocytosis-promoting factor, respectively, decrease the glycemic response to epinephrine, sensitize the recipients to histamine, and cause leukocytosis. At present, it is certain that these activities are associated, but it is not yet clear whether the alteration of insulin secretion is causally related

to the others. In light of this information, however, it is clear that caution should be observed in using *B. pertussis* cells or pertussis vaccine as a nonspecific adjuvant for other proteins as has been commonly done in the past.

A final issue is the relevance of these findings on alteration of insulin secretion to patients with clinical pertussis and recipients of pertussis vaccine. Hannik and Cohen found that the mean serum insulin level was slightly elevated in 14 children 8 h after receiving diphtheria-tetanus-pertussis-polio vaccine (16 opu of pertussis vaccine) as compared with unimmunized controls and diphtheria-tetanus vaccine recipients (17). This change was not associated with an alteration in blood glucose. In light of the data presented here and by Furman et al. (14), however, it is clear that an acute stress in such vaccinees or in children with clinical whooping cough could be sufficient to elevate the insulin further and result in hypoglycemia. There are other data to support the potential importance of this phenomenon in humans. Badr-El-Din et al. (2) showed that children with whooping cough have a diminished glycemic response to epinephrine. Although insulin levels were not measured, this is indirect evidence for the occurrence of this phenomenon in clinical disease. In addition, Dhar and co-workers have described a reduction of insulin requirements in a group of adult-onset diabetics after intramuscular administration of pertussis vaccine (11). It should be noted that the vaccine used in the present study is equivalent in most biological activities to commercially available pertussis vaccines in general use. Therefore, it is possible that both phases of the alterations in insulin metabolism described here may occur in humans, and it is imperative that these phenomena be further characterized so that their relevance may be appreciated.

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