The Effect of Pertussis Vaccine on the Insulin-Dependent Diabetes Induced by Streptozotocin in Mice

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Summary

Protective effect of pertussis vaccine (PV) against the development of insulin-dependent diabetes (IDD) induced by streptozotocin (STZ) in young CD-1 male mice were studied. When PV at a dose of 1.2×10^{10} microorganism was administered on -10, 0, or $+4$ d relative to a single injection of STZ (60 mg/kg body wt) on d 0, it completely aborted the development of IDD after 140 d. When PV was given on $+30$ d after STZ injection and followed by PV booster injections, 66% of mice reverted to normoglycemic state. Intact islet cells in the pancreas were confirmed by histologic findings and normal plasma insulin values. The effect of PV was compared with that of boiled PV in another model of IDD induced by multiple injections of STZ (40 mg/kg for five doses). In this model, 60% of mice either remained or reverted to normoglycemic state with PV and booster injections whereas boiled PV protected 40% of mice from developing IDD. The protective effect appeared to reside in the biologic property of both pertussigen and endotoxin of *Bordetella pertussis*.

Abbreviations

bPV, boiled pertussis vaccine
HSF, histamine sensitizing factor
IAP, islet cell activating protein
IDD, insulin-dependent diabetes
LPF, lymphocyte promoting factor
PV, pertussis vaccine
STZ, streptozotocin

STZ, given either as a single dose (8, 16) or in multiple doses (13, 14, 22), may produce a permanent form of IDD in young mice. These models have been useful in studying the pathogenesis of IDD also known as juvenile onset diabetes because it resembles many biochemical, immunologic, and histologic changes reported in recent onset IDD in man (8, 13, 22). It also provides the opportunity for the study of intervention at early stages of the disease which invariably carries high frequency of complication in patients in spite of insulin treatment. Using a single dose STZ model, we previously showed that *a prior* injection of whole PV before STZ injection would protect susceptible, young CD-1 mice from developing IDD (9). The protection was confirmed by findings of normal blood glucose level, normal insulin level, normal glucose tolerance test, and the histology of islets in pancreas. In this study, we chose the single dose STZ model and injected PV at various time intervals after STZ injection in order to determine the time course of this protection from IDD. In another experiment, we chose also the multiple dose STZ model to compare the effect of PV *versus* bPV and to evaluate the role

of endotoxin in the protection. Whenever it was indicated, successive booster injection of PV or bPV was employed to assess its effect on established hyperglycemia. This report describes the results of serial blood glucose changes in these groups and histologic findings in islet cells in pancreas in each animal and compare the results with the overall incidence of IDD in each group at the end of the experiment.

MATERIALS AND METHODS

Animals. Young CD-1 male mice (aged 35 d) (Charles River Laboratory, Wilmington, MA) were given laboratory chow and water *ad libitum* for 10 d before experimentation. Their weights were determined weekly throughout the experiments.

STZ. STZ (a gift from Dr. Paul W. O'Connell of Upjohn Co., Kalamazoo, MI; Lot No. 60, 273-2) was dissolved in citrate buffer, pH 4.2, immediately before the experiment.

PV. PV (a gift from Dr. Charles R. Manclark of Bureau of Biologics, FDA, Rockville, MD; Lot 76) in the form of whole-cell *Bordetella pertussis* vaccine was given at a dose of 0.25 ml containing approximately 1.2×10^{10} organisms by intraperitoneal injection. The control animal received 0.25 ml of 0.01% merthiolate solution which was used as a diluent for the vaccine. bPV was prepared by incubating the reconstituted PV at 80°C for ½ h. This would destroy most of the heat labile biologic factor but would preserve the endotoxin (19).

Blood glucose and insulin. The whole blood glucose was measured as previously described (17) and the radioimmunoassay of insulin has also been described (9).

Examination of the pancreas. The mice were exsanguinated under ether anesthesia. The pancreas were removed and immediately fixed for paraffin sectioning in the mixture of 4% formaldehyde and 1% glutaraldehyde in a buffer of 176 mosmol/L (18). After H&E stain, at least 45 islets from different portion of each pancreas were examined for histologic changes compatible with the insulitis or the development of IDD (9).

Experiment I. Animals were divided into five groups ($n = 6$ each). To each animal STZ was administered at a dose of 60 mg/kg on d 0. PV was injected on d -10, 0, $+4$, and $+30$ relative to STZ injection in four separate groups of mice. The control group received STZ and 0.01% merthiolate solution. Serial blood glucose was measured and plasma insulin was assayed at the termination of the experiment. Repeated injections of PV were given to mice that developed hyperglycemia (blood glucose above 200 mg%). The pancreas of each mouse was examined at the end of the experiment.

Experiment II. In this experiment, STZ was administered daily on 5 successive d at a dose of 40 mg/kg. PV or bPV was injected 3 d before STZ injection in two groups ($n = 10$ each). Another group ($n = 10$) received 0.01% merthiolate solution before STZ

injection. Control group ($n = 6$) only received 0.01% merthiolate solution. Blood glucose was measured serially and booster injection of either PV or bPV was given in respective groups whenever hyperglycemia developed in mice. At the termination of the experiment, histology of pancreas was examined in each mouse.

Statistical Analysis. We employed a t test for small samples and chi-square test for the analysis of the data.

RESULTS

Figure 1 illustrates the time course of blood glucose levels of mice injected with a single dose of STZ (60 mg/kg) and with PV which was given at different schedules. The group that received only STZ developed hyperglycemia 4–6 wk after the injection. The outcome of the other group of mice receiving PV either before (-10 d), simultaneously (0 d), or after ($+4$ d) STZ injection appeared to be the same. All mice that received PV, experienced a hypoglycemic state followed by normoglycemia that lasted for at least 20 wk. In no instance did any of those mice develop hyperglycemia. In the group of mice that received PV when they were already hyperglycemic on d 30 after STZ injection, normoglycemia returned and lasted for nearly 8–10 wk before hyperglycemia reappeared in the 12th wk. After a booster dose at that time of PV, four of six mice (66%) in the hyperglycemic group returned to a normoglycemic state which lasted more than 6 wk whereas the remaining two mice had developed irreversible hyperglycemia (Fig. 1). It thus appeared a timely injection of PV might abort STZ-induced IDD in those experimental conditions.

The plasma insulin values are presented in Table 1. The control mice which received STZ uniformly developed hypoinsulinemia (15.7 μU/ml) by 30 d after single injection of STZ. Those mice protected by PV all retained normal insulin values.

Table 1. *Determinations of plasma insulin values in mice of experiment 1**

Injection of PV relative to STZ (in d)	Days after STZ injection	Insulin (μU/ml) (mean $\pm$ SEM)
-10	140	53.4 ± 1.0
0	140	55.2 ± 1.1
$+4$	140	50.0 ± 1.6
$+30$	30	17.9 ± 4.0
$+30$	36	81.0 ± 2.3
$+30$	140	54.6 ± 3.0
STZ only (no PV)	30	15.7 ± 3.6
STZ only (no PV)	140	6.5 ± 1.1
Control (no PV or STZ)	140	59.2 ± 1.7

* $n = 6$ in each group. Each mouse received streptozotoan (STZ) (60 mg/kg) on d 0. PV, pertussis vaccine. Significant difference was present ($P < 0.01$) when compared with animals receiving no intervention.

The mice which received STZ developed hypoinsulinemia on d 30 but the injection of PV reversed the plasma insulin values toward normal until the end of the experiment. As previously described, those mice that received STZ uniformly showed a significant decrease in size as well as changes of morphology of islets in pancreas (8, 9) (Fig. 2) as compared with controls (Fig. 3). Less than 10% of islets in those diabetic mice still showed traces of inflammatory cell infiltrates (Fig. 4). Those histologic changes were consistently compatible with a significant decrease in their plasma insulin values.

Figure 5 shows serial blood glucose changes in mice that received PV or bPV before multiple injection of STZ (40 mg/kg
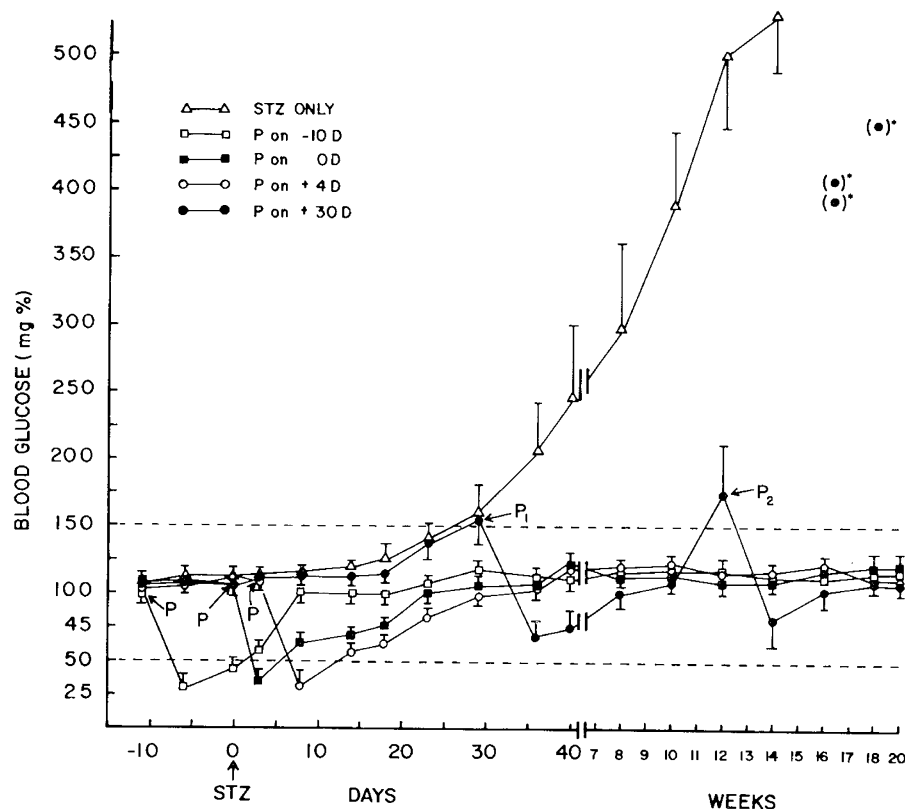

Fig. 1. The sequential changes of blood glucose levels in five groups of mice ($n = 6$ each) receiving injection of pertussis vaccine (PV) and/or streptozotocin (STZ) respectively with varying schedule in Experiment 1. PV was administered IP in 0.25 ml containing 1.2×10^{10} organisms. STZ was injected IP at single dose of 60 mg/kg body wt per mouse. The *vertical bars* indicate 1 SD from the mean values. The normal range of blood glucose levels is expressed by *two broken lines*. P_1 indicates the first injection of PV given on the 30th d after STZ (●——●); P_2 is the booster injection given after 12 wk. *Asterisk* indicates separate blood glucose values from two mice of the same group (●——●) that continued to develop significant hyperglycemia inspite of the booster PV injection.
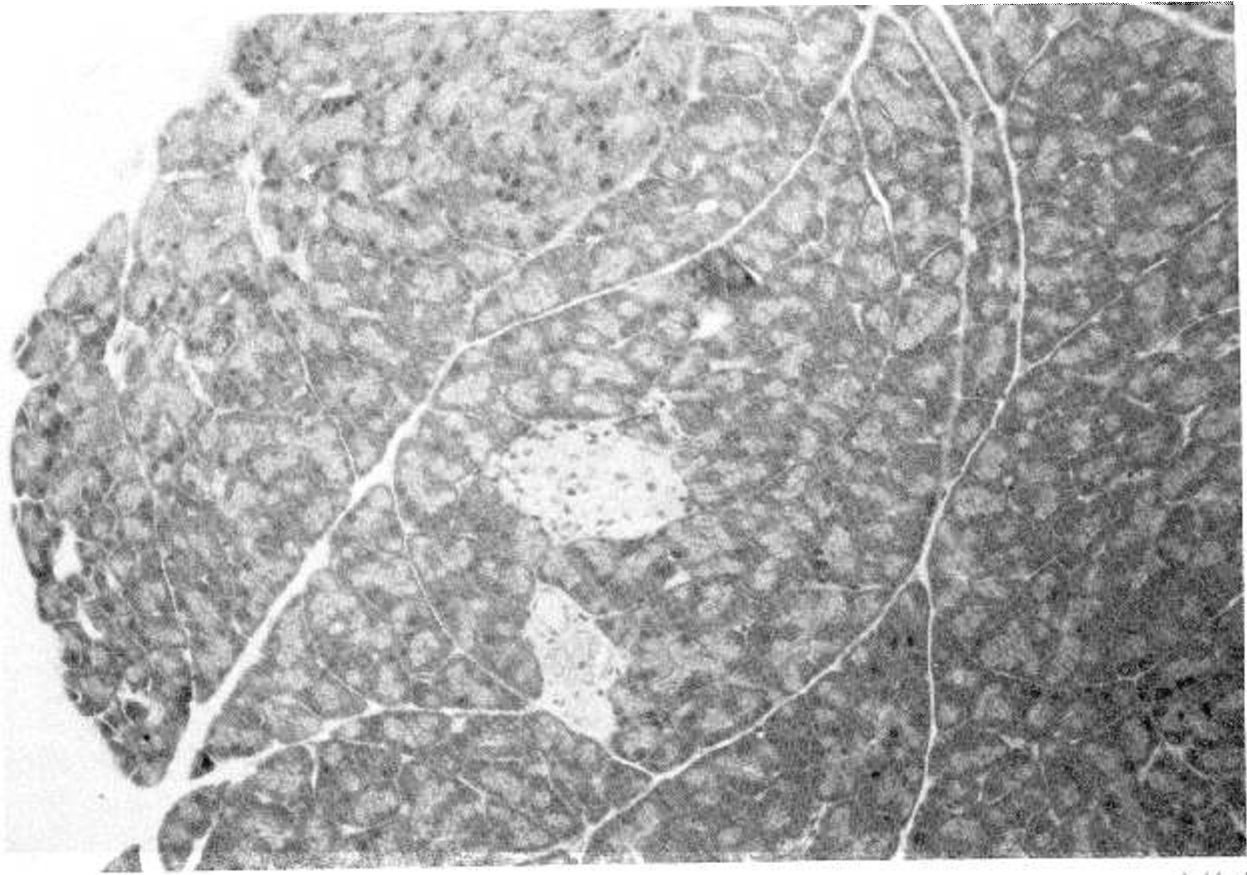
Fig. 2. The pancreatic tissue was obtained on d 60 after a single injection of streptozotocin in Experiment 1 (islets show a remarkable decrease in size and its appearance).
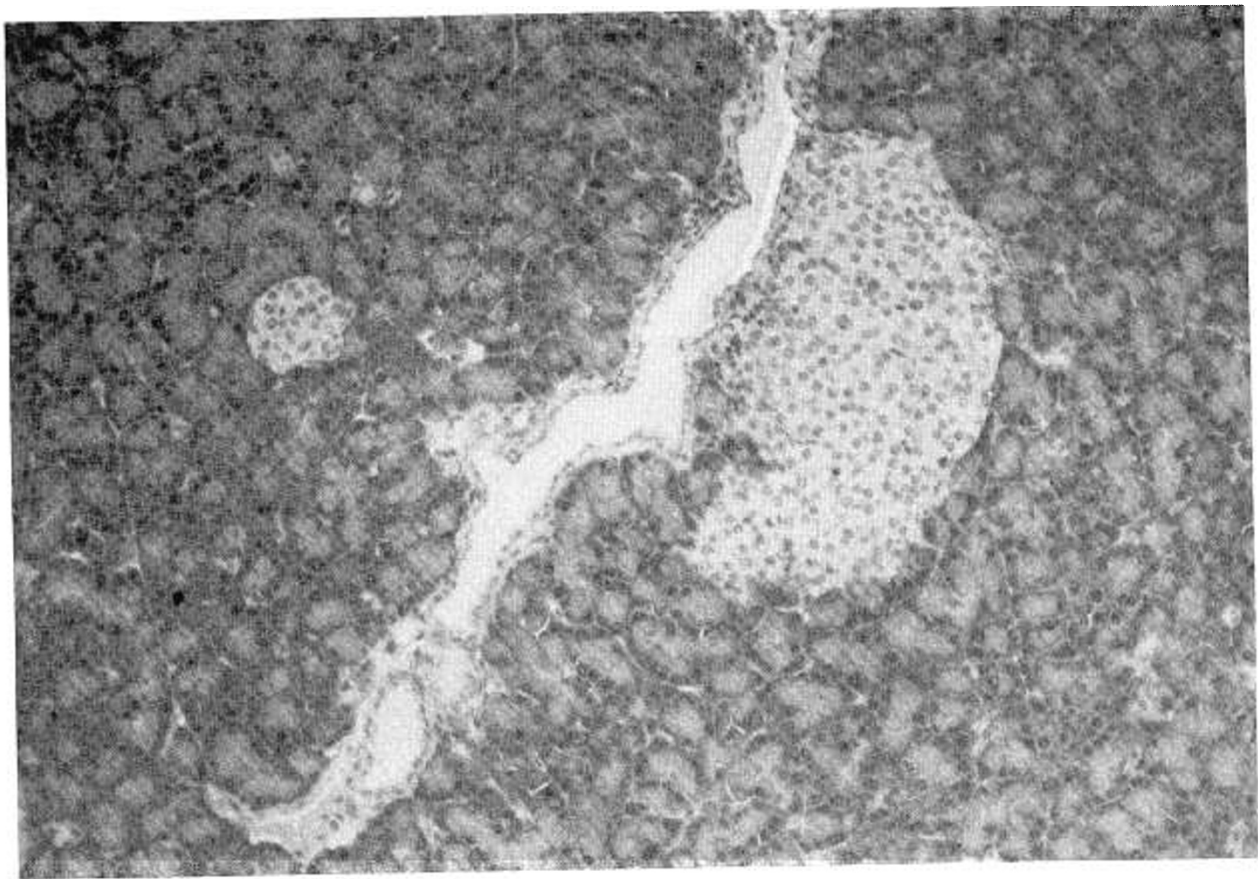

Fig. 3. The pancreatic tissue was obtained on d 60 from one of the control mice not receiving streptozotocin injection in Experiment 1. The islet cells were much more populated as compared with Figure 2.
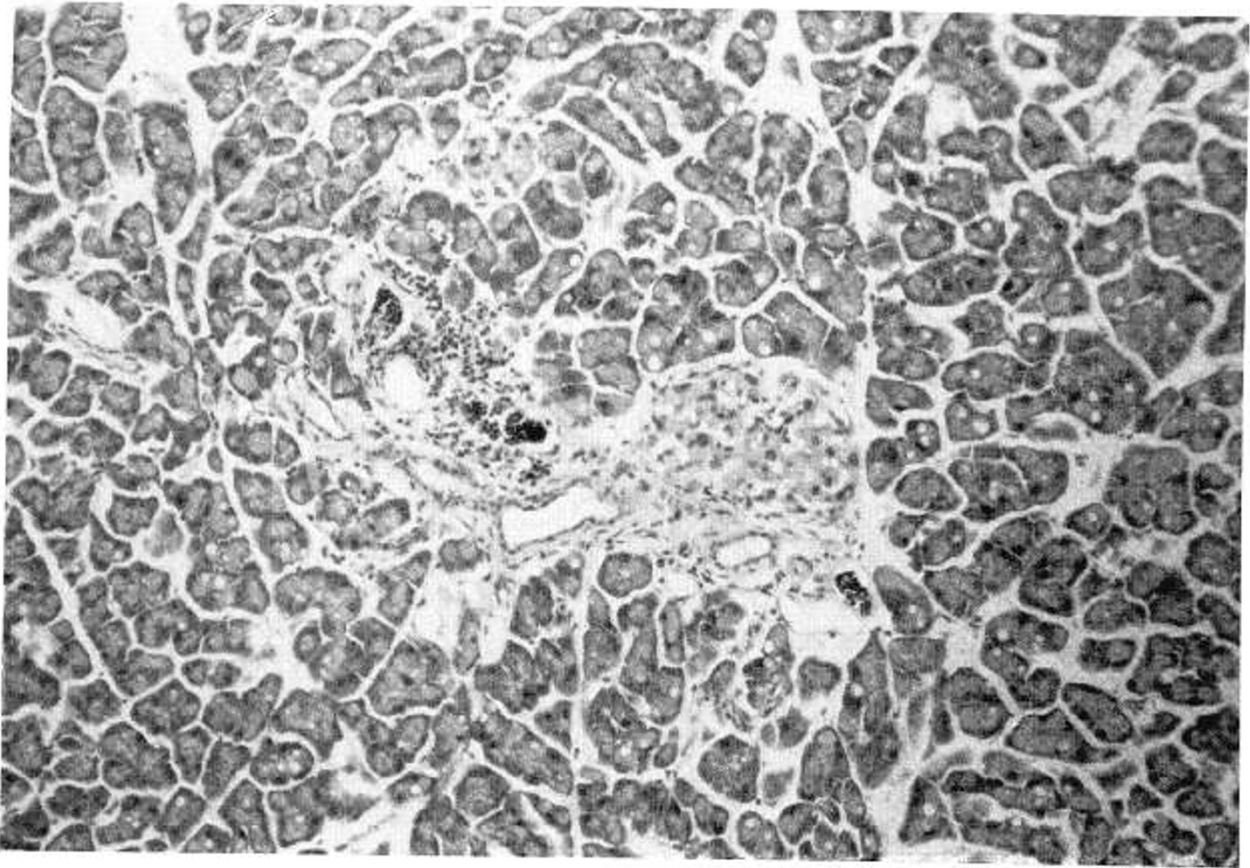
Fig. 4. Illustration of insulitis in the pancreas from a mouse that received streptozotocin alone in Experiment I. The pancreas was obtained on d 60. There were still mild infiltration of mononuclear cells still visible in one of the islets while the infiltration was starting in the other.
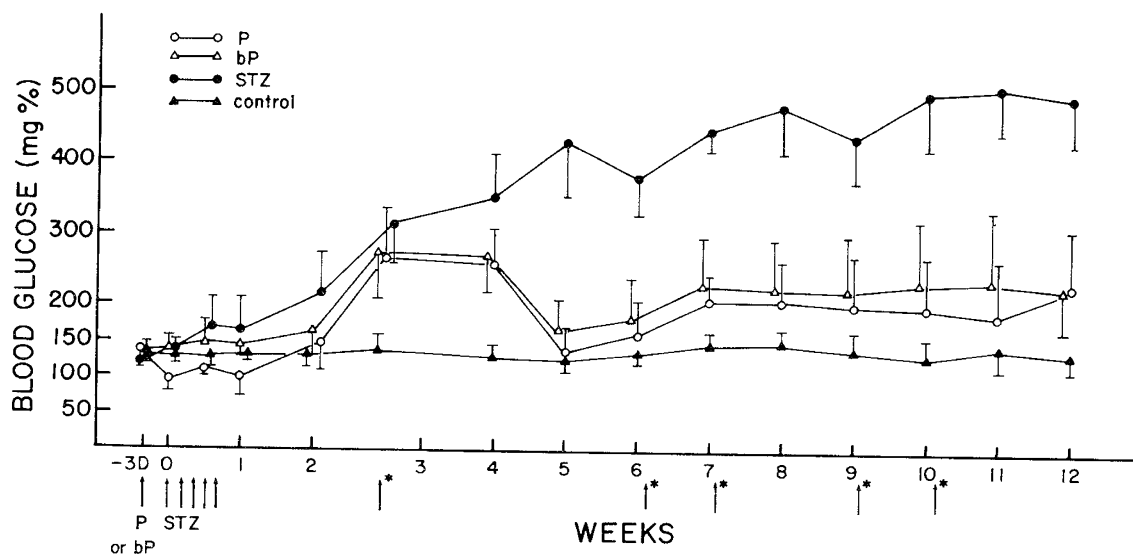

Fig. 5. The sequential changes of blood glucose levels in four groups of mice in Experiment II ($n = 10$ each, except for the control group which had six mice). In one group (O——O) pertussis vaccine (PV) was given 3 d before streptozotocin (STZ) injection at a dose of 0.25 ml containing 1.2 $\times 10^{10}$ organisms. Another group (△——△) received the same dose of boiled PV (bPV) 3 d before the STZ injection. The third group (●——●) only received STZ. Each mouse in these three groups received STZ for 5 consecutive d. The control group (▲——▲) only received 0.25 ml of 0.01% merthiolate solution. * sign indicates the time of injection of the booster of PV (O——O) or boiled PV (△——△) in each group. The *vertical bars* indicate 1 SD from the mean values. P or bPV indicates initial injection of PV or bPV (see "Materials and Methods" for details).

each time). A hypoglycemic effect of PV was still noticeable; however, single injection of either PV or bPV failed to protect effectively the mice from developing hyperglycemia in each group (the difference between them was not statistically significant, $P > 0.15$), although they had significantly lower glucose levels at the end of the experiment as compared with mice either treated with STZ only or control group ($P < 0.05$ in either case). Repeated booster injections did, however, reverse hyperglycemia in four of eight mice in PV group or in two of eight mice which received bPV. The result of the number of booster injections required and the number of mice which returned to normoglycemia was listed in Table 2. It appeared that PV was relatively

Table 2. *Effect of booster injections on the return of normoglycemia in diabetic mice**

Vaccine	n	No. of booster injections					
		0†	1	2	3	4	5
PV	10	2	2‡ (8)	1‡ (6)	1‡ (5)	0 (4)	0 (4)
Boiled PV	10	2	0‡ (8)	1‡ (7)	1‡ (6)	0 (6)	0 (6)

* Each mouse received five successive, multiple injections of stepto-zotocin (STZ) initially (40 mg/kg body wt). One dose of either pertussis vaccine (PV) or boiled pertussis vaccine (bPV) was given before STZ injection. Normoglycemia was defined as blood glucose level before 200 mg%.

† Two mice in each group of PV or bPV never developed hyperglycemia (blood glucose above 200 mg% in two successive measurements).

‡ Indicates the number of diabetic mice which returned to normoglycemia. () indicates the total number of diabetic mice which received the booster injection.

effective when it was given at the early stage (between 2–6 wk after the onset) of hyperglycemia whereas bPV was only effective at the mid-stage (between 6–9 wk after the onset) of hyperglycemia. After 8 wk of continued hyperglycemia, neither PV nor bPV would be effective to reverse hyperglycemia (Fig. 5).

DISCUSSION

Katada and Ui (10) first reported that prior immunization with *Bordetella pertussis* vaccine reversed hyperglycemic state of diabetic mice injected with STZ. Toyota *et al.* (25) subsequently observed the reversal of hyperglycemia even in spontaneously occurring diabetic rats after they were immunized with a by-product of *Bordetella pertussis* recovered from culture fluid. We recently reported that immunization of CD-1 young mice with a dose of PV 3 d before STZ injection completely protected them from developing diabetes induced by a single injection of 60 mg/kg STZ (9). The full protective effect was evidenced by the persistence of normoglycemia, normal results of glucose tolerance tests, normal plasma insulin values, and the lack of appearance of nucleic acid antibodies in the mice immunized with PV (9). Furthermore, there was absence of insulitis in those vaccinated animals as compared with control mice in which inflammation of pancreatic islets were frequently observed (8, 9). The inflammation in the islets is thought to be the primary cause of the permanent damage to β-cells and is an important pathogenetic mechanism leading to the development of IDD.

Our study in Experiment I indicated the anti-diabetogenic effect of PV against STZ was quite extensive. If the vaccine was given from −10 d to +4 d relative to the day of STZ injection, the outcome of the protective effect appeared to be identical. If the PV was given 30 d after STZ which usually would be sufficient to induce hyperglycemia in mice, two-thirds of mice would still revert to normoglycemia after booster injections. As judged from the plasma insulin values and the histologic findings, it is conceivable that in this model using a single STZ injection, the progression of β-cell damage in islets occurs more slowly than the multiple injection model so that interventions can be more effective in the former. Usually, the onset of hyperglycemia in this model occurs in 2 wk (13, 14, 22) whereas the former model takes 4–6 wk after STZ injection (8, 16). In the multiple dose model employed in Experiment II, the protection against the development of hyperglycemia was less complete. After numerous booster injections, the outcome was somewhat improved but was significantly less effective as compared with the results in Experiment I. This suggests that even the simple exposure of the animal to toxin such as STZ was considered subdiabetogenic, a repeated or successive exposure would greatly shorten the latency of the development of IDD. This would significantly reduce the optimal time required to practically halt the development of IDD.

The exact mechanism of the protective effect of PV against STZ remains speculative. At least two important biologic components (Pertussigen and endotoxin) contained in cell membrane of *Bordetella pertussis* may be linked to anti-diabetogenic effect. Pertussigen consists of biologic properties of LPF, HSF, and IAP (20). Although LPF is known to redistribute circulating lymphocytes and thus modifies lymphocyte activity (1, 7), IAP would act on β-cells in islets to enhance its insulin release (26). We confirmed the action of IAP in both Experiment I and II as we noticed the temporary development of hypoglycemia in immunized mice (Fig. 1 and 5). Currently it is believed that the mechanism of the action of IAP on β-cells in islets appears to be related to its enhancement of adrenergic beta-receptor-mediated process. It seems reasonable, therefore, to speculate that the chain of events timely occurs in β-cells after the stimulation of IAP effectively reverses or prevents the damage incurred by STZ. This would explain the fact that the immunization with whole vaccine immediately before the exposure of cell toxin such as STZ may be more effective as illustrated in Table 2.

By incubating the whole cell PV at 80°C for 30 min, we were able to distinguish between the effect of the pertussigen and the effect of endotoxin of *Bordetella pertussis*. The pertussigen is known to be heat labile, therefore, the remaining effect after the treatment only reflects the biologic effect of endotoxin which is heat stable. This was observed by the lack of visible hypoglycemia in mice immediately after the injection of bPV in contrast to the blood glucose change in mice receiving PV (Fig. 5); however, the protective effect of endotoxin was still noticeable as the booster was repeated. As shown in the Table 2, the effect of endotoxin on hyperglycemia was not as prompt as that of pertussigen. This endotoxin of *B. pertussis* is known to possess all the usual biologic components of enterobacterial endotoxins (2) and may act as an immunoregulator by altering the immune response in the host (5). There is also new evidence that suggests phagocyte impotence may be caused by an invasive bacterial adenyl cyclase of *B. pertussis* (4). This may further ameliorate the inflammatory process in the islets by reducing the biochemic outburst of the inflammatory cells, such as macrophages.

The results of the study on the prevention of experimental (11, 15) or human diabetes (3, 6, 12, 21, 23), such as ours and others offer an opportunity to re-assess the current management of diabetes in man. Insulin and other supportive care have undoubtedly decreased the mortality caused by IDD as a result of acute ketoacidosis in new patients; however, the prevention of morbidity and mortality resulting from various complications of diabetes occurring in later years has not been satisfactory. This grows out of the fact that the pathogenesis of IDD in man is complex and poorly understood. In some patients there are indications for early intervention to halt the disease process and this early intervention is probably more justified in patients whose early disease process are accompanied with immunologic injury to β-cells. There are already reports of attempts to halt the disease by employing various immunosuppressants in new patients (3, 6, 12, 21, 23). The results of those studies were still too early to assess but more efforts are likely to be attempted as long as the incidence of IDD in man remains unchanged. Future studies focused on the prevention of diabetes should consider: *1*) developing meaningful parameters to accurately predict the stage of the disease, thus allowing us to determine optimal time for intervention, and *2*) evaluating the efficacy of using biologic products such as those produced by *B. pertussis* which is not only an effective immunosuppressant but it also carries other biologic effect which tend to preserve functioning β-cells.

REFERENCES AND NOTES

1. Andersen, V., Hertz, J. B., Nicolaisen, E. M., and Christeson, P. E.: Lymphocytosis promoting factor for B. pertussis: a mitogen for human T-lymphocytes. In: C. R. Manclark and J. C. Hill: Publ. No. (NIH), 79-1830, p. 156 (NIH, Bethesda, Maryland, 1978).
2. Chaby, R., Ayme, G., Caroff, M., Donikian, R., Haeffner-Caraillon, N., Ledur,

A., Moreau, M., Mynard, M.-C, Roumiantzeff, M., and Szabo, L.: Structural features and separation of some of the biological activities of the B. pertussis endotoxin by chemical fractionation. in: C. R. Manclark and J. C. Hill. International Symposium of Pertussis, Publ. No. (NIH) 79-1830, p. 195 (NIH, Bethesda, Maryland, 1978).

3. Cobb, W. E., Molitch, M., and Reichlin, S.: Levamisole in insulin-dependent diabetes mellitus. N. Engl. J. Med., *303:* 1065 (1980).

4. Confer, D. L. and Eaton, J. W.: Phogocyte importance caused by an invasive bacterial adenyl cyclase. Science, *217:* 948 (1982).

5. Dresser, D. W. and Phillips, J. M.: The cellular targets for the action of adjuvants: T-adjuvants and B-adjuvants. Ciba Found. Symp., *18:* 3 (1973).

6. Elliott, R. B., Crossley, J. R., Berryman, C. C., and James, A. G.: Partial preservation of pancreatic β-cell function in children with diabetes. Lancet, *2:* 1 (1981).

7. Ho, M-K., Kong, A. S., and Morse, S. I.: The in vitro effect of Bordetella pertussis lymphocytosis-promoting factor on murine lymphocytes. V. Modulation of T-cell proliferation by helper and suppressor lymphocytes. J. Immunol., *24:* 36 (1980).

8. Huang, S-W., and Taylor, G. E.: Immune insulitis and antibodies to nucleic acid induced with streptozotocin in mice. Clin. Exp. Immunol., *43:* 425 (1981).

9. Huang, S.-W. and Taylor, G. E.: Pertussis vaccine inhibits immune insulitis induced with streptozotocin. Clin. Exp. Immunol., *48:* 375 (1982).

10. Katada, T. and Ui, M.: Spontaneous recovery from streptozotocin induced diabetes in rats pre-treated with pertussis vaccine or hydrocortisone. Diabetologia, *13:* 521 (1976).

11. Laupacis, A., Stiller, C. R., Gardell, C., Keown, P., Dupre, J., and Wallace, A. C.: Cyclosporin prevents diabetes in BB Wistar rats. Lancet, *1:* 10 (1983).

12. Leslie, R. D. G. and Pyke, D. A.: Immunosuppression of acute insulin-dependent diabetes. In: W. J. Irvine: Immunology of Diabetes. p. 345 (Edinburgh: Teviot Scientific Publications, 1980).

13. Like, A. A. and Rossii, A. A.: Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus. Science, *193:* 415 (1976).

14. Like, A. A., Appel, M. C., William, R. M., and Rossini, A. A.: Streptozotocin-induced pancreatic insulitis in mice. Morphologic and physiologic studies. Lab. Invest., *38:* 470 (1978).

15. Like, A. A., Rossini, A. A., Guberski, D. L., Appel, M. C., and Williams, R. M.: Spontaneous diabetes mellitus: reversal and prevention in the BB/W rat with antiserum to rat lymphocytes. Science, *206:* 1421 (1979).

16. Maclaren, N. K., Newfeld, M., McLaughlin, J. V., and Taylor, G. E.: Androgen sensitization of streptozotocin-induced diabetes in mice. Diabetes, *29:* 710 (1980).

17. Mark, V.: An improved glucose-oxidase method for determining blood, CSF and urine glucose levels. Clin. Chem. Acta, *4:* 395 (1959).

18. McDowell, E. M. and Trump, B. F.: Histologic fixatives suitable for diagnostic light and electron microscopy. Arch. Pathol. Lab. Med., *100:* 405 (1976).

19. Munoz, J. J. and Bergman, R. K.: Bordetella pertussis-immunological and other biological activities. Immunology Series, Vol. 4, pp. 1–235 (Marcel Dekker, New York, 1977).

20. Munoz, J. J. and Bergman, R. K.: Biological activities of Bordetella pertussis. In: Manclark, C. R. and J. C. Hill. International Symposium of Pertussis. Publ. No. (NIH) 79-1830, p. 143, (NIH, Bethesda, Maryland, 1978).

21. Rand, K. H., Rosenbloom, A. L., Maclaren, N. K., Silverstein, J. H., Riley, W. J., Butterworth, B. E., Yoon, J. W., Rubenstein, A. H., and Merigao, T. C.: Human leukocyte interferon treatment of two children with insulin-dependent diabetes. Diabetologia, *21:* 116 (1981).

22. Rossini, A. A., William, R. M., Appel, M. C., and Like, A. A.: Complete protectin from low-dose streptozotocin-induced diabetes in mice. Nature, *276:* 182 (1978).

23. Stiller, C. R., Laupacis, A., Dupre, J., Jenner, M. R., Keown, P. A., Rodger, W., and Wolfe, B. M. J.: Cyclosporin for treatment of early type 1 diabetes: preliminary results. N. Engl. J. Med., *308:* 1226 (1983).

24. Sumi, T. and Ui, M.: Potentiation of the adrenergic beta-receptor-mediated insulin secretion in pertussis-sensitized rats. Endocrinology, *97:* 352 (1975).

25. Toyota, T., Kakizaki, M., Kimura, K., Yagima, M., Okamoto, T., and Ui, M.: Islet cell activating protein (IAP) derived from the culture supernatant fluid of Bordetella pertussis: Effect on spontaneous diabetic rats. Diabetologia, *14:* 319 (1978).

26. Ui, M., Katada, T., and Yajima, M.: Islet-activating protein in Bordetella pertussis: Purification and mechanism of action. In: Manclark, C. R. and J. C. Hill: International Symposium of Pertussis, Publ. No. (NIH) 79-1830, p. 166 (NIH, Bethesda, Maryland, 1978).

Erratum

The Publishers regret the misplacement of the important announcement in January issue. The announcement was inadvertently placed on the inside cover instead of page 2.