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Adjuvant Effect of *Bordetella pertussis* Vaccine to Sheep Erythrocytes: Enhancement of Antibody Formation by Using Subcutaneous Administration of Adjuvant and Antigen

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The subcutaneous route (s.c.) was used to study the adjuvant effect of *Bordetella pertussis* vaccine (PV) on the primary antibody response to sheep erythrocytes. The reasons for using the s.c. route are discussed. PV, besides enhancing the hemagglutinin response, also markedly increased the number of plaque-forming cells in the draining lymph nodes. A heated preparation of PV was tested and found to possess significant adjuvant activity. Interestingly, the enhancement occurred in the absence of marked enlargement of the lymph nodes, which was characteristic of the unheated preparation. In addition, a crude solubilized cell-free preparation of PV was tested and also found to possess significant adjuvant activity. The activity was only partially abolished by heat. Hence, it was concluded that both heat-labile as well as heat-stable factors contributed to the adjuvanticity of PV. The studies also support the view that the draining lymph nodes represent a principal locus of action of PV and that the s.c. route of administration of adjuvant and antigen provides a suitable model for studying and assaying the adjuvanticity of PV.

Since it was first observed that the addition of *Bordetella pertussis* vaccine (PV) to diphtheria toxoid resulted in the enhancement of antitoxin production in guinea pigs (11) and in children (10), many investigators have been concerned with the nature of this adjuvant effect.

PV has been shown to potentiate the antibody response of mice to both soluble (13, 22) and particulate (6, 8, 12) antigens. The intraperitoneal (i.p.) injection of PV with sheep erythrocytes (SRBC) into mice results in increased numbers of splenic plaque-forming cells (PFC) as compared with the response to SRBC alone (6, 8, 9). Similar results have been observed in rats after the intravenous (i.v.) injection of PV and SRBC (24).

However, to date only a few studies have used the footpad or subcutaneous (s.c.) route to study the adjuvanticity of PV. The footpad injection of PV plus antigen into mice (27) and rats (29) results in a marked enlargement of the regional lymph nodes. Taub and Gershon (26) have attempted to relate the adjuvanticity of PV with the histological changes in the draining lymph nodes of mice after the footpad injection of adjuvant and antigen. They concluded that PV enhanced the antibody response by inducing hyperplasia and increasing the rate of deoxyribonucleic acid synthesis in the regional lymph nodes. However, their assay procedure for adjuvanticity involved the i.p. administration of these substances. This inconsistency between the route used for the adjuvanticity assay and that used for the histological studies may be significant, since the i.v. or i.p. injection of PV results in a marked lymphocytosis and neutrophilia that does not occur when the s.c. route is used (17). Furthermore, these investigators did not attempt to determine the PFC response in the lymph nodes reacting to the footpad injection of adjuvant and antigen.

The purpose of the present investigation was to study the adjuvant effect of PV on the primary antibody response of mice to SRBC, using exclusively the s.c. route. In addition to the serum hemagglutinin response, the adjuvant effect of PV specifically on the draining lymph nodes was studied by using the PFC response as an index of local antibody production. Lastly, these assay procedures were used to determine the effects of a heated preparation of PV and a crude solubilized cell-free preparation of PV on the antibody response to SRBC.

(This work was done in partial fulfillment of the requirements for the M.Sc. degree at the State University of New York, Downstate Medical Center, Brooklyn, by Anthony J. Murgo.)

MATERIALS AND METHODS

Animals. Swiss Webster (SW) and C57Bl/6J (C57Bl) mice were obtained from Sprague-Dawley, Madison, Wis., and Jackson Laboratories, Bar Har-

bor, Me., respectively. The mice were 2 to 3 months of age at the time of use.

B. pertussis vaccine. Fluid *B. pertussis* vaccine (lot no. 6WE37A), purchased from Eli Lilly and Co., Indianapolis, Ind., contained approximately 2.9×10^{10} organisms per ml as measured by optical density against an NIH standard (courtesy of Stephen I. Morse of the Department of Microbiology and Immunology).

Antigen. SRBC in Alsever solution (Microbiological Associates, Bethesda, Md.) were washed three times in physiological saline or Hanks balanced salt solution (Grand Island Biological Co., Grand Island, N.Y.) before use.

LPS. Purified *B. pertussis* lipopolysaccharide (LPS) was kindly supplied by Stephen I. Morse and was prepared by phenol extraction (30).

Preparation of solubilized PV. A crude solubilized preparation of PV (SOL-PV) was prepared by ultrasonication. Ten milliliters of PV was sonicated for 1 min with a Brosnik Ultrasonicator (Bronwill Scientific Co., Rochester, N.Y.) using a 13-mm-diameter probe and 80% probe intensity. This procedure was repeated 10 times. The sonicated material was centrifuged at $14,000 \times g$ for 15 min at 4 C and the supernatant material (SOL-PV) was carefully removed.

Injections and bleedings. All injections of adjuvant and antigen were given via the s.c. route into the forelimb in a total volume of 0.1 ml. Sera were obtained from infraorbital sinus blood.

Hemagglutinin assay. Individual heat-inactivated sera were doubly diluted with 0.9% saline in a total volume of 0.1 ml. To each dilution was added 1 drop of a 2% suspension of SRBC, and the mixture was then shaken. After 2 h of incubation, the titer of each serum was recorded as the greatest dilution giving macroscopic agglutination. 2-Mercaptoethanol (2-ME)-resistant antibody titers were determined in a similar manner except that each serum was initially incubated with 0.1 M 2-ME at 37 C for 45 min. 2-ME-sensitive titers were determined by subtracting the 2-ME-resistant titers from the total hemagglutinin titers. Unless otherwise stated, hemagglutinin titers refer to the total hemagglutinins.

Preparation of lymph node cell suspensions. On various days after injection the mice were sacrificed and the draining lymph nodes (axillary and brachial) were removed. Single-cell suspensions were prepared by gently teasing the tissue and passing the cells through a no. 60 steel mesh. The suspensions were washed in Hanks balanced salt solution, and the number of viable nucleated cells was determined by trypan blue exclusion.

Hemolytic plaque assay. The number of PFC was determined by using the microslide monolayer technique of Cunningham and Szenberg (4). For the development of indirect PFC, 1:100 rabbit anti-mouse gamma globulin (Miles Laboratories, Kankakee, Ill.) was added to the system. Direct plaques were inhibited by such treatment, a phenomenon reported by others (3, 21). The recorded number of indirect PFC represents the total number of PFC counted after the addition of the anti-gamma globulin serum.

Splenectomies. Splenectomies and sham operations were performed under ether anesthesia 12 to 20 days before immunization.

Statistical analysis. The Student *t* test was used to compare the experimental data, and a *P* value of less than 0.05 was considered significant. Hemagglutinin titers and PFC numbers were converted to $\log_2$ and $\log_{10}$, respectively, before statistical analysis.

RESULTS

Effect of PV on the hemagglutinin response to simultaneously injected SRBC. Table 1 illustrates the hemagglutinin response of SW mice injected s.c. with various doses of SRBC with or without 1.5×10^9 organisms of PV. PV had no effect on the response to the high dose (2×10^8) of SRBC. PV did enhance the hemagglutinin response to the lower doses of the antigen; the adjuvant effect was best elicited when PV was injected with 2×10^6 SRBC. Hence, in subsequent experiments low doses of SRBC (2.0×10^6 to 4.0×10^6 cells) were used. Also, hemagglutinin titers were usually determined 3 weeks after injection, at which time peak titers were reached. In addition, the adjuvant effect was dependent upon the dose of PV; a dose of 1.5×10^9 organisms was found to be optimal, whereas doses equal to or less than 3.0×10^8 were not effective in enhancing the hemagglutinin response.

No naturally occurring hemagglutinins were

TABLE 1. *Hemagglutinin response to various doses of SRBC with or without pertussis vaccine*

Weeks after injection	SRBC dose								
	2×10^8			2×10^7			2×10^6		
	SRBC-PV	SRBC	*P*	SRBC-PV	SRBC	*P*	SRBC-PV	SRBC	*P*
1	11.0 ± 0[a]	11.0 ± 0.3	NS[b]	8.6 ± 0.4	8.0 ± 0.9	NS	5.0 ± 0.6	2.2 ± 0.8	0.02
2	14.6 ± 0.2	13.4 ± 0.5	NS	11.0 ± 0.4	11.6 ± 0.5	NS	6.4 ± 1.1	6.0 ± 1.1	NS
3	12.6 ± 0.2	12.2 ± 0.4	NS	11.8 ± 0.2	10.6 ± 0.8	NS	9.2 ± 0.9	5.0 ± 0.9	0.01
4	13.8 ± 0.6	13.0 ± 0	NS	12.4 ± 0.2	10.6 ± 0.2	0.001	8.8 ± 0.8	4.2 ± 1.2	<0.025

[a] Mean titer ($-\log_2$) ± standard error of the mean of five mice.
[b] NS, Not significant ($P > 0.05$).

found in the sera of nonimmunized SW mice (Table 2). Nonimmunized C57BL mice, in contrast, did have low titers of hemagglutinins to SRBC. It should be noted that the injection of PV without SRBC had no effect on the hemagglutinin titers of either of these strains of mice.

Besides increasing the total hemagglutinin response, PV significantly enhanced both the 2-ME-resistant and -sensitive hemagglutinin titers to approximately the same extent (Table 2).

Importance of the time and site of injection of PV in relation to SRBC. PV was injected on various days before or after or simultaneously with SRBC. The hemagglutinin titers were significantly enhanced only when PV was given at the same time as SRBC (Table 3). The response to SRBC was suppressed when PV was administered 3 days after the antigen; however, the difference from the control group (SRBC alone) was of only borderline significance ($P < 0.1$).

In another experiment, PV was injected at the same s.c. site as SRBC or s.c. into the contralateral forelimb. Determination of hemagglutinin titers 3 weeks later indicated that the response to SRBC was enhanced only when PV was injected into the same s.c. site as SRBC (Table 4).

Effect of splenectomy. SRBC with and without PV was injected s.c. into a total of 29 splenectomized, sham-operated, and unoperated SW mice, and hemagglutinin titers were determined 21 days later. Splenectomy had no effect

TABLE 3. *Effect of the time of injection of pertussis vaccine on the hemagglutinin response to SRBC*

Day[a]	No. of mice	Mean titer ± SEM[b] ($-\log_2$)	P[c]
No PV	9	4.8 ± 1.0[d]	
−2	8	6.4 ± 1.2	NS[e]
−1	8	4.0 ± 1.2	NS
0	9	8.9 ± 1.3	<0.025
1	8	3.5 ± 1.1	NS
3	8	1.9 ± 1.0	NS

[a] Time of injection of PV in relation to that of SRBC.

[b] SEM, Standard error of the mean.

[c] P value relates to the group that received no PV.

[d] Titers were determined 3 weeks after the injection of SRBC in SW mice.

[e] NS, Not significant ($P > 0.05$)

TABLE 4. *Effect of the site of injection of pertussis vaccine on the hemagglutinin response to SRBC*

Site of injection of PV as compared with that of SRBC	No. of mice	Mean titer ± SEM ($-\log_2$)[a]	P[b]
No pertussis	11	3.6 ± 0.9	
Same	11	6.7 ± 0.7	<0.02
Contralateral	11	2.5 ± 0.8	>0.1

[a] Titers were determined 3 weeks after the injection of SW mice. SEM, Standard error of the mean.

[b] P value relates to the group that received no pertussis vaccine.

TABLE 2. *Effect of pertussis vaccine on the total, 2-ME-resistant, and 2-ME-sensitive hemagglutinin response to SRBC*

Expt. no.	Strain	Day	Group	No. of mice	Mean titer ± SEM[a] ($-\log_2$)		
					Total	2-ME resistant	2-ME sensitive
31	SW	7	SRBC-PV	5	5.0 ± 0.6	0	5.0 ± 0.6
			SRBC	5	2.2 ± 0.8	0	2.2 ± 0.8
			PV	3	0	0	0
		21	SRBC-PV	5	9.2 ± 0.9	7.6 ± 1.3	6.8 ± 1.5
			SRBC	5	5.0 ± 0.9	3.2 ± 1.0	4.6 ± 0.8
			PV	4	0	0	0
			Not injected	5	0	0	0
33	SW	7	SRBC-PV	9	3.7 ± 0.8	1.1 ± 0.4	3.6 ± 0.8
			SRBC	9	1.3 ± 0.4	0.1 ± 0.1	1.3 ± 0.5
		21	SRBC-PV	5	8.4 ± 0.5	6.6 ± 0.2	7.8 ± 0.7
			SRBC	5	4.3 ± 1.3	2.2 ± 1.3	3.9 ± 1.1
41	C57	21	SRBC-PV	5	9.8 ± 0.6	7.0 ± 0.6	9.4 ± 0.7
			SRBC	6	5.8 ± 0.5	2.5 ± 0.7	5.7 ± 0.5
			PV	5	1.2 ± 0.8		
			Not injected	6	1.5 ± 0.7		

[a] SEM, Standard error of the mean.

on the response to SRBC or on the adjuvant effect of PV.

Effect of PV on the size of the draining lymph nodes. On various days after the s.c. injection of PV and/or SRBC, the weights of the draining nodes were determined and are illustrated in Fig. 1. The administration of PV with or without SRBC resulted in a marked enlargement of the draining nodes, which reached a peak by day 7. Cell counts (not illustrated) indicated that this enlargement or increase in weight was paralleled by an increase in the number of nucleated lymph node cells.

Effect of PV on the PFC response of the draining nodes. On various days after the injection of SRBC and/or PV, the draining nodes were removed and the numbers of direct and indirect PFC were determined. The results with C57BL mice are illustrated in Fig. 2. There were no PFC in the lymph nodes of uninjected mice or in mice injected with PV alone. The injection of PV markedly enhanced the direct and indirect response to simultaneously injected SRBC. Since direct (immunoglobulin [Ig] M) PFC predominated, the identification (IgM or IgG) of the indirect PFC could not be extrapolated from this data.

Equivalent results were obtained in other experiments using SW mice.

Effect of heating PV on the adjuvanticity. Heated PV was prepared by heating a suspension of organisms for 40 min at 100 C (22). C57Bl mice were injected s.c. with SRBC alone, SRBC plus PV, or SRBC plus heated PV. Hemagglutinin titers were determined 3 weeks after injection and are illustrated in Fig. 3.

The hemagglutinin response to SRBC plus heated PV was significantly lower ($P = 0.005$) than that to SRBC plus unheated PV. But the administration of heated PV still had an enhancing effect ($P < 0.02$) when compared with the antibody response to SRBC alone. It is of interest that the enlargement of the draining nodes caused by PV did not occur in the mice that received heated PV, despite the fact that a significant adjuvant effect was achieved.

Figure 4 illustrates the weights of the draining lymph nodes 6 and 12 days after the injection of SRBC with and without PV, heated PV, or purified *B. pertussis* LPS into SW mice. The ability of PV to cause an increase in the weight of the draining nodes was markedly diminished by heating the vaccine. The injection of SRBC plus 10 μg of LPS caused a moderate increase in lymph node weight as compared with SRBC alone; 2 μg of LPS had no effect. The increased lymph node weight after the injection of unheated PV was maintained 12 days after injection, whereas this response appeared to be tapering in the other groups.

Figure 5 illustrates the direct PFC response of the draining nodes 6 and 12 days after the injection of SRBC with and without PV, heated PV, or 10 μg of LPS. Unheated PV and LPS

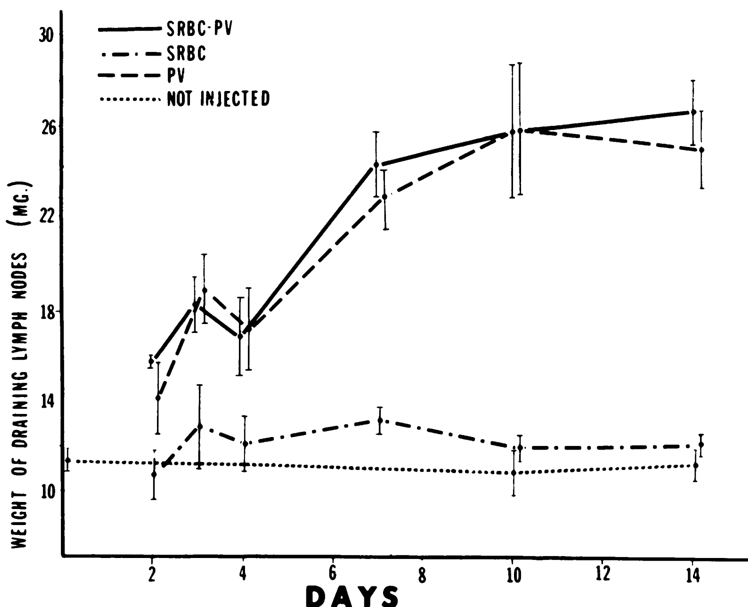

FIG. 1. *Effect of PV and SRBC on the weight of the draining lymph nodes. Each point represents the mean of four SW mice. Vertical lines indicate ±1 standard error of the mean.*

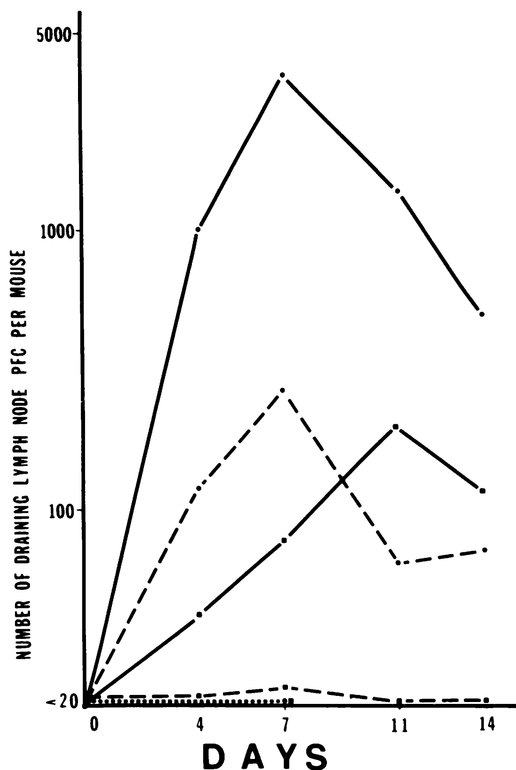

FIG. 2. *Effect of PV and SRBC on the number of PFC in the draining lymph nodes. Each point represents the mean of three to four C57B1 mice. Symbols:* ●, *Direct PFC;* ■, *indirect PFC;* ——, *SRBC-PV;* - - -, *SRBC;* -----, *PV.*

were responsible for a marked enhancement of the PFC response 6 days after injection; heated PV caused only a moderate and statistically insignificant enhancement at day 6. By 12 days after injection of SRBC alone no PFC were detectable in the draining nodes; at this time the PFC response to SRBC plus unheated PV persisted and was markedly enhanced, whereas the response to SRBC plus heated PV or SRBC plus LPS was only moderately increased, but much diminished from the response of day 6.

Adjuvanticity of SOL-PV and heated SOL-PV. SW mice were injected with SRBC with and without SOL-PV (each dose prepared from 1.5×10^9 organisms) or SOL-PV that was heated to 100 C for 40 min.

SOL-PV was responsible for a marked increase in the number of draining lymph node cells on day 6 as compared with SRBC alone (Fig. 6). This increase did not occur after the injection of heated SOL-PV. In addition, SOL-PV markedly enhanced the direct PFC response to SRBC. The PFC response to SRBC was also increased by heated SOL-PV but to a much lesser extent than the unheated preparation.

DISCUSSION

The enhancing effect of PV on the antibody response to SRBC has been extensively studied. However, previous reports involved either the i.p. (5, 6, 8) or i.v. (24) routes of administration of adjuvant and antigen. The present investigation is unique in that the s.c. route was utilized exclusively to quantify the adjuvanticity of PV on the antibody response of mice to SRBC.

The s.c. route was preferred because it is not associated with some of the other biological or systemic side effects of PV such as the intense lymphocytosis and neutrophilia (17) which may not be necessary for the adjuvant effect but which may complicate interpretation of the results. It has been documented that i.v. and i.p., but not s.c., injection of PV or cell-free supernatant fluids derived therefrom results in a mobilization of small lymphocytes from the lymphoid organs and an inhibition of lymphocyte migration from the blood back to these organs (1, 17, 18). This situation culminates in an intense sustained lymphocytosis that is primarily due to a redistribution of recirculating small lymphocytes (17). Moreover, and further complicating this picture, recent studies (1) on the kinet-

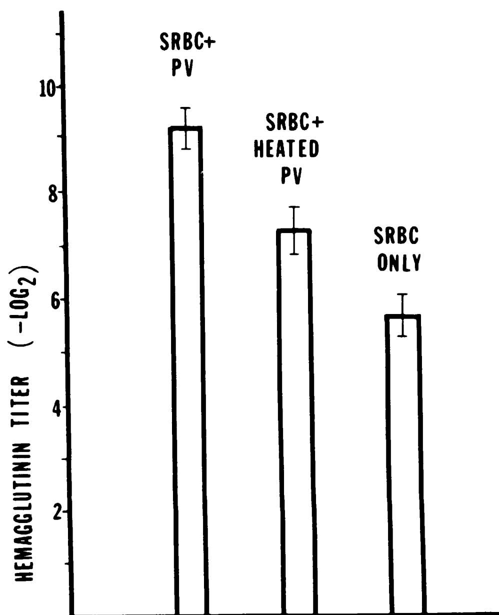

FIG. 3. *Effect of heated PV on the hemagglutinin response to SRBC. Each point represents the mean titer of 11 C57B1 mice bled 3 weeks after injection. Vertical lines indicate ±1 standard error of the mean.*

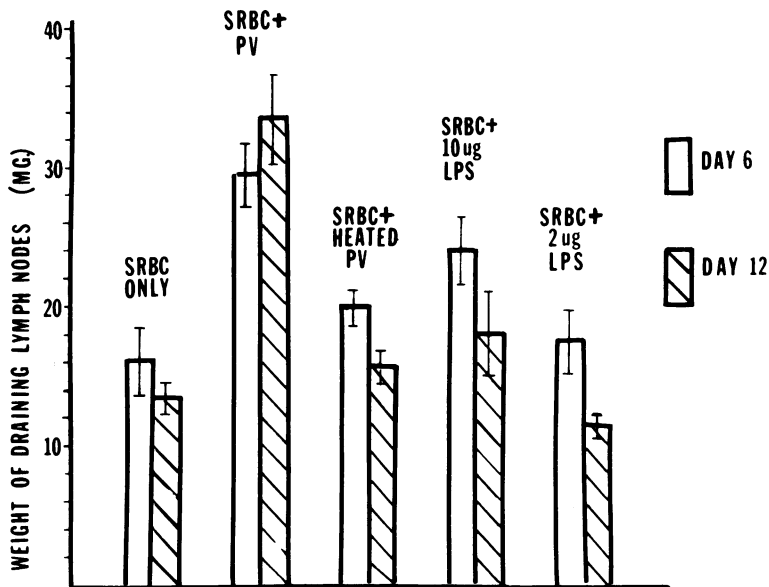

FIG. 4. *Effect of SRBC with and without PV, heated PV, or LPS on the weight of the draining lymph nodes. Each point represents the mean of three to seven SW mice. Vertical lines indicate ±1 standard error of the mean.*

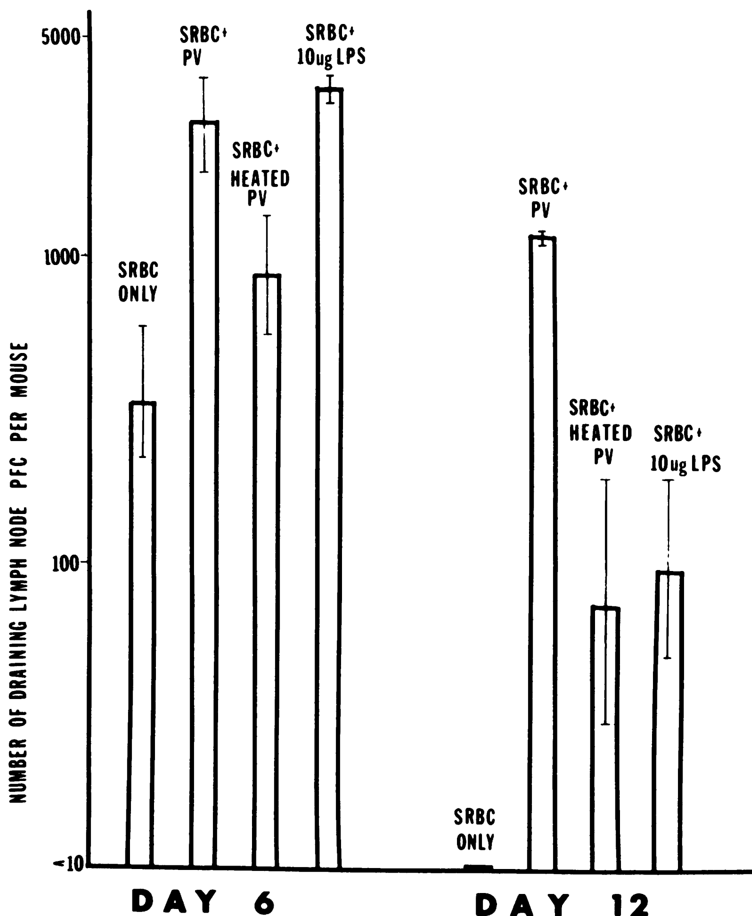

FIG. 5. *Effect of SRBC with and without PV, heated PV, or LPS on the number of direct PFC in the draining lymph nodes of SW mice. The results are the means of three experiments (six and nine mice per point). Vertical lines indicate ±1 standard error of the mean.*

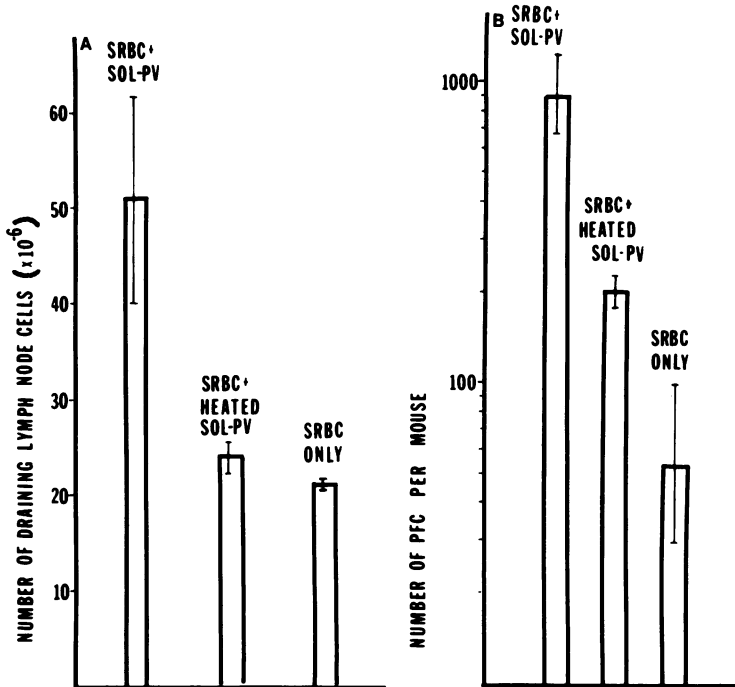

FIG. 6. *Effect of SRBC and solubilized PV or heated solubilized PV on the number of nucleated cells (A) and direct PFC (B) in the draining lymph nodes of SW mice 6 days after injection. The results represent the means of three experiments (nine mice per point) and four experiments (12 mice per point) in (A) and (B), respectively.*

ics of this phenomenon using cell-free supernatant fluids of PV have shown that the mobilization and repopulation of lymphocytes in the spleen and lymph nodes during the lymphocytosis does not occur in a synchronous manner. That is, during the early phase of lymphocytosis, days 1 and 2, when the white pulp of the spleen is maximally and drastically depleted of both T- and B-lymphocytes, the lymph nodes have not yet begun to depopulate. However, between days 4 and 6 of the lymphocytosis, when the splenic white pulp is becoming progressively repopulated, the lymph nodes are undergoing progressive depopulation. By day 7, when the spleen appears almost repopulated, the lymph nodes appear maximally depleted of T- and B-lymphocytes. Hence, it is quite possible that these profound changes in the small lymphocyte content of the blood and lymphatic tissues that are avoided when PV is given s.c. can only serve to complicate those studies on adjuvanticity using other routes.

Although it has been reported that PV enhances the splenic PFC response to doses of SRBC that were previously found to be optimal or even maximal (5), we found that PV increased the primary hemagglutinin response of mice to simultaneously injected suboptimal doses of SRBC but not to optimal doses. The greatest enhancement of the antibody response was noted when PV was administered at the same time as SRBC. These results are in agreement with the findings of others following the i.p. administration of PV and SRBC (5).

After the footpad injection of SRBC into rats, the major site of appearance of direct and indirect PFC has been reported to be in the draining lymph nodes (28). Moreover, the subsequent appearance of indirect PFC in the spleen was shown to depend on the migration to this site of a radiosensitive cell population derived from the draining lymph nodes. In the present investigation the s.c. injection of SRBC was also followed by the development of antibody or PFC in the draining lymph nodes, and the direct or IgM PFC predominated. This response was accelerated, intensified, and prolonged by the simultaneous injection of PV. Two additional findings from our study were that splenectomy had no effect on the hemagglutinin titers or on the adjuvanticity of PV, and that the administration of PV at a remote s.c. site from that of SRBC did not enhance the antibody response. These data suggest that the

draining lymph nodes represent a major site of adjuvant action of PV. Indeed, the results of Levine and Wenk (15) also suggest that the locus of adjuvant activity of PV with regard to the induction of hyperacute experimental allergic encephalomyelitis in rats is in the draining lymph nodes.

Evidence is also presented suggesting that at least two components of PV are involved in the enhancement of antibody formation. This is contrary to the results of studies in guinea pigs (7), which suggest that the adjuvant activity of PV can be entirely replaced with equivalent amounts of purified *B. pertussis* LPS, and some studies in mice (22, 23), which suggest that the adjuvant activity is related only to a heat-labile component, probably the histamine-sensitizing factor, and not to the heat-stable component, LPS. In our experiments the ability of PV, and that of a crude soluble cell-free preparation derived therefrom, to enhance the antibody response to SRBC was decreased by heat but not completely abolished. Hence, both heat-labile and heat-stable factors would appear to contribute to this activity. This is not unexpected since the ability of LPS from other gram-negative bacteria to enhance the antibody response of mice to SRBC (19) and to other antigens (2, 20) is well documented. But in the present investigation the marked enlargement of the draining lymph nodes caused by PV was not a property of LPS or heated PV. Also, the enhancement of the PFC response caused by LPS and heated PV was less prolonged than that caused by unheated PV. Hence, it is proposed that both the prolongation of the PFC response and the enlargement of the draining nodes are essentially a property of the heat-labile component, probably the histamine-sensitizing factor. In addition, it has been shown that this heat-labile component, which is as yet inseparable from the lymphocytosis-promoting factor, also possesses the ability to increase reaginic antibody production (14, 25) and to enhance the induction of hyperacute experimental allergic encephalomyelitis (16).

Besides the marked enlargement and increased PFC response, PV resulted in a variety of histological changes in the draining lymph nodes, some of which may be related to its adjuvanticity (A. J. Murgo, M.Sc. dissertation, State University of New York, Downstate Medical Center, Brooklyn, 1974). Similar histological changes have been reported by others (26, 27). Studies are currently in progress to determine the nature of these changes and to elucidate their relationship to the adjuvanticity of PV. In addition, PV and its various products are also presently being assayed for their adjuvant effect on the cell-mediated immune response to SRBC by using a sensitive in vivo procedure whereby antigens are injected intracutaneously into the mouse ear and the inflammatory swelling is measured with calipers (T. J. Athanassiades, Fed. Proc. 33:650, 1974).

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