THE OCCURRENCE AND PROPERTIES OF LEUKOCYTOSIS AND LYMPHOCYTOSIS-STIMULATING MATERIAL IN THE SUPERNATANT FLUIDS OF BORDETELLA PERTUSSIS CULTURES*

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In previous communications, various aspects of the leukocytosis and lymphocytosis produced in mice by *Bordetella pertussis* cells have been described (1, 2). The bacterial substance which caused this reaction was not defined, and subsequent attempts to isolate active material from bacterial cells proved to be unsuccessful except under rather special conditions which yielded only very small amounts of a highly active particulate fraction. In view of the inefficiency of the methods thus far used, experiments were performed to determine whether potent leukocytosis-stimulating material was released into the extracellular environment of growing cultures of *B. pertussis*, and if so to determine some of its properties.

It had been previously established that cell-associated activity was tightly bound to agar-grown organisms, and that if active material had diffused into the agar it could not be subsequently recovered (1). Therefore, studies were performed with fluid-grown cultures. These studies showed that under the conditions employed, the supernatant fluids were highly effective in producing leukocytosis and lymphocytosis. The specific activities were manyfold greater than those of intact microbial cells.

A water-insoluble fraction, consisting predominantly of protein, was isolated from supernatant fluids. This material was highly active and induced significant leukocytosis in mice in doses of 1 μg or less. Histamine-sensitizing factor but not protective antigen, was also found in culture supernatant fluids and was localized to the same fraction as the leukocytosis-stimulating material.

Material and Methods

Culture Media.—The liquid culture medium used was essentially that described by Sutherland and Wilkinson (3). Modifications included: a decrease in the amount of $CaCl_2$, an increase in the amount of tris (hydroxymethy) aminomethane (Tris) and of nicotinamide, the addition of NaCl, and also of glutamic acid as recommended by Rowatt (4).

Composition of 1 Liter of the Medium

	g
Tris[1]	6.0
Casamino acids, technical[2]	10.0
NaCl	2.0
Dowex 1-X1, 50-100 mesh[3]	1.0
KH_2PO_4	0.5
$MgCl_2 \cdot 6H_2O$	0.4
DL-Glutamic acid	0.2
Nicotinamide	0.03
$CaCl_2$	0.01
$FeSO_4$-$7H_2O$	0.01
Reduced glutathione	0.01

The Tris was added to approximately 750 ml of H_2O and the pH was adjusted to 7.4 with HCl. The remainder of the ingredients, with the exception of glutathione, were then added. The Dowex 1-X1 resin had been washed with 1 N NaOH followed by distilled H_2O and was dried at 45–50°C overnight before use. The pH was readjusted to 7.0 if required and the medium was brought to volume. The medium was, when in small volumes, autoclaved at 15 lb. pressure for 20 min; the time was extended to 60 min for volumes of 1 liter or greater.

Reduced glutathione was dissolved in distilled H_2O and the solution was sterilized by filtration through a Millipore filter, 0.45 μ pore size (Millipore Filter Corp., Bedford, Mass.). Appropriate volumes were added to cool, autoclaved medium to give the desired concentration of 0.001%.

Solid medium was prepared by adding 200 mg of Norite A and 2 g of agar-agar to 100 ml of glutathione-free medium. After autoclaving the agar medium was held at 45°C and glutathione was then added prior to pouring of slants or plates.

Organism and Growth Conditions.—Slants of the phase I *B. pertussis* strain 3779B were originally obtained through the courtesty of Dr. J. M. McGuire from Eli Lilly Co., Indianapolis, Ind. This strain was utilized in the studies previously reported (1, 2). It has been maintained by semimonthly passage on solid media. On two occasions in the past few years, new seed slants of the same strain have been obtained from Eli Lilly Co. and no biologic differences have been noted. At intervals, the cultures were checked for change of phase by plating heavy suspensions onto penassay agar (Difco).

Liquid culture was performed in stationary, shallow layer cultures, e.g. 20 ml in 8 oz Owens oval prescription bottles; 100 ml in 1 liter Blake bottles, and 1 liter in 5 liter Povitzky diphtheria toxin bottles. All were incubated horizontally at 36°C.

For culture volumes up to 100 ml, inocula were prepared by suspending the growth from 3 to 4-day-old agar cultures in 2% casamino acids. The concentration of organisms was determined by comparison of the OD_{530} of the suspension with The National Institutes of Health opacity standard which was kindly supplied by Dr. Margaret Pittman. Appropriate corrections were made for the intrinsic optical density of the suspending medium. A sufficient volume of the suspension was aded to achieve an initial concentration of bacterial cells of 0.5–5.0 $\times$ 10^8/ml. In the case of 1 liter cultures, the inoculum consisted of 10 ml aliquots of 3–4 day liquid growth with titers of 0.9–2.0 $\times$ 10^{10} bacilli/ml.

Enumeration of Blood Leukocytes.—A model F Coulter Counter (Coulter Electronics Inc.,

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[1] Sigma 7–9, Sigma Chemical Co., St. Louis, Mo.

[2] Difco Laboratories, Detroit Mich.

[3] Baker Chemical Co., Phillipsburg, N. J.

Hialeah, Fla.) was used to determine the total leukocyte count (WBC) in the peripheral blood of mice. Blood from the distally severed tail was allowed to flow into a disposable capillary pipette with a capacity of 5 μl (Drummond Scientific Co., Philadelphia, Pa.). The blood was expelled into a vial contining 10 ml of 0.85% NaCl and 10 units (0.1 mg) of heparin/ml (Bio-heprin, 1000 USP units/ml, Ries Biologicals Inc., Los Angeles, Calif.), and the pipette was rinsed several times. 0.05 ml of 0.5% saponin (Coulter Electronics) in 85% NaCl was added with a calibrated dropper (Cooke Engineering Co., Alexandria, Va.). Stock 1% solutions of saponin were made weekly and diluted before use.

After mixing the vial contents, leukocyte counts were performed between 12 and 25 min later. A 100 mμ aperture tube was used. The threshold setting was 26 and the attenuation and aperture settings were 1 and 8 respectively. Raw counts were corrected for dilution, coincidence, and excessive background.

Under these conditions, the WBC's determined by means of the electronic counter were generally between 70 and 85% of counts determined by methods previously described (1). The counts were internally consistent. Spot differential cell counts were performed in situations in which major alterations in cell types in the blood might affect interpretation of the results obtained with total counts alone.

When parallel total and differential leukocyte counts were desired, disposable Unopettes (Becton-Dickinson & Co., Rutherford, N. J.) were used. Tail vein blood was drawn into 13 μl pipettes and the pipette contents were diluted in 1.3 ml of Turk's solution. The leukocytes were quantitated and differentiated microscopically using a standard hemacytometer and magnification of 430.

Mice.—Mice were from the NCS colony of Swiss mice maintained at The Rockefeller University. Male mice weighing 25–30 g were usually employed.

Chemical Analyses.—Nitrogen, phosphorus, and elemental analyses were performed by Mr. T. Bella, microanalyst of The Rockefeller University. Carbohydrate was determined by the phenol-sulfuric acid method (5) after hydrolysis in 2 N HCl at 100°C for 2–3 hr *in vacuo*. Hexosamine was determined by a modification of the Elson-Morgan reaction after hydrolysis under similar conditions (6). Amino acid analyses were kindly performed by Dr. Roger Lundblad using the automatic recording equpment of Spackman, Stein, and Moore (7). Fatty acids were determined by gas-liquid chromatography (GLC) after acid hydrolysis and extraction of the , fatty acids into ether and chloroform as described by Taylor, Knox, and Work (8). Dr. Norton Spritz graciously performed the GLC analyses. Protein was estimated by a modified Folin method using muramidase (Worthington Biochemical Corp., Freehold, N. J.) as the standard (9); nucleic acid by UV absorption characteristics either of material in solution or, in the case of insoluble material, of extracts obtained by heating the unknowns in 6% perchloric acid for 60 min.

RESULTS

The Distribution of Leukocytosis-Promoting Activity in Fluid Cultures of B. pertussis.—A number of initial experiments were performed in order to determine the distribution of leukocytosis-promoting activity in fluid cultures of *B. pertussis*. The general design of the experiments was as follows:

8 oz Owens oval prescription bottles containing 20 ml of media were inoculated with organisms washed from agar slants which had been incubated for 3 days. In any given experiment the initial concentration of organisms was identical, and was in the range of 0.5–5.0 $\times$ 10^8/ml. The bottles were incubated horizontally at 36°C. At various time periods individual cultures were removed and the concentration of organisms was determined by optical density measurements.

A 4.0 ml resin-free aliquot of the culture was centrifuged at 8000 g for 10 min.[4] The supernatant fluid was decanted and the bacterial pellet was suspended in 4.0 ml of 0.5 M Tris–HCl buffer, pH 7.4, or of sterile media. 1% thimerosal was added to the supernatant fluid, suspended bacterial pellet, and an aliquot of the uncentrifuged culture. The final concentration of thimerosal was 0.002%. The samples were held at 4°C for 3–7 days. Groups of four mice were then injected intravenously with 0.2 ml of each sample and leukocyte counts were determined 3 days later.
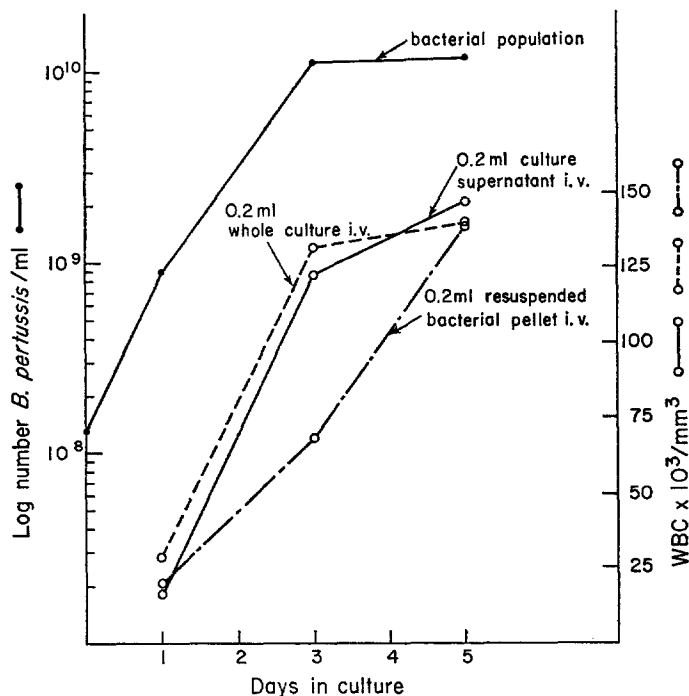

Fig. 1. Growth of B. pertussis in fluid medium and leukocytosis induced by culture fractions injected intravenously in mice. WBC's were performed 3 days after inoculation.

Fig. 1 illustrates both the characteristic growth curve of the organism under the conditions employed, and the leukocyte counts of mice 3 days after injection of the various culture fractions. It can be seen that the rapid phase of bacterial division lasted for more than 48 hr. Near maximum numbers were achieved by the 3rd day of culture, although some increase in bacterial concentration often occurred over the next 2 days. The peak titer of organisms was between 1.0 and 2.0 × 10^{10}/ml. There was little or no change in bacterial density in cultures incubated for another 2 wk.

Leukocytosis-promoting activity of the intact cultures was marked in those

[4] In all instances resin was first removed from the cultures or from aliquots of the cultures, by passage through fine gauze before further processing.

harvested after 2 days of growth and was maximal after 3–5 days of growth. Cultures incubated for 17 days were equal in potency to those harvested early in the stationary phase.

The distribution of leukocytosis-producing activity of *B. pertussis* is also depicted in Fig. 1. After 1 day of growth, the bacterial population had increased approximately sixfold. Injection of the culture fractions did not produce leukocytosis as the values seen were in the range of the normal values for these mice.

Intravenous injection of 0.2 ml of the thimerosal-killed culture, harvested after 3 days of incubation, produced an average leukocytosis amounting to 133,000 cells/mm^3 when the blood was assayed 3 days after injection. It is readily seen that the supernatant fluid contained the bulk of the activity. In subsequent studies, it was shown that on the basis of dry weight analyses, the supernatant fluids were greater than 50 times more active than the bacterial cells.

After 5 days in culture, it is seen in Fig. 1 that there was a slight increase in total activity of the whole culture. Two interesting phenomena are apparent. The first is that the sum of the activities of the supernatant fluid and pellet greatly exceeded the leukocytosis-promoting property of the intact culture. This was shown to be due to the fact that at the dosages employed, the response in mice was maximal.

It is also seen that there was an apparent increase in the activity of the bacterial cells between the 3rd and 5th day of culture. However, when equal numbers of bacteria were injected there was no difference in the leukocytosis produced, indicating that the observed potency increase was due to the approximately 50% increase in bacterial density, rather than to any intrinsic enhancement of bacillary activity.

Comparison of Leukocytosis-Promoting Activity of B. pertussis Liquid Cultures Containing Dowex-1 with those Containing Starch.—Soluble starch has been used frequently in liquid media for the growth of *B. pertussis* (10) and it was of interest to compare its effect with that of the anion exchange resin on bacterial growth and on the production of leukocytosis-promoting activity by *B. pertussis*

Parallel 100 ml cultures in Blake bottles were inoculated with slant washings from 3 day agar growths. One of the bottles contained 0.1% Dowex-1 and the other 0.1% soluble starch (Amend Drug and Chemical Co., N.Y.). The initial concentration of bacterial cells was 6.0 and 6.3 × 10^8/ml respectively. Samples were removed at various time intervals. The optical density was measured and thimerosal was then added. The samples were centrifuged, the supernatant removed, and the pellets resuspended in 0.05 M Tris, pH 7.4, to achieve a cell concentration of 2.5 × 10^{10}/ml. 0.2 ml of the supernatants and resuspended pellets were injected intravenously into groups of four mice whose leukocyte counts were determined 3 days ater.

The growth curves of the organisms in the two media, identical except for the presence of Dowex-1 or soluble starch, are shown in Fig. 2. It is clear that with virtually identical inocula, high bacterial density was reached earlier in Dowex-1

media. Moreover, the culture supernatant fluid of the Dowex-1 medium had far greater leukocytosis-inducing activity even when, at day 5, the bacterial densities were equal. With respect to the activity of the cell pellets, which were tested at equal concentrations, in each instance there was little activity and the leukocyte counts were not raised to levels greater than 60,000/mm³. There were no significant differences among the activities of the various cell pellets harvested from the two media. It should be noted that the concentrations used were
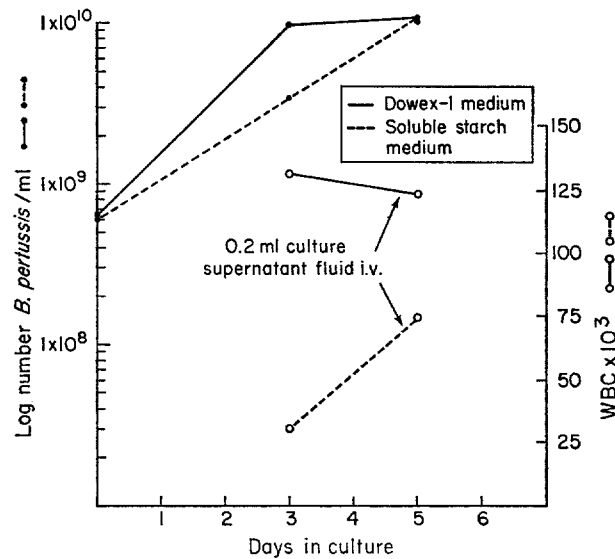

FIG. 2. Growth of *B. pertussis* in fluid medium containing an anion exchange resin or soluble starch and the leukocytosis induced by supernatants of the cultures.

greater than those achieved during growth (2.5 × 10¹⁰/ml vs. 1.04–1.06 × 10¹⁰/ ml).

The Effect of Ampicillin and Mitomycin C on the Growth and Leukocytosis-Promoting Properties of B. pertussis Cultures.—The result of the experiments described above strongly suggested that the bulk of the leukocytosis-promoting factor was rapidly excreted into the media and that very little was cell-bound. This was further supported by the observation that prolonged storage of washed bacterial cells in medium containing 1:5000 merthiolate did not result in the release of active material. However, it was possible that the active material could be in part bound and in a relatively inactive state when within the cell.

Sodium ampicillin (Polycillin-n, Bristol Laboratories, Syracuse, New York) was dissolved and diluted in sterile water immediately before use. Three flasks were inoculated with *B. pertussis* cells and 24 hr later 0.4 ml of the ampicillin dilutions was added to two of the flasks

to achieve concentrations of 1 μg/ml and 10 μg/ml respectively. 0.4 ml of sterile H_2O was added to the third. Aliquots were removed, and then the cultures were returned to the 36°C incubator. Samples were removed 24 and 48 hr later. Thimerosal was added to all the samples which were held at 4°C before testing.

It was found that 1 μg/ml of ampicillin inhibited growth to the same extent as 10 μg/ml. The effect of the former concentration is indicated in Fig. 3. 24 hr
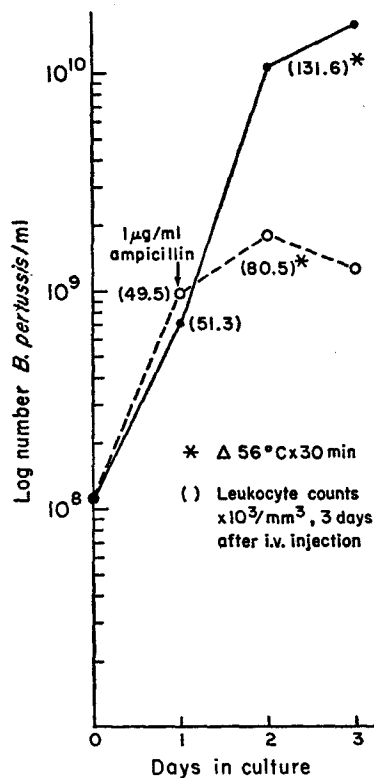

FIG. 3. The effect of ampicillin on bacterial growth and production of leukocytosis-stimulating material by *B. pertussis*.

after addition of the drug there was found only a twofold increase in the cell density as determined by optical density measurement. In contrast, the bacterial density of the control culture had increased more than tenfold.

Phase contrast examination of the ampicillin-treated cultures revealed virtually total replacement of small coccobacillary forms by pleomorphic, phase-lucent bodies together with ballooning cells and cells with the characteristic "rabbit-ear" deformity seen when cell-wall inhibitors are added to bacterial cultures in media which is not hypertonic.

The ability of the cultures to induce leukocytosis was next studied. Samples taken immediately after addition of the ampicillin to the 24 hr culture produced a low level of leukocytosis comparable to that induced by the control culture (Fig. 3). However, the ampicillin-treated cultures harvested 24 hr after addition of the drug were markedly toxic and upon intravenous injection of 0.2 ml into mice there was an 80-90% mortality. The control culture proved to be non-toxic and it produced an expected hyperleukocytosis.

It was possible that the toxicity was related to excess production or release of leukocytosis-promoting material. Heating culture supernatant fluids at 56°C for 30 min had been found to diminish but not abolish that activity. Therefore, the control and ampicillin-containing cultures obtained after 48 hr of culture and 24 hr after addition of the drug were heated under those conditions. As seen in Fig. 3, the leukocytosis was far less following injection of heated ampicillin-treated supernatant.

The nature of the heat-labile toxin was not investigated but it is known that B. pertussis does produce such an agent which is presumed to be a protein (11). This experiment shows that it is clearly separate from the leukocytosis-promoting material. It would have been of interest to establish viable lines of ampicillin-induced protoplasts in order to obtain more information on the heat-labile toxin as well as on leukocytosis-inducing factor. However, it was found that the parent organism was incapable of adequate growth in the presence of concentrations of NaCl or sucrose required to maintain the osmotic integrity of the protoplasts. Thus, valid comparisons could not have been made.

The widespread phenomenon of lysogeny made it important to determine whether the production of leukocytosis-stimulating material was related to the existence of the lysogenic state in the organism used. There was no observed lysis during prolonged growth of B. pertussis 3779 B, and centrifugation experiments suggested that mature bacteriophage was not being produced by the strain. Nevertheless, further evidence was necessary. The antimicrobial drug, Mitomycin C is a potent inducer of bacteriophage development in lysogenic bacteria (12). Therefore this agent (obtained from Calbiochem, Los Angeles, Calif.) was added at a final concentration of 1.25 μg/ml at various times during the growth of B. pertussis in liquid medium. Marked reduction in bacillary multiplication occurred without lysis and without an increase in ability of the culture to induce leukocytosis. Use of other concentrations of Mitomycin C similarly did not yield any evidence of lysogeny.

Leukocytosis as a Function of the Administered Dose of Supernatant Fluids of B. pertussis Cultures.—

The supernatant fluid from a 4-day culture of B. pertussis was obtained by centrifugation of the culture after the growth had been sterilized by addition of 1/50th vol of 1% thimerosal. The fluid was diluted in 0.05 M Tris-0.5 M NaCl, pH 7.4. The high concentration of NaCl was

found necessary to prevent loss of activity, presumably as a result of aggregation in dilute solution. 0.2 ml of the dilutions was injected intravenously into groups of four mice and leukocyte counts were determined 1, 3, 4, 7, and 10 days after injection. In this instance WBC's were performed manually rather than electronically.

Fig. 4 illustrates the average blood leukocyte concentrations at the various time periods. It was apparent, as previously noted, that there was a maximum effect at a dilution of 1:2 which was probably not significantly different from that of the undiluted culture. It was possible that this was a manifestation of
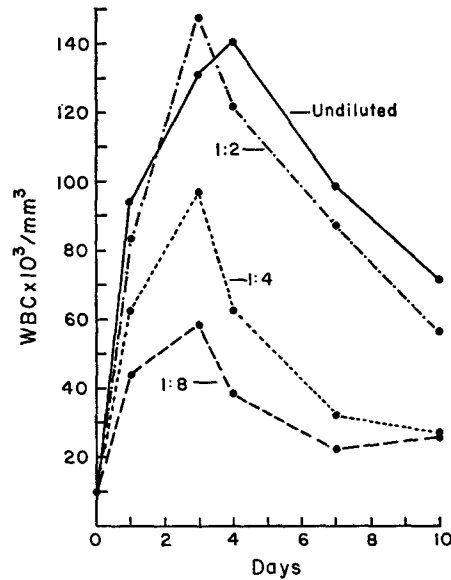

FIG. 4. Time course of the leukocytosis induced in mice by intravenous injection of 0.2 ml of various dilutions of *B. pertussis* culture supernatant.

intrinsic susceptibility of groups of mice to the active principle. However, it was more likely that an inhibitor was present in the undiluted fluid. This was supported by the observation that supernatant fluids from different culture batches when tested in parallel produced quite different peak leukocyte responses. Thus, individual animal reactions would not seem to be responsible.

The differential leukocyte counts observed after the injection of the undiluted and the 1:2 dilution of culture supernatant fluid are presented in Fig. 5. At the time of maximal response, small lymphocytes accounted for over 60% of the circulating leukocytes. The cells appeared to be morphologically mature. By the 7th day after injection the number of circulating small lymphocytes had decreased sharply to between 50 and 60% of the peak values. The falloff in the number of circulating PMN's was more gradual.

The dynamics of the changes in the blood leukocyte population during the first 72 hr after injection of the same culture supernatant fluid was also studied. The preparation was diluted 1:2 in 0.05 M Tris–0.5 M NaCl and 0.2 ml was injected intravenously into six mice. Total and differential leukocyte counts were performed 0, 2, 6, 24, 48, and 72 hr after inoculation. The spectrum of changes is shown in Fig. 6 which depicts the total and differential leukocyte counts in three of the mice.

2 hr after injection, leukopenia was found which was primarily lymphopenia. 6 hr after injection, there was marked leukocytosis. The increases ranged in the six mice from 6.0 to 9.9 times the 2 hr value and from 2.7 to 7.2 times the initial
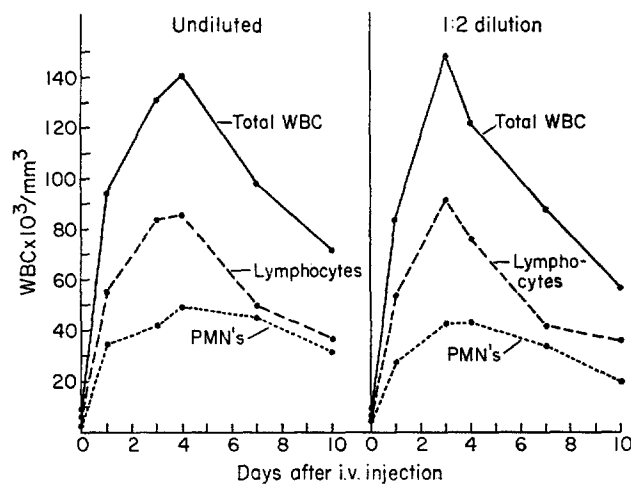

Fig. 5. Total and differential leukocyte counts of mice injected intravenously with 0.2 ml of *B. pertussis* supernatant fluid.

value. The major cause of the leukocytosis was a striking granulocytosis. These cells were increased from 7- to 18-fold greater than normal and constituted over 70% of the circulating cells. In contrast, although the number of lymphocytes had risen from their lowered value at 2 hr to near normal, in no instance had they increased more than 40% over the base line figure.

24 hr after injection, the picture had markedly altered from that seen at the 6 hr period. There was a marked decrease in the number of circulating granulocytes, although they were still elevated over base line values. Concomitantly the number of small lymphocytes increased, so that they were again the predominant cell. The total WBC varied between 79×10^3 and 119×10^3 cells/mm^3. The total number of cells had either fallen, stayed approximately the same, or risen with respect to the 6-hr counts–depending upon the magnitude of the 6 hr count, the reduction in the number of granulocytes, and the extent of the lymphocytosis. The interplay of these factors is well illustrated in Fig. 6. The pro-

gression of events between the 1st and 3rd days after injection was similar to that shown in Fig. 5. The large mononuclear cells, composed of both monocytes and large lymphocytes, behaved as a group in a manner similar to the granulocytes, although of course at much lower numbers.
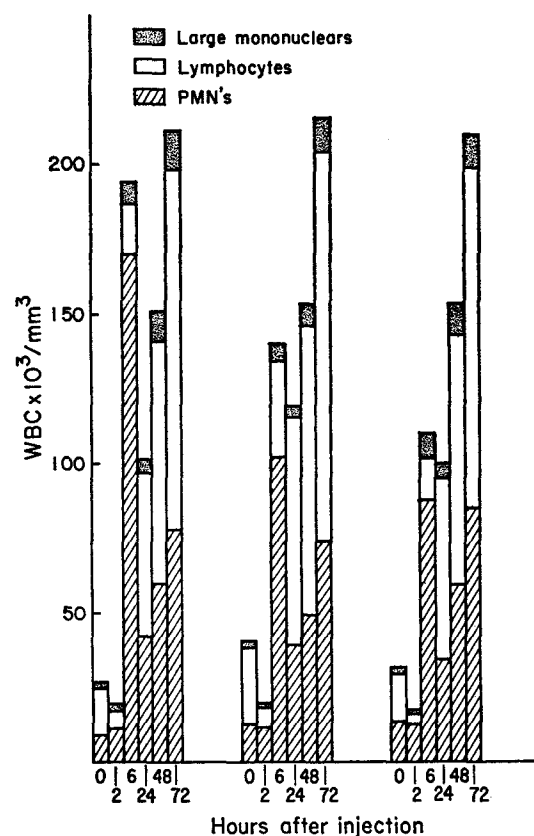

FIG. 6. Total and differential leukocyte counts of mice during the first 72 hr after injection of *B. pertussis* supernatant fluid.

Morphologically, both granulocytes and lymphocytes appeared mature during the various stages of the reaction as shown in Fig. 10.

The Effect of the Route of Injection on the Response to B. pertussis Supernatant Fluids.—

Culture supernatant fluid from a 4 day growth of *B.pertussis* was diluted 1:2 in 0.05 M Tris-0.5 M NaCl, pH 7.4. One group of four mice was injected with 0.2 ml intravenously and another group with the same dose intraperitoneally. At intervals total and differential WBC's were performed. As seen in Fig. 7, the qualitative aspect of the changes in the leukocyte popu-

lations in the two groups was similar, but quantitatively the response to the intravenous inoculations was greater.

When a similar experiment was performed to determine the efficacy of the subcutaneous route it can be seen in Fig. 8 that this route of administration produced very little response.

The Effect of Antiserum on the Leukocytosis Produced by B. pertussis Supernatant Fluids.—

B. pertussis culture supernatant fluid was mixed with normal rabbit serum or various amounts of rabbit antisera raised by repeated subcutaneous injections of suspensions of
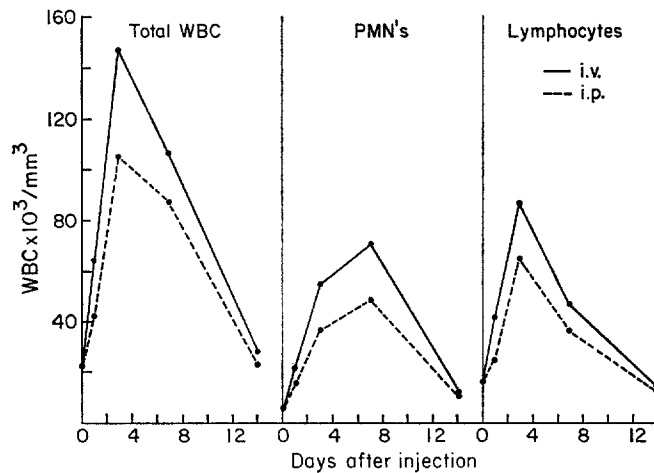

Fig. 7. Total and differential leukocyte counts of mice injected intravenously or intraperitoneally with equal amounts of *B. pertussis* culture supernatant fluid.

lyophilized organisms. All sera were heated at 56°C for 30 min before use. The tubes were incubated at 37°C for 60 min and then overnight at 4°C. Any precipitate that had formed was removed by centrifugation at low speed at 4°C, and 0.2 ml of the resulting supernatant fluids was injected intravenously into groups of four mice. Leukocyte counts were performed 3 days later.

As is seen in Table I the addition of 25% immune rabbit serum markedly inhibited the production of leukocytosis, whereas 25% normal rabbit serum had no effect. 5% immune serum caused slight inhibition and 1% had no effect. Thus it was clear that the leukocytosis-promoting factor was serologically active.

Active Immunity and Pertussis-Induced Leukocytosis.—

In order to determine whether the culture supernatant fluid itself was capable of inducing a refractory state with respect to the production of leukocytosis, one group of mice was given a

single injection of 0.1 ml of supernatant fluid intravenously, another group received the same dose subcutaneously, and a third group was not injected.

24 days later, leukocyte counts were obtained on all mice and when it was found that all were in the same range they were injected intravenously with dilutions of the supernatant fluid. WBC's were determined 3 days later. The results of this experiment are presented in Table II.
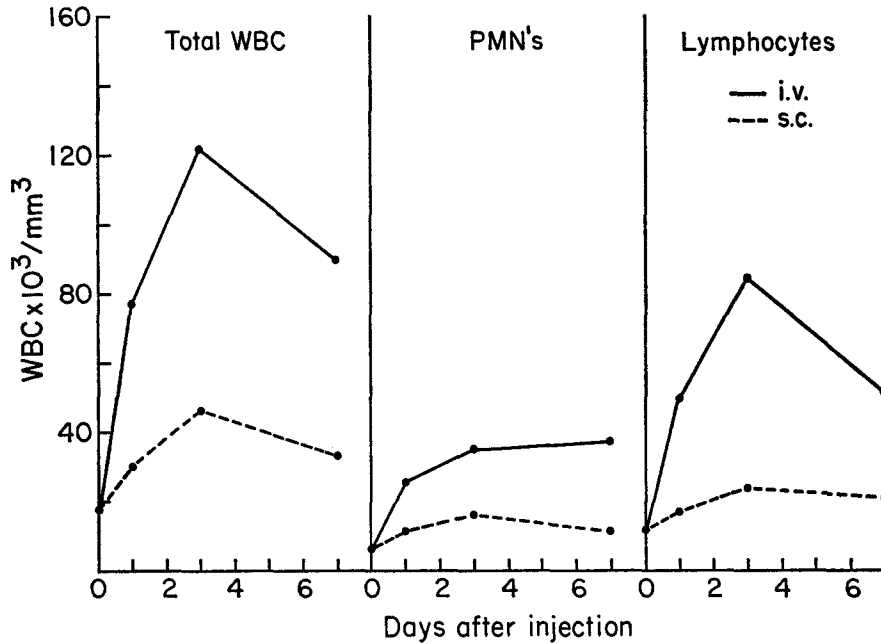

FIG. 8. Total and differential leukocyte counts of mice injected intravenously or subcutaneously with equal amounts of *B. pertussis* culture supernatant fluid.

TABLE I

The Effect of Immune and Normal Rabbit Serum on the Leukocytosis Produced by B. pertussis Culture Supernatant Fluids

Final dilution of supernatant fluid	Final concentration of:		Precipitate	Average WBC/mm³
	Antiserum	Normal serum		
	%	%		
1:2	—	25	±	164,700
1:2	25	—	++++	73,900
1:2	5	—	++	137,900
1:2	1	—	+	164,400
1:2	—	—	0	179,800
1:4	—	—	0	147,100

It can be readily appreciated that a single subcutaneous injection produced complete protection against a subsequent intravenous challenge. In contrast there was essentially no protection engendered by an equivalent intravenous injection.

The lack of induction of active immunity following intravenous injection may be a manifestation of the depletion of lymphocytes from lymphoid tissue.

The Distribution of Protective Antigen and Histamine-Sensitizing Factor (HSF) in Liquid Cultures of B. pertussis.[5]—Questions regarding the localization of certain biologic factors of B. pertussis have not been fully answered. Knowing that the bulk of the leukocytosis-promoting activity resides in the supernatant fluid, it was of interest to determine whether mouse protective activity and HSF followed the same distribution pattern.

TABLE II

The Effect of Active Immunization with B. pertussis Culture Supernatants on the Leukocyte Response

Route of immunization	Prechallenge WBC*	WBC* 3 days after intravenous challenge with supernatant fluid	
		0.05 ml‡	0.025 ml‡
Intravenous	37,600	96,400	70,100
Subcutaneous	44,700	44,100	42,700
No immunization	39,900	116,000	76,300

* Cells/mm³.

‡ All doses were administered in 0.2 ml vol in 0.05 M Tris–0.5 M NaCl, pH 7.4.

The materials for the protection test were derived from 3 day cultures of the organism to which 1% thimerosal had been added to a final concentration of 1:5000. The concentration of organisms was 10^{10}/ml. A portion of the killed culture was centrifuged at 13,000 g for 5 min. The supernatant fluid was decanted and the pellet was resuspended to initial volume in medium containing thimerosal.

The average leukocyte counts of mice 3 days after intravenous injection of 0.2 ml of the fractions were 189,000/mm³ for the whole culture, 159,600 mm³ for the supernatant fluid, and only 65,800/mm³ for the pellet.

The preparations were diluted to appropriate volumes in 0.5 M NaCl and 0.5 ml was injected intraperitoneally into groups of 10–14 mice weighing 12–14 g. 16 days later the mice were challenged intracerebrally with 1.0 × 10⁵ organisms, approximately 550 times the LD₅₀.

Although toxic deaths prior to challenge followed the injection of whole culture and prevented accurate grading of the ED₅₀, significant protection was

achieved (5 survivors out of 10 challenged) when an equivalent dose of 6×10^7 organisms was used for immunization. The ED_{50} of the bacterial pellet was 8.9×10^8 organisms. On the other hand, the supernatant fluid induced no protection whatever even when the immunizing dose was equivalent to 1.5×10^9 organisms or greater.

The protection test was repeated using the subcutaneous route of injection in order to rule out vagaries of the immune response related to the mode of immunization which were found to occur with respect to active immunity against leukocytosis-promoting factor (vide supra).

Equivalent doses of 1.5×10^9 cells of a whole culture, cell pellet, and culture supernatant were injected subcutaneously into mice which were subsequently challenged by intracerebral inoculation of living organisms. The whole culture protected 2/8 mice, the pellet 5/9, and the supernatant 0/8. Thus this study confirmed that, unlike leukocytosis-stimulating activity, protective antigen was a cellular component and did not appear in supernatant fluids to any appreciable extent.

Studies on the distribution of HSF were performed in a similar manner. An aliquot of a 4 day growth of *B. pertussis* was adjusted to a cell concentration of 5×10^9/ml with culture medium. A portion of this sample was centrifuged at 26,000 g for 10 min. The supernatant fluid was removed and the bacterial pellet was resuspended in medium to the original volume. 0.002% thimerosal was present in all samples. The preparations were diluted fourfold in 0.05 M Tris–0.5 M NaCl and 0.2 ml was injected intravenously into 25–30 g male mice. Uninjected mice served as controls.

3 days later WBC's were determined and the mice were injected intraperitoneally with 2 mg of histamine dihydrochloride (Mann Research Laboratories, New York) contained in 0.4 ml of physiological saline. Control mice were also injected with 10 and 20 times this amount. Although all deaths occurred within 1–2 hr after histamine injection, the animals were observed for a further 24 hr.

As can be seen in Table III the distribution of histamine-sensitizing factor, unlike protective antigen, was similar to that of leukocytosis-stimulating ac-activity. The supernatant fluid contained virtually all of the HSF as well as the leukocytosis-stimulating material and was more than 10 times as potent as the bacterial cell fraction.

The Effect of Heat and pH on the Leukocytosis-Promoting Activity of Culture Supernatant Fluids of B. pertussis.—

Aliquots of a culture supernatant fluid were heated for 30 min at temperatures of 56°, 80°, and 100°C in a water bath. 0.2 ml of the undiluted preparations or preparations diluted in 0.05 M Tris–0.5 M NaCl were injected intravenously into mice and WBC's were determined 3 days later.

As indicated in Fig. 9, heating the supernatant fluid at 80° and 100°C for this time period completely destroyed activity. Approximately 50–75% of the activity was lost at 56°C.

Samples of the supernatant fluid were dialyzed for 18 hr at 4°C against 100-fold volumes of 0.05 M Tris buffer at pH 7.4 and pH 9.2 and against 0.05 M acetate buffer at pH 4.0. All of the buffers contained 0.5 M NaCl. The appropriate samples were then neutralized with HCl or NaOH and diluted in 0.05 M Tris–0.5 M NaCl, pH 7.4. 3 days after intravenous injection it was found that there was slight loss of activity after exposure to both the alkaline and acid conditions.

TABLE III

Distribution of Histamine-Sensitizing Factor in Fluid Cultures of B. pertussis

Pretreatment	Dilution	Histamine dose	S/T*	Average WBC $\times 10^3/mm^3$‡
		mg		
Uncentrifuged culture	0§	2	0/5	150.8
"	1:4	2	0/5	69.4
"	1:16	2	1/5	47.3
Culture supernatant	0§	2	0/5	120.6
"	1:4	2	0/5	62.7
"	1:16	2	2/5	51.5
Bacterial pellet	0§	2	1/5	48.9
"	1:4	2	5/5	44.2
"	1:16	2	5/5	Not tested
None	—	2	5/6	32.5
"	—	20	6/6	32.5
"	—	40	1/6	32.5

* Survivors/Total.

‡ Before histamine challenge. 3 days after pretreatment.

§ Culture adjusted to 5×10^9 bacteria/ml before fractionation and pellet resuspended in initial volume. 0.2 ml amounts injected intravenously.

The assay system did not permit accurate estimation of losses of 40% or less so that the magnitude of the loss of activity could not be ascertained precisely.

The Effect of Dialysis on the Leukocytosis-Promoting Activity of B. pertussis Supernatant Fluids.—The medium utilized in these experiments was chosen because it was dialyzable and would therefore facilitate the isolation of active material if that also were nondialyzable. In preliminary experiments it was found that when supernatant fluids were dialyzed against distilled water the fluid within the dialysis sac became turbid. Concomitantly there was greater than 50% loss in activity. Similarly if 0.05 M Tris, pH 7.4, was used as the dialyzing medium aggregation also occurred indicating that a pH shift was not responsible for the alteration in physical properties. Addition of 0.001 M EDTA to the Tris buffer did not prevent aggregation nor did dialysis against deionized or

glass-distilled water, suggesting that divalent cations were not involved. Sulf-hydryl reagents also were not effective inhibitors of aggregation. Addition of NaCl in concentrations greater than 1% (w/v) to the Tris buffer, however, prevented aggregation and full activity of the supernatant was maintained indicating that the active factor was in fact nondialyzable.

It was not clear whether loss of activity after H_2O dialysis was due to irreversible changes in the active material per se; to mechanical alterations related to
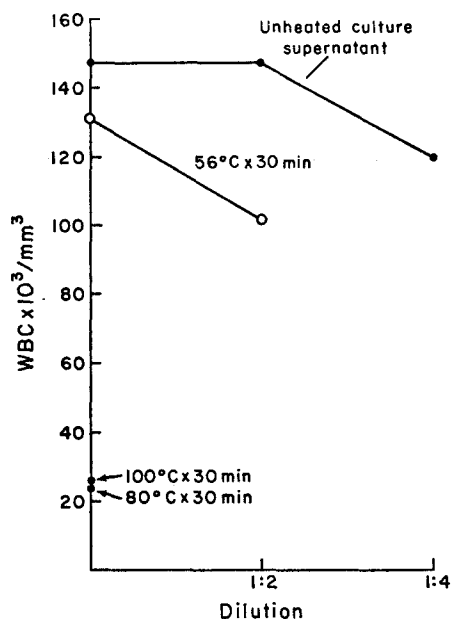

FIG. 9. The effect of heat on leukocytosis production by *B. pertussis* culture supernatant fluid.

aggregation which prevented homogeneous distribution; to a different distribution of the material after injection into the test animal; to physical masking of the reactive sites; or to actual losses of material.

It was observed that after H_2O dialysis the bulk of the remaining activity was associated with the aggregates which were readily centrifugable. Pellets isolated after prolonged H_2O dialysis were not rendered soluble by addition of buffered NaCl at concentrations up to 2 M. However, from the precipitate which formed after overnight dialysis, 80% of the activity was solubilized in 0.05 M Tris–0.5 M NaCl, pH 7.4.

Many attempts were made to solubilize the aggregates which had formed after prolonged dialysis of culture supernatant fluids against H_2O. In addition to strong NaCl solutions, reagents used included deoxycholate; anionic, cationic,

and neutral detergents; ethylene glycol; and butanol. Both sodium dodecyl sulfate and cetylpyridinium chloride at concentrations of less than 1%, solubilized the pellets to an extent greater than 75%. However, the material was rendered inactive whether or not the detergents were removed before testing. Nonionic detergents neither solubilized nor inactivated the pellets. Deoxycholate at concentrations greater than 2.5% also caused over 75% solubilization but again activity was diminished. Ethylene glycol caused approximately 50% solubilization but inactivation also paralleled the degree of solubilization. The material was not resolved in butanol.

The results of these experiments were similar to those found when attempts were made to extract active material from intact organisms. Thus, sodium dodecyl sulfate at concentrations of 1% or less caused marked lysis of intact organisms, but complete loss of leukocytosis-stimulating activity. In the same way, both 6 M urea and 5 M guanidine at both neutral and alkaline pH had been shown to destroy activity. Other reagents which inactivated leukocytosis-stimulating activity of intact organisms included 2% aqueous phenol, aqueous ether, 50% pyridine, and acetone-ether.

The Distribution of Leukocytosis-Stimulating Activity in Culture Filtrates Subjected to Ultracentrifugation.—

Samples of culture supernatant fluids were centrifuged at 100,000 g at 4°C in the preparative ultracentrifuge (Model L-2, Beckman Instruments Inc., Palo Alto, Calif.). The supernatant fluid was decanted and the pellet resuspended in 0.05 M Tris–0.5 M NaCl, pH 7.4 to the original volume. Samples of the 100,000 g supernatant and pellet, as well as the starting material were tested for induction of leukocytosis in mice.

After 1 and 3 hr of centrifugation under these conditions, the total activity of the starting material remained in the 100,000 g supernatant. However, after 18 hr there was redistribution of the active material (Table IV). The activity was found in both the supernatant fluid and in the pellet which had formed; slightly more was in the latter. In a separate experiment when the two fractions were recombined, less than total activity was recovered. As in the case of pellets formed after prolonged dialysis against media of low osmotic strength, the pellet formed after ultracentrifugation could not be readily resolved.

The Effect of Various Enzymes on the Leukocytosis-Stimulating Activity of B. pertussis Supernatant Fluid.—

For all enzyme experiments, culture supernatant fluids were throughly dialyzed against 0.5 M NaCl in the appropriate buffer. This was done in order to remove thimerosal and low molecular weight constituents of the supernatant fluid which might inhibit enzyme action. Ribonuclease and deoxyribonuclease (pancreatic ribonuclease, Mann Research Laboratories, New York; pancreatic deoxyribonuclease, Worthington Biochemical Corp., Freehold, N. J.) were tested in 0.05 M Tris–0.5 M NaCl, pH 7.4. In the case of the latter enzyme, 0.001 M MgCl was also present. The mixtures were incubated 3–5 hr at 37°C and even at concentrations

greater than the calculated concentration of the nondialyzable components of the supernatant fluid neither nuclease had any effect. Wheat germ lipase (Worthington) also had no effect on the activity.

The proteolytic enzymes trypsin (Worthington), pronase (Calibochem), chymotrypsin (Sigma Chemical Co., St. Louis, Mo.), Subitilisin (Sigma), and activated papain (Sigma) were also tested under similar conditions and at concentrations approximately one-half that of the protein present as determined by the Folin reaction. Pepsin was not tested since inactivation occurred in buffer at pH 2.5 alone. Loss of activity was variable, and when it occurred was of a low order so that the significance was uncertain. Trypsin and pronase were also tested in supernatant fluids dialyzed against 0.01 M PO_4–0.5 M NaCl, pH 7.4, and again questionable enzymatic effect was observed. In addition, casein was added to identical mixtures of supernatant fluid and enzyme and incubated under the same conditions. On the basis of the increase in trichloracetic acid–soluble, Folin-reacting material, it was calculated that both trypsin and pronase were present in 20-fold excess, in terms of their caseinolytic activity and that no inhibitor was present.

TABLE IV

Distribution of Leukocytosis-Stimulating Activity in B. pertussis Supernatant Fluids Centrifuged at 100,000 g for 18 Hr

Sample	Equivalent volume injected and WBC's* 3 days later			
	0.07 ml‡		0.035 ml‡	
	Mean	Range	Mean	Range
100,000 g supernatant	94.0	85.2–104.8	81.0	65.6–94.0
100,000 g pellet	133.0	108.0–151.6	85.4	74.0–100.8
Uncentrifuged	225.3	214.4–238.8	139.4	127.6–152.0
Reconstituted	122.0	108.4–136.8	99.9	88.0–114.0
Uncentrifuged	153.2	126.4–191.2	124.8	101.6–144.8

* WBC $\times$ 10^3/mm^3.

‡ Total volume injected was 0.2 ml in 0.5 M Tris–0.5 M NaCl, pH 7.4.

Both trypsin and pronase were then restudied over longer time periods and it was found that after 24 hr at room temperature the leukocytosis-stimulating property was reduced to 25–50% of that present in preparations to which no enzyme had been added. No further loss of activity occurred during the subsequent 48 hrs.

It was therefore concluded that the active material was incompletely susceptible to proteolytic enzymes and that proteolysis occurred at a slow rate.

Attempts to Concentrate the Active Leukocytosis-Stimulating Material in B. pertussis Supernatant Fluids.—

Solid $(NH_4)SO_4$ was added to culture supernatant fluids while maintaining the pH at neutrality by the addition of NH_4OH. At 60% saturation a fine precipitate formed and was col-

lected on filter paper. The precipitate did not fully redissolve even in media containing 1.0 M
NaCl. Leuocytosis-promoting activity was present in this fraction but the total activity re-
covered was less than 25%, despite the fact that no activity could be detected in the 60%
$(NH_4)SO_4$ supernatant fluid.

Concentration of preparations in a glass rotary evaporator (Buchler Instruments, Fort Lee,
N. J.) was attempted. Untreated culture supernatant fluids and those dialyzed against fluids
with sufficient salt concentration to prevent aggregation were used. In all cases, there was loss
of activity estimated to be between 60 and 70%. Concomitantly, approximately the same
amount of nondialyzable protein could not be recovered. Similarly, pressure or vacuum con-
centration procedures employing collodion bags, cellophane dialysis sacks, and membrane filters
all resulted in parallel losses of activity and protein. It was shown that these losses were not the
result of catalytic activity. Thus, preparations to which 0.5% formaldehyde, 1.0% phenol, or
10^{-2} M iodoacetamide had been added, lost over 60% of the initial protein present when they
were concentrated by vacuum in cellophane sacks; the same loss occurred when the super-
natant fluids were first heated at 100°C for 30 min. In addition, no proteolytic activity against
casein could be detected.

These results indicated that when the fluids were concentrated, the active
material was readily adsorbed onto a variety of materials including some
generally inert in this regard. It was also of interest that the effluent after
passage of supernatant fluids through 0.45 μ Millipore filters (Millipore Corpo-
ration, Bedford, Mass.) had lost the bulk of the original activity. On the basis of
the ultracentrifugation data one would not have expected the active fraction
to have been retained on the filter by virtue of molecular size. Activity was also
adsorbed onto alumina, cellulose, and diatomaceous earth. No activity could
be removed from these with acid or neutral buffers containing 0.5 M NaCl, but
small amounts were eluted at alkaline pH. Thus far, no suitable procedure has
been devised which would yield an efficient absorption-elution method of con-
centration.

Attempts to precipitate active material by the addition of Mg, Zn, and Cu
ions also were unsuccessful.

*Isolation of a Leukocytosis-Stimulating Fraction from B. pertussis Culture
Supernatant Fluids by Precipitation in a Hypotonic Milieu.*—Since no other
satisfactory method of isolation of the active fraction could be devised, further
studies were performed on material which precipitated when culture superna-
tant fluids were dialyzed against solutions of low ionic strength.

2 liters of culture supernatant fluid were dialyzed first against running tap water for 18 hr
and then against several changes of large volumes of distilled water at 4°C. An aliquot of the
turbid solution was saved, and the remainder was centrifuged at 28,000 g for 30 min. The
supernatnat fluid was removed and the pellet was resuspended in distilled H_2O. All three sam-
ples were then lyophilized.

The materials were then taken up in 0.05 M Tris–0.5 M NaCl, pH 7.4. The uncentrifuged and
pellet fractions were in suspension, not solution, and care was taken to achieve effective disper-
sion by mixing them repeatedly with the aid of a 26 gauge hypodermic needle. The preparations
were diluted in the same medium and 0.2 ml of the appropriate dilutions were injected into
groups of four mice.

It is clear from Table V that the bulk of the activity in the culture supernatant fluid was in the H_2O-insoluble fraction. This fraction had slightly more than five times the specific activity of the uncentrifuged dialyzed culture supernatant fluid, a figure commensurate with almost complete recovery of activity, since it approximated the weight fraction which the water-insoluble material represented. (It should be noted, however, that in terms of the untreated supernatant, only 15–25% of activity was recovered.) Moreover, this fraction was approximately 25 times more active than the water-soluble components. In addition, in tests on lyophilized bacteria from liquid cultures, WBC's greater than $150,000/mm^3$ were not induced unless 200 μg or more was injected.

TABLE V

Blood Leukocyte Counts of Mice 3 Days after Intravenous Injection of Fractions from Dialyzed B. pertussis Supernatant Fluids

Fraction	Yield	Amount injected	WBC $\times 10^3/mm^3$	
			Average	Range
	mg/L	*μg*		
	70.8	200	*	*
Uncentrifuged		40	145.6	135.2–175.6
		8	100.9	90.8–125.2
H_2O-soluble	56.4	200	161.0	149.2–179.6
		40	91.6	79.2–104.4
		8	39.4	35.6– 44.0
H_2O-insoluble	19.3	200	*	*
		40	*	*
		8	173.6	162.4–187.6

* 4/4 Mice dead within 3 days after injection.

The active pellet was quite dark in color as though extraneous particulate contaminants had adhered to it. Cleaner preparations were obtained in the following manner. After overnight dialysis against running tap H_2O the dialyzed fluid was centrifuged in 500 ml polyethylene bottles at 9000 g for 45 min. The supernatant fluid was decanted and 20 ml of 0.05 M Tris–0.5 M NaCl, pH 7.4, was added to each bottle. The bottles were shaken and the fluid was centrifuged at 12,000 g for 10 min. The resulting pellet was dark colored and had little activity. The supernatant fluid was dialyzed against running tap H_2O overnight. The precipitate was harvested by centrifugation, washed with distilled H_2O, and lyophilized. The product now was pale yellow in color and was still highly active. Injection of 5 μg per mouse resulted in an average leukocytosis of $150,800$ cells/mm^3 3 days later.

The time course of events with respect to the qualitative and quantitative changes in the blood leukocyte population of mice was identical to that observed after the injection of crude culture supernatant fluids (Fig. 6).

Occurrence of Histamine-Sensitizing Factor in Fractions Containing Leuko-cytosis-Stimulating Activity.—

7 liters of supernatant fluid from a *B. pertussis* culture were dialyzed against running tap H_2O overnight. An aliquot was saved and the remainder was centrifuged at 9000 g for 30 min. A portion of the supernatant was saved. The precipitate was taken up in ⅕th vol of 0.05 M Tris–0.5 M NaCl, pH 7.4. The inactive insoluble fraction was removed by centrifugation at 28,000 g for 10 min. The supernatant fluid was dialyzed against running tap H_2O overnight and the resultant precipitate was harvested and washed with distilled H_2O on the centrifuge.

The aliquots of the dialyzed culture supernatant fluid and of the initial supernatant fluid were dialyzed thoroughly against distilled H_2O. The bacterial pellet was also washed with distilled H_2O. All four fractions were then lyophilized.

The original dialyzed culture supernatant fluid and the water-soluble fraction were not soluble in 0.05 M Tris–0.5 M NaCl, pH 7.4, but were brought into solution in the cold in the same buffer at pH 10 with the aid of brief sonication. The water-soluble fraction readily dissolved in 0.05 M Tris–0.5 M NaCl at pH 7.4 and the lyophilized bacteria were brought into fine suspension in the same medium. The samples were diluted and 0.2 ml vol were injected intravenously into groups of four mice. 3 days later, WBC's were determined, and then the mice were injected intraperitoneally with 1 mg of histamine dihydrochloride in 0.4 ml of physiologic saline. Deaths were recorded at 24 hr.

As seen in Table VI, the H_2O-insoluble fraction of the culture supernatant fluid had more than five times the leukocytosis-stimulating activity on a weight basis than the uncentrifuged specimen, it had more than 25 times the specific activity of the H_2O-soluble fraction, and more than 100 times that of the bacteria from the same culture.

Histamine-sensitizing activity paralleled that of leukocytosis-stimulating activity and clearly the H_2O-insoluble fraction had the greatest specific activity (Table VI). Under the conditions employed the end point was not reached even at a dose of 1.6 μg/mouse.

It can also be seen in Tables V and VI that a lethal effect paralleled the degree of leukocytosis. On the basis of the results of the experiments employing ampicillin, this effect was believed to be intrinsic to the H_2O-insoluble fraction and not due to contamination of the preparation with heat-labile toxin.

*Properties and Composition of the H_2O Insoluble Active Fraction.—*Immune diffusion analysis of the active fraction dissolved at 1 mg/ml in 0.05 M Tris–0.5 M NaCl, pH 10, was performed in 1 % agarose gel in the same buffer. The antiserum had been prepared against intact organisms. Three bands of immune precipitate developed. Separation of these immunologically distinct factors proved difficult.

The chemical composition of a representative preparation (corrected for

moisture and ash) was as follows: nitrogen, 14.5%; carbon, 51.2%; hydrogen, 7.4%; phosphorus, 0.6%; nucleic acid, <0.3%; carbohydrate (excluding hexosamine), 3.8% as glucose equivalents; hexosamine, 1.2%; fatty acids, 1.0%; and amino acids, as determined on the basis of recovery of the weight of material applied to the column, 74–80%. On the evidence of the elemental and group

TABLE VI

Blood Leukocyte Counts and Histamine Susceptibility of Mice 3 Days after Intravenous Injection of Dialyzed Culture Supernatant Fractions and B. pertussis Cells

Fraction	Amount injected	WBC $\times$ 10^3/mm^3		Survivors/Total after 1 mg of histamine i.p.
		Average	Range	
	μg			
Bacteria	200	79.6	62.4– 90.0	0/4
	40	38.0	31.6– 44.0	3/4
	8	38.9	32.4– 46.0	4/4
	1.6	—	—	4/4
Dialyzed uncentrifuged super-natant	200	*	*	0/4
	40	139.9	114.8–159.6	0/4
	8	88.3	64.4–107.6	0/4
	1.6	53.6	45.6– 64.8	2/4
H$_2$O-soluble supernatant fraction	200	120.9	105.2–147.6	0/4
	40	102.6	70.8–121.2	0/4
	8	41.1	23.6– 59.2	2/4
	1.6	31.3	26.8– 36.8	3/4
H$_2$O-insoluble supernatant fraction	200	*	*	0/4
	40	*	*	0/4
	8	166.3	144.4–196.4	0/4
	1.6	76.5	60.8– 94.0	0/4

* 4/4 Mice dead within 3 days after injection.

analyses, it is likely that the figure for amino acids is a minimum and that there was loss on the columns.

The amino acid analysis was striking in that no cysteine was present. The total amount of fatty acid present was too small to permit accurate indentification of the individual components, but probably myristic and/or hydroxymyristic acids were present.

DISCUSSION

B. pertussis cells when injected into mice result in hypersensitivity to a variety of agents and conditions including histamine, serotonin, anaphylaxis, endotoxins, viral infections, X-irradiation, cold and anoxia (reviewed by Kind

[13] and Munoz [14]). Another striking manifestation of the injection of pertussis cells into mice is the production of hyperleukocytosis. As in the case of the leuko-cytosis which accompanies clinical pertussis, the unique feature of this response is that the mature small lymphocyte is the predominant cell in the circulation at the time the white blood count is maximal (1).

The phenomenon has been utilized to explore various aspects of lymphocyte physiology (2). However, in order to pursue further studies it would be advantageous to utilize material less complex and more readily manipulated than suspensions of intact bacteria. Fractionation of the bacteria to this end was generally unsuccessful and therefore the possibility that the active material might be liberated extracellularly in fluid medium was explored.

Fluid growth of *B. pertussis* to high titers generally requires the presence of starch, charcoal, or albumin. Presumably these substances, as well as whole blood, neutralize factors toxic for the organism. Unfortunately, they cannot be effectively removed from the culture fluids by centrifugation or dialysis. This problem was circumvented by utilizing a modification of the culture medium described by Sutherland and Wilkinson (3) in which an anion exchange resin is employed as the "detoxifying" agent. The resin was readily removed by filtration through fine gauze, thereby permitting subsequent independent study of the bacterial cells and supernatant fluids. Moreover, the remainder of the medium was completely dialyzable.

In contrast to the results of previous studies on agar-grown organisms (1), the bulk of the leukocytosis-promoting material was found to be extracellular. The discrepancy is most likely explained by the binding of the active fraction to the agar. Binding of activity of the supernatant fluids to a variety of substances was frequently seen, yet there was no evidence of binding to the anion exchange resin in the medium. It was proposed, but not proven, that the active material was basic.

The effects of the supernatant fluids on the leukocyte counts of mice completely paralleled those found after injection of intact bacterial cells (1). Of note was the inability of these soluble preparations to induce leukocytosis by the subcutaneous route of administration, a phenomenon also seen when killed bacteria were used. This suggested that local inactivation rather than difficulty in transport was responsible for the latter finding.

Neither nucleases nor lipase diminished activity, but the active fraction was affected by the presence of the proteolytic enzymes trypsin and pronase in relatively high concentrations over a long period of time.

Preparation of immunologically homogenous material was hampered by two characteristics of the active substance. The first was aggregation with concomitant loss of activity during exposure to media which did not contain a high salt concentration and under certain conditions, such as prolonged dialysis against distilled H_2O or istonic buffers, no solubilizing reagents could be found. Sec-

ondly, there was a marked tendency for activity to be adsorbed to a variety of materials and efficient recovery could not be achieved.

Partial purification was achieved by taking advantage of the water insolubility of the active material. After dialysis of the supernatant fluid against water, the specific activity of the isolated water-insoluble preparation was 25-fold greater than that of the water-soluble material. Virtually all of the activity in the water-dialyzed supernatant fluid was recovered in the insoluble fraction. However, such dialyzed preparations had already lost 75–85% of the activity of the undialyzed culture supernatant fluid. There is reason to believe that this was due to an alteration in the active fraction, but alternatively actual loss of material due to adsorption could have occurred.

The main constituent of the active fraction was protein as evidenced by the nitrogen values and the content of amino acids. There was no cysteine present and there was only 1.1 mM of methionine per 100 mM of amino acids. This indicated that disulfide bond formation was not responsible for insolubility and that SH groups were not required for activity. The thesis was supported by the lack of effect of sulfhydryl reagents on solubility and activity. Total carbohydrate, including hexosamine, amounted to less than 6% of the fraction, and fatty acid accounted for approximately 1.0%.

Several lines of evidence suggested that a protein was the active moiety. These included reduction in activity of supernatants by proteolytic activity, sensitivity to heating at 56°C for 30 min, and loss of activity when various detergents or protein denaturants were added. Furthermore, it had been shown that both urea and guanidine destroyed the activity of intact cells.

The presence of both carbohydrate and fatty acids suggested that small amounts of endotoxin might be present. However, it was clear that lipopolysaccharide endotoxin was not the active factor since attempts at extraction of lipopolysaccharide from whole cells with reagents such as phenol-water, aqueous ether, diethylene glycol, and aqueous pyridine resulted in complete loss of leukocytosis-stimulating activity. "Native" endotoxin is a term used to describe a lipopolysaccharide-peptide complex from which lipopolysaccharide with endotoxic properties can be isolated. Such complexes are liberated into the medium during growth of lysine-requiring *Escherichia coli* strains under lysine-limiting conditions (15) and perhaps to some extent during normal growth of Gram-negative organisms. However, though it was possible that the peptide moiety of a complete lipoglycopeptide of this nature was the responsible factor, it seemed unlikely on quantitative grounds. Resolution of the problem must await separation of the various components in the active fraction.

The results of the studies reported here show that histamine-sensitizing factor (HSF) and leukocytosis-stimulating activity are closely associated. Both were found primarily in the supernatant of fluid cultures of *B. pertussis* and both were precipitated when the supernatants were dialyzed against water. On the

basis of studies on whole cell extracts HSF is thought to be a basic substance (16); is susceptible to but not completely inactivated by trypsin (17); is more effective when administered by the intravenous route (18); can be given repeatedly by the intravenous route without dulling of the response (18); and contains no cysteine (19). These are also properties of the leukocytosis-stimulating material suggesting that its association with HSF may not be merely fortuitous.

On the other hand, mouse protective antigen was not associated with leukocytosis-producing activity and was essentially confined to the bacterial cells. These results are at variance with the findings of Kuwajima et al. (20). These workers found that supernatant fluids induced greater protection than the bacterial cells. Whether the difference is related to the use of different strains and media, or to the fact that Kuwajima et al. gave two immunizing injections of the supernatant fluid and only one of the bacterial pellet, is not clear.

The results of our studies provide further evidence that HSF and mouse protective antigen are unrelated (21–23), rather than associated or identical (e.g. 17, 24). Nevertheless, ultimate resolution of this controversy awaits isolation of homogenous materials.

SUMMARY

1. Leukocytosis- and lymphocytosis-stimulating activity was present in fluid cultures of B. pertussis. The activity was found primarily in the culture supernatant fluid.

2. The sequential changes in the leukocyte response were similar to those previously observed following injection of intact bacteria into mice.

3. Activity was destroyed by heat and was diminished, but not abolished, by prolonged treatment with proteolytic enzymes.

4. A water-insoluble fraction of the culture supernatant fluid was isolated which contained virtually all of the activity. The specific activity was more than 100-fold greater than that of the intact bacteria, and injection of microgram quantities produced a response.

5. The distribution of histamine-sensitizing factor followed that of leukocytosis-stimulating activity. In contrast, mouse protective antigen was localized to the bacterial pellet.

BIBLIOGRAPHY

1. Morse, S. I. 1965. Studies on the lymphocytosis induced in mice by Bordetella pertussis. J. Expt. Med. 121:49.
2. Morse, S. I. 1966. The effect of hydrocortisone and X-irradiation on the lymphocytosis induced by Bordetella pertussis. J. Exp. Med. 123:283.
3. Sutherland, I. W., and J. J. Wilkinson. 1961. A new growth medium for virulent Bordetella pertussis. J. Pathol. Bacteriol. 82:431.
4. Rowatt, E. 1957. Some factors affecting the growth of Bordetella pertussis. J. Gen. Microbiol. 17:279.

5. Dubois, M., K. A. Giles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Color-imetric method for determination of sugars and related substances. *Anal. Chem.* **28**:350.

6. Rondle, C. J. M., and W. T. J. Morgan. 1955. The determination of glucosamine and galactosamine. *Biochem. J.* **61**:586.

7. Spackman, D. H., W. H. Stein, and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino acids. *Anal. Chem.* **30**:1190.

8. Taylor, A., K. W. Knox, and E. Work. 1966. Chemical and biological properties of an extracellular lipopolysaccharide from *Escherichia coli* grown under lysine-limiting conditions. *Biochem. J.* **99**:53.

9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.

10. Rowatt, E. 1957. The growth of *Bordetella pertussis*. A review. *J. Gen. Microbiol.* **17**:297.

11. Banerjea, A., and J. Munoz. 1962. Antigens of *Bordetella pertussis*. II. Purification of heat-labile toxin. *J. Bacteriol.* **84**:269.

12. Kageyama, M. 1964. Studies of a pyocin. I. Physical and chemical properties. *J. Biochem.* (*Tokyo*) **55**:49.

13. Kind, L. S. 1958. The altered reactivity of mice after inoculation with *Bordetella pertussis* vaccine. *Bacteriol. Rev.* **22**:173.

14. Munoz, J. 1964. Hypersensitivity reactions induced in mice treated with *Bordetella pertussis*. *In* Bacterial Endotoxins. M. Landy and W. Braun, editors. Institute of Microbiology, Rutgers, The State University, New Brunswick. 460.

15. Bishop, D. G., and E. Work. 1965. Extracellular glycolipid produced by *Escherichia coli* grown lysine-limiting conditions. *Biochem. J.* **96**:507.

16. Munoz, J., and B. M. Hestekin. 1962. Method of preparing histamine sensitizing factor. *Nature.* **196**:1192.

17. Pieroni, R. E., E. J. Broderick, and L. Levine. 1965. The soluble protective antigen and the histamine-sensitizing factor of *Bordetella pertussis*. *J. Immunol.* **95**:643.

18. Munoz, J., and R. K. Bergman. 1966. Some histamine sensitizing properties of soluble preparations of the histamine sensitizing factor (HSF) from *Bordetella pertussis*. *J. Immunol.* **97**:120.

19. Munoz, J., and B. Hestekin. 1968. Properties of Mg SO$_4$ precipitated histamine sensitizing factor from *Bordetella pertussis*. *Fed. Proc.* **27**:267.

20. Kuwajima, Y., M. Masui, A. Asano, and T. Matsui. 1956. A soluble antigen of phase I *Hemophilus pertussis*. I. Mouse protection test of filtrate or supernatant of cultures in a medium without soluble starch or carbon particles. *J. Immunol.* **76**:175.

21. Nagel, J. 1967. Isolation from *Bordetella pertussis* of protective antigen free from toxic activity and histamine sensitizing factor. *Nature.* **214**:96.

22. Preston, N. W., and P. Garrity. 1967. Histamine-sensitizing factor of *Bordetella pertussis* differentiated from immunogens by neutralization and passive protection tests. *J. Pathol. Bacteriol.* **93**:483.

23. Sato, Y., and N. Kin-ichiro. 1967. Isolation of protective antigen from *Bordetella pertussis*. *Biochem. Biophys. Res. Commun.* **27**:195.

24. Munoz, J. J. 1963. Immunological and other biological activities of *Bordetella pertussis* antigens. *Bacteriol. Rev.* **27**:325.
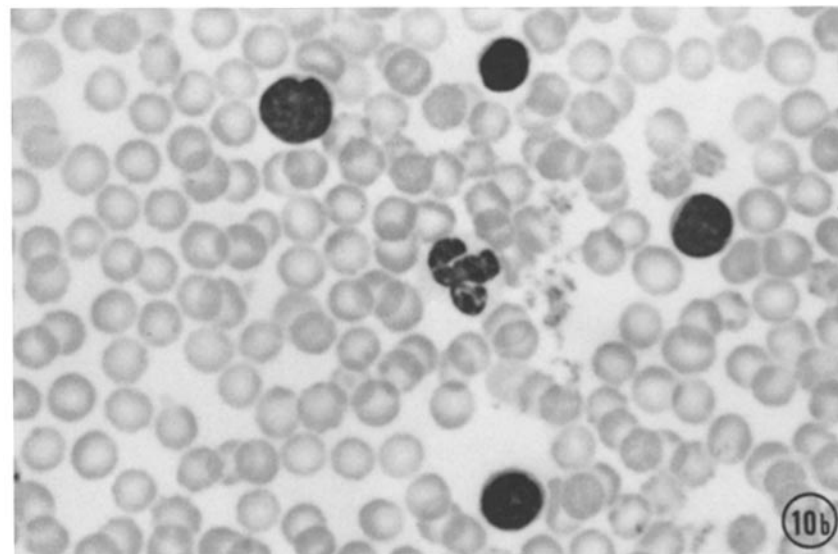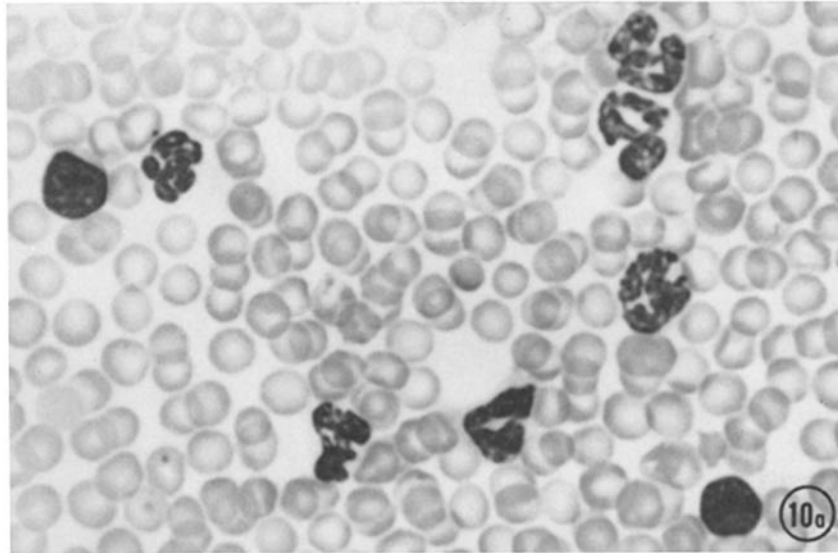
FIG. 10 a. Blood film of a mouse 5 hr after injection of B. pertussis culture supernatant fluid. b. Film at 24 hr. Wright-Giemsa stain. × 1000.