Studies on the Leukocytopenia Due to Acute Effects of Supernatant Fluids of *Bordetella Pertussis* Cultures[1] (35281)

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While studying the mechanism of lymphocytosis induced in mice following the injection of *Bordetella pertussis* vaccine or the supernatant fluid of *B. pertussis* cultures, Morse (1), and Morse and Bray (2) observed a pronounced, but transient leukocytopenia during the first 2 hr after such an injection. A hyperleukocytosis was observed about 24 hr later. Morse (1) considered that the initial leukocytopenia was unlike the response to endotoxin, for his animals exhibited a marked lymphocytopenia and only a slight neutropenia. However, our observations with sheep, calves, and goats suggest that this acute leukocytopenic response involves neutrophils and to a lesser extent lymphocytes. The present communication is a report of these and other data from isotope labeling studies, which indicates that this initial reaction is due to endotoxin.

Materials and Methods. Preparations of *B. pertussis* were kindly provided by Dr. S. I. Morse of the Rockefeller University, New York. The detailed method of preparation has been described by Morse and Bray (2), but is briefly outlined as follows: Organisms of phase I *B. pertussis* strain 3779 B were cultured in liquid media to obtain 0.9–2 $\times$ 10^{10} bacilli/ml. They were then treated with thimerosal (1:5000), filtered, and centrifuged at 900*g* for 45 min. The clear, "pertussis supernatant" (PS) was removed and stored at 4° until used. This supernatant fluid (PS) was shown to contain the bulk of the leukocytosis producing activity of the whole culture (2). We found that 0.02 ml/kg of body weight of PS, when administered intravenously (iv) in sheep, calves, and goats, produced an optimal blood lymphocytosis of 2–2.5 times the initial base line count without causing any observable anaphylactoid reactions. Larger doses resulted in an increased incidence of morbidity and mortality as well as a greater degree of lymphocytosis. Therefore, all the animals in the present series were given 0.02 ml/kg iv. Seventeen adult sheep, 6 adult goats and 5 calves, all in apparently normal health and belonging to either sex were administered PS.

Blood counts, on external jugular venous blood samples collected in sodium EDTA tubes (Vacutainer DB, Becton, Dickinson & Co., Rutherford, New Jersey), were done at 1 or 2-day intervals 3 to 5 times during the 2 weeks preceding the PS treatment to establish the initial base line mean levels, and at 1, 2, 3, 4, 5, 6, 12, 24 hr postinjection and daily thereafter. In some sheep and calves all eight samples were not obtained during the first 24 hr, but all had at least 3 samples in this period, while in goats, the blood samples were drawn only at 1 and 24 hr postinjection. Three calves were studied for diurnal variation in their blood count prior to PS treatment by obtaining blood samples at different times of the day, with 6–10 counts in a 24-hr period. The time of PS injection in all animals was kept between 8:30 a.m. and 10:00 a.m. Hematocrits were measured by the standard capillary technic, total white blood cell counts were done using Model F Coulter counter (Coulter Electronics, Hialeah, Florida) and a 200-cell differential was done on Wright's stained coverslip smears.

Rectal temperatures were recorded daily or more frequently as indicated.

In order to determine whether the leukocytes marginate or are sequestered in the large capillary beds of lungs and other organs accounting for the marked leukocytopenia, the following experiments were performed.

[1] Research supported by U.S. Atomic Energy Commission.

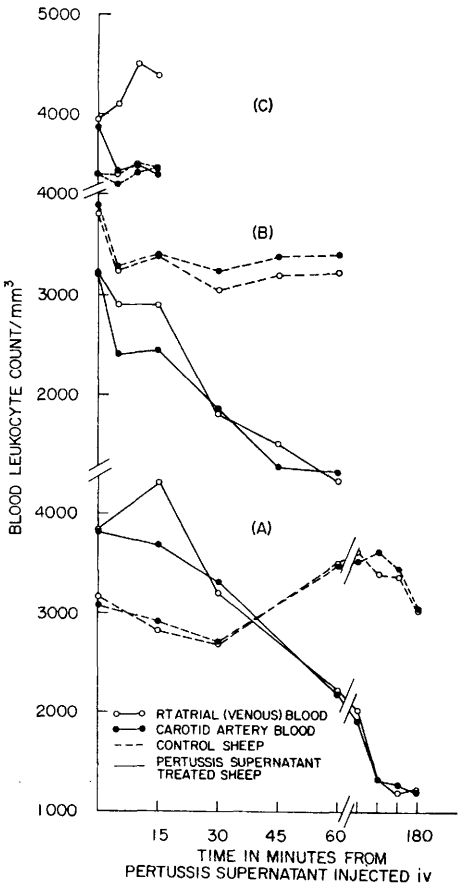


FIG. 1. Leukocyte count of blood from the right atrium (venous) and the carotid artery in sheep: (- - -) saline control; (—) intravenous pertussis supernatant. Pair A blood samples obtained up to 3 hr, Pair B up to 1 hr, and Pair C up to 15 min.

Tritiated thymidine (^{3}HTdR) obtained from Schwarz Bio Research, Orangeburg, New York (sp act 1.9 Ci/mmole) was given iv to sheep in two doses of 0.25 μCi/g of body weight, 12 hr apart. Twelve hr following the second injection of ^{3}HTdR, general anesthesia was induced with iv thiamylal (Surital, Parke, Davis Co., Detroit, Michigan) and maintained by endotracheal halathane (Fluothane, Ayerst Lab., Inc., New York, N.Y.). A catheter was inserted via the external jugular vein into the right atrium and another catheter was inserted into the common carotid artery. Samples of blood were withdrawn simultaneously from the two catheters and then PS 0.02 ml/kg was given iv. Control animals received 0.9% saline instead of PS. Simultaneous arterial and venous blood samples were obtained at frequent intervals until the termination of the experiment. Sheep were divided in three pairs, marked A, B, and C (Fig. 1). Blood samples were obtained for 3 hr in A, 1 hr in B, and for 15 min in C. One animal of each pair was used as a control and received 0.9% saline, while the other received PS. From each blood sample the total white blood cell count was done six times and the mean was calculated. Differential counts of 200 cells and hematocrits were recorded.

When the last sample of blood had been obtained, the animals were sacrificed by iv Surital and exsanguinated. A complete autopsy was performed and the proper placement of cannulas in the carotid artery and the right atrium was confirmed. Whole organ wet weights of heart, lungs, spleen, liver, thymus, adrenals, kidneys, and popliteal nodes were recorded, and all autopsy tissues were processed for histology (10% buffered formalin fixative) and for radioactive counts by liquid scintillation (Unilux I, Model No. 6850, Nuclear Chicago Corp., Des Plaines, Ill.) for tritium activity. The ratio of counts due to tritium in an organ of saline-treated control animal (considered as percentage of total activity injected) to the corresponding counts in the same organ of the PS-treated animal was calculated (Table II).

Results. The diurnal variation studies in 3 calves showed a fluctuation of 11 to 23% around the mean lymphocyte count and of 16 to 50% around the mean neutrophil count. There was a mean elevation in the rectal temperature of 1.7° (SD ± 0.13) at 1 hr post-PS injection. The temperature gradually returned to preinjection level by 24 hr. The hematocrit did not show any significant change during the period of observation. The absolute counts of neutrophils and lymphocytes at the various times are given in Table I. As shown, within the first 3 hr there was a pronounced leukocytopenia. In the sheep there was an average reduction of over 90% in the number of neutrophils and of about 75% in the number of lymphocytes. In calves, there was a reduction of about 97% in the number of neutrophils and of 85% in the

TABLE I. Blood Leukocyte Count During the First 72 hr After Injection (iv) of *B. pertussis* Supernatant Fluid.[a]

	Sheep		Calves		Goats	
(hr)	Neutrophils	Lymphocytes	Neutrophils	Lymphocytes	Neutrophils	Lymphocytes
Initial	4150	4750	3200	4800	7050	5400
1	330	1820	75	1385	830	3200
2	385	1720	270	940		
3	500	1200	200	725		
4	1250	1450	220	750		
5	1930	1530	460	1170		
6	3950	2230	1230	1550		
12	11,150	5110	7460	5270		
24	12,400	7460	7720	7965	9400	7000
48	10,400	9150	6370	8370		
72	4000	9200	6630	17,100		

[a] Average of data (cells/mm³) from: 9 to 17 sheep at each time interval; 3 to 5 calves at each time interval; and 6 goats at each time interval.

number of lymphocytes. In goats the corresponding values were 88 and 40%, respectively at 1 hr.

The leukocyte counts of the sheep in the three pairs A, B, and C listed under Material and Methods are shown in Fig. 1. It is evident that the arterial and venous blood leukocyte counts remain stable and approximate to each other in the control animals during the period of observation. In all PS-treated animals the arterial blood during the first 15 min had an obviously lower leukocyte count as compared to the simultaneously drawn venous blood sample ($p<0.01$ by X^2). At later time intervals the arterial and venous blood counts are again close to each other. The leukocytopenia in arterial blood becomes evident within 15 min after PS; whereas the venous blood shows this effect only after 30 min.

Table II shows the results of tritium radioactivity in the various tissues. The lungs and liver of the PS-treated animals sacrificed 3 hr (Pair A) and 1 hr (Pair B) post-PS, had 1.4 to 1.5 times the activity of the same organ of the corresponding control animals. The bone marrow of the PS-treated animal in Pair A had 1.74 times the activity of the control bone marrow. Pair C, sacrificed at 15 min after PS or saline, had no remarkable difference in the activity in any organ; nor did the other organs (spleen, popliteal node, thymus, adrenal, kidney, and heart) of Pairs A and B show any significant difference.

Microscopic examination of lungs revealed a marked aggregation of leukocytes surrounding the alveoli as well as bronchioles in the animal sacrificed at 3 hr following PS, compared to its control (Fig. 2a and b). There was no significant difference in histopathological and autoradiographic appearance of other tissues at 3 hr nor in any tissues from animals sacrificed at earlier times.

Discussion. Endotoxin administration iv has been known to cause a regularly reproducible sequence of events in both laboratory

TABLE II. Effect of Pertussis Supernatant (PS) in Sheep, Prelabeled with ^{3}HTdR.

Ratio between radioactivity in the organs of PS-treated animal and saline-treated control.

	A[a]	B[a]	C[a]
Lung	1.51	1.43	1.04
Liver	1.40	1.40	1.08
Spleen	1.04	0.93	1.08
Bone marrow	1.74	0.90	0.89
Popliteal node	0.85	1.00	—
Thymus	1.03	—	1.10
Adrenal	1.12	1.00	1.00
Kidney	0.97	1.03	0.86
Heart	0.92	0.90	1.24

[a] Pair A sacrificed at 3 hr, B at 1 hr, and C at 15 min post-PS or saline injection iv.

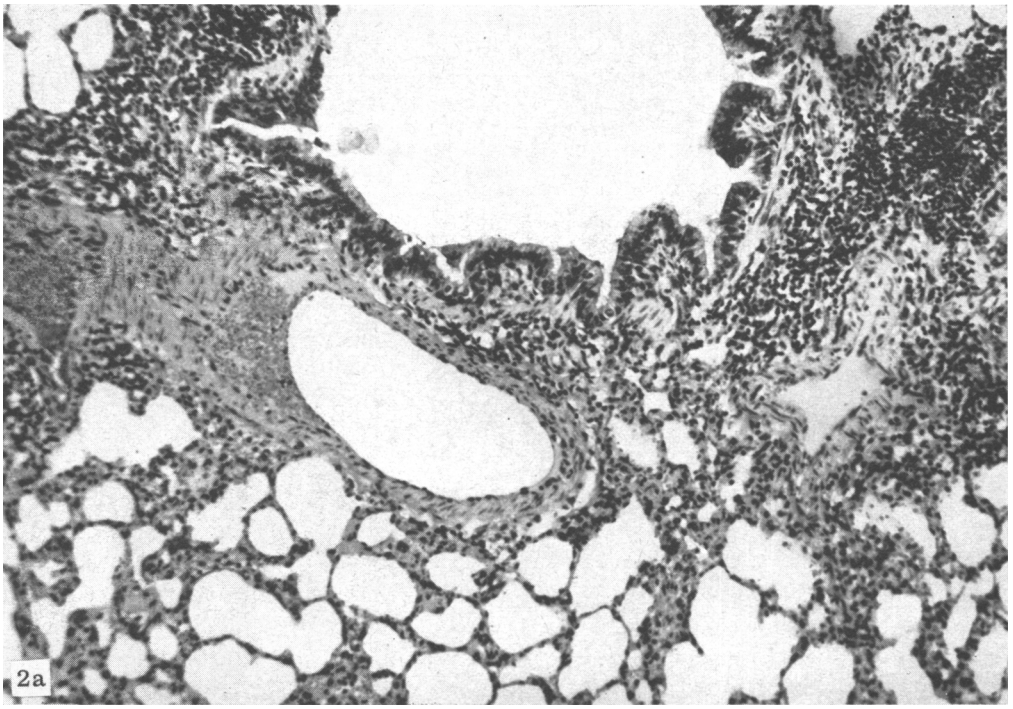
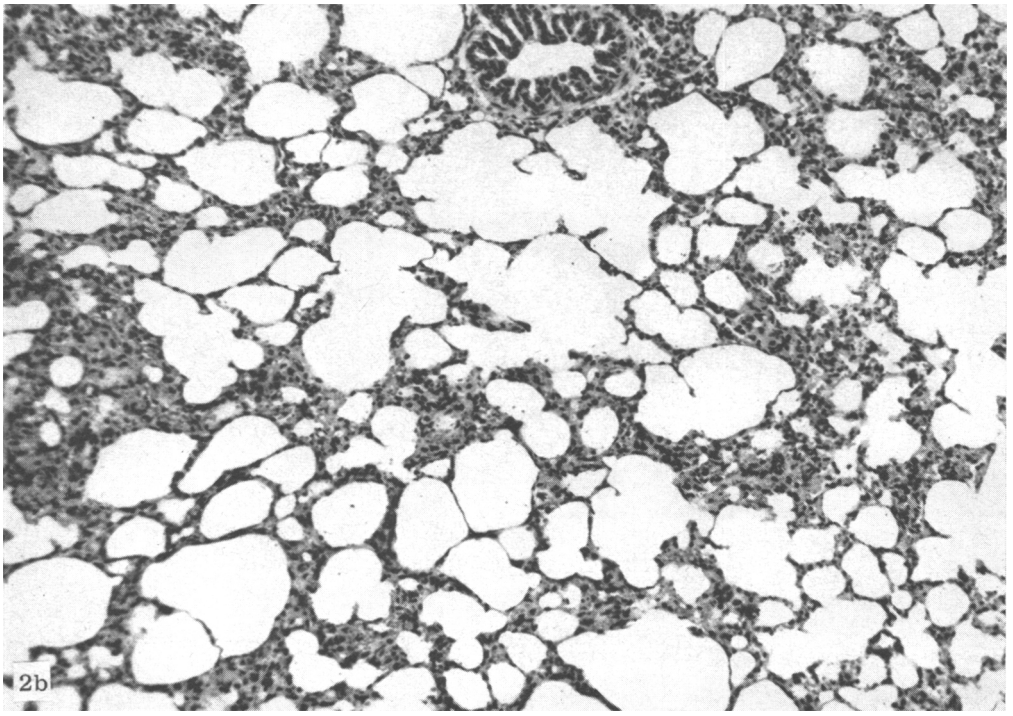


FIG. 2. Histological section of the lung of sheep 3 hr after iv injection of pertussis supernatant (a) showing a marked aggregation of leukocytes surrounding the bronchiole and alveoli compared to the saline-treated control (b). (×100, H & E).



animals and man. These events include fever (3), and marked granulocytopenia within 1 hr followed by granulocytosis (4) at about 24 hr. Our results with PS in the three species studied are consistent with such an endotoxin-induced response. The leukocytopenia occurring within 1 hr after PS was not due to diurnal variation. The observation (1, 2) that the initial leukocytopenia in mice following iv pertussis vaccine or PS is due almost entirely to a lymphocytopenia may be a species characteristic. Similar effects of endotoxin have been reported in man (5), showing an absence of significant granulocytopenia but a definite fall in the mononuclear cells, eventually followed by granulocytosis. The presence of small amounts of endotoxin in the PS used by Morse (material used for the present studies) has already been suggested (2) on the basis of the finding of carbohydrates and fatty acids in it.

Mulholland and Cluff (6) have demonstrated that during the leukopenic phase in rabbits following iv endotoxin, large numbers of leukocytes become adherent to the blood vessel endothelium, with marked migration into perivascular tissue after 3 hr. This may explain our observations in sheep of (i) aggregation of leukoyctes in the lungs on histopathological examination 3 hr after PS iv; (ii) lower leukocyte numbers during 15 min post-PS in the arterial blood compared to the venous blood, suggesting the trapping of leukocytes in the lungs; and (iii) higher tritium radioactivity in the lungs, liver, and bone marrow at 3 hr and in lungs and liver at 1 hr post-PS in animals given ^{3}HTdR 12 and 24 hr before PS. Stetson (7) also observed a rapid accumulation of large numbers of leukocytes in the pulmonary capillaries during the acute leukopenic stage in rabbits at 1 hr post-iv endotoxin. The pathological basis for such accumulation of leukocytes outside the circulating blood is not clear and is beyond the scope of the present study; however, the subject has been investigated and reviewed (6, 8) by several workers recently.

The leukocytosis caused by *B. pertussis* at 2–3 days after the iv administration is mainly due to a pronounced lymphocytosis, which is attributable to the lymphocytosis promoting factor (LPF) of *B. pertussis* (1, 2). Our interest in PS is chiefly in its LPF activity (9) and in obtaining the fraction of LPF which is free of other toxic factors of *B. pertussis*, *viz.*, the endotoxin, the histamine sensitizing factor (HSF) and the heat labile (dermatonecrotic) toxin (HLT). Kurokawa, Ishida, Iwasa and co-workers have done pioneer work in this direction (10–13) and have obtained by ultracentrifugation and gelfiltration of PS a fraction which possesses LPF but has no demonstrable endotoxin and HLT. However their LPF [as also of Morse (2)] was found to be closely associated with HSF (13). We were able to separate HSF and LPF on Bio Gel fractionation of PS at cold temperature (9). The present results indicate the desirability of isolation of endotoxin free LPF.

Summary. Supernatant fluid of a liquid culture medium growing phase I *Bordetella pertussis* strain 3779 B was found to possess a marked leukocytosis stimulating property when injected intravenously in sheep, calves, and goats. At high doses, the animals also showed anaphylactoid reactions and death. Doses which produced a leukocytosis also caused a marked leukocytopenia and transient fever within the first 3 hr following injection. This acute, transient leukocytopenia was mainly due to a severe neutropenia. Arterial blood leukocyte counts were lower than the venous blood counts during the initial leukocytopenic phase in the sheep. Aggregations of leukocytes were found surrounding the alveoli and the bronchioles in the lung of sheep at the time of blood leukocytopenia. These animals had been given tritiated thymidine prior to pertussis supernatant; and the tritium radioactivity in the lungs, liver, and bone marrow was found to be significantly higher at 3 hr post-pertussis dose compared to the saline-treated control. The results suggest that the acute leukocytopenic and febrile response was due to an endotoxin of *B. pertussis*.

We are grateful to Dr. Horton Johnson for reviewing the histopathology slides, and to Mr. Clyde Sipe and Mr. Thomas Weldon for their excellent technical support.

1. Morse, S. I., J. Exp. Med. **121**, 49 (1965).

2. Morse, S. I., and Bray, K. K., J. Exp. Med. **129**, 523 (1969).

3. Atkins, E., Physiol. Rev. **40**, 580 (1960).

4. Herion, J. C., Walker, R. I., and Palmer, J. G., Amer. J. Physiol. **199**, 809 (1960).

5. Wolff, S. M., Rubenstein, M., Mulholland, J. H., and Alling, D. W., Blood **26**, 190 (1965).

6. Mulholland, J. H., and Cluff, L. E., "Bacterial Endotoxins" (M. Landy and W. Braun, eds.), p. 224. Inst. Microbiol., Rutgers Univ., New Brunswick, N.J. (1964).

7. Stetson, C. A., Jr., J. Exp. Med. **93**, 489 (1951).

8. Nowotny, A., Bacteriol. Rev. **33**, 72 (1969).

9. Okuyama, S., Aronson, R. B., Chanana, A. D., Cronkite, E. P., Rai, K. R., and Schiffer, L. M., Proc. Soc. Exp. Biol. Med. **133**, 723 (1970).

10. Kurokawa, M., Iwasa, S., and Ishida, S., Jap. J. Med. Sci. Biol. **18**, 161 (1965).

11. Ishida, S., Jap. J. Med. Sci. Biol. **21**, 115 (1968).

12. Kurokawa, M., Ishida, S., Iwasa, S., Asakawa, S., and Kuratsuka, K., Jap. J. Med. Sci. Biol. **21**, 137 (1968).

13. Iwasa, S., Ishida, S., Asakawa, S., and Kurokawa, M., Jap. J. Med. Sci. Biol. **21**, 363 (1968).

Received Sept. 2, 1970. P.S.E.B.M., 1971, Vol. 136.