Studies on Lymphocytes. XVIII. Mechanism of Lymphocytosis Induced by Supernatant Fluids of *Bordetella pertussis* Cultures

By Kanti R. Rai, Arjun D. Chanana, Eugene P. Cronkite, Darrel D. Joel, and Jerry B. Stevens

Supernatant fluid from liquid medium cultures of phase I *Bordetella pertussis* organisms (pertussis supernatant) when given to calves and sheep by intravenous injection produces a marked lymphocytosis. Thoracic duct and splenic vein cannulation was performed in some animals to study the mechanism of production of this lymphocytosis. There was a transient increased mobilization of lymphocytes from the lymphoid organs as seen by the increase in output of lymphocytes from thoracic duct and splenic vein within a few minutes following administration of pertussis supernatant. This was followed by a decrease in the thoracic duct cell output as long as the blood lymphocyte count remained elevated, indicating an inhibition of recirculation of lymphocytes from blood to lymph. There was no evidence of increased new cell production.

As a part of the studies on lymphocyte migration pathways and regulation of lymphocyte concentration in blood, several agents have been used to induce an increase in the blood lymphocyte count in experimental animals. A proper interpretation of the data derived from the use of such agents requires a clear understanding of the mechanism by which a blood lymphocytosis has been produced. Heparin¹ and heparinoid compounds² as well as synthetic polyanion polymethacrylic acid³ have been reported to induce lymphocytosis by a mobilization of cells from the lymphoid organs. A review of current literature reveals that *Bordetella pertussis* is being used by several workers in the investigations on lymphocyte physiology. *B. pertussis*

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was used by Guyer and Crowther as a nonspecific immune stimulant in efforts to prolong chemotherapeutically achieved remission in human acute leukemia. Similarly, Finger and Emmerling found increased immunological activity in mice after pertussis treatment. On the other hand, *B. pertussis* was found to have an immunosuppressive effect as evidenced by an augmented growth of transplanted lymphoma and a reduction in the formation of circulating antibody in mice. In a recent study on thymus dependent and independent populations of lymphocytes, Kalpaktsoglou et al. used pertussis vaccine to perturb the lymphocyte kinetics. Although the mechanism of induction of blood lymphocytosis by *B. pertussis* is not clearly understood, Morse and Riester suggested two possibilities: (1) the circulating cells are unable to leave the blood stream to recirculate via the lymph; and (2) the lymphocytes from the lymphoid organs may enter the circulation directly without passing through the lymphatics. The studies reported here suggest that *B. pertussis* causes an initial increased mobilization of lymphocytes from the lymphoid organs and then the lymphocytosis is sustained by an inhibition of recirculation from blood to lymph.

**Material and Methods**

Preparations of *B. pertussis* were kindly provided by Dr. S. I. Morse of the Rockefeller University, New York City (now of the Downstate Medical Center, Brooklyn, N.Y.). The detailed method of preparation has been described by Morse and Bray, 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 × 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°C until used. This PS was shown to be potent in inducing lymphocytosis in mice.

Calves of about 100 kg body weight, and adult sheep of both sexes were used. Blood counts, on external jugular venous blood samples collected in sodium EDTA tubes (Vacutainer BD, Becton, Dickinson & Co., Rutherford, N.J.), were done at 1- or 2-day intervals three to five times during the 2 weeks preceding the PS treatment, and baseline mean preexperimental level (MPEL) of hematocrit, and total and differential white cell counts were established for each animal. Hematocrits were measured by the standard capillary technic, total white blood cell counts were performed using Model F Coulter Counter (Coulter Electronics, Hialeah, Fla.) and 200 cell differential counts were done on Wright’s-stained coverslip smears. Absolute counts of neutrophils and lymphocytes were calculated. Following the intravenous (i.v.) administration of PS, blood counts were obtained at appropriate intervals for up to 2 wk.

**Thoracic Duct Cannulation**

Three calves and one sheep were used in these experiments. The thoracic duct (TD) was cannulated and connected to the jugular vein with an extracorporeal shunt by the procedure previously described. Two to three days were allowed for postoperative recovery. The blood count, and TD lymph and lymphocyte output were recorded at 1 to 2 hourly intervals for 24 hr to determine the diurnal pattern of each animal. At the end of 24 hr, PS (0.02 ml/kg) was injected i.v. In calves 1 and 2, lymph was collected quantitatively at 2–5 min intervals into separate test tubes for a period starting 20 minutes prior to PS injection to 60–95 min post-PS and the average fluid and cell output per minute was calculated. At all other times during the TD experiments lymph was returned to the animal via the jugular vein shunt. Volume outflow from TD was automatically recorded with the use of a SCIRT pump (Biomed Systems, Danbury, Conn.). Two-ml lymph samples were collected aseptically, when required, via a T-connection in-
serted on the TD side of the extracorporeal shunt tubing. Average fluid and cell output per unit time was calculated. Blood and lymph samples were incubated for 1 hr with tritiated thymidine (3HTdR, 2.5 μCi/ml, specific activity 1.9 Ci/mM, Schwarz Bio-Research, Orangeburg, N.Y.) at various times prior to and following the PS injection. Smears were processed for autoradiography using Kodak NTB-2 emulsion and an exposure time of 10 days.

Intrasplenic Artery Injection of PS

Two sheep were used in a study to determine if PS mobilizes lymphocytes from the spleen. General anesthesia was induced with i.v. thiamylal (Surital, Parke Davis Co., Detroit, Mich.) and maintained by endotracheal halothane (Fluothane, Ayerst Lab., New York, N.Y.). A catheter was introduced via the jugular vein into the right atrium to sample pooled venous blood, a second catheter was inserted into the carotid artery to sample the arterial blood, and through an upper abdominal laparotomy incision, a third catheter was inserted through the portal vein into the splenic vein. Blood samples were collected through the three cannulae simultaneously into separate test tubes. PS was then injected into the splenic artery of one animal, while the other animal received 0.9% saline via the splenic artery. At frequent intervals following injections, blood samples were simultaneously collected for blood leukocyte count from the systemic arterial, pooled venous and splenic venous blood for 1 hr. When the last sample of blood had been obtained, the animals were sacrificed by i.v. Surital and exsanguinated. A complete autopsy was performed, the proper placement of cannulae was confirmed, and tissues were processed for histology.

RESULTS

Dose of PS

Twenty-eight sheep and 11 calves were studied for sequential changes in blood leukocyte concentrations following varying doses of PS to determine the dose most effective in inducing a predictable lymphocytosis without causing any observable morbidity. It is seen in Table 1 that doses of 0.002 and 0.01 ml/kg body weight were ineffective, whereas 0.05 ml/kg and higher doses

<table>
<thead>
<tr>
<th>PS (ml/kg)</th>
<th>Number of Animals</th>
<th>Maximum Response†</th>
<th>Number of Animals</th>
<th>Maximum Response†</th>
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<tbody>
<tr>
<td>0.002</td>
<td>1</td>
<td>1.0</td>
<td>Not tested</td>
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<tr>
<td>0.01</td>
<td>1</td>
<td>1.0</td>
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<tr>
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<td>1.9</td>
<td>2.0</td>
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<td>(±0.3)†</td>
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<td>0.05</td>
<td>2 §</td>
<td>1.2</td>
<td>1.1</td>
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<td>0.20</td>
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<td>2.5</td>
<td>2.6</td>
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<td></td>
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<td>(±0.4)†</td>
<td>(±2.1)†</td>
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<tr>
<td>0.50</td>
<td>3 §</td>
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<td>1.00</td>
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* Anaphylactoid death.
† The ratio of the lymphocytes or neutrophils count at peak leukocytosis to the base-line count.
‡ Mean of the maximal response of all animals in the same dose group ± SD.
§ The survivors had marked anaphylactoid reaction.
produced lymphocytosis, but also caused deaths due to severe anaphylactoid respiratory distress.

The optimal dose was found to be 0.02 ml/kg. Sixteen sheep showed a lymphocytosis 1.5 to 3 times the MPEL and an elevation in neutrophils from 0.9 to 3.3 times the MPEL at 1–3 days. The same dose was also effective in eight calves, but higher doses induced anaphylactoid death (Table 1). There was no significant change in hematocrit in the animals given 0.02 ml/kg. Figures 1A and B show the sequential changes in blood leukocytes in one calf and one sheep, respectively, as representative response in each species studied.

**Thoracic Duct Cannulation Studies**

The MPEL of thoracic duct lymphocyte output (obtained from the average of the data for 3 days prior to PS injection) in calf 1 was $178 \times 10^6$/min with a fluid output of 8 ml/min. The lymphocyte output of this calf was $112 \times 10^6$/min just prior to i.v. administration of PS (Fig. 2A). A slight decline was observed in the first sample at 2 min post-PS, followed by a threefold increase in the cell output within 20 min. The fluid output also increased at this time to 20 ml/min. At 40 min, both fluid and cell output returned to MPEL. Between 60 and 95 min the cell output fell appreciably below the MPEL, whereas the fluid output remained normal (fluid output not shown in the figures).
Fig. 2.—The immediate effect of i.v. pertussis supernatant on the thoracic duct (TD) output of lymphocytes and the blood lymphocyte count in calf 1 (A) and calf 2 (B). There is a more than threefold increase in TD output within 20 min in both calves. (See text for explanation of decline in the blood count.)

lymphocyte output in calf 2 (Fig. 2B) showed an almost sixfold increase at 20 min. The fluid output increase from 3–4 ml/min pre-PS to 20 ml/min at 20 min, returning close to pre-PS level by 50 min. As in all other animals studied before, and shown in Figs. 1A and 1B the blood lymphocyte count declined also in these calves during the first hour post-PS.

The TD output and blood lymphocyte levels for 3 to 6 days after the i.v. injection of PS were studied in three calves and one sheep and the results on two calves are shown in Fig. 3. The immediate effects of PS have already been shown in Figs. 2A and 2B; therefore, many of the early points were omitted and the scale on the abscissa of these figures was chosen to demonstrate the effect of PS for later intervals. Accordingly, the initial increase in the TD lymphocyte output is not evident. It will be seen that calf 1 (Fig. 3A) had an appreciable decline from 1.5 to 4 days post-PS in TD lymphocyte output which is from the MPEL of $178 \times 10^6$/min. The fluid output during the entire post-PS period remained more than twice the MPEL of 8 ml/min. The blood lymphocytes remained about 2.5 times the MPEL. It is interesting to note that concomitant with the decline in the blood lymphocyte count after day 4 post-PS, there was a steep rise in the TD output of lymphocytes. The study of calf 2 was performed several days prior to the acute observations study shown in Fig. 2B and at this time the MPEL of TD lymphocyte output was $40-65 \times 10^6$/min (Fig. 3B), with a fluid output of 3–5.5 ml/min. It is quite obvious that although the blood lymphocytes increased to more than 2.5
times pre-PS level on day 2 post-PS, the TD output of lymphocytes dropped precipitously to less than 25% of pre-PS levels. Similar results in TD output were noted in calf 3: the blood lymphocytes rose from 5500 to 14,000/cu mm, whereas the TD output of lymphocytes remained well below the pre-PS level. The TD output, in the one sheep studied, fell from a pre-PS level of about $65 \times 10^6$/min to $25-40 \times 10^6$/min for 2-6 days post-PS while the blood lymphocytes remained more than 1.75 times the MPEL of 4400/cu mm. Incubation of $^3$HTdR with calf lymph or blood was performed in vitro with samples obtained pre-PS and at various times post-PS up to 3 days. The labeling indices as obtained by autoradiography did not show any significant increase at any time post-PS over the pre-PS values ($p \geq 0.70$ by $\chi^2$).

**Injection of PS Into Splenic Artery**

Figure 4 shows the results on the two sheep in which simultaneous blood samples were obtained from the right atrium (pooled venous blood), carotid artery, and the splenic vein. As early as 1 min after the administration of PS via the splenic artery the lymphocyte count rose in the splenic vein blood and was greater than threefold at 3 min. Thereafter the count returned to the pre-
**Fig. 4.**—Effect of intra-splenic artery injection of pertussis supernatant (PS) on the lymphocyte count in the blood sampled from the splenic vein, right atrium, and the carotid artery as compared with a control sheep (bottom) given saline instead of PS. There is a more than threefold increase in the lymphocyte count of splenic vein blood (top) within 3–4 min following PS injection.

injection level at 30 min and was appreciably below that level at 60 min, when the experiment was terminated. The right atrial and carotid arterial blood counts remained stable for 30 min postinjection, then fell to about 50% of the preinjection level. This latter response was ascribed to the eventual effect of PS in systemic circulation, and is similar to the findings within 1–3 hr following i.v. PS, as shown in Fig. 1. The counts on corresponding simultaneous blood samples (Fig. 4, bottom) in the control sheep given saline by the intrasplenic arterial route show no significant variation from the preinjection level. Among the tissues examined at autopsy (at 1 hr post-PS), only the spleen had observable changes. Figure 5A and 5B demonstrate the histopathological appearances of the representative areas of the PS-treated and control spleen respectively. There is a marked reduction in density of lymphocytes in the periarterial dense white pulp in the PS-treated spleen.

**DISCUSSION**

The blood leukocyte response to intravenous PS in sheep and calves, as shown in Table 1 and Figs. 1A and 1B, is similar to the previously reported response in mice. However, the optimal dose of PS in inducing a marked lymphocytosis in sheep and calves is less than 1% of the effective dose in mice. The pronounced lymphocytosis induced by *B. pertussis* may be caused by one or more of the following mechanisms: (1) an increased rate of entry of lymphocytes into the circulation via the efferent lymphatics; (2) a decreased rate of exit of lymphocytes from the blood stream via postcapillary venules, thus increasing the average residence time in the blood; (3) entry of lymphocytes into the blood compartment by unusual routes in addition to lymphatics—that is, direct entry into blood vessels; and (4) increased new cell production. The data on thoracic duct lymph of mice seem to favor strongly the prolonged blood residence time, suggesting an inhibition of
Fig. 5.—Microphotograph of a representative area of (A) spleen of sheep sacrificed at 1 hr after the intrasplenic artery injection of pertussis supernatant and of (B) a control sheep given saline instead of PS. Note the marked depletion of periarteriolar lymphocytes in the white pulp in the PS-treated spleen, while the control shows a normal cellularity. Hematoxylin and eosin (× 100).
recirculating ability of lymphocytes from blood to lymph, while also indicating a possibility of direct entry of lymphocytes.

Cannulation of the thoracic duct in large animals permits a quantitative measurement of output of lymph and lymphocytes per unit time by recycling lymph to the veins, thus almost completely avoiding the stress on the animal due to the loss of cells and lymph. The maximum period of external drainage of lymph in our studies was 2 hr each in calves 1 and 2.

These studies clearly demonstrate the extreme rapidity with which intravenously administered PS increases the mobilization of lymphocytes from lymph nodes into the TD lymph. Within 20 min of injection, the output increased threefold in one calf and nearly sixfold in the other calf. In our earlier work on heparin, an increase in TD lymphocyte output in calves was not seen until 2–8 hr after heparin injection. The effectiveness of a synthetic polyanion, polymethacrylic acid, in increasing TD lymphocyte output3 by about twofold in mice was also noticeable only between 2–3 hr after its administration. A further confirmation of a prompt mobilization of lymphocytes from the lymphoid organs is provided by the experiment with injection of PS via the splenic artery. The histological appearance of this spleen is similar to the appearance of the white pulp of the rat spleen 3 hr after intraperitoneal dextran sulfate, which also caused increased mobilization of lymphocytes. Morse and Riester did not observe this early and transient mobilization via TD since they cannulated the TD only 2–3 days after the injection of pertussis vaccine and did not study the splenic vein lymphocytes. The influence of this increased mobilization of lymphocytes within a few minutes post-PS is not evident in the blood. On the contrary, a marked leukocytopenia involving neutrophils and lymphocytes has been noted during the first hour post-PS (Fig. 1). Recent studies from this laboratory indicate that endotoxin of B. pertussis is responsible for this leukocytopenia, and would, therefore, mask the expected lymphocytosis due to increased mobilization.

The fact that the blood lymphocytosis was sustained in the mice following pertussis vaccine, even though there was a marked decrease in the TD output of lymphocytes indicated to Morse and Riester that the mechanism of lymphocytosis must be due to an inhibition of recirculation. Our findings in calf and sheep of a similarly decreased TD output of lymphocytes for 3 to 6 days post-PS with blood lymphocytes count remaining as high as 2.5 times MPEL support this interpretation and, in addition, show that there is an initial short-lived increased input through efferent lymphatics and the splenic vein. The integral of the increased TD output of cells would cause an increase in the blood lymphocyte count by approximately 10% (calculated from the data on calf 1). This is a minimum estimate of contribution from the lymphatics by mobilization because it does not include the input of cells from lymphatics other than the thoracic duct, nor does it take into account the mobilization from the spleen. It is, therefore, difficult to calculate precisely the extent of the contribution of the inhibition of recirculation in the eventual lymphocytosis. Bradfield and Born have recently shown that heparin-induced lymphocytosis in the rat is due to an inhibition of recirculation, but the effectiveness of heparin lasts less than 12 hr after an intravenous injection.
The possibility of direct entry of lymphocytes into the blood compartment was considered by Morse and Riester\textsuperscript{10} as a partial explanation of lymphocytosis, but these authors also added that a pathway for such entry has not been demonstrated in normal animals. Our data can neither support nor deny the existence of a direct entry pathway.

An increase in the new cell production certainly could not be invoked to explain the early increase in TD output during the first few minutes following PS. Although an increased production is not necessarily reflected as an increase in the in vitro $^{3}$HThDR labeling index of lymphocytes in circulation, a greater proportion of cells in the stage of DNA synthesis (and therefore labeled in vitro) is more likely to be seen in blood and lymph during a period of stimulated proliferation than is seen normally. Results of the labeling indices of lymphocytes in blood and lymph of calves at various times after PS do not show any significant increase over the pre-PS values. Morse and Riester,\textsuperscript{16} using $^{3}$HThDR in vivo, also found no evidence of increased production of lymphocytes in the \textit{B. pertussis}-treated mice.

These findings demonstrate that the supernatant fluid of liquid cultures of \textit{B. pertussis} is an extremely potent agent in producing blood lymphocytosis by the dual mechanism of initial mobilization of lymphocytes from the lymphoid organs and subsequent inhibition of recirculation of lymphocytes from blood to lymph. This dual mechanism renders PS more effective in sustaining prolonged lymphocytosis than is possible with a single administration of heparin or heparinoids, and therefore it has a greater potential value wherever such lymphocytosis is desired, as an adjunct with antilymphocyte serum or extracorporeal irradiation for depletion of lymphocytes from blood and tissues for immunosuppression and treatment of chronic lymphatic leukemia.

It will be of much interest to examine further whether the inhibition of recirculation is due to the action of the agent on the lymphocytes or on the lymph node cortex postcapillary venule, and marginal sinus in splenic pulp, the sites of recirculation.\textsuperscript{17,18} Morse and Barron\textsuperscript{19} in a recently reported study used circulating lymphocytes (from PS-treated and normal mice) which were radioactively labeled in vitro and transfused into PS-treated and normal isogenic mice. Their results clearly demonstrate that the inhibition of lymphocyte emigration is due primarily to some alteration of the lymphocyte, which, according to these workers, was presumably a surface phenomenon. However, their observations do not exclude the possibility of alteration of the sites of emigration.

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