STUDIES ON THE LEUKOCYTOSIS AND LYMPHOCYTOSIS INDUCED BY BORDETELLA PERTUSSIS

III. The Distribution of Transfused Lymphocytes in Pertussis-Treated and Normal Mice*

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In previous studies from this laboratory, it has been shown that the lymphocytosis induced in mice by *Bordetella pertussis* is due to the presence in the circulation of an increased number of preexisting lymphocytes (1). Cellular proliferation of lymphocytes is not responsible for the effect. Thus, in experiments in which tritiated thymidine was administered during the course of the reaction, it was found that the percentage of circulating lymphocytes with nuclear label was identical in pertussis-treated mice to that found in normal animals. Moreover, at the time of maximal lymphocytosis all lymphoid organs, as well as the bone marrow, were depleted of small lymphocytes without there being any histological evidence of increased mitotic activity (2).

These results indicated that the lymphocytosis was due to a redistribution of lymphocytes between the circulating and tissue pools. As shown by Gowans (3) in the rat, there is a continuous flux of lymphocytes between the lymphoid organs and the blood, with the thoracic duct serving as the main conduit for cellular traffic between these two pools. Similarly, in the mouse, depletion of small lymphocytes from lymphoid organs can be achieved by thoracic duct drainage. It can be estimated that during 24 hours 7–12 times as many lymphocytes enter the blood as are present at any one time (4, 5).

However, in mice with pertussis-induced lymphocytosis the normal lymphocyte circulatory pathway is altered. Cannulation of the thoracic ducts of mice with pertussis-induced lymphocytosis revealed that the 24 hr output of lymphocytes in the drainage was less than normal despite the fact that the concentration of circulating lymphocytes was 3–8 times normal (5). Moreover, in several of the animals the blood lymphocyte count increased markedly during the 24 hr period in which a patent thoracic duct cannula was draining externally.

The results of the cannulation experiments clearly showed that the normal pathway of circulation and recirculation of small lymphocytes was altered by *B. pertussis*. In

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addition, the observation that the circulating lymphocyte count could increase despite the presence of an indwelling thoracic duct cannula suggested that the lymphocytes might enter the blood by an alternate route or routes.

These findings could be explained by an abnormality of the blood or lymphatic vessels or by a change in the lymphocytes themselves, or by a combination of both effects.

In order to gain more information on this question, circulating lymphocytes obtained from pertussis-treated and normal mice were radioactively labeled and transfused into pertussis-treated and normal isogenic mice. The distribution of the cells in various tissues and the blood was then assayed.

Materials and Methods

Mice. 4–6 wk old female Balb/c mice weighing 19–24 g were obtained from the Jackson Memorial Laboratory, Bar Harbor, Maine, and held for at least 10 days before use.

Induction of Lymphocytosis by B. pertussis.—Strain 3779B, obtained from the Eli Lilly and Co., Indianapolis, Ind. was cultured in liquid medium as described previously (6). Bacteria-free supernatant fluids containing 1:5000 thimerosal were diluted 1:4, and 0.2 ml was injected intravenously. 24 hr later the leukocyte counts were elevated to between 90,000 and 110,000/mm^3 and 40–60% of the cells were mature small lymphocytes.

These animals served as both donors and recipients of lymphocytes as did normal mice. Thus, peripheral blood lymphocytes from both pertussis-treated mice and normal mice were injected into normal and pertussis-treated animals.

Isolation of Lymphocytes from Mouse Blood.—Anesthesia was induced with chloroform and blood was drawn from the severed axillary vessels into a Pasteur pipette which had been rinsed in a solution of 5000 USP units/ml of heparin (Liquaemin sodium "50," Organon, Inc., West Orange, N. J.). An equal volume of 3% dextran (Dextran 250, pharmacia, Uppsala, Sweden) containing 10 units/ml of heparin was added. The tubes were then placed at an angle of approximately 45°. After 30 min at room temperature, the layer containing the white blood cells (WBCs) and platelets was aspirated and centrifuged at 700 rpm in an International Clinical Centrifuge (International Equipment Co., Needham Heights, Mass.) for 10 min at room temperature. The platelet-rich supernatant fluid was discarded. The pellet was resuspended in saline containing 10 units of heparin per ml. The tubes were centrifuged at 700 rpm for 10 min and the pellet was washed again on the centrifuge. The final pellet was resuspended at a cell concentration of 15–20 $\times$ 10^6/ml in Dulbecco's phosphate buffered saline (PBS) containing 50% fresh Balb/c plasma.

1 ml samples were placed on prewarmed (37°C) absorbent cotton columns of this capacity prepared in 5¾ inch disposable Pasteur pipettes. After incubation at 37°C for 60 min, each column was eluted with 1.2 ml of PBS-20% Balb/c plasma.

The eluted cells, which consisted of more than 95% small lymphocytes, were pelleted by low-speed centrifugation and resuspended in Eagle's minimal essential medium for labeling.

To 1.7 ml of lymphocyte suspension containing 6–10 $\times$ 10^6 cells, 0.1 ml of Balb/c serum and 0.2 ml of a 10 μCi/ml solution of tritiated uridine (S.A. 20 Ci/mmole, Schwarz Bioresearch, Inc. Orangeburg, N. Y.) were added. The suspension were incubated at 37°C for 60 min. The suspensions were then diluted 0.5-fold with medium 199 (Microbiological Associates Inc., Bethesda, Md.) containing 5% Balb/c serum. They were then centrifuged at 700 rpm for 10 min at room temperature. The cells were washed twice more and resuspended to the original volume used for labeling in medium 199–5% Balb/c serum. They were then postincubated for 20–30

min at 37°C, centrifuged, and resuspended in the same medium at a cell concentration of 1–2 × 10^6/ml. 0.2 ml portions were slowly injected intravenously into recipient mice.

After labeling and processing, cell viability was greater than 92% in all cases as determined by nuclear exclusion of trypan blue. Moreover, the cells still possessed normal motility.

Samples of the inocula were frozen for subsequent processing.

Processing of Samples.—Samples of blood, lung, thymus, spleen, mesenteric lymph node, and liver were obtained 5 and 60 min after injection. Three mice were examined at each time period.

0.3 ml of heparinized blood obtained from the axillary vessels of the recipient mice was added to heparinized saline; and the cells were deposited by centrifugation, frozen in dry ice–acetone, and kept at −20°C. The various organs were removed immediately after bleeding, trimmed, and quick-frozen on a block of dry ice, and held in the frozen state.

Immediately upon thawing, 1.0 ml of H_2O and 1.0 ml of 10% trichloroacetic acid (TCA) was added to the blood cells. In the case of the lymphocyte inocula, 1 ml of 0.1% bovine serum was added and then 1.0 ml of 10% TCA was added.

The organs were weighed in the frozen state. They were then homogenized after thawing in an ice bath with a motor driven Teflon pestle. The livers were homogenized in 5.0 ml of distilled H_2O and the lungs, thymuses, mesenteric lymph nodes, and spleens in 2 ml. Portions of the homogenates were then taken and mixed in 12 × 75 mm disposable plastic tubes with TCA to achieve a final concentration of 5% in a total volume of 2 ml.

All samples were held at 4°C for 1 hr and then washed with 5% TCA into a microanalysis filtering apparatus with a 0.45 μ filter (Millipore Corp., Bedford, Mass.). The precipitates were washed with cold 10% TCA and then ether.

The filters containing the dried precipitates were then combusted and samples counted by the procedure described by Gupta (7). A Packard 3003 liquid scintillation counter was employed.

The dpm's were expressed in terms of the intact organs or of total blood, with the blood volume of the mice assumed to be 0.055 ml/g body weight (8).

RESULTS

For each treatment group, observations were made in triplicate after 5 min and at the end of 1 hr from samples of blood, liver, lung, mesenteric node, and spleen. Insignificant levels of thymic radioactivity were found in all groups and results for this organ are not included.

The summary statistics for each of these sets of data are shown in Tables I–IV. In the first table the mean, standard deviation, slope (5–60 min) and the proportional change are shown for each of the compartments for each of the four classes of experiments.

In comparing the results obtained for normal cells in normal animals with normal cells in pertussis animals (Table I, A and B), a significant difference was found between the amount of radioactivity present in the lymph nodes at the two observation periods, 5 and 60 min. This difference cannot be attributed to a difference in the base line value, for when expressed as proportional change and slope, the differences are still significant. Thus, although the proportional change in content of acid-precipitable radioactivity in the node of animals receiving lymphocytes from pertussis-treated mice is greater, the amount of label is considerably less (Table II). The differences in the slope and propor-

tional change for blood between these two experiments while not as marked as those for the node, are complementary to them. The large positive slope seen for the node with normal cells in normal animals is decreased for normal cells in pertussis animals. The absolute value for this measure in blood also decreases.

TABLE I

*Summary Statistics for the Five Compartments for all Experiments**

	Blood	Liver	Lung	Node	Spleen
A. *Normal lymphocytes in normal animals*					
Mean	2.68 ± 2.34	25.93 ± 8.21	42.93 ± 28.46	4.47 ± 5.03	22.92 ± 21.28
Slope	−4.23	−13.67	−51.13	7.33	36.78
Proportional change	−0.88	−0.42	−0.75	0.17	9.04
B. *Normal lymphocytes in pertussis animals*					
Mean	2.48 ± 0.97	22.17 ± 3.92	38.62 ± 26.31	1.64 ± 1.10	20.48 ± 18.72
Slope	−1.70	−6.27	−47.77	1.98	32.30
Proportional change	−0.51	−0.35	−0.76	3.05	7.45
C. Pertussis lymphocytes in normal animals					
Mean	2.73 ± 1.07	15.78 ± 4.80	30.43 ± 14.67	0.28 ± 0.16	15.68 ± 13.27
Slope	−1.87	−7.83	−25.73	0.23	23.83
Proportional change	−0.51	−0.40	−0.59	1.41	3.89
D. Pertussis lymphocytes in pertussis animals					
Mean	5.93 ± 1.42	17.03 ± 5.30	28.28 ± 10.13	0.32 ± 0.24	28.08 ± 17.95
Slope	−2.07	−8.83	−17.17	0.34	31.77
Proportional change	−0.30	−0.41	−0.47	1.40	2.60

* The data are based upon the total amount of acid-precipitable radioactivity present in the blood or organ expressed as a percentage of the amount injected. The mean represents the average percentage of injected radioactivity found at both the 5 and 60 min sampling periods The slope of the regression line (coefficient of regression) and the proportional change refer to the differences between the 5 and 60 min values.

TABLE II

Radioactivity in the Mesenteric Node and Blood

Source of lymphocytes	Recipient	Percentage of injected radioactivity in							
		Mesenteric node				Blood			
		5 min		60 min		5 min		60 min	
		Avg.	Range	Avg.	Range	Avg.	Range	Avg.	Range
Normal	Normal	0.8	0.4-1.4	8.1	5.0-13.6	4.8	4.4-5.3	0.57	0.4-0.8
Normal	Pertussis-treated	0.65	0.62-0.67	2.6	2.4-2.9	3.3	3.0-3.8	1.6	1.5-1.7
Pertussis-treated	Normal	0.16	0.08-0.28	0.39	0.31-0.54	3.7	3.3-4.0	1.8	1.5-2.2
Pertussis-treated	Pertussis-treated	0.14	0.09-0.19	0.49	0.3-0.76	7.0	6.5-7.8	4.9	3.8-6.1

The opposite relationship holds when these contrasts are made for the observed proportional change.

These same comparisons can be made between the data for normal cells in pertussis animals and pertussis cells in normal animals (Table I, B and C). Again, the most marked and consistent differences can be seen in the data from

the node. It should be noted that the results for pertussis cells in normal animals are more similar to normal cells in pertussis animals than for normal cells in normal animals.

The data obtained for pertussis cells in pertussis animals are presented in Table I, D. The results for the node are found to be consistent with those observed for pertussis cells in normal animals. Again, there is a notable difference in the blood or circulating pool when contrasted with the corresponding data from the other experiments presented here.

The amounts of acid-precipitable radioactivity found at the two time periods in the node and blood, expressed as percentage of injected label, are shown in Table II.

Although there are some differences among the results shown in Table I for the other compartments studied (liver, lung, and spleen) the changes seen

TABLE III

*Correlation Coefficients for Normal Lymphocytes in Normal Animals and Normal Lymphocytes in Pertussis Animals**

	Blood	Liver	Lung	Node	Spleen
Blood	1	0.912	0.956	−0.813	−0.952
Liver	0.834	1	0.856	−0.932	−0.947
Lung	0.929	0.877	1	−0.954	−0.916
Node	−0.937	−0.891	−0.993	1	0.942
Spleen	−0.875	−0.877	−0.964	0.982	1

* Values above the diagonal line formed by the numeral 1 are correlation coefficients between compartments for normal lymphocytes in normal animals; those below the diagonal are for normal lymphocytes in pertussis animals.

are small and not clearly consistent or ordered. The most interesting and significant differences are seen in the circulating pool and the node. Based on these observations it was of interest to examine the interrelationship between the different compartments studied here.

The correlation coefficient was selected as the statistic used to measure such an association. This coefficient, which can range from $+1$ to -1, was calculated for the six pairs of values for the five compartments taken two at a time. These results are shown in Tables III and IV. With $n = 6$, the probability of observing a correlation coefficient of $|0.8114|$ or greater by chance alone, if in fact the true value is zero, is less than 1 in 20 or 5%. The critical value for the 1% significance level is $|0.9172|$. When a correlation coefficient is found to be statistically significantly different from zero based on a small sample, it can be taken as strong evidence of the existence of an interdependence between the two variables.

In Table III the correlation coefficients between compartments for normal

cells in normal animals are shown above the diagonal and those for normal cells in pertussis animals are shown below the diagonal. Although the signs of the coefficients change, it should be noted that the results for any compartment are in every case for both experiments statistically significantly different from zero. In addition, there are no consistent differences between the two experiments with respect to this measure within any given compartment as related to all the others. In no case are two corresponding correlation coefficients for the two experimental situations significantly different.

The results for pertussis cells in normal animals are shown above the diagonal in Table IV and those for pertussis cells in pertussis animals below. Although the sign of the coefficients here agree in every case with those in Table III, it should be noted that for the node these measures are *not* significantly different from zero for any pair of compartments in either experiment. The lack of dependence between the pairs of compartments together with the differences noted in Table I suggest that these cells are not handled in the node as are the normal cells and are not sequestered but retained in the circulating pool or in some other compartment. From the data obtained for pertussis cells in pertussis animals (below the diagonal in Table IV) it can be seen that *no* correlation coefficient is significantly different from zero.

TABLE IV

*Correlation Coefficients for Pertussis Lymphocytes in Normal Animals and Pertussis Lymphocytes in Pertussis Animals**

	Blood	Liver	Lung	Node	Spleen
Blood	1	0.980	0.987	−0.647	−0.928
Liver	0.810	1	0.939	−0.618	−0.867
Lung	0.639	0.786	1	−0.629	−0.913
Node	−0.632	−0.686	−0.654	1	0.802
Spleen	−0.665	−0.806	−0.738	0.763	1

* Correlation coefficients above the diagonal line formed by the numeral 1 are values between compartments for pertussis lymphocytes in normal animals; those below the line are for pertussis lymphocytes in pertussis animals.

The structure and interdependence seen with normal cells for the compartments surveyed does not exist when pertussis cells are studied. It is of considerable interest to see that this lack of structure is seen for pertussis cells in normal animals (above the diagonal in Table IV) and is not seen in the data for normal cells in pertussis animals (below the diagonal in Table III).

DISCUSSION

Small lymphocytes recirculate from blood to lymphoid tissue and then after passing through the peripheral lymphatics reenter the circulation through

the major lymphatic vessels, particularly the thoracic duct. In pertussis-induced lymphocytosis, despite the high level of circulating lymphocytes, the number of cells passing through the thoracic duct are actually fewer than in normals. This finding suggested that lymphocyte recirculation might be impaired, either by an anomalous function of the vasculature, e.g., the post-capillary venules, or through an abnormality of the lymphocyte itself such as an alteration in surface properties which would inhibit normal migration.

For testing these alternative hypotheses peripheral blood lymphocytes were isolated from mice with pertussis-induced lymphocytosis and from normal mice. After labeling in vitro with tritiated uridine, the cells were transfused into normal and pertussis-treated mice.

Striking differences were found in the percentage of injected label recovered in the mesenteric node (Table II). 60 min after transfusion of labeled normal cells into normal animals, an average of 8.1% of the acid-precipitable radioactivity was recovered in the mesenteric node. In contrast, when cells from pertussis-treated mice were injected into normal mice, less than 5% of this amount, i.e. less than 0.4% of the injected label, was recovered in the node; similarly, when lymphocytes from pertussis-treated mice were injected into pertussis-treated mice a markedly lower percentage of the injected label, less than 0.5%, was found in the node (Table II). These results clearly suggested that the lymphocytes in the blood of animals undergoing pertussis-induced lymphocytosis have a profound alteration in their capacity to emigrate from the blood.

When normal lymphocytes were injected into pertussis-treated mice, the recovery of label in the node was approximately one-third of that found when normal cells were transfused into normal mice. This was still greater than five times that recovered when cells from pertussis-treated animals were injected into either host. It may be that cells transfused into pertussis-treated animals also will have their migratory properties altered. In addition, a secondary, and certainly less consequential, abnormality of the vascular endothelium may occur. This is particularly likely since it is believed that initial entry of lymphocytes into the circulation of pertussis-treated mice may be via an abnormal route.

These results were paralleled by the findings with respect to the blood. That is, there tended to be greater retention of label in the blood when cells from pertussis-treated mice were injected into treated or normal recipients than when normal lymphocytes were transfused into normal recipients. The retention of cells in the blood of pertussis-treated mice injected with normal lymphocytes was more like that found when pertussis cells were injected. This again suggests that these cells may have been altered in the recipient.

Insight into the dynamics of the system was obtained by examining the interrelationships between the different compartments for which data were

obtained; blood, mesenteric node, liver, lung, and spleen. The correlation coefficient was used as the measure of these relationships. When normal lymphocytes were injected into either normal or pertussis-treated recipients, the correlation coefficients were significant in all cases (Table III). That is, there was an interrelationship between any two compartments.

When pertussis cells were injected into normal mice there was no interrelationship found between the node and any other compartment (Table IV). This was not the case when normal lymphocytes were transfused into pertussis-treated mice. For the case of pertussis lymphocytes transfused into pertussis-treated mice *no* two compartments showed any interdependence. Thus, other factors may play a role in addition to an aberration of the lymphocytes themselves.

Several considerations were taken into account in evaluating the data obtained. The radioactivity was expressed in terms of the total organ or total blood volume to avoid variations produced by variations in organ weight. As it turned out there were in fact no consistent or significant differences in organ weights between the groups of mice used.

Recovery of total label in all groups was essentially the same arguing against some sort of mechanism involving cell destruction which might affect the results. It could also be argued that the circulating lymphocytes in pertussis-induced lymphocytosis might contain an abnormal percentage of a subpopulation of lymphocytes with intrinsically different migrating properties. In previous experiments it had been shown that the proportion of circulating cells which label with thymidine is the same in both normal and pertussis-treated mice, so that in terms of long- and short-lived cells there is no difference (1). Analysis of grain counts and percentage of labeled cells of normal and pertussis lymphocytes in the experiments reported here were essentially the same in both, indicating that there was no difference in the extent of labeling with uridine. Similarly, viability of the cells in the inocula and after in vitro incubation for 90 min was the same for cells from both sources, as was the stability of the label. Radioactive uridine was injected in amounts equal to the total amount of acid-soluble and acid-insoluble label injected with the labeled cells. Acid-precipitable radioactivity found 1 hr later was negligible. Therefore differences in reutilization of the label did not affect the results.

Finally, it should be noted that after transfusion of labeled lymphocytes, a large proportion of the injected cells were found in extra lymphoid tissue as well as in the spleen, i.e., trapping takes place. However, again, no consistent or significant differences between the groups were found indicating that the results were not related to differences in initial trapping and secondary release of cells.

These results suggested that at least one mechanism responsible for the lymphocytosis which follows the injection of pertussis bacilli or their products involves a decreased capacity of the lymphocytes to emigrate from the blood.

This abnormality must involve a change in the cells, presumably a surface alteration, for lymphocytes obtained from the blood of pertussis-treated mice showed markedly diminished entry into the mesenteric node of normal mice. This finding does not exclude a concomitant role of the vasculature in the process but clearly such a role would be independent of the effect on the cells themselves.

Bradfield and Born (9) have shown that heparin-induced lymphocytosis is also the consequence of inhibition of lymphocyte emigration. However, it is not clear whether this is due to a primary effect on the lymphocytes. We would tend to agree with their thesis that it is "unlikely that lymphocytosis can occur without a diminution in the efficiency of the emigration mechanism."

Preincubation of lymphocytes with glycosidases (10), neuraminidase (11), or trypsin (12) is known to affect their subsequent emigration from the blood. Studies are now in progress to determine whether preincubation of lymphocytes with products of *B. pertussis* will alter their migration pattern.

SUMMARY

Peripheral blood lymphocytes were isolated from normal mice and mice undergoing pertussis-induced lymphocytosis. After labeling in vitro with tritiated uridine the cells were transfused into normal or pertussis-treated mice. It was found that the lymphocytes from pertussis-treated mice entered the lymph nodes of both normal mice and pertussis-treated mice to a significantly lesser extent than did normal lymphocytes which had been transfused into either class of recipient. In addition, an interdependence of changes in the various body compartments examined was found when normal lymphocytes were injected into either type of recipient. However, when pertussis lymphocytes were injected into normal mice there was no interrelationship between the changes in the node with those in the blood, liver, lung, or spleen. In the case of pertussis lymphocytes transfused into pertussis-treated mice *no* interrelationship between any two compartments was observed. It was concluded that in pertussis-treated mice there is an inhibition of lymphocyte emigration which is primarily the consequence of an effect on the cell.

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