STUDIES ON THE LEUKOCYTOSIS AND LYMPHOCYTOSIS INDUCED BY BORDETELLA PERTUSSIS*

II. The Effect of Pertussis Vaccine on the Thoracic Duct Lymph and Lymphocytes of Mice

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Plates 75 and 76

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The striking lymphocytosis induced in mice by *Bordetella pertussis* has been shown to be due to an increase in the circulating number of preformed lymphocytes rather than to an increase of newly formed cells (1). There are several possible mechanisms by which the lymphocytosis could occur. There might be an increase in the rate of entry of lymphocytes into the circulation via the normal route. This should be reflected in an increase in the cell content of thoracic duct lymph since in several animal species it has been shown that the thoracic duct serves as the major lymphatic conduit into the blood (2, 3). Alternatively, an increased number of lymphocytes might enter the circulation by unusual routes in addition to, or instead of, lymphatics; e.g., direct entry into blood vessels. Finally, the rate of entry of lymphocytes into the blood stream might be normal, but the rate of egress might be dampened. If the number of lymphocytes which enter and leave the circulation of the mouse each day is greater than the number present at any one time, as is true in other animals (4–6), a diminished rate of exit of the lymphocytes from the blood might, by itself, lead to an increase in the lymphocyte count.

In order to answer this specific question, as well as to attempt to derive information on the mechanisms which control the level of circulating lymphocytes, studies were performed in which the cell content of the thoracic duct fluid of normal and pertussis-treated mice was examined.

Materials and Methods

Thoracic Duct Cannulation.—The basic operative procedure described by Gesner and Gowans (7) was modified, and also simplified by the use of Eastman 910 Adhesive (Armstrong Cork Co., Lancaster, Pa.) to secure the catheter in place in the thoracic duct. Recently, Boak and Woodruff (8) have also reported success in the use of this agent in their studies on the cannulation of the thoracic duct in mice.

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Male Swiss mice of the NCS colony, maintained at The Rockefeller University, weighing 18–20 g were fed 0.2–0.3 ml of olive oil by intragastric instillation. 45–90 min later, 2.0 mg of sodium pentabarbital in 0.2 ml of saline was injected intraperitoneally. Relatively light anesthesia was subsequently maintained by ether inhalation.

A left anterolateral incision was made and the left kidney and adrenal gland were gently retracted inferiorly. The thoracic duct was exposed by blunt dissection and overlying fascia removed from the duct from just below the diaphragm to the level of the cisterna chyli.

The polyethylene catheters which were used had an inside diameter of 0.011 inches (Intramedic, PE 10, Clay-Adams, Inc., N. Y.). A hairpin curve at the end was found to be readily made by bending the catheter to the desired position, fixing it in that position with cellophane tape, and briefly immersing it into hot water. The catheter was then flushed with heparin solution, 5000 units/ml, and dried by drawing air through it. The catheter was cut at an angle approximately 1 cm distal to the curve, and after fixing the duct in a stable position with cotton swabs, the beveled end was plunged caudally into the duct just above the cisterna for a length of about 4 mm. The adhesive was applied over the point of entrance of the catheter into the duct and along the catheter as it curved anterosuperiorly and then inferiorly along the abdominal wall. The catheter exited at the lower end of the wound where it was fixed by a silk suture. Before closure, the abdominal contents were gently massaged and the site examined for leakage of thoracic duct fluid. The wound was closed with 9 mm clips (Autoclips, Clay-Adams, Inc.) in a single layer and 0.5 ml of saline was injected subcutaneously.

The trunk of the mouse was then swathed in adhesive tape, and the animals placed in a modified restraining cage (Arthur H. Thomas, Co., Philadelphia, Pa.) which allowed restricted movement (Text-Fig. 1). There was free access to food, and to 10% dextrose in saline (9). The catheter drained into a flask containing 1 ml of saline to which 500 units of heparin and 2 mg of streptomycin had been added. The flask temperature was maintained below 20°C by immersing it in a bath of ice water.

Leukocyte Counts and Morphology.—Peripheral white blood counts (WBC) and cell counts of thoracic duct fluid were performed by conventional methods. Differential counts were carried out by direct examination in the counting chamber or by inspection of films stained with Wright-Giemsa. In the case of lymph, satisfactory films were made by first depositing cells from an aliquot of the drainage by low speed centrifugation. The cells were then gently resuspended in a small amount of newborn calf serum and the suspension was painted on microscope slides with a fine sable brush.

Viability of the cells in the thoracic duct drainage was established by the nuclear exclusion of trypan blue and by observing the cells for motility under phase-contrast optics.

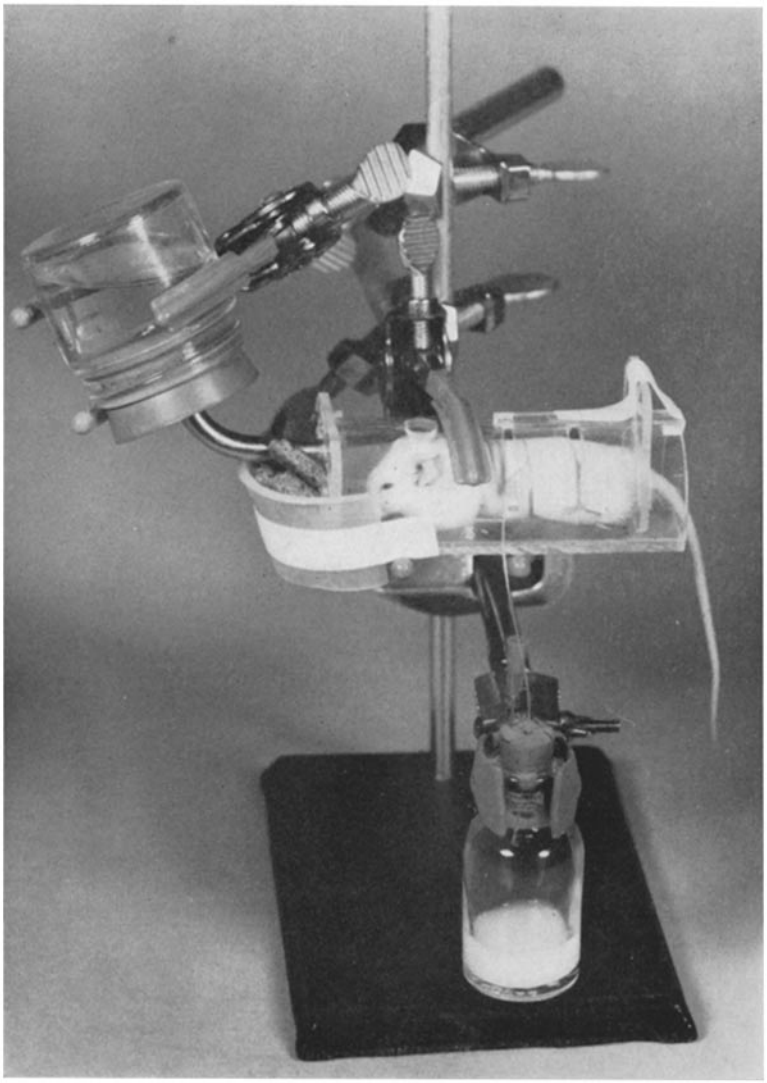
Specimens of thoracic duct cells were fixed for electron microscopic studies by allowing 1.0–2.0 ml of thoracic duct fluid to drain directly into 2.5% glutaraldehyde in 0.1 M phosphate (pH 7.4) at 4°C with intermittent mixing. After a further 30 min, the cells were sedimented by low speed centrifugation, washed with cold saline, postfixed in osmium in the cold, and washed again with saline. The blocks were then embedded in Epon and thin sections were stained with uranyl acetate and lead citrate (10). The grids were examined in a Siemens Elmiskop I electron microscope. We are indebted to Dr. James G. Hirsch for his assistance in this phase of our studies.

RESULTS

Thoracic Duct Output of Normal Mice.—Table I presents the volume flow and cell contents of thoracic duct lymph from 16 normal NCS mice. The average number of cells shed in the first 24 hr after drainage was 123.6×10^6; in 10 of the 16, the output of cells was greater than 100×10^6 per day and in none was

the output less than 62×10^6. In general the volume and cell concentration were inversely related.

The vast majority of cells present in the thoracic duct fluid 24 hr after cannulation were small and medium lymphocytes, but when drainage was continued, a greater proportion of cells were found which were classified as large



TEXT-FIG. 1. The apparatus used for housing mice and collecting thoracic duct fluid from cannulated animals.

lymphocytes (Table II). 0–2.0% of the cells were typical monocytes whereas polymorphonuclear leukocytes (PMN's) were rarely found. Less than 1% of the cells were large primitive-looking cells, which were unable to be classified, and even cells in mitosis were rarely seen. Plasma cells were noted infrequently. The light and electron microscopic appearance of the various types of cells found are shown in Figs. 1 *a*–1 *c*, 2, and 3.

It is difficult to calculate precisely the total number of circulating lymphocytes in the mouse because of marked differences in cell count between the central and peripheral blood (11). However, by assuming the tail vein WBC to be two-thirds of the mean WBC and the circulating blood volume of the mouse to be 2.0 ml, a minimum estimate could be made that during the first 24 hr after cannulation the number of small and medium lymphocytes which were found in the thoracic duct fluid averaged 3.9 (range 1.4–8.8) times the number present in the circulation immediately prior to the operative procedure.

TABLE I

Output of Lymph and Cells from the Thoracic Duct during the first 24 hr after Cannulation of Normal Mice

Animal	Volume	Concentration of cells	Total cells
	ml	× 10^6 *per ml*	× 10^6
1	13.5	6.00	81.0
2	20.0	3.75	75.0
3	17.0	10.00	170.0
4	19.5	5.25	102.4
5	12.0	5.63	67.6
6	7.6	21.38	162.2
7	20.0	3.13	62.6
8	12.4	13.80	171.6
9	11.5	7.80	89.7
10	28.0	5.72	160.2
11	16.5	7.03	115.9
12	7.6	22.40	170.3
13	17.5	10.94	191.5
14	15.0	10.56	158.4
15	8.7	11.50	100.0
16	15.0	6.61	99.2

After prolonged drainage, large lymphocytes accounted for approximately 20% of the cells present and there was also an absolute increase in their number as compared to those found the 1st day of flow (Table II). Lymphocytopenia and depletion of tissue lymphocytes accompanied prolonged thoracic duct drainage.

Thoracic Duct Drainage of Mice Undergoing Pertussis-Induced Leukocytosis.—

Mice injected with the dose of pertussis which we have previously employed (12) did not tolerate the operative procedure. Consequently a lesser number of organisms (between 5×10^9 and 1×10^{10} cells) was injected intravenously in the present studies. Under these circumstances the peak leukocytosis occurred between the 2nd and 4th day after injection and cannulation was performed during this time period.

10 mice with striking lymphocytosis as the result of injection with pertussis vaccine 2–4 days previously, were successfully cannulated. Table III presents

TABLE II

Prolonged Drainage of Thoracic Duct Lymph in Normal Mice

Animal	Day	Volume	Concentration of cells	Total cells	Percentage of cells*			
					Lympho-cytes	Large lympho-cytes	Mono-cytes	PMN's
		ml	× *10⁶/ml*	× *10⁶*				
4	1	19.5	5.25	102.4	97.4	2.0	0.3	0.0
	2	17.8	5.50	97.9	95.0	4.6	0.0	0.0
	3	7.6	3.25	24.7	92.9	6.0	0.5	0.0
5	1	12.0	5.63	67.6	95.0	4.0	1.0	0.0
	2	20.0	2.38	47.6	92.7	5.2	2.0	0.0
	3	27.5	2.18	59.8	92.0	7.0	1.0	0.0
7	1	20.0	3.13	62.6	97.4	2.0	0.3	0.0
	2	20.0	1.75	35.0	85.0	4.3	0.6	0.0
	3	25.9	2.08	53.7	93.3	6.0	0.6	0.0
	4	27.8	1.78	49.5	90.9	7.4	1.0	0.0
	5	37.0	0.13	4.75	86.0	13.0	1.0	0.0
	6	36.0	0.51	18.5	62.3	19.6	1.3	0.5

* At least 300 cells counted.

data on the thoracic duct drainage during the first 24 hr after thoracic duct cannulation of the pertussis-treated mice as well as the increment in the number of circulating lymphocytes existing at the time of operation.

It can be seen in Table III that although all of the mice in the pertussis-treated group had significant lymphocytosis, the volume of thoracic duct output, the lymph cell concentration, and the total cell output were all strikingly less than the values obtained in normals. None of the injected mice drained more than 10.0 ml during the first 24 hr after cannulation, but on autopsy there was no evidence of cryptic cannula or duct obstruction. In addition the transit time for fat, fed by gavage to cannulated mice, to appear in the catheter was normal.

More importantly, in contrast to the normals, reduction in volume output was not accompanied by a reciprocal increase in cell concentration. Thus, 5 of the 10 pertussis-treated mice drained between 7.0 and 10.0 ml of thoracic duct fluid, but the cell concentration ranged between 0.86 and 5.9 $\times$ 10^6 cells/ml (animals 3, 5, 8, 9, and 10). In two normal animals (6 and 12) the drainage was 7.6 ml over a similar time period but the cell concentrations were 21.4 and 22.4 $\times$ 10^6/ml; animal 15 drained 8.7 ml and the cell concentration was 11.5 $\times$ 10^6/ml (Table I). Differential counts of thoracic duct cells were similar however in both groups. In addition, two mice which had been injected subcutaneously with per-

TABLE III

*Output of Lymph and Cells from the Thoracic Duct during the First 24 Hr after Cannulation of Mice Undergoing Pertussis-Induced Hyperlymphocytosis**

Animal	Volume	Concentration of cells	Total cells	Increase in circulating lymphocytes‡
	ml	$\times$ 10^6 *per ml*	$\times$ 10^6	
1	5.3	0.81	4.3	6.4
2	4.2	1.36	5.7	4.9
3	7.0	.86	6.0	7.4
4	5.8	1.30	7.5	3.8
5	9.0	1.20	11.0	3.7
6	2.4	6.80	16.3	3.8
7	5.4	3.60	19.4	12.5
8	10.0	2.30	21.6	7.1
9	7.2	4.80	34.9	2.8
10	8.2	5.90	48.4	5.5

* Pertussis vaccine injected 2–4 days before cannulation.

‡ $\frac{\text{No. of circulating lymphocytes per mm}^3 \text{ at time of cannulation}}{\text{No. of circulating lymphocytes per mm}^3 \text{ before injection of pertussis vaccine}}$

tussis vaccine, a procedure which does not induce lymphocytosis, were cannulated 2 days after inoculation. Drainage was reduced in amount but cell concentration was increased and the total output of cells was only slightly less than the average output of normals.

Thus it appeared that the decrease in both volume and cell concentration which resulted in a markedly diminished total yield of cells in the pertussis-treated mice was a relevant finding. Table IV presents a summary of the findings in the normal mice and mice undergoing pertussis-induced lymphocytosis.

In all normal animals the circulating lymphocyte count decreased during the first 24 hr after thoracic duct cannulation, and in 12 of the 16 normal mice the fall amounted to greater than 70%. This decline was in part related to the operative procedure alone. It was therefore of great interest to determine whether changes would occur in the circulating lymphocyte count of pertussis-treated animals with indwelling thoracic duct cannulae.

In only 6 of the 10 pertussis-treated animals had the circulating lymphocyte count decreased 24 hr after thoracic duct cannulation (Table V). In five of these six the percentage decrease was less than 20%, and in the sixth

TABLE IV

Average and Range of Thoracic Duct Outflow in Normal and Pertussis-Treated Mice during the First 24 Hr after Cannulation

Group	No. of mice	Thoracic duct outflow 24 hr after cannulation			No. of blood lymphocytes at time of cannulation
		Volume	Cell concentration	Total cells	
		ml	$\times 10^6$ *per ml*	$\times 10^6$	
Normal	16	15.1 (7.6–28.0)	9.46 (3.13–21.38)	123.6 (62.6–191.5)	Normal
Pertussis-treated	10	6.4 (2.4–10.0)	2.89 (0.80–6.80)	17.5 (4.3–48.4)	5.36 × normal (2.8–12.5)*

* Average: 113,700 lymphocytes/mm^3. Range: 35,300–147,700 lymphocytes/mm^3. (Tail vein blood WBC's).

TABLE V

Changes in the Circulating Lymphocyte Counts of Pertussis-Treated Mice in the First 24 hr after Thoracic Duct Cannulation

Animal	Circulating lymphocytes, $\times 10^3/mm^3$*		Per cent change
	At cannulation	24 hr after cannulation	
1	128.7	124.7	(−) 3.1
2	97.2	60.3	(−) 38.0
3	147.7	119.4	(−) 19.2
4	76.8	69.7	(−) 9.2
5	73.4	69.7	(−) 5.1
6	76.2	142.3	(+) 87.0
7	107.1	132.4	(+) 23.8
8	49.7	132.8	(+)167.5
9	35.3	65.7	(+) 86.1
10	69.7	57.3	(−) 17.8

* Tail vein concentration.

the lymphocyte count declined by 38 %. More importantly, in four of the animals the number of circulating lymphocytes increased. The number of lymphocytes in two of the animals increased by 86.1 and 87.0%, and in one by more than 2½-fold. These increments represented a total increase in the number of circulating lymphocytes of approximately 40.5, 80.1, and 110.8 × 10^6 cells respectively. The hematocrits of the animals were determined from tail

vein blood and were normal. Therefore the increase in concentration of blood lymphocytes was not due to hemoconcentration.

DISCUSSION

In an effort to determine the mechanisms responsible for the lymphocytosis induced by killed *Bordetella pertussis* cells, experiments were performed in which the thoracic ducts of normal and pertussis-treated mice were cannulated. Volume flow, cell concentration, total output of cells, and changes in the number of circulating small lymphocytes were measured.

The volume of thoracic duct drainage during the first 24 hr after cannulation of normal animals averaged 15.1 ml (range 7.6–28.0 ml). The average cell concentration was 9.46×10^6/ml (range 3.13–21.38 $\times 10^6$/ml), and the total output of cells averaged 123.6×10^6 (range 62.2–191.5 $\times 10^6$). These values were in accord with those found by other workers employing different strains of mice (7, 8). Over 95% of the cells found in the thoracic duct fluid during this time were small lymphocytes.

Because of the great variation in leukocyte concentration between the central and peripheral circulation in the mouse, it was difficult to estimate accurately the total number of lymphocytes in the blood of these rodents. This prevented accurate assessment of the relationship between the circulating lymphocyte pool at the time of cannulation to the number of cells found in the thoracic duct fluid drainage. However, by taking into account the known disparities between the leukocyte count of tail vein blood and left ventricular blood a *minimum* estimate was that 3.9 times the number of circulating lymphocytes present in the blood at the time of catheterization passed through the thoracic duct fluid in 24 hr. Almost surely the actual figure was greater. This finding suggested that recirculation of lymphocytes, shown by Gowans and his coworkers to occur in the rat (4–6), also occurs in the mouse.

During the first 24 hr after thoracic duct cannulation of pertussis-treated mice, the average volume outflow was 43% of that found in normal mice. It was striking that the average cell concentration, despite the diminished volume flow, was decreased to 30.5% of normal. There was no evidence of mechanical obstruction of the duct or catheter. It is pertinent that the average circulating lymphocyte concentration was 5.4 times normal and even if the lower volume outflow was related to pathophysiologic effects of pertussis vaccine on the circulation it would be difficult to account for the lower cell concentration. Therefore, these results indicated clearly that the rise in the concentration of circulating lymphocytes which was induced by the injection of *B. pertussis* was not reflected by an increase in the number of lymphocytes carried by the thoracic duct. Indeed, there was a marked decrease in the cellular output of thoracic duct lymph per unit time.

The further observation that the number of circulating lymphocytes increased markedly in three of the pertussis-treated mice even though the thoracic

duct was cannulated, suggested the possibility that lymphocytes may have entered the circulation through other routes following the injection of *B. pertussis* cells. It was unlikely that the channels were other lymphatics since one would expect the thoracic duct cell content to reflect occurrences in other lymphatics. Moreover, during pertussis-induced lymphocytosis, histologic examination of lymphoid organs which drain into thoracic duct, such as mesenteric lymph nodes, revealed depletion of lymphocytes. The possibility therefore existed that the cells entered into the circulation without passing through the normally intermediate major lymphatic channels. Although many investigators, including Ehrlich (13), have proposed that lymphocytes might gain access to the blood stream directly (eg. 3, 14, 15), thus far a pathway for so called "direct entry" lymphocytes has not been demonstrated in normal animals.

A second question which arises as a result of these studies concerns the fate of the lymphocytes in the blood of pertussis-treated mice. In normal rats, it is apparent that there is a continuous recirculation of lymphocytes from the blood to lymphoid tissue to lymphatics and then back to blood with the major lymphatic channel being the thoracic duct. The 24 hr output of cells from the thoracic duct of normals suggests this also occurs. In the mice with pertussis-induced lymphocytosis, however, the thoracic duct was obviously not even carrying the complement of cells found in normal animals despite a severalfold increase in the number of circulating lymphocytes. Either the circulating cells were unable to leave the blood stream to recirculate, or once having entered the lymphatic tissue from the circulation they reentered the blood directly. Future experiments in which labeled lymphocytes are to be injected into pertussis-treated animals may resolve this question.

SUMMARY

The 24 hr volume flow, cell concentration, and total cell output of thoracic duct fluid from mice with pertussis-induced hyperlymphocytosis were markedly reduced when compared with values obtained in normal animals.

An increase in the number of circulating lymphocytes occurred in several of the pertussis-treated mice despite the presence of an indwelling thoracic duct cannula. The drainage from such animals also showed a reduced cell concentration and total cell output.

It is suggested that lymphocyte recirculation may be minimal in pertussis-induced lymphocytosis, and the evidence obtained also suggests that lymphocytes may enter the blood stream by direct routes during the course of the reaction.

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EXPLANATION OF PLATES

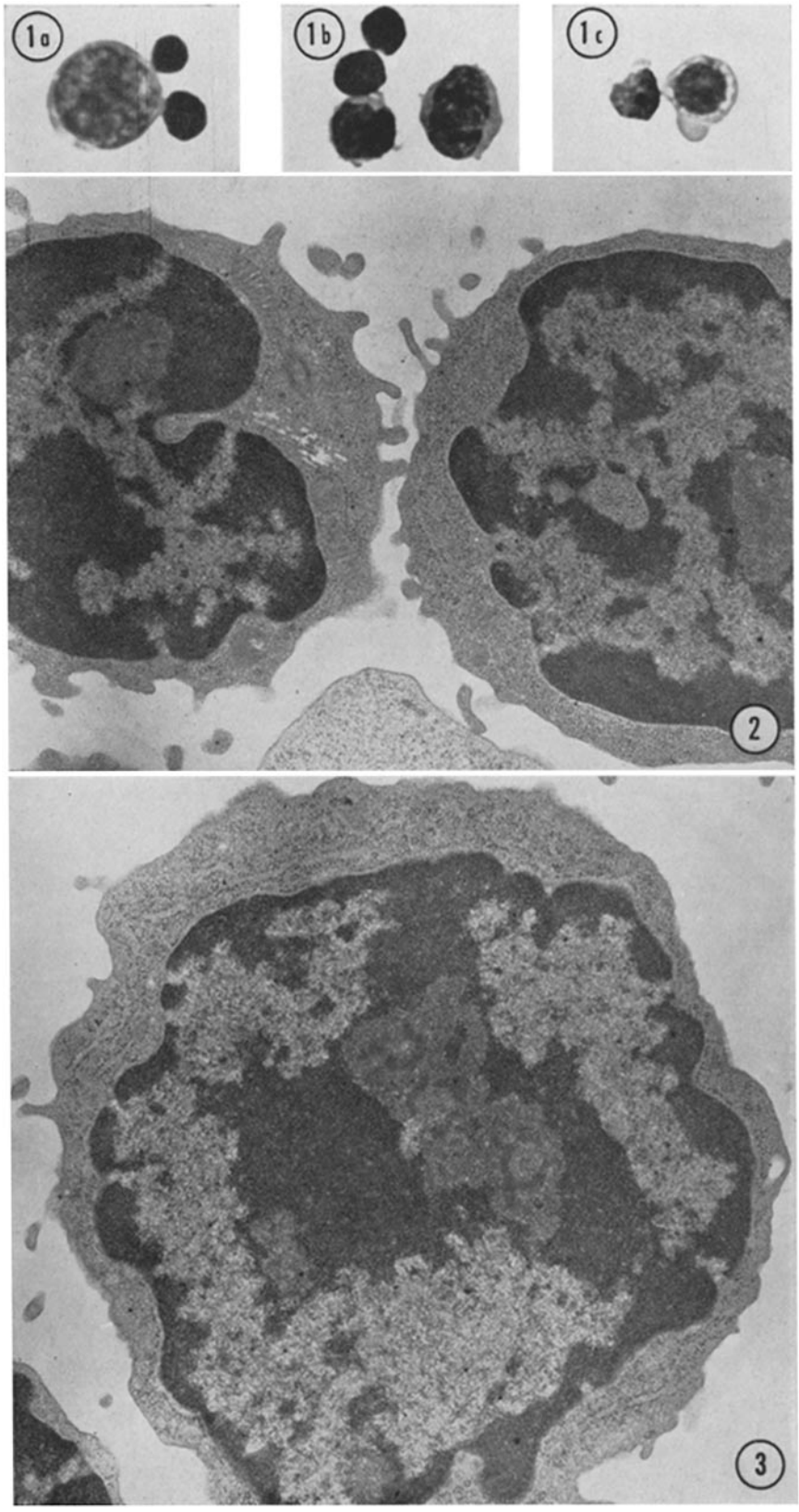
PLATE 75

FIGS. 1 *a*–1 *c*. Photomicrographs of cells found in the thoracic duct fluid during the first 24 hr after cannulation. Small and medium sized lymphocytes are seen together with a large lymphocyte (Fig. 1 *a*); a large cell with a monocytoid nucleus (Fig. 1 *b*); and a plasma cell (Fig. 1 *c*). Wright-Giemsa, × 1200.

FIGS. 2 and 3. Electron micrographs of some of the cell types found in the thoracic duct fluid of normal mice during the first 24 hr after cannulation. × 17,000.

FIG. 2. Two small cells whose nuclei show prominent nucleoli and condensation of nuclear material. The cytoplasm is scant and neither the Golgi region nor the endoplasmic reticulum are prominent. Free ribosomes are present. These features are characteristic of small lymphocytes.

FIG. 3. Except for large size and the more evident rough endoplasmic reticulum this cell is similar in morphology to those shown in Fig. 2. It is most likely a large lymphocyte.



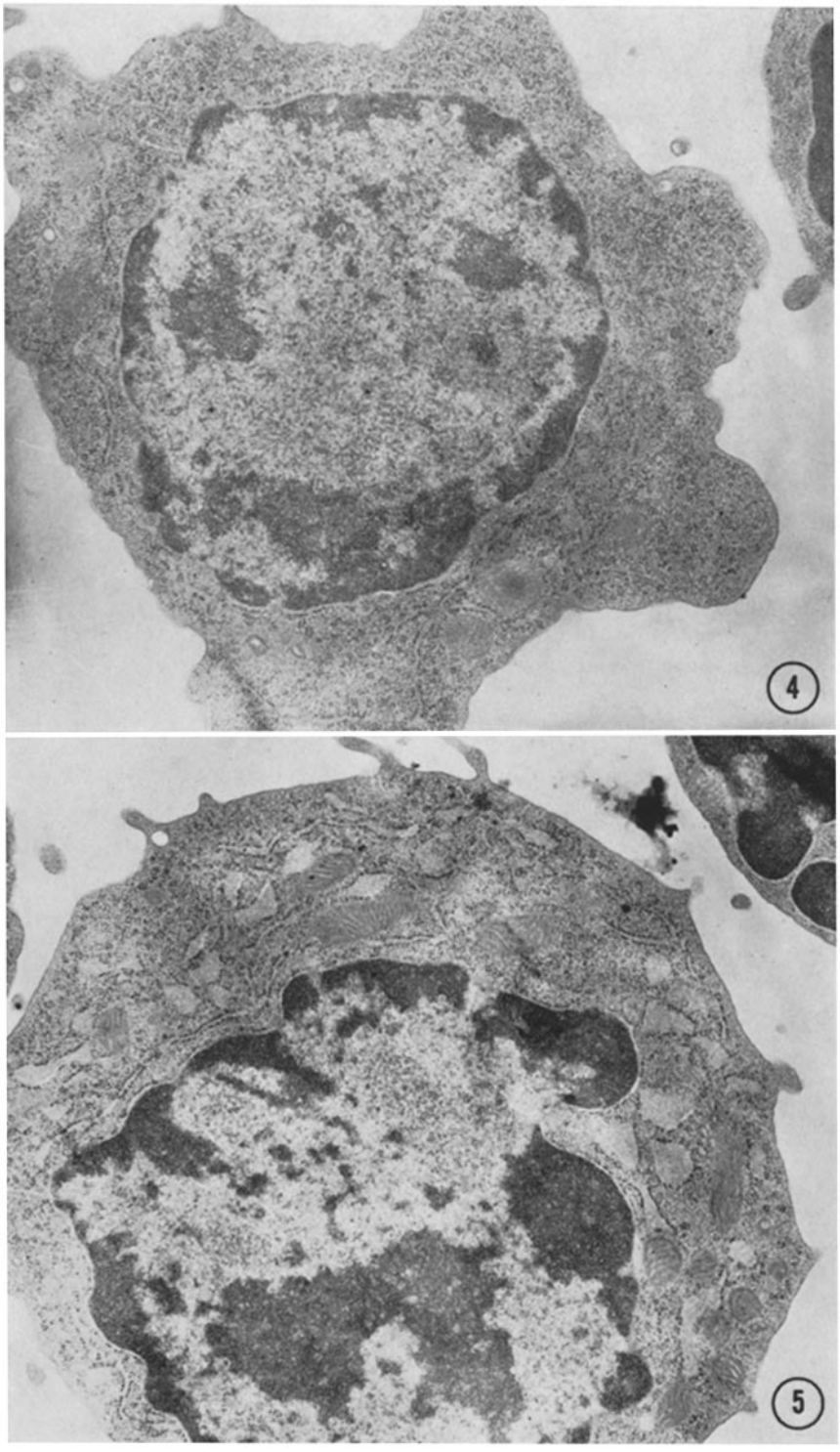
(Morse and Riester: Pertussis)

PLATE 76

FIGS. 4 and 5. Electron micrographs of some of the cell types found in the thoracic duct fluid of normal mice during the first 24 hr after cannulation. × 17,000.

FIG. 4. The cell is also large in size but has abundant cytoplasm and a low nucleus-to-cytoplasm ratio when compared to the cells illustrated in Figs. 2 and 3. There is little condensation of nuclear material. Profiles of rough endoplasmic reticulum are seen, as well as free ribosomes and polyribosomes. The cells can not be readily classified and is probably a primitive cell.

FIG. 5. The most striking feature of the cell is the abundant rough endoplasmic reticulum which in many instances is dilated to form cisternae which appear to contain amorphous material. These features are characteristic of actively synthesizing cells and the overall appearance would suggest that it is a cell in the plasmacyte series.



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