STUDIES ON THE LEUKOCYTOSIS AND LYMPHOCYTOSIS INDUCED BY BORDETELLA PERTUSSIS

I. Radioautographic Analysis of the Circulating Cells in Mice Undergoing Pertussis-Induced Hyperleukocytosis*

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Plates 23 and 24

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In previous studies, it was shown that the intravenous injection of killed *Bordetella pertussis* cells into experimental animals resulted in hyperleukocytosis which was uniquely characterized by the predominance of normal appearing small lymphocytes (1, 2). Lymphocytosis was maximal 4–5 days following injection of the bacilli. At that time period it was noted on histological examination that the population of lymphocytes in lymphoid tissue, including spleen, lymph nodes, and thymus, was depleted (Figs. 1 *a* and 1 *b*). There was no apparent increase in the number of cells with mitotic figures, nor were primitive cells more abundant than in normal lymphoid tissue. From these observations, it was hypothesized that the lymphocytes which had entered the circulation in response to the administration of pertussis vaccine were cells initially present in the extravascular tissues, rather than newly formed entities.

More precise evaluation of the source of the circulating cells in pertussis-induced leukocytosis could be made by the use of the DNA label tritiated thymidine, and the results of studies utilizing this compound are reported herein.

Materials and Methods

Mice.—The male Swiss mice, weighing 22–25 g, employed in these studies were from the NCS colony maintained at The Rockefeller University.

B. pertussis Suspensions.—Concentrated suspensions of phase I *B. pertussis* cells in 1:10,000 thimerosal were purchased from Eli Lilly and Company, Indianapolis, Ind. The organisms were diluted in physiologic saline to achieve a concentration of approximately 3×10^{10}/ml. 0.2 ml was injected intravenously into a lateral tail vein. White blood counts were performed in standard hemacytometers, and differential counts were performed by examining the cells directly in the counting chamber as well as by examination of stained blood films.

Dosage and Administration of Tritiated Thymidine.—Tritiated thymidine (specific activity 6.7 c/mM) was obtained from New England Nuclear Corporation, Boston, Mass. The isotope

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was diluted in physiologic saline buffered to pH 7.2 with 0.01 M phosphate. 0.5 μc/g of body weight was injected intraperitoneally into mice in a volume of approximately 0.2 ml every 4 hr for a total of 16 injections commencing 30 min after the intravenous injection of pertussis vaccine. Control animals, which received intravenous injections of saline instead of pertussis vaccine were paired with the pertussis-treated group and received the same dosage of tritiated thymidine according to the same schedule.

Radioautographic Techniques.—At appropriate intervals, thin films of tail vein blood were prepared on 1 × 3 inch microscopic slides, which were then fixed in cold absolute methanol for 15 min and air dried. Slides were coated with emulsion in the following manner: K5 emulsion (Ilford), at a concentration of 1 g/ml of distilled water, was dissolved in a Wheaton jar at 43°C and held at that temperature. The slides were then prewarmed for 30 sec at this temperature, slowly dipped into and out of the emulsion, and then permitted to drain vertically until the coating of emulsion was thoroughly dry. They were then stored in a light-tight box containing desiccant for 3 wk at room temperature.

TABLE I

*Leukocyte Counts of Mice Given Pertussis Vaccine and Tritiated Thymidine**

Animal	Day		
	0	3	8
1	17,500‡	114,000	—
2	12,500	144,000	170,000
3	13,500	177,000	176,000
4	14,500	172,500	165,000

* 0.5 μc/g every 4 hr from day 0 to day 3.

‡ Leukocytes/mm^3.

The emulsions were developed by immersing the slides in developer (Dektol) for 2 min and after fixing for 5 min they were thoroughly washed. Cells were stained through the emulsion with Giemsa at pH 5.75.[1]

The stained autographs were examined at 1000 × with an oil immersion objective and the nuclear grain counts were scored after correction for background.

RESULTS

Nuclear Labeling by Tritiated Thymidine of Small and Medium Lymphocytes in the Blood of Normal and Pertussis-Treated Mice.—In carefully prepared thin films of mouse blood, it is very difficult to make a distinction between small and medium lymphocytes. There is a gradual gradation in size rather than any sharp distinction. Moreover, in both stained films and in viable preparations examined under phase microscopy, there do not appear to be any distinguishing nuclear or cytoplasmic characteristics which might be useful in classification. Finally, when an arbitrary system was employed which was based upon size, there was no appreciable difference in the labeling pattern of those cells which

[1] We are indebted to Dr. V. P. Bond for his advice regarding the radioautographic and staining procedures.

were termed small lymphocytes and those which were called medium size lymphocytes. For these reasons these mononuclear cells were considered together, as has been done by other investigators.

Four mice were injected with 0.2 ml of pertussis vaccine intravenously and the changes which occurred in the total leukocyte counts are shown in Table I. As is noted, one of the four mice died between the 3rd and 8th day. Four control mice were injected with 0.2 ml saline intravenously and the leukocyte counts of these animals ranged between 9000 and 35,000 cells/mm^3. Tritiated thymidine was administered every 4 hr to all mice until the first blood samples for radioautography were taken 3 days after intravenous inoculation.

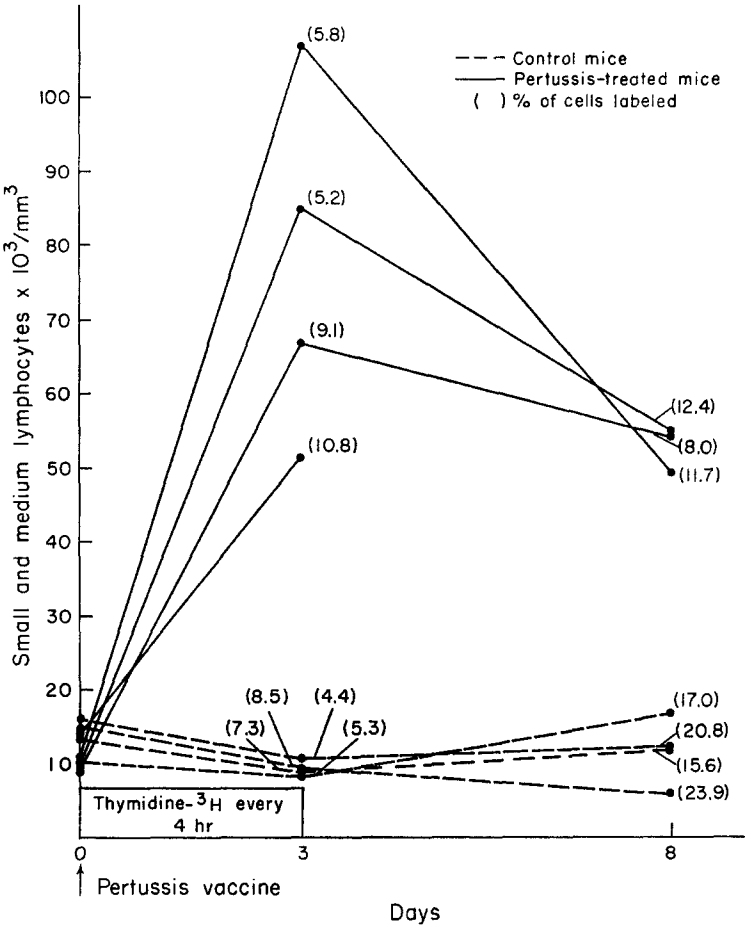
Specimens for autoradiography were obtained on the 3rd and 8th day and at least 200 lymphocytes from each animal were examined. As can be seen in Text-fig. 1, 3 days after the injection of pertussis vaccine the number of circulating lymphocytes rose from 3.7 to greater than 10 times the initial value. Nevertheless, the percentage of lymphocytes labeled in the blood of pertussis-treated animals was essentially the same as that in the controls. In all, 849 lymphocytes from the blood of pertussis-treated animals at this time period were examined and 66 (7.8%) had nuclear label above background. 12 of these cells had more than 50 grains/nucleus. 53 (6.4%) of 830 lymphocytes from the normal animals which were examined had nuclear label above background, but none had more than 50 nuclear grains. Therefore, the slight increment in the percentage of cells labeled in the experimental animals appeared to be accounted for by the heavily labeled cells.

8 days after the intravenous injection of pertussis vaccine (5 days after cessation of thymidine treatment), the number of circulating lymphocytes had decreased from the values found 3 days after the injection of vaccine. At this time period the percentage of labeled lymphocytes had risen slightly but was now less than in the control animals. There was still a higher proportion of heavily labeled cells in the blood of the experimental animals than in the control mice.

Nuclear Labeling of Polymorphonuclear Leukocytes (PMN's) in Normal and Pertussis-Treated Mice.—3 days after the injection of pertussis vaccine, the numbers of circulating PMN's had risen from 15 to over 30 times the initial values (Text-fig. 2). It was found that over 70% of the cells were labeled at this time; in the control animals only between 27 and 41% of the PMN's were labeled. Between the 3rd and 8th day, during which time no thymidine was given, the number of circulating PMN's in the experimental group continued to increase, but the percentage of labeled cells decreased slightly. In contrast, over 95% of the circulating PMN's in the control group were now labeled.

In terms of the extent of labeling at the 3 day time period, 25 of the 306 labeled PMN's in the experimental group had more than 50 nuclear grains (8.2%), whereas only 3 of the 136 labeled PMN's in the control group had

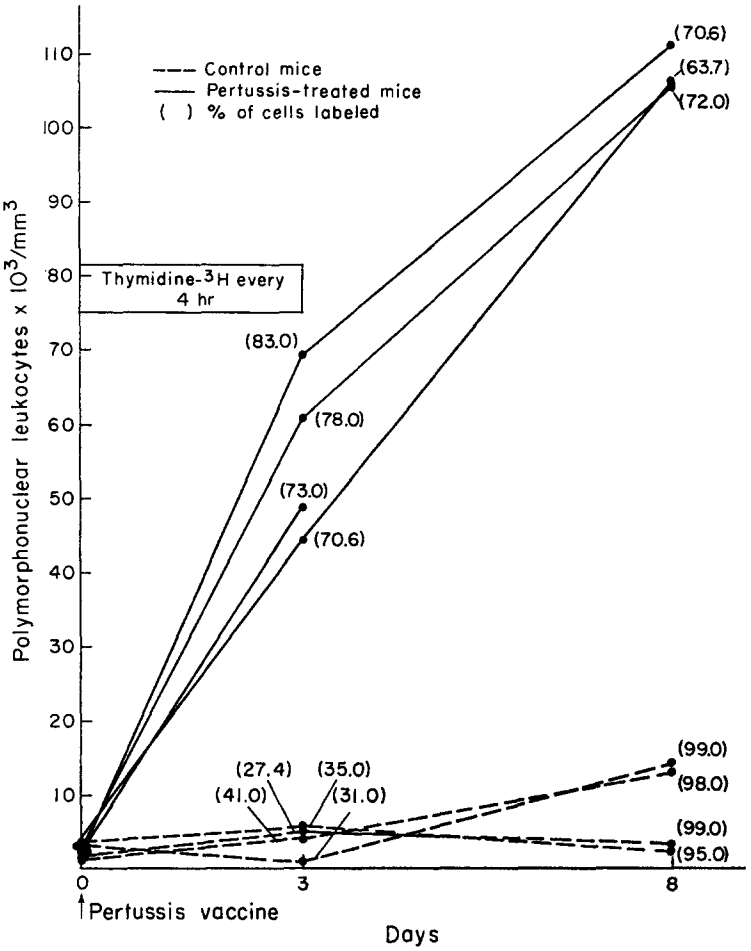
greater than 50 nuclear grains (2.2%). PMN's with more than 50 grains/nucleus were not found in either group at the 8 day sampling period.



TEXT-FIG. 1. The number of circulating lymphocytes and the percentage of cells with nuclear labeling in normal mice and in mice injected with pertussis vaccine.

Further information regarding the dynamics of the PMN response was also obtained by examination of the femoral bone marrow of mice at the time of peak response to pertussis vaccine (3–4 days after intravenous injection). In these mice the myeloid/erythroid ratio was greater than four times that found in normal marrow and the ratio of mature PMN's to erythroid elements was two to three times greater than the normal ratio. At the same time it was noted that cells with the morphologic appearance of small lymphocytes had markedly

decreased in the marrow of pertussis-treated mice (1–2% of cells counted compared with 29–38% of cells in normal marrow).



TEXT-FIG. 2. The number of circulating PMN's and the percentage of cells with nuclear labeling in normal mice and mice injected with pertussis vaccine.

Examples of nuclear labeling of leukocytes in the blood of mice 3 days after pertussis administration are shown in the photomicrographs which comprise Figs. 2 *a*–2 *c*. It will be noted that the heavily labeled PMN's do not have immature morphology.

As has been previously noted, large mononuclear cells, including both monocytes and large lymphocytes, appear in increased numbers in the circulation of

mice which have received pertussis vaccine (2). Unfortunately it is often difficult to distinguish between these cells unless they possess typical morphology and many of them are not typical. For this reason, quantitative studies were not performed. However, qualitative examination of radioautographs obtained 3 days after the injection of pertussis vaccine revealed that an average of 75.3% (235/312) of cells which appeared to be typical monocytes were labeled while in the control group, 79.7% (243/305) had nuclear grains above background. At the same time period 59 of 75 cells with the characteristic morphology of large lymphocytes were labeled (78.7%) whereas only 41 of 106 cells of this type (38.7%) were labeled in the blood of the control animals.

DISCUSSION

In the experiments reported herein, 0.5 μc/g of body weight of tritiated thymidine was injected into mice every 4 hr beginning at a time coinciding with the intravenous injection of pertussis vaccine. Injections of the label were continued until the time of near maximal leukocytosis 3 days later when the first blood samples were obtained for radioautographic analysis. Control animals received an intravenous injection of saline and the same dosage of nuclear label.

The rationale for this experiment design was based upon the thesis that in the presence of a continuously available isotopic label, every cell in the circulation which was newborn during that time would possess labeled DNA which could then be detected by radioautographic techniques. It was therefore of critical importance to ensure that label would be available to any dividing cell during the course of pertussis-induced leukocytosis. The schedule of administration of tritiated thymidine was chosen on the basis of the known DNA-synthesizing periods for mammalian cells, particularly with relationship to the small lymphocyte.

It is becoming increasingly apparent that the majority of small lymphocytes, at least 80%, have a long life span of 100–200 days and perhaps even of years, whereas a minority population has a much shorter mean life (3–7). The long life span of the bulk of the population of circulating small lymphocytes is reflected in the low order of labeling of these cells after a single injection of tritiated thymidine into an animal (4, 8). However, after 80 days of continuous infusion of tritiated thymidine into rats, 93% of small lymphocytes could be labeled (5). The increase in the percentage of cells during continuous infusion of thymidine was commensurate with that achieved when the label was administered every 12 hr during a similar time period (5, 9). Little et al. (5) therefore suggested a duration of DNA synthesis in small lymphocytes, or their precursors, of at least 12 hr. By administering the label every 4 hr in the present study it seemed unlikely that a significant number of dividing cells would escape labeling.

During the 3 day period of time when the label was injected every 4 hr, the number of circulating small and medium lymphocytes in the pertussis-treated

animals rose from 3.7 to over ten times the initial level. However, the percentage of labeled cells averaged 7.8% and ranged between 5.2 and 10.8%. In the control animals, the average labeling of lymphocytes was 6.4% (range 4.4–8.5%). Therefore, despite the dramatic increase in circulating lymphocytes in the pertussis-treated mice there was little difference in terms of the degree of lymphocyte labeling between the two groups of animals, and no evidence could be adduced that the lymphocytosis caused by pertussis vaccine was due to an increased rate of lymphocyte production (8). Rather the phenomenon appeared to be due to a redistribution of lymphocytes.

Of note was the finding of a small proportion of heavily labeled lymphocytes in the blood of pertussis-treated mice at day 3, whereas such cells were rarely found in the blood of the normal animals. It is known that bone marrow cells which are morphologically indistinguishable from small lymphocytes take up DNA label more rapidly than lymphocytes from other sites (10). These cells may not be true lymphocytes but rather may be stem cells (11). In pertussis-treated animals there was a drastic reduction in the bone marrow population of such cells and they may be the heavily labeled cells found in the circulation.

5 days after the first sample, during which time label was not given, the numbers of circulating lymphocytes in the experimental group of mice decreased. The percentage of labeled cells increased slightly more than twofold in two of three instances. In the normal animals there was a greater increase in the percentage of labeled cells, but the numbers of animals used was insufficient to evaluate the significance of the differences between the two groups.

The increase in circulating PMN's 3 days after injection of pertussis vaccine, under circumstances in which tritiated thymidine was administered virtually continuously, was primarily due to the influx of newly formed cells. There was an average labeling of the PMN'S of 76.1% whereas in the normal animals the labeled PMN's represented only 27.4–41.0% of the total. That true myeloid proliferation had occurred was also demonstrated by bone marrow examination.

In the label-free interim between the 3rd and 8th day, the number of circulating PMN's in the pertussis-treated mice continued to increase but the percentage of labeled cells decreased to between 63.7 and 72.0%. In contrast, 95% or greater of the circulating PMN's in the normal animals were now labeled. This difference most likely was due to ongoing myeloid proliferation and dilution of the label in the pertussis-treated group. Because of the relatively short life span of the PMN, a less tenable explanation was that preformed mature PMN's, present before the administration of thymidine, continued to enter the circulation of mice in the experimental group.

SUMMARY

By the use of radioautographic techniques it was shown that the lymphocytosis induced by *Bordetella pertussis* in mice was not caused by an increased production of lymphocytes but was primarily due to the entry into the circula-

tion of mature cells from tissue pools. The accompanying polymorphonuclear leukocytosis was due to both proliferation of myeloid elements and entry of mature cells from tissue reserves, with the former the predominant mechanism.

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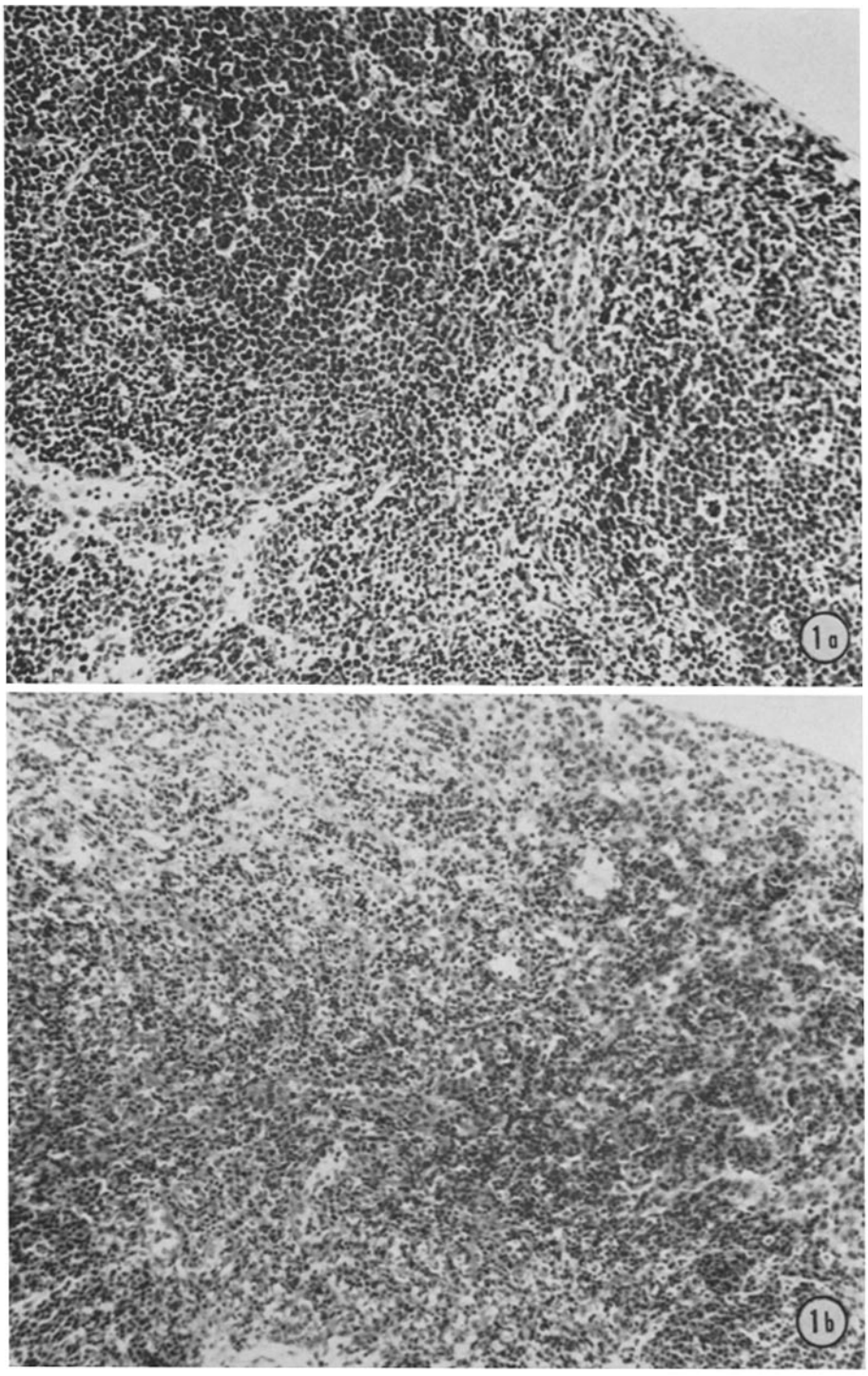
EXPLANATION OF PLATES

PLATE 23

FIGS. 1 *a* and 1 *b*. Hematoxylin-eosin stain. × 190.

FIG. 1 *a*. Photomicrograph of the mesenteric lymph node (pancreas of Aselli) of a normal mouse.

FIG. 1 *b*. Photomicrograph of the same organ of a mouse injected with pertussis vaccine 4 days previously. The lymphocytes are decreased in number around the cortical sinuses, in the perifollicular area, and in the medullary cords.



(Morse and Riester: Pertussis)

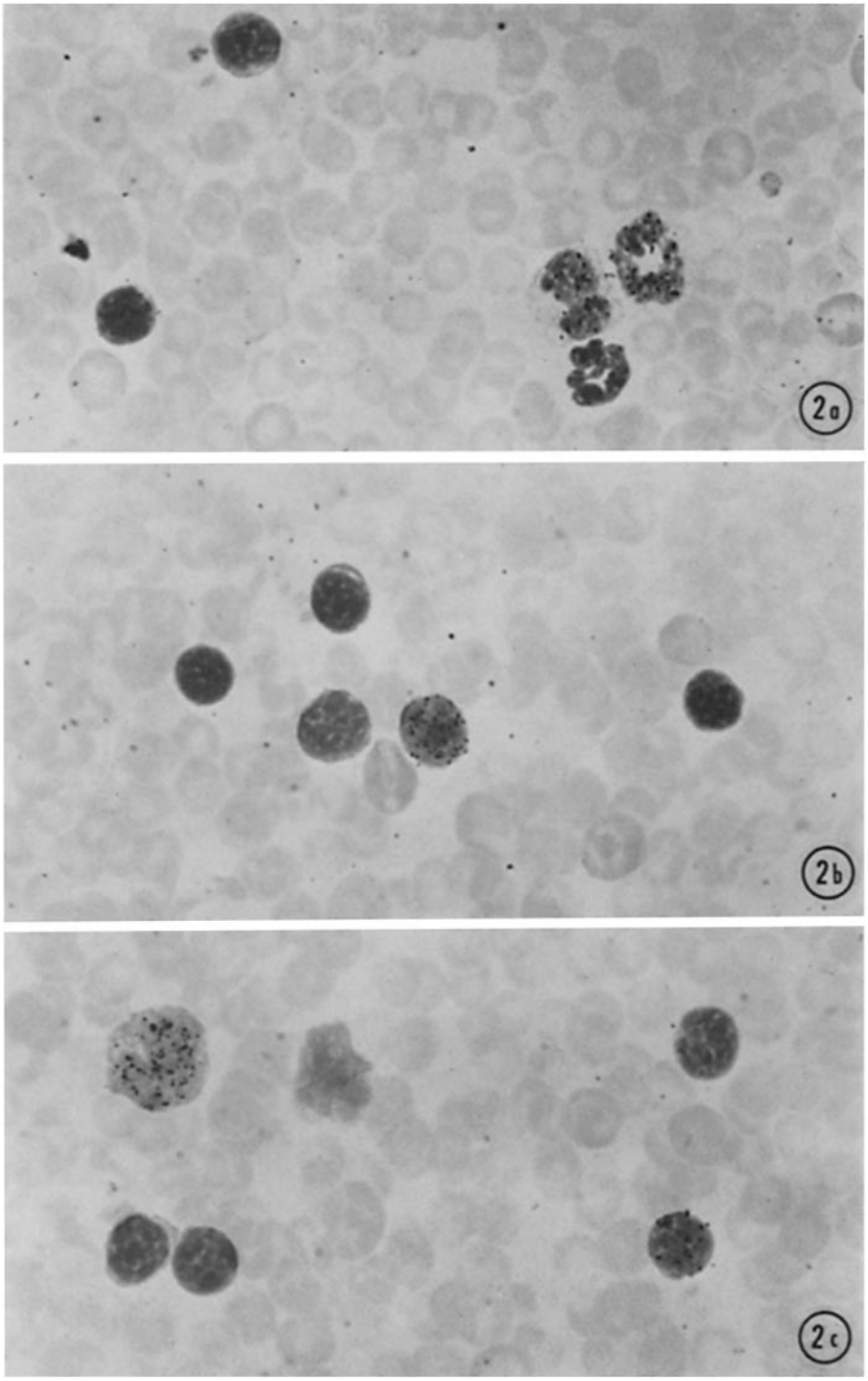
PLATE 24

FIGS. 2 *a*–2 *c*. Photomicrographs of radioautographs of the blood of mice given pertussis vaccine 3 days previously and tritiated thymidine every 4 hr. Giemsa stain. × 1200.

FIG. 2 *a*. Two PMN's with moderately heavily labeled nuclei are present together with one PMN and two lymphocytes which do not have nuclear label.

FIG. 2 *b*. Four unlabeled and one heavily labeled lymphocyte are present.

FIG. 2 *c*. A heavily labeled monocyte and one labeled lymphocyte are seen together with three unlabeled lymphocytes.



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