Quantitative Studies on the Rate of Cell Production in the Thymolymphatic Organs

I. A Simple Method for Determination of the Mitotic Index in Suspension of Cell Nuclei*†

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Quantitative estimation of the rate of lymphocyte production in the thymolymphatic organs has hitherto been made by using either one of the following three methods: (1) cannulation of thoracic-duct lymphocytes (and right lymphatic-duct lymphocytes) (*Yoffey*, 1935–36; *Sanders* et al., 1940; *Reinhardt*, 1946; *Mann* and *Higgins*, 1950; *Whaler* and *Widdicombe*, 1956; *Hughes* and *Widdicombe*, 1956; *Reinhardt* and *Yoffey*, 1957); (2) determination of the mitotic indices in lymphoid organs (*Kindred*, 1940, 1942, 1955); (3) determination of the rate of desoxyribonucleic acid formation in lymphoid organs using radioactive tracers (*Andreasen* and *Ottesen* 1945; *Yoffey* et al., 1958).

Among these, the second method seems to be the most rigorous and direct one. It should be emphasized, however, that the quantitative approach of this type in tissue sections, in reality, meets with difficulties because of irregular distribution of mitotic figures and wide variations in the cellular density in different regions of lymphoid organs. This may cause uncertainty in the results. Considering these points, *Andreasen* and *Christensen* (1949) have elaborated a new method for counting the cells in mitosis in suspension of cell nuclei, which undoubtedly may yield more accurate and reliable results than any method for mitotic counts in tissue sections. The principle of the method employed is as follows: The finely divided organ is treated with 5 % citric acid to free the nuclei. The suspension of nuclei is stained by gallocyanine and the ratio of dividing nuclei to resting nuclei is determined in a counting chamber using benzyl benzoate as diluent. The count is performed directly on a small part of the suspension which is transferred to a blood-counting chamber. From each specimen 10,000 nuclei are counted, and the number of mitosis is recorded.

In order to make the method more accurate and convenient, the following procedure was devised by *Christensen* (1950): A suspension of cell nuclei is prepared from

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organs fixed in N/4 HCl, instead of treating with 5 % citric acid. After determination of the concentration of nuclei in suspension, a measured volume of the suspension, having about 30,000 nuclei, is transferred to a slide, and, after evaporation, all the mitotic figures seen on the slide are counted. This procedure makes it possible to count a much greater number of mitotic figures with ease. For this reason, the method seems to be more accurate than the original method.

However, the present study, which was intended to improve the method for mitotic counts in suspension of nuclei prepared from lymphoid organs, indicated that a modification of the original method of *Andreasen* and *Christensen* to be described below would be preferable to the procedure proposed by *Christensen.*

In the present study, young adult male albino rats of a subline of the *Wistar* strain, weighing around 200 g, were used. All were fed on a standard laboratory diet and water *ad libitum*, supplemented once a week with cabbage or other vegetables. They were divided into two series, the normal untreated ones and colchicine-treated ones. All rats of both series had been starved since the previous evening.

The reason why colchicine technique was used in the present research is to facilitate counting mitotic figures. This is based on the fact that colchicine stops normally occurring cell division at metaphase without influencing the rate at which mitoses occur[1]. Each animal was given a subcutaneous injection of 0.10 mg (or 0.15 mg) of colchicine[2] per 100 g of body weight. In most instances, the adrenal glands of the animals had been removed, according to the technique described by *Farris* and *Griffith* (1949), before colchicine injection; because colchicine, when injected in above doses into intact animals, produced extensive destruction of proliferating lymphocytes in the lymphoid tissues, the secondary nodules in particular (see Figs. 1–2).

Injections of colchicine were made at 10:00 a.m. and the animals were killed 4 or 8 hours after colchicine injection without having been fed during the intervening 4 or 8 hours. The normal untreated animals were killed at 10:00 p.m. Each animal was killed by cervical dislocation and bleeding followed immediately by removal of the tissues.

The lymphoid organs to be studied (the thymus, mesenteric lymph nodes and other lymph nodes) were removed separately[3] and immediately fixed in N/4 HCl for 2–3 hours, according to the method of *Christensen* (1950). The fixed organs were then wrapped up in a four-folded gauze and squeezed between two fingers,

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[1] It is important to bear in mind, however, that the rate of entrance into mitosis is somewhat diminished by the colchicine and, in addition, that not all the mitoses are arrested in the metaphase.

[2] The colchicine solution was prepared by dissolving the powder in distilled water or normal saline. Stored in a refrigerater it will maintain its potency for several weeks.

[3] A small portion of each of the excised organs was fixed in *Zenker*-formol for histological examination.

after being dipped into a vessel containing a small amount of distilled water. The nuclei pass through the filter cloth freed from their cytoplasm, so that they are suspended evenly in distilled water. After staining with *Mayer*'s acid hemalum and washing by centrifugation, the nuclei were resuspended in a small amount of alcohol. The final concentration was adjusted to about 20,000 nuclei/1mm^3 by varying the amount of alcohol to be added.

The suspension of nuclei obtained was then used for determination of the mitotic index. The method employed in the present study is a modification of the original method of *Andreasen* and *Christensen* (1949). From a small portion of the suspension of nuclei film preparations on slides were made and, after counting 5,000 nuclei from each film, the ratio of the number of nuclei in mitosis to the total number of nuclei present (the mitotic index) was determined. It is essential to make sufficiently thin films from the suspension, so that the cell in mitosis can be counted with accuracy

Table 1. Comparison of values of the mitotic index* obtained by the author's method with those obtained by the *Christensen's* method on the same material.

Rat No.	Treatment	Organs	Author's method	*Christensen's* method
81	Without colchicine injection	Thymus	1.10	0.30 (72/23,900)
		Mesenteric lymph nodes	0.78	0.20 (48/23,000)
		Other lymph nodes	0.70	0.51 (33/21,600)
82	Without colchicine injection	Thymus	0.98	0.47 (128/22,800)
		Mesenteric lymph nodes	0.80	0.21 (54/25,000)
		Other lymph nodes	0.92	0.20 (48/23,600)
83	Without colchicine injection	Thymus	1.32	0.47 (99/21,000)
		Mesenteric lymph nodes	1.12	0.30 (47/15,000)
		Other lymph nodes	0.98	0.23 (39/16,500)
62	4 hours after colchicine injection	Thymus	3.68	2.60 (540/20,600)
		Mesenteric lymph nodes	2.20	1.16 (246/21,200)
		Other lymph nodes	1.98	1.09 (230/21,000)
63	4 hours after colchicine injection	Thymus	3.34	2.76 (498/18,000)
		Mesenteric lymph nodes	2.16	1.03 (280/27,000)
		Other lymph nodes	1.16	1.46 (272/18,600)

* Expressed in percentage of the total nucleated cells.

Table 2. Values of the mitotic index* determined in sections from the different regions of the thymus and mesenteric lymph nodes 4 hours after colchicine injection (unpublished observations of *Kawamura*).

Thymus			Mesenteric lymph nodes			
Superficial cortex	Deep cortex	Medulla	Germinal center	Dark-staining mantle	Internodular region	Medullary cords
4.11	3.03	1.27	6.95	0.78	1.36	1.93

* Expressed in percentage of the total nucleated cells.

(see Figs. 3–5). This is the most important point of the method.

Another small portion of the same suspension of nuclei was used for mitotic counts by the *Christensen*'s method. As already mentioned, the principle of this technique is to count all the mitotic figures occurring in a measured volume of the suspension, the concentration of which had been determined previously. In the present study a known volume of the suspension, containing a known number of nuclei (about 20,000 nuclei) per mm^3, was transferred to a slide by means of a calibrated pipette (a volume of 1.0 mm^3). As pointed out by *Christensen*, the surface of the slide should be kept a little greasy to prevent spreading of the droplet deposited on it. This does not, however, facilitate counting of cells in mitosis.

Some examples, which show a comparison of values of the mitotic index obtained by the author's method with those obtained by the *Christensen*'s method on the same material are given in Table 1. It can be seen from the table that the author's method generally gives higher values than the *Christensen*'s method, with respect to the mitotic index in the lymphoid organ. The reason for this discrepancy is not apparent. However, in view of the fact that, as *Patt* (1957) has recently pointed out, the results of mitotic counts tend to be in the direction of an underestimate, the author's method seems to be preferable to the *Christensen*'s method, since the former tends to yield higher values than the latter. Of special interest in this connection are the values of the mitotic index determined in sections from the different regions of the thymus and mesenteric lymph nodes, which are shown in Table 2 (*Kawamura*, unpublished observations). From a comparison of the figures in this table with the corresponding values given in Table 1, it can be seen that the relatively high values of the mitotic index obtained by the author's method in the lymphoid organs are quite reasonable.

SUMMARY

A simple method for determination of the mitotic index in suspension of cell nuclei prepared from lymphoid organs has been described. This method tends to yield higher values than a similar method previously described by *Christensen* (1950). The recommended procedure is as follows: the lymphoid organ to be examined is fixed in N/4 HCl for 2–3 hours to prepare a suspension of nuclei from the whole organ. After staining the nuclei with *Mayer*'s acid hemalum, film preparations on slides are made from the suspension. The mitotic index is then determined after counting 5,000 nuclei from each film. It is essential to make sufficiently thin films from the suspension, so that cells in mitosis can be counted with accuracy.

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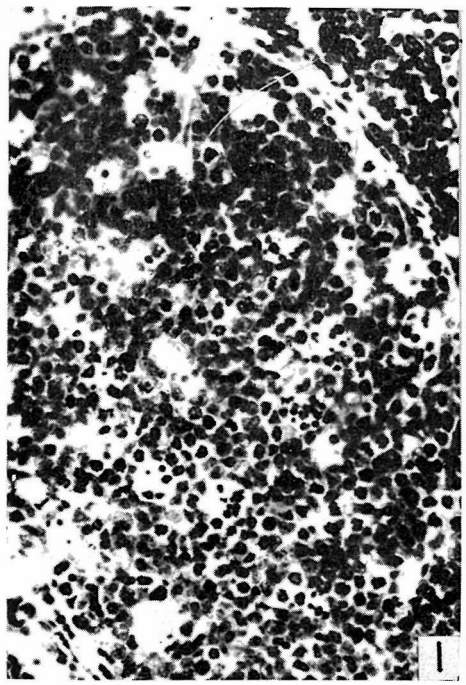
EXPLANATION OF PLATE-FIGURES

Fig. 1. Secondary nodules of the *Flemming* type of mesenteric lymph node. Rat No. 13, 8 hours after injection of colchicine in a dose of 0.10 mg per 100 g of body weight after adrenalectomy. Notice numerous mitotic figures with a small amount of nuclear debris. Fixed in *Zenker*-formol and stained with *Mayer*'s hemalum and eosin. × 400.

Fig. 2. Secondary nodule of the *Flemming* type of mesenteric lymph node. Rat No. 15, 8 hours after injection of colchicine in a dose of 0.10 mg per 100 g of body weight without adrenalectomy. Notice a few mitotic figures with a large amount of nuclear debris. Fixed in *Zenker*-formol and stained with *Mayer*'s hemalum and eosin. × 400.

Figs. 3–5. Mitotic figures in the film preparations made from the suspension of cell nuclei, which had been prepared according to the procedure described in the text. Rat No. 4, 4 hours after injection of colchicine in a dose of 0.10 mg per 100 g of body weight. Notice that all mitoses are arrested at pyknotic metaphase and easily distinguishable from the resting nuclei. × 1000.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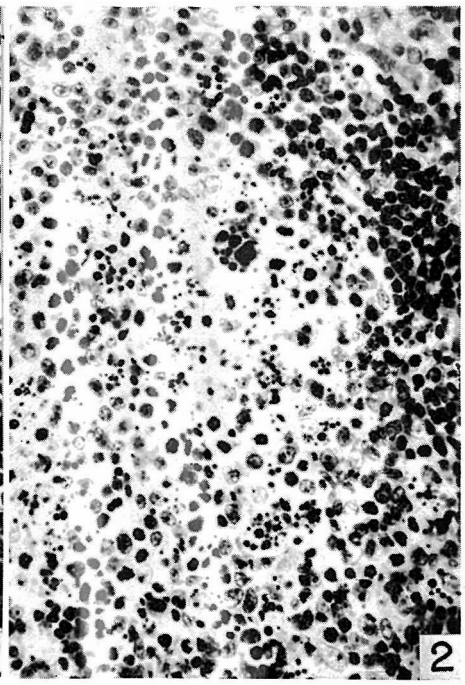
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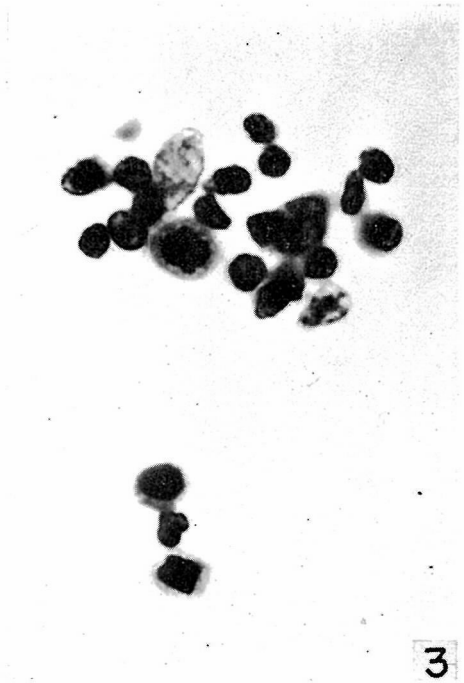
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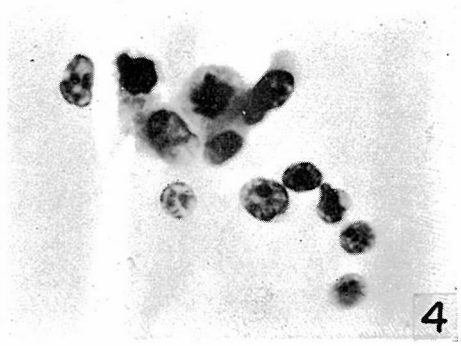
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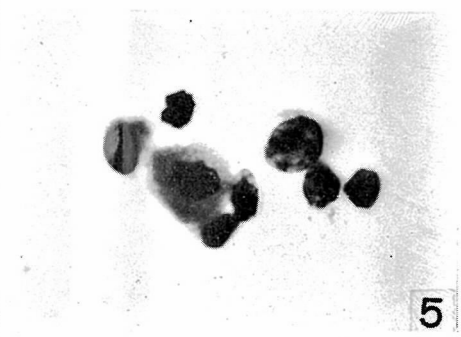
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