

## Studies of Phagocytic Activity of Lymphocytes

### III. Phagocytosis of Intravenous India Ink in Human Subjects

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**T**HE CURRENT THEORY ascribes the phagocytic activity of the blood to the monocytes and polymorphonuclear leukocytes. The lymphocytes are regarded as non-phagocytic in nature.<sup>1-3</sup> The few indirect findings which would indicate their phagocytic properties seemed to be inconclusive.<sup>4-6</sup>

Recently it has been shown that the lymphocytes are capable of ingesting hemosiderin,<sup>7-9</sup> but there was a need for more experiments in the nature of phagocytosis by lymphocytes. The following data are submitted to serve in establishing that human lymphocytes are able to phagocytize India ink injected intravenously.

#### MATERIALS AND METHODS

Eight patients, chronic intractable cases, were selected at random from the hospital service for these studies. The quantity of India ink used and the diagnosis of the cases are recorded in table 1. Each patient received daily intravenous injection of 10 to 20 cubic centimeters of India ink. The suspension was prepared by using Higgins India ink diluted to 10 per cent by volume with physiologic saline. After being filtered, it was sterilized for 15 minutes at 15 pounds pressure in an autoclave.

The antecubital veins were selected as sites of injection. The ink suspension was injected very slowly into the antecubital veins, care being taken to prevent hematoma or infiltration around the injection site because escape of the material into the surrounding tissue leaves a permanent tattooing effect.

The ink injections did not conspicuously affect any of the disease processes present in the patients. Hanzlik and Karsner<sup>10</sup> emphasized that colloid substances cannot be given intravenously without hazard, but India ink seems to be tolerated relatively well in human beings. Untoward effects as seen in our series consisted of chills lasting an average of ten to fifteen minutes, the longest being thirty minutes in duration. They were promptly brought under control by use of antihistaminics. In the few cases in which the reactions persisted (cases 2 and 8), the injections were discontinued early.

Before the injections were started, the blood of each patient was examined at regular intervals for a period of two weeks to obtain basic values. During the experimental period, it was checked every two days for a total of thirty days. The examination consisted of leukocyte and erythrocyte counts on the Neubauer hemocytometer and hemoglobin determinations with the Fisher Electro-Hemometer. Hematocrits were measured by Wintrobe's method.<sup>6</sup> Differential leukocyte count was done on slides stained by Wright solution. A second blood film was stained with 1 per cent safranin to clearly show the presence of carbon particles in the white blood cells. This stain makes the differentiation of the granulocytes impossible, but allows for easy identification of lymphocytes, monocytes, band and segmented cells.

#### RESULTS

There were essentially no quantitative changes in the blood values of the patients receiving the India ink injections. Minimal variations in hemoglobin, erythrocyte and leukocyte counts and differential shifts were attributed to the

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TABLE 1.—*Diagnosis of the Cases Used in the Studies with Intravenous India Ink*

Exper. No.	Age	Sex	Race	Diagnosis	Total number of injections	Total ink used
1	15	M	C	Chronic ulcerative colitis	10	100 ml.
2	37	F	R	Neurofibromatosis transverse myelitis	6	120 ml.
3	37	F	W	Serological lues, Laennee's cirrhosis	10	200 ml.
4	41	M	W	Laennee's cirrhosis	8	160 ml.
5	52	F	C	Generalized sarcoma	9	90 ml.
6	68	F	C	Cerebrospinal lues	10	190 ml.
7	74	M	C	Luetic heart failure	13	240 ml.
8	79	M	W	Chronic duodenal ulcer	6	120 ml.

clinical course of the patient undergoing treatment for chronic illness. The only noticeable change in the morphology of the leukocytes was the addition of the India ink particles in the cytoplasm.

All eight cases showed ingestion of carbon particles by the circulating leukocytes. Figure 1 illustrates the behavior of the white blood cells in a typical case (case 3). The first inclusions occurred after two days and the number of the phagocytizing cells increased progressively with continued injections. The polymorphonuclear leukocytes were the first to show phagocytic response with the lymphocytes following closely. The monocytes rarely showed ingestion of India ink. No inclusions were seen in eosinophils or basophils.

The neutrophils were the most efficient cells in the process of phagocytosis.

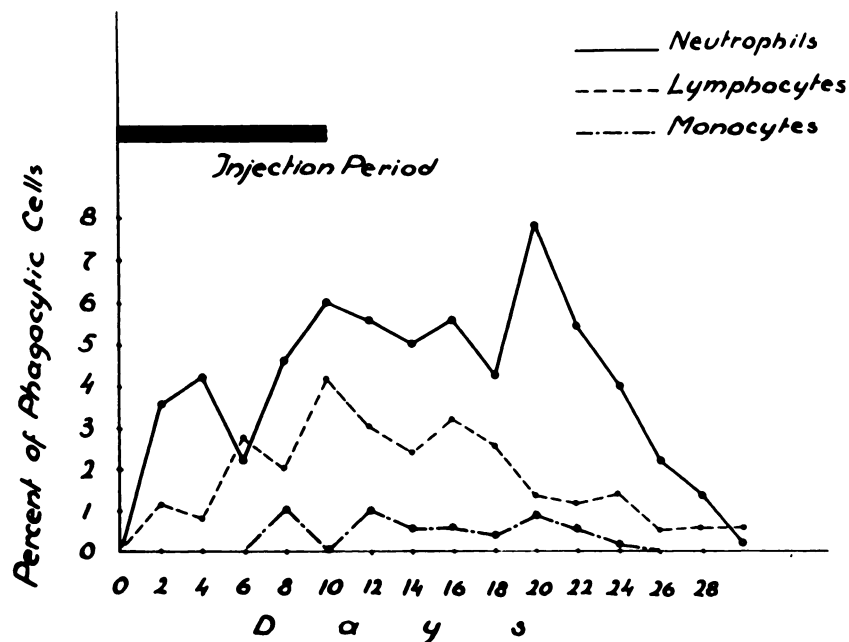


FIG. 1.—Percentage of the neutrophils, lymphocytes, and monocytes phagocytizing intravenous India ink. Case #3.

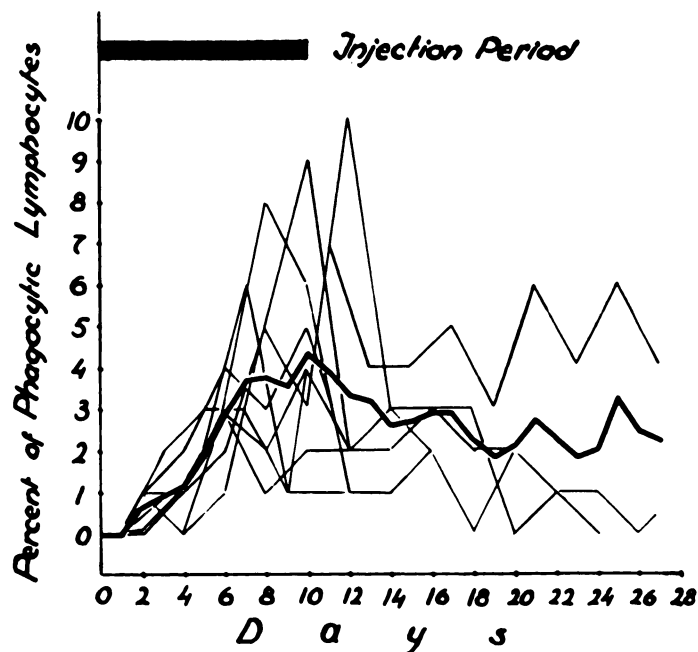


FIG. 2.—Variations of the percentage of phagoctyzing lymphocytes following the intravenous injections of India ink for the average period of 10 days. The thick line presents the middle values.

In all cases the percentage of polymorphonuclear cells which took up India ink was higher than that of lymphocytes, and they contained more inclusions in the cytoplasm than the other cells. The particles picked up by the granulocytes seemed also to be larger than those ingested by the lymphocytes.

The rate of appearance of phagoctyzing lymphocytes varied with the individual and also varied from day to day in the same individual (fig. 2). The first inclusions occurred in two cases on the second day; in the others on the fourth or sixth day. There was a rather rapid rate of appearance of the inclusion bodies and then a slower and more prolonged disappearance. The mean peak was reached on the tenth day following the onset of injections. The inclusions occurred with such regularity that a phagoctytic activity of lymphocytes seemed to be well ascertained. The artifacts, which may be caused by staining with anilin dyes, (Wright stain), could be excluded by the regular use of safranin with which India ink appeared as jet black particles against the pink cell body (fig. 3).

In some cases as much as 30 per cent of all lymphocytes showed India ink in the cytoplasm. Most of the cells contained only one inclusion, but some of them had two or more particles. The phagoctyzing cells did not appear different from the inclusion-free elements. Their nuclei in the Wright's stain were round and deep purple with dense chromatin pattern and prominent nuclear membrane (fig. 4). Both morphologic variations, the small strongly basophilic lymphocyte, as well as the intermediate and/or large, slightly basophilic cell contained India ink in their cytoplasm. Only a few phagoctyzing cells had a large basophilic body and an indented coarse nucleus, thus resembling Downey's Type I

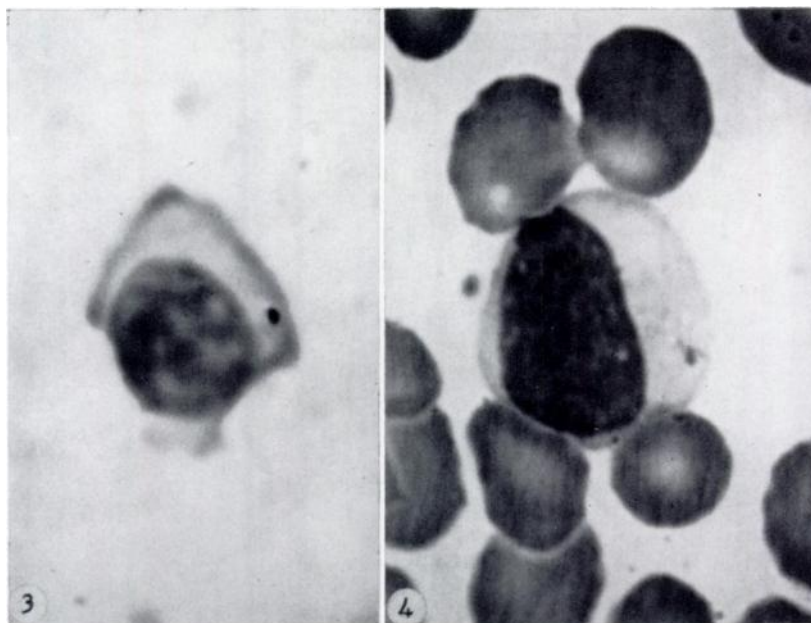


FIG. 3.—Photomicrograph of a small lymphocyte containing India ink particle in the cytoplasm ( $\times 2800$ ). Safranin stain.

FIG. 4. Photomicrograph ( $\times 2800$ ) demonstrating phagocytosis of India ink by a medium-sized lymphocyte. Wright's stain.

lymphocytes.<sup>11</sup> They could be easily distinguished from monocytes which have finely reticulated chromatin in the nucleus and a grayish cytoplasm with azurophilic granulation.

#### DISCUSSION

The role of the lymphocytes in the body is obscure, although they contribute from 20 to 30 per cent of the circulating white blood cells. By those who accept the unitarian theory of hemopoiesis they are hemocytoblasts, or stem cells of all the blood elements and may also develop into macrophages and fibroblasts.<sup>12</sup> For those who adhere to the dualistic or polyphyletic theory, they represent mature cells which cannot develop progressively, except perhaps into plasma cells.<sup>4</sup> Their function is regarded as chiefly mechanical in nature since they may help in walling off chronic inflammatory lesions, but they are considered definitely not to be bactericidal or phagocytic.<sup>1-6</sup>

Bergel<sup>13</sup> repeatedly pointed out that the lymphocytes are connected with digestion of fat. The intraperitoneal, or intrapleural injections of fatty oil brought about a marked mononuclear exudation, but a closer study by means of supravital technic demonstrated the cells to be clasmatoocytes (macrophages) and monocytes.<sup>14</sup> Kuczynski<sup>15</sup> thought that the lymphocytes are concerned with the handling of foreign protein which enter the tissues undigested. Ehrlich<sup>16</sup> went on to assign to them an enzymatic function, suggesting that because they are rich in adenosinase, they may serve the function of destruction of toxic products by splitting adenosine.

The fact that lymphocytes participate in the defense mechanism has been demonstrated by Metalnikov and Toumanoff,<sup>17</sup> Rich et al.,<sup>18</sup> Ehrlich and Harris,<sup>19</sup> and Dougherty and White.<sup>20</sup> Their belief was that they are involved rather in the production, or transportation of the antibodies than in the actual process of phagocytosis.

The theory of phagocytosis as first propounded by Metschnikoff<sup>21</sup> stated that animal cells are able to incorporate and to digest solid foodstuffs in their cytoplasm. In the human organism bacteria and other harmful elements would be attacked and destroyed by special cells called phagocytes and the contest between these elements and the phagocytes would produce inflammation. The cells were divided into microphages, i.e., the polymorphonuclear leukocytes and macrophages, i.e., large mononuclear cells of different origin. This definition found a widespread acceptance although it was a teleologic rather than a morphologic concept. The situation was complicated by the introduction of colloidal dyes into cellular research which made the differentiation between ultra-microscopic phagocytosis and the absorption of dye necessary.<sup>22</sup> Both processes can be observed in vivo, but only the ingestion of particulate substance should be regarded as true phagocytosis.<sup>23, 24</sup>

In the early days of intravital dye injections a distinction was made between the storage action of the reticulo-endothelial elements and the phagocytic activity of the leukocytes and macrophages.<sup>25, 26</sup> Only the monocytes seemed to be able to ingest vital dyes and, therefore, represent reticulo-endothelial elements of the peripheral blood.<sup>27</sup> This concept was not supported by other investigators who could easily prove that the leukocytes were able to store colloidal dyes in their cytoplasm.<sup>28, 29</sup> According to Downey,<sup>30, 31</sup> ingestion of dye by leukocytes seldom occurs within the general blood stream, but is very intensive in stagnated blood of isolated vessels. Under these conditions the polymorphonuclear leukocytes are more active than the macrophages which show dye inclusions only after most of them disappeared from the white blood cells.

The question of whether or not the lymphocytes are phagocytic was complicated by statements that these cells are able to transform into other elements, such as monocytes, polyblasts, free histiocytes, and reticular or resting wandering cells.<sup>32, 33</sup> Maximow<sup>22</sup> stated repeatedly that the lymphocytes are able to incorporate vital dyes after having undergone transformation in tissue to polyblasts. His observations were doubted as they were to a great extent based on cultures from the lymph nodes, omentum and subcutis where the mixture of different cells renders the field of observation very complex.<sup>34</sup> No such obvious transformation and dye ingestion was encountered by others who used blood explantates<sup>35, 36</sup> or stored blood.<sup>37, 38</sup> Recently Rebeck et al.<sup>39</sup> proved beyond doubt by using the tissue technic that a large part of the polyblasts are of lymphocytic origin.

Timofejewski and Benewolenskaja<sup>40</sup> observed phagocytosis of tubercle bacilli by lymphocytes in tissue culture. However, other investigators had not succeeded in finding bacterial phagocytosis by lymphocytes.<sup>41, 42</sup> The only positive report came from Hertzog,<sup>43</sup> who observed phagocytosis of nonvirulent streptococci and staphylococci in incubated blood. Seitzer and Sandkuehler<sup>44</sup> have seen cytoplasmic inclusions in lymphocytes after incubating non-hemolytic

staphylococci and blood, but they were unsuccessful with streptococci, meningococci, subtilis and coliform bacilli.

Animal experiments did not help in deciding whether the lymphocytes, as such, or their transition forms exhibit phagocytic activity. Ingestion of particles by lymphocytes had been observed by Downey<sup>31</sup> in rabbit's blood, and by Stillwell<sup>45</sup> in frog's blood after they used intravenous India ink. Bloom<sup>46</sup> in his experimental studies about monocytes also pictured many typical small and medium sized lymphocytes engulfing India ink at the later stages of storage. He concluded that phagocytosis of India ink is not a dependable criterion for the origin of the monocytes, or other cells, as claimed by McJunkin<sup>47</sup> and Foot.<sup>48</sup> These observations were disregarded as it was believed that in animals there is no sharp distinction between the lymphocytes and small monocytes.<sup>48-50</sup>

Koszewski<sup>7</sup> observed hemosiderin inclusions in the lymphocytes of patients with hemochromatosis and the same phenomenon could be produced in animals and human beings who received suspensions of iron oxide parenterally in excess.<sup>8, 9</sup> The hemosiderin granules appeared in as many as 25 per cent of the lymphocytes.

The present results with India ink injections on humans provide further proof that lymphocytes are capable of phagocytic activity. The polymorphonuclear leukocytes were shown to be the most active phagocytic cells, but many lymphocytes also ingested carbon particles in their cytoplasm. The inclusions of the lymphocytes occurred as early as the second day. There was a gradual increase of phagocytic lymphocytes during the course of injections and a prolonged decrease after the injections were discontinued. In some cases as many as 30 per cent of the total lymphocytes contained India ink.

The high percentage of phagocytizing lymphocytes speaks against the possibility that the carbon particles were incorporated in some passive way. The amount of India ink injected daily (1.0 Gm. per 60-70 Kg. of body weight) was insufficient to cause "contact inclusions." The total dosis of 10-14 Gm. was also below the dosis used in animals where oversaturation with colloid dyes caused inclusions even in fibroblasts. The inclusions in our cases cannot be the result of absorption as India ink is a particulate substance and can be incorporated only by active phagocytosis.

There is no reason to assume that the phagocytizing cells consisted of elements other than the lymphocytes. The injections of India ink as used in our experiments were not extensive and did not alter the blood cell count or differential of the leukocytes. Transitional forms looking like Maximow's polyblasts were not present although a few cells resembled Downey's "stress" lymphocytes.<sup>51, 52</sup> Histiocytes or endothelial cells which may appear in the blood at the progressed stages of storage<sup>9, 27, 49</sup> were absent. These cells are usually larger, contain numerous particles in the pale blue cytoplasm and have an indented nucleus which shows considerably less dense chromatin pattern than that of the lymphocytes.<sup>8, 38</sup> The ingesting cells showed no morphologic changes as compared with the ink-free lymphocytes. Therefore, it must be assumed that they belong to the same species as the nonphagocytizing elements. Phagocytosis should be added to the limited list of possible functions of the lymphocytes in the human body.

## SUMMARY

Eight subjects with chronic intractable disease were subjected to intravenous injections of India ink and the peripheral blood was studied for evidence of phagocytosis. Carbon particles were seen not only in the granulocytes and monocytes, but also in lymphocytes. Through these intravital studies, further proof is established for the theory that human lymphocytes are capable of phagocytosis.

## SUMMARIO IN INTERLINGUA

Octo subjectos con intractabile morbos chronic esseva subicite a injectiones intravenose de tinta de China e lor sanguine peripheric esseva studiate pro indicios de phagocytosis. Particulas de carbon esseva trovate non solmente in le granulocytos e le monocytos sed etiam in le lymphocytos. Per medio de iste studios intravital, provas additional es establite pro le theoria que leucocytos human es capace de phagocytosis.

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