Studies on the Fate of Lymphocytes. I. Labeling Small Thymic Lymphocytes with Tritiated Thymidine

By Raymond G. Murray and Assia Murray

In spite of extensive and intensive investigations, reviewed recently by Trowell and by Gowans, the function of lymphocytes, particularly the small lymphocyte, remains an enigma. One approach to a solution of this problem has been to label lymphocytes, inject them into an animal, and follow their distribution and subsequent behavior. Radioactive phosphorus, radioactive chromium, radioactive gold, and fluorescent dyes have been employed to label lymphocytes. None of these methods provides the precise cellular localization combined with long-term stability that is possible with H3 thymidine labeling of nuclear DNA, which also has been employed in a number of recent investigations. We agree with Everett et al. that for autographic studies, labeling of lymphocytes by the last named technic is the method of choice.

This paper deals with a series of trials of various time schedules and dosages designed to develop efficient methods to label adequate numbers of small lymphocytes. A procedure is described whereby 0.5 ml. suspensions containing 3-5 x 10⁶ small lymphocytes per ml., nearly 50 per cent of which were labeled, have been prepared, and brief mention is made of preliminary injections of these preparations into recipient rats.

MATERIALS AND METHODS

Wistar rats ranging in weight from 60 to 100 Gm. were injected intraperitoneally with H3 thymidine (Schwarz Laboratories or New England Nuclear Corp.). The specific activity ranged from 0.36 to 1.9 c/mm, and the original solution of 1 mc/ml. was diluted to 0.2 mc/ml. and made isotonic. After one or several injections and the appropriate time intervals, the rats were anesthetized with ether, the thymus excised and placed in Tyrode solution. Additional Tyrode was injected into the thymus with a fine needle and the cells expressed by gentle pressure, according to the suggestion of Richtelius. The total number of cells was estimated by the usual white cell counting technic in a hemocytometer. At the same time, a part of the thymus and other organs were fixed in Bouin's fluid, touch preparations of thymi and spleen, smears of circulating blood and of the cell suspensions from the thymi were prepared, air-dried and fixed for 10 minutes in absolute ethyl alcohol. Heavily overstained paraffin sections of the fixed organs, and the various smears and touch preparations, were coated with liquid NTB-2 emulsion (Eastman Kodak), essentially in the manner of Messier and Leblond, but omitting the thin celloidin protective coating. The emulsion was diluted by one or two parts of distilled water to reduce the background grain count. After storage from 3 to 12 weeks in dessicated boxes, the slides were developed in Eastman D-11 developer. The smears of cell suspensions, stained with Wright's...
stain after photographic processing, were counted for positive nuclear labeling in two categories, small lymphocytes and all other lymphocytes (see Discussion for criteria used to delimit the categories). Figure 1 illustrates the type of preparation used for the counts. Autoradiographs of the sections and of touch preparations were also studied, but no quantitative evaluation was attempted except in the one instance specifically mentioned below. At least 400 cells were counted for each determination.

Only cells with two or more silver grains were considered labeled. This value was based on a background grain count never higher than 0.5 grains per 50 square microns (approximately the area of the largest small lymphocyte). This low background, achieved by dilution of the emulsion, while it made possible the recognition of weakly labeled cells, also required long autoradiographic exposure periods, in some cases as long as 92 days, in order to give these weakly positive cells a chance to register on the emulsion. A similar criterion for positive labeling was used by Everett et al.17

For the preliminary injection experiment, 10 rats were injected by tail vein with 0.5 ml of a suspension of cells prepared as indicated above, containing approximately 3 x 10^8 cells per ml. The rats had been irradiated several hours previously with 700 r of 200 KVP x-ray to destroy antibodies which might affect the reaction of the host to foreign cells. Sections of various organs of these recipients, sacrificed in pairs at 15 and 60 minutes, 12, 24 and 48 hours, were examined by the same autoradiographic technic as that used for the tissues of the donor rats.

RESULTS

 Autoradiographs of Smears of Lymphocyte Suspensions

Table 1 lists the rats included in the study, arranged according to the total time between the first injection and sacrifice, with dose and time intervals for each. In the right hand columns are given the results of the counts of the

![Fig. 1.—Autoradiograph of a smear of a thymus-cell suspension from rat #358 which received a total of 5 μc/Gm. of H^3 thymidine in two intraperitoneal injections; total time from first injection to sacrifice was 37 hours. Note large lymphocyte (L), medium lymphocytes (M), and small lymphocytes (S). One cell (?) is presumably a partially ruptured and flattened small lymphocyte. Exposure 67 days. (x 1500)]
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Table 1.—Dose, Time Relations and Per Cent Labeled Thymic Lymphocytes for Individual Rats

<table>
<thead>
<tr>
<th>Animal number</th>
<th>Injection pattern</th>
<th>Intervals*</th>
<th>Total dose μg/Gm.</th>
<th>Total time to sacrifice</th>
<th>% of small lymphocytes labeled</th>
<th>% of all lymphocytes labeled</th>
</tr>
</thead>
<tbody>
<tr>
<td>347</td>
<td>3.0-Sac.</td>
<td>1/2 hr.</td>
<td>10.0</td>
<td>11/2 hr.</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>350</td>
<td>3.4-3.4-3.4-Sac.</td>
<td>1/2 hr.</td>
<td>10.0</td>
<td>1 1/2 hr.</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>341</td>
<td>3.4-3.4-3.4-3.4-Sac.</td>
<td>1 hr.</td>
<td>10.2</td>
<td>2 hr.</td>
<td>6</td>
<td>19</td>
</tr>
<tr>
<td>340</td>
<td>3.4-3.4-3.4-Sac.</td>
<td>1 hr.</td>
<td>10.2</td>
<td>2 hr.</td>
<td>6</td>
<td>23</td>
</tr>
<tr>
<td>359</td>
<td>2.0-Sac.</td>
<td>1 hr.</td>
<td>10.2</td>
<td>1 hr.</td>
<td>6</td>
<td>23</td>
</tr>
<tr>
<td>351</td>
<td>1.9-Sac.</td>
<td>2 hr.</td>
<td>10.2</td>
<td>2 hr.</td>
<td>6</td>
<td>23</td>
</tr>
<tr>
<td>358</td>
<td>3.0-2.0-2.0-Sac.</td>
<td>17, 20 hr.</td>
<td>10.0</td>
<td>17, 20 hr.</td>
<td>44</td>
<td>53</td>
</tr>
<tr>
<td>350</td>
<td>3.0-2.0-Sac.</td>
<td>17, 21 hr.</td>
<td>10.0</td>
<td>17, 21 hr.</td>
<td>44</td>
<td>49</td>
</tr>
<tr>
<td>352</td>
<td>4.0-2.0-2.0-Sac.</td>
<td>20, 18, 18 hr.</td>
<td>8.0</td>
<td>20, 18 hr.</td>
<td>48</td>
<td>51</td>
</tr>
<tr>
<td>353</td>
<td>4.0-2.0-2.0-Sac.</td>
<td>20, 18, 18 hr.</td>
<td>8.0</td>
<td>20, 18 hr.</td>
<td>48</td>
<td>51</td>
</tr>
<tr>
<td>356</td>
<td>3.0-2.0-2.0-Sac.</td>
<td>18, 24, 18 hr.</td>
<td>7.0</td>
<td>18, 24 hr.</td>
<td>48</td>
<td>51</td>
</tr>
<tr>
<td>361</td>
<td>3.0-2.0-2.0-Sac.</td>
<td>18, 24, 18 hr.</td>
<td>7.0</td>
<td>18, 24 hr.</td>
<td>48</td>
<td>52</td>
</tr>
</tbody>
</table>

*Intervals between injections, or from last injection to sacrifice.
†Small lymphocytes varied between 80 and 90 per cent of the whole.
‡Between 60 and 80 per cent of medium and large lymphocytes were labeled, with no relation to the time interval.
§Only touch preparations were available, so accurate counting was not possible. However, many positive large and medium lymphocytes were seen.

Autoradiographs of the smears of cell suspensions. No small lymphocytes were labeled at 30 minutes, which agrees with our observations in the sections and with reports of most other investigators. In spite of intensive treatment, few small lymphocytes were positive at the short intervals, but at 19 and 22 hours a substantial number was seen, and at 1 1/2 days 44 per cent were labeled. In 3 of the 4 rats at 2 1/2 days, essentially half the small lymphocytes were labeled, but this was only a moderate increase for an additional day. Labeling of medium and large lymphocytes, not shown separately on the table, varied between 60 and 80 per cent. Since these constituted only 10–20 per cent of all lymphocytes, the percentage of all cells labeled did not differ appreciably from that of small lymphocytes labeled, except when absolute numbers of labeled cells were low, as in the earliest intervals.

In table 2 the same rats, with a few additional, are grouped by major time intervals, with additional figures indicating the rise in labeled lymphocytes in the blood. The same data are shown graphically in figure 2. When thus grouped, the pattern is more clearly indicated. The number of labeled small lymphocytes in the thymus increased more or less linearly with time until about 1 1/2 days and only slightly thereafter. The value for labeled cells in the blood rose more slowly, but showed no sign of leveling off within the period of the experiment. Since in the rat the thymus is said to be a major source of supply of blood lymphocytes, this suggests that many newly formed small lymphocytes remain for a time in the thymus.

Autoradiographs of Touch Preparations

Rough counts of the number of labeled cells in these preparations confirmed in general the values arrived at from the smears, but because of the many
Table 2.—Per Cent Labeled Lymphocytes in Thymus Smears and Blood, with Respect to Time

<table>
<thead>
<tr>
<th>Total time</th>
<th>Number of rats</th>
<th>% of small lymphocytes labeled</th>
<th>% of all lymphocytes labeled</th>
<th>% of circulating lymphocytes labeled</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min.</td>
<td>1</td>
<td>0</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>2–5 hr.</td>
<td>4</td>
<td>6</td>
<td>27</td>
<td>9</td>
</tr>
<tr>
<td>19–22 hr.</td>
<td>2</td>
<td>23</td>
<td>27</td>
<td>9</td>
</tr>
<tr>
<td>32 hr.*</td>
<td>3</td>
<td>39</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>37–38 hr.</td>
<td>2</td>
<td>44</td>
<td>51</td>
<td>16</td>
</tr>
<tr>
<td>57–60 hr.</td>
<td>4</td>
<td>47</td>
<td>51</td>
<td>27</td>
</tr>
</tbody>
</table>

*Not included in the listing of individual rats (table 1) because the cells from all three rats were pooled and counted together.
†Also based on evidence from the tissue sections.

additional variables to be considered, little value can be attached to these estimations.

Autoradiographs of Tissue Sections of Donor Rats

The distribution of labeled cells was studied in sections of thymus, spleen, bone marrow, lymph node and duodenum. The pattern illustrated in figures 3, 4 and 5 represents the appearance after single or repeated injections of H3 thymidine. In figure 3 the margin of the cortex of the thymus illustrates the high percentage of labeled cells, but with none of them showing a very heavy label. It is clear that quantitative evaluation of the number of labeled cells would not be very reliable in such sections. This is in contrast to the labeling of cells in the adjacent connective tissue, in the diffuse tissue of the lymph node (fig. 4) and splenic white pulp (fig. 5) where a few cells are very heavily labeled and most are without label. The nodules of the lymph node differ from the diffuse lymphatic tissue in that the germinal centers are full of lightly labeled cells, much like the thymic cortex, while the zone immediately around the active center is packed with small lymphocytes, a modest number of which are lightly labeled. Heavily positive cells are rare in the center and in the rim. Germinal centers in the white pulp of the spleen, although not illustrated, had much the same appearance as those of the lymph node. The margin of the splenic white pulp (fig. 5) resembles the diffuse lymphatic tissue of the lymph node, but the adjacent red pulp is heavily populated with cells with a moderately heavy label. These cells represent a concentration of extramedullary erythropoiesis which is characteristic of the splenic red pulp of these rats. The bone marrow showed a pattern of labeled cells similar to that of the splenic red pulp, and the duodenum showed heavily labeled cells in the crypts and less heavily labeled ones migrating toward the tips of the villi, as reported by Leblond and Messier.23

Autoradiographs of Tissues of Rats Receiving Labeled Cells

These results will be reported in detail in a later paper. Only those features pertinent to this report will be mentioned here. With respect to the viability of the injected cells, it was important to establish whether they were im-
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Fig. 2.—Graph of the variation in percentage of H$^3$ thymidine-labeled lymphocytes in thymus-cell suspensions and in peripheral blood, with respect to time between first injection and sacrifice.

...immediately engulfed by the phagocytic cells of the body, as dead or damaged cells presumably would be, or whether they survived in apparently normal condition.

At 15 minutes, large numbers of labeled small lymphocytes were found in the lung. They were in good condition, within the alveolar wall, but not engulfed by macrophages, and at 60 minutes had largely disappeared. A few labeled lymphocytes were found in bone marrow (fig. 6), liver, mesenteric lymph node and spleen at all the intervals from 15 minutes to 48 hours, and although occasional pyknotic labeled cells were seen, the vast majority appeared to be in good condition and only rarely was labeled debris seen in the macrophages. There were no concentrations of labeled cells anywhere, except in the lung as mentioned, although the spleen always had several times as many as the other organs with the exception of the lung. They were as frequent in the white pulp as in the red pulp, and were no more frequently dead or phagocytized here than elsewhere.
Fig. 3.—Autoradiograph of the margin of thymus cortex from rat #363 which received a total of 8 μC/Gm. of H³ thymidine in three intraperitoneal injections, total time from first injection to sacrifice was 57 hours. Exposure 92 days. (x 435)

Fig. 4.—Autoradiograph of the cortex of a mesenteric lymph node of rat #351 which received one intraperitoneal injection of 1.9 μC/Gm. of H³ thymidine 22 hours before sacrifice. Arrows designate a germinal center. Exposure 92 days. (x 435)
Fig. 5.—Autoradiograph of the margin between white pulp (below) and red pulp of the spleen of the same rat as that of figure 3. Exposure 92 days. (x 435)

Fig. 6.—Autoradiograph of the bone marrow of a rat irradiated one day previously with 700 r and shortly thereafter injected intravenously with a suspension of H3 thymidine-labeled lymphocytes similar to that of figure 1. An apparently unaltered small lymphocyte is present among the marrow cells. Exposure 87 days. (x 2275)
DISCUSSION

The choice of thymus as a source of lymphocytes raises the question of the identity of thymocytes and lymphocytes. Although Diderholm and Fichtelius\textsuperscript{24} have recently presented evidence to the contrary, the bulk of evidence seems to support their identity,\textsuperscript{21,25-28} and no indisputable evidence of difference has ever been offered. Certainly they cannot be separated on morphologic criteria, as illustrated by the fact that the thymus contributes large numbers of cells to the blood which cannot be distinguished there from those contributed by lymph nodes and other lymphatic tissue. The suggestion of Diderholm and Fichtelius\textsuperscript{24} is based on the different treatment by the host of injected suspensions of thymus cells as opposed to lymph node cells. Suspensions of cells from lymph nodes contain many cells which are not lymphocytes, and the quantitative distribution of lymphocyte sizes is different from that of thymus cell suspensions, which might account for the differences without postulating a basic difference between thymus lymphocytes, and those in the lymph nodes. Moreover, if it were shown that the thymocyte was indeed a separate cell type, we would be left with the no less baffling question of the disposition and function of the vast number of thymocytes that are known to be produced each day.

The classification of cells into small and medium lymphocytes as used in this report is necessarily somewhat arbitrary, there being no sharp boundaries between these categories. However, only those cells seven microns or less in diameter and displaying only a very narrow rim of cytoplasm were included in the category "small lymphocyte." The size of smeared cells varies with the degree of spreading of each cell on the glass. Since a small difference in the diameter between two spherical cells may be reflected in a larger difference when the cells are spread, it is possible that excessive spreading may have shifted some small lymphocytes into the medium category, but unlikely that a significant number of cells counted as small lymphocytes belonged in the medium sized category. We believe the classification procedures used here have led to a minimal rather than maximal value for per cent of small lymphocytes. If this is true, it follows that the per cent of labeled small lymphocytes would also be a minimum value because there was always relatively greater labeling of medium lymphocytes than of small lymphocytes.

Two other considerations support these minimal values: (1) Whereas we found the small lymphocytes to constitute from 80 to 90 per cent of the cells in our suspensions, Schooley and Bryant\textsuperscript{13} have reported 90 to 95 per cent small lymphocytes in smears of the thymus. (2) These same authors report a significant number of labeled cells in their category "small lymphocyte" in the first 15 minutes of in vitro exposure to H\textsuperscript{3} thymidine, most of these being cells at the top of the size distribution. Since we found no labeled small lymphocytes at the earliest interval, we may have counted some labeled cells of marginal size as medium sized lymphocytes. In view of the arbitrary nature of the classification based on size, it may eventually prove more valuable to use the presence or absence of DNA synthesis as measured by H\textsuperscript{3} thymidine uptake as a criterion to separate medium from small lymphocytes.
The value of approximately 50 per cent labeled small lymphocytes we report here appears to be at variance with the work of others who have attempted to label lymphocytes with H<sup>3</sup> thymidine. Everett et al.<sup>13</sup> counted labeled cells in the thoracic duct after six weekly doses of 1 μc/Gm. and found a maximum of 30 per cent labeled, not all of which were small lymphocytes. The cellular composition of thoracic duct lymph, however, is influenced by many factors which might affect the per cent of labeled cells. The immediate source of these cells is apparently the lymph nodes, rather than thymus, and the possibility of recirculation must be considered. Schooley et al.<sup>18</sup> counted smears of thymus cells and reported only 15–20 per cent of small lymphocytes labeled one day after 1 μc/Gm. They did not use multiple injections, however, and their figure compares almost exactly with our value of 21–25 per cent labeled at 19–22 hours after 2.0 μc/Gm. (<i>rats #350, 351, table 1</i>). Cronkite<sup>29</sup> reports the labeling of 40 per cent of small cells in the thymus after injections every 12 hours for 22 days. He does not state what dose was used, but it is possible that the label was diluted out by the successive mitotic divisions which produce these cells,<sup>22</sup> as suggested by Everett et al.<sup>17</sup> In his paper and in the discussion that followed, a number of reasons were suggested to account for the low per cent of labeled lymphocytes usually observed. Very rapid DNA synthesis, and mitosis without DNA synthesis were suggested by Leblond, but both were largely ruled out in the discussion. Relatively small surface area for absorption of H<sup>3</sup> thymidine and geometric considerations in the smears were suggested by Cronkite as factors likely to reduce the apparent number of positive small lymphocytes. Everett had previously suggested that dilution by intense mitotic activity was a likely cause.

There is another possible explanation why small lymphocytes of the thymus are difficult to label. In our autoradiographs of sections of thymus, the large number of lightly labeled cells in the cortex (<i>fig. 3</i>) suggests that in such areas, where a large portion of the cells are engaged in DNA synthesis, there is competition for the H<sup>3</sup> thymidine resulting in a reduction in the amount available to any one cell. This hypothesis is supported by the fact that in the germinal centers of the lymph node where mitosis is intense, the same pattern is found. The splenic red pulp is mitotically active, but not to the extent of the thymic cortex and germinal centers, and the degree of concentration of label in cells is correspondingly intermediate between that of the germinal centers and the less mitotically active diffuse lymphatic tissue. Since the rat of figure 3 received its last injection 19 hours before sacrifice, it is possible that the two or three mitotic generations intervening before sacrifice might account for the dilution of label. However, the difference in density of label between thymic cortex and diffuse lymphatic tissue cells is so great that a much larger number of mitotic generations would appear to be necessary. In addition, at 30 minutes after injection of H<sup>3</sup> thymidine in mice<sup>30</sup> the density of label in thymus cells is much less than that of lymphocytes in adjacent lymph nodes. This concept of competition for available thymidine would account for the relatively high doses required to label thymus lymphocytes.
We have not used numbers of animals at each interval sufficient to allow a rigorous statistical treatment. The general conclusion that several days are required to allow maturation of large numbers of labeled small lymphocytes seems justified on the basis of the consistency of direction of the curve (fig. 2), and is consistent with the concept that many mitotic generations are required between stem cell and mature small lymphocyte, as reported by Sainte-Marie and Leblond. In view of the small number of animals it is not impossible that longer time might produce a higher percentage, but 50 is an adequate percentage for most purposes, and the leveling of the curve after 1½ days is at least suggestive that not much more is to be gained by longer intervals. Since the counts of grains over labeled cells were often so low as to approach the background density, it is possible that some cells were too weakly labeled to be counted and larger doses of H thymidine might result in higher counts of positive cells. For example, one of the small lymphocytes adjacent to the large lymphocyte at the right margin of figure 1 has only one grain over it, but in view of the low background, it might be a positive cell. Such cells were not counted as positive. On the other hand, there is some danger of damage to cells by radiation from the tritium so that indefinite increase in the dose is not possible. We occasionally saw labeled, dead lymphocytes in our sections of donor rats, suggesting that the upper limit of safe dosage had perhaps nearly been reached. This raises the question of how much damage, short of killing a noticeable percentage of the lymphocytes, had been done to the cells in the suspensions. Although dead cells, either labeled or unlabeled, were not recognizable in the smears, it is possible that many of the cells were affected in more subtle ways rendering them susceptible to early degeneration. It is fortunate, in this connection, that the small lymphocytes are among the least heavily labeled cells. In order to produce the degree of label shown by the small lymphocytes in figure 1, an exposure of two months was required, and most of the cells were not as heavily labeled as those shown. These cells probably contain less than one-tenth as much radioactivity as the spermatogonia illustrated by Johnson and Cronkite.

The preliminary injection experiment provides a check on how much damage the lymphocytes have sustained by reason of the radiotoxicity, as well as by the handling processes. The injected cells did not appear in the macrophages in large numbers, as one would expect if many dead or damaged cells were in the suspensions, and they were found in apparently good condition in the spleen, bone marrow and lymph nodes as late as two days after injection.

The absence of labeled small lymphocytes at 30 minutes confirms the widely held belief that these cells rarely, and perhaps never, undergo mitosis, but are a maturation product of somewhat larger cells. The period of maturation is apparently quite short, at least in some cells, as indicated by the appearance of a moderate number of labeled small lymphocytes as early as two hours after injection. A more precise answer to this question might be found by employing larger numbers of animals and making a rigorous
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statistical analysis of the number of labeled cells in relation to their size and grain count, which is beyond the scope of this report.

The usefulness of these labeled lymphocytes will be limited by several factors. Autologous transplants are not feasible because all the DNA-producing cells throughout the animal will be labeled, and confusion between these cells and the re-injected labeled cells will prevent recognition of the latter. In homologous or heterologous transplants there will be some interaction between the injected lymphocytes and the antibodies of the host which will complicate interpretation of results. Even if the injected cells are not immediately destroyed by the host, their distribution and subsequent development into other cell types may be affected. Use of inbred littermate hosts might reduce this effect, and irradiation of the host to destroy the antibodies might largely eliminate it. Both approaches are under investigation in this laboratory.

Summary

A procedure is described whereby 0.5 ml. suspensions containing 3-5 x 10^8 small lymphocytes per ml., nearly 50 per cent of which were labeled with H^3 thymidine, have been prepared from rat thymus. Repeated doses of thymidine totaling 7-8 μG/ml. and from two to three days were required to produce this percentage, and it is suggested that longer times would not be much more effective. Preliminary results indicate that cells of such suspensions injected in irradiated hosts are widely distributed, do not appear to disintegrate rapidly or to be massively ingested by the macrophages of the reticulo-endothelial system, and are still present in good condition one and two days after injection.

Summary in Interlingua

Es describite un technica per medio del qual il esseva possibile preparar, ab thymo de ratto, suspensiones de 0,5 ml continente inter 3 e 5 x 10^8 micre lymphocytos per ml, inclue quasi 50 pro cento marcate con thymidina a H^3. Repetite doses de thymidina amontante a un total de 7 a 8 μG/g e un lapso de tempore de duo a tres dies esseva requisite pro producer iste procentage. Il pare que plus prolongate intervallos non esseva plus efficace. Observationes preliminari indica que cellulas ab tal suspensiones, quando illos es injicite in irradiate hospites, es distribuite extensemente, que illos – apparentemente – non se disintegra rapidemente e non es ingerite in massa per le macrophagos del systema reticulo-endothelial, e que illos remane presente in bon condition un o duo dies post le injection.

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