An Autoradiographic Study of the Effect of Neuraminidase or Trypsin on Transfused Lymphocytes

Bertram M. Gesner, MD*, Judith J. Woodruff, MD, and Robert T. McCluskey, MD

In previous studies evidence was obtained that treatment of lymphocytes with either trypsin or neuraminidase alters their capacity to migrate normally in vivo.[1,2] In these studies Cr^{51}-labeled rat thoracic duct lymphocytes were incubated with either enzyme and then injected intravenously into syngeneic or allogeneic rats. The distribution of radioactivity was measured at intervals up to two days in the tissues of recipients.

Each enzyme affected the distribution of radioactivity in a distinctive way. In recipients of neuraminidase-treated lymphocytes, large amounts of radioactivity were found in the liver and reduced amounts in lymph nodes and spleen at early intervals; these findings suggested that the altered cells became trapped in the liver and failed to "home" to lymph nodes and spleen. At later intervals, the pattern of radioactivity indicated that many of the lymphocytes subsequently left the liver, homed to lymph nodes, and then recirculated in the normal fashion.

The distribution of radioactivity in recipients of trypsin-treated lymphocytes showed a different pattern. At early intervals, almost no radioactivity was found in lymph nodes, although the amount found in the spleen was similar to that found after injection of untreated lymphocytes. Increased radioactivity was not found in the liver. At later intervals normal amounts of radioactivity were found in lymph nodes. These results suggested that treatment with trypsin selectively interfered with the capacity of lymphocytes to home to lymph nodes without affecting their homing to the white pulp of the spleen. However, it was also possible that most of the cells in the spleen represented damaged cells which were trapped in the red pulp, rather than cells which had migrated normally to the white pulp. In order to investigate this possibility

From the Department of Medicine, New York University School of Medicine and the Department of Pathology, School of Medicine, State University of New York at Buffalo, NY.

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Address for reprint requests: Dr. Robert T. McCluskey, Department of Pathology, School of Medicine, State University of New York at Buffalo, NY 14214.

* Deceased.

and to define more precisely the localization of lymphocytes altered by trypsin or neuraminidase, an autoradiographic study was performed, employing H^3-adenosine-labeled lymphocytes.

Materials and Methods

Highly inbred Fisher strain rats and stock Sprague-Dawley rats were used. All were males weighing between 250 and 350 g.

Reagents

Neuraminidase (500 units/ml) derived from Vibrio cholera, and trypsin (twice crystalized) were obtained from General Biochemicals, tissue culture Medium 199 (M199) from Grand Island Biological Company, and H^3-adenosine (specific activity 8.76c/mm, in sterile water) from Tracerlab.

Collection and Radioactive Labeling of Lymphocytes

Rat thoracic duct lymphocytes were collected as previously described.[3] Lymph was collected at room temperature in 125 ml flasks containing 10–15 ml of saline and 20 units of heparin/ml. The cells to be used for injection were obtained during an 8–12 hr period within the first 24 hr after cannulation. Following collection, the cells were washed once in M199 or saline and resuspended in a concentration of 75–100 $\times$ 10^6 lymphocytes/ml. In order to label the cells, 10μc H^3-adenosine/ml was added to the cell suspension, which was then incubated at 37° C for 1 hr.

Preparation of Lymphocytes for Transfusion

For experiments in which neuraminidase was used, the labeled cells were washed three times with M199, resuspended so as to contain 10^8 lymphocytes/ml, and then incubated with neuraminidase for 15 min at 37° C. Neuraminidase was used in a concentration of 25 units/10^8 lymphocytes. For experiments in which trypsin was used, the labeled lymphocytes were washed three times in saline and resuspended so as to contain 10^8 lymphocytes/ml saline. The cells were then incubated with trypsin (0.02 mg/10^8 lymphocytes) for 5 min at 37° C and injected immediately. Control cells were treated in the same fashion, except that neither enzyme was added to the mixture. In some experiments labeled lymphocytes were washed in saline and killed by incubation at 45° C for 15 min before transfusion into recipients. Smears for autoradiographs were prepared by suspending a small drop of the pellet in serum after the final washing. Injections were given into the tail vein under light ether anesthesia. In all experiments, recipients were transfused with 200–600 $\times$ 10^6 lymphocytes.

Autoradiographic Studies

Recipients were killed at 30 min, 2, 4, or 24 hours; at least 3 recipients in each group were studied at these times. Tissues were fixed in buffered formalin, embedded in paraffin and sectioned at 4μ. Sections of lymph nodes—(superficial and deep cervical nodes and mesenteric nodes), spleen, thymus, liver and lung were examined. Autoradiographs were prepared using NTB-3 liquid photographic emulsion (Eastman Kodak), with an exposure time of 2–4 weeks at 4° C. After development, the sections were stained with either methyl green pyronin or hematoxylin and eosin. In addition, autoradiographs were prepared of smears of donor lymphocytes following labeling and incubation with or without enzyme.

Results

Labeling of Donor Cells

Autoradiographs of smears of thoracic duct cells which had been incubated with H^3-adenosine showed that a majority of the small lymphocytes (about 75%) and almost all of the large lymphocytes (about 95%) were labeled. There was no change in the percentage of labeled cells or in the intensity of labeling in smears of cells which had been incubated with trypsin or neuraminidase following labeling.

Effects of Neuraminidase on the Distribution of H^3-adenosine-labeled Lymphocytes

As expected, recipients of H^3-adenosine-labeled lymphocytes that had not been treated with enzyme showed selective accumulation of labeled lymphocytes in lymph nodes and white pulp of the spleen at 30 min, 2 hr, and 24 hr after transfusion (Fig 1 and 2). Relatively few labeled cells were found in the red pulp of the spleen. A few labeled lymphocytes were seen in the liver sinusoids and in pulmonary capillaries, an extremely rare cell in the kidney, and none in the thymus. In contrast, in recipients of neuraminidase-treated cells, substantially fewer labeled lymphocytes were found in the lymph nodes and white pulp of the spleen at 30 min and at 2 hr after transfusion (Fig 3) (Table 1). The few labeled cells in these sites showed no unusual localization. Occasional labeled lymphocytes were found in the liver sinusoids, in the same location as in recipients of untreated lymphocytes (Fig 4). Despite the large increase in radioactivity found in the liver at 30 min and 2 hr after transfusion of Cr^{51}-labeled neuraminidase-treated lymphocytes,[2] an increase in the numbers of labeled lymphocytes was not obvious in autoradiographs of the liver. As in recipients of untreated cells, only a few labeled lymphocytes were found in the lung and virtually none in the kidney or thymus.

In contrast to the findings at 30 min and 2 hr, recipients of neuraminidase-treated lymphocytes which were killed at 24 hr showed large numbers of lymphocytes in lymph nodes (Fig 5) and white pulp of the spleen, similar to those found in recipients of untreated lymphocytes (Table 1).

Essentially the same distribution of labeled lymphocytes was observed when Sprague-Dawley rats were used as donors and recipients as when both were inbred Fisher rats.

Effect of Trypsin on the Distribution of H^3-adenosine Labeled Lymphocytes

At 30 min and 2 hr after injection of trypsin-treated lymphocytes, the distribution of labeled lymphocytes differed from that found with either

Table 1. Concentration of Labeled Cells in Recipients of Neuraminidase-Treated, Trypsin-Treated, or Untreated Lymphocytes

Donor lymphocytes	Spleen		Lymph nodes	
	2 hr	24 hr	2 hr	24 hr
Untreated	48.1	19.6	29.6	64.3
Neuraminidase-treated	17.9	21.7	9.6	57.8
Trypsin-treated	54.7	25.0	0.025	59.6

Counts represent average number of labeled cells per field (× 900 for the spleen, × 450 for lymph nodes), 20 fields in each of 2 recipients counted. In the spleen, counts were made of the white pulp. In lymph nodes, counts were made of the cortical and paracortical regions, where most of the labeled cells were found. Germinal centers were excluded, since they never contained labeled cells.

neuraminidase-treated or untreated lymphocytes, in that virtually no labeled cells were found in the lymph nodes (Table 1). In marked contrast to the findings in the nodes, large numbers of labeled lymphocytes were found in the white pulp of the spleen, both at 30 min and 2 hr (Fig 6). In fact, the number appeared to be slightly greater than in recipients of untreated lymphocytes (Table 1). As in recipients of untreated cells, relatively few labeled cells were found in the red pulp. The distribution and extent of accumulation of labeled cells in the liver, lung, kidney, and thymus in the recipients of trypsin-treated lymphocytes did not differ from that found in recipients of untreated cells.

In contrast to the findings at early intervals, recipients of trypsin-treated lymphocytes sacrificed 24 hr after transfusion showed large numbers of labeled lymphocytes found in lymph nodes (Fig 7). The distribution and extent of accumulation of labeled lymphocytes in the nodes at this time appeared to be similar to that found in comparable recipients of untreated lymphocytes (Table 1). The distribution of labeled cells in the spleen, liver, kidney, and thymus also appeared to be no different than that found in recipients of untreated lymphocytes. The same effects on the distribution of cells were seen whether inbred Fisher rats or Sprague-Dawley rats were used as donors and recipients.

Since virtually no labeled cells accumulated in the lymph nodes in the first 2 hr after injection of trypsin-treated lymphocytes, it was of interest to determine if the circulation to the nodes was intact during this interval. For this purpose, 2 recipients were transfused with either unlabeled trypsin-treated lymphocytes or with an equal number of unlabeled, untreated lymphocytes. Five min later, each recipient was given a transfusion of equal numbers of H^3-adenosine-labeled untreated lymphocytes and then sacrificed 2 hr later. Autoradiographs of the nodes of these animals showed a normal accumulation of labeled lymphocytes, indicating that the circulation to the nodes was intact after the injection of trypsin-treated lymphocytes.

Distribution of H^3-adenosine-labeled Heat-killed Lymphocytes

Recipients of heat-killed lymphocytes were killed at 2 or 24 hr. Virtually no labeled cells were found in the lymph nodes or in the white pulp of the spleen, and only extremely rare labeled cells were found in the liver, lung, and red pulp of the spleen.

Discussion

The present report deals with an autoradiographic study of the effects of neuraminidase or trypsin treatment of lymphocytes (labeled with H^3-adenosine) on their distribution in allogenic or syngeneic recipients following intravenous injection. There is no reason to doubt that the labeled lymphocytes found in the recipients represent transfused cells, since there is clear evidence that labeled RNA precursors incorporated into lymphocytes in vitro remain associated with the cells for at least 24 hr.[4] Furthermore, the changes in accumulation of labeled lymphocytes in the lymph nodes and spleen caused by neuraminidase and trypsin are in agreement with the changes in distribution of radioactivity in these organs in studies using Cr^{51}-labeled lymphocytes,[1,2] and it is highly probable that Cr^{51} incorporated into lymphocytes remains largely associated with the cells, at least during the first 24 hr.

In substantiation of previous studies,[1,2] the results of the present investigation demonstrate that when lymphocytes are incubated with neuraminidase or with trypsin and then injected intravenously, their distribution is different from that of untreated lymphocytes. Each enzyme led to a different pattern of distribution. In the case of neuraminidase treatment, there was moderate reduction in the number of cells which homed to lymph nodes and the white pulp of the spleen at 30 min and 2 hr after transfusion, whereas at 24 hr the accumulation of cells at these sites approached or equaled that seen following injection of untreated lympocytes. When trypsin was used, virtually none of the transfused lymphocytes homed to lymph nodes within 2 hr after transfusion, although during this period, the accumulation of trypsin-treated cells in the white pulp of the spleen was not impaired. However, by 24 hr, the number of labeled cells found in the lymph nodes appeared equal to that seen in recipients of untreated lymphocytes. When lymphocytes were killed by heat before transfusion, virtually no labeled cells were found in the lymph nodes or the white pulp of the spleen at 2 and 24 hr.

In previous studies concerning the effects of neuraminidase on the migration of lymphocytes employing Cr^{51}-labeled cells, the distribution of radioactivity indicated that, in the first few hours, large numbers of the cells became trapped in the liver and that fewer than normal cells

accumulated in lymph nodes and spleen. At later intervals (24 and 48 hr) the distribution of radioactivity indicated that many of the lymphocytes had emigrated from the liver and selectively accumulated in lymph nodes. The present autoradiographic findings support this interpretation. However, the increase in accumulation of labeled neuraminidase-treated lymphocytes in the liver at early intervals after transfusion indicated by the previous studies [2] with Cr^{51} was not apparent in routine examination of autoradiographs of the liver. This is not too surprising, since the amount of increase of Cr^{51} per unit weight of liver suggests that the increase in number of labeled cells in a section of liver would not be great, and probably could not be detected unless labeled cells in many sections were counted. The labeled cells which were seen in the livers of recipients of neuraminidase-treated cells appeared to be within the sinusoids, most of them apparently in contact with endothelial or Kupffer cells. The present findings again do not provide an answer to the question whether the early decrease in accumulation of lymphocytes in the lymph nodes and white pulp of the spleen is due solely to entrapment of the cells in the liver. However, it is certain that some lymphocytes incubated with neuraminidase do reach these areas and retain the ability of localizing in them at early intervals after transfusion.

The most remarkable effect of trypsin treatment was that during the early period almost none of the cells homed to lymph nodes, whereas their homing to the white pulp of the spleen was unimpeded. Studies with Cr^{51}-labeled cells showed the almost complete absence of homing to the nodes to last for at least 4 hr after transfusion of the cells.[1] During this early interval, it is clear that blood flow through the node was intact, since transfusion of unlabeled, untreated lymphocytes did not impair the accumulation of labeled cells in the node. It therefore seems highly probable that the failure of trypsin-treated lymphocytes to accumulate selectively in lymph nodes was not due to the failure of the cells to reach the nodes.

It can be concluded that the alterations in the distribution of neuraminidase and trypsin-treated lymphocytes were due to the action of the enzymes on the transfused lymphocytes, rather than on the recipient rats, since separate sequential transfusions of Cr^{51}-labeled lymphocytes and either enzyme caused no effect on the distribution of radioactivity in recipients.[1,2,5] The exact changes, induced by either enzyme, which were responsible for the altered circulation of the cells are unknown. However, it is clear that effects were not due to killing the cells, since the present evidence, taken together with the results obtained using Cr^{51}-labeled cells, indicate that lymphocytes treated with either neuramini-

dase or trypsin regained the property of homing to lymph nodes and recirculating to the lymph by 24 hr after injection. Furthermore, it has been shown that treatment of lymphocytes with either enzyme does not abolish the ability of the cells to produce graft-against-host reactions.[5] Presumably, each enzyme produces changes in the surface and/or metabolism of the cells which lead to their altered circulation. In the case of neuraminidase it is likely that the effect depends upon removal of sialic acid containing components of the lymphocyte surface. Thus, the effects can be prevented by addition of neutralized sialic acid to the in-vitro reaction mixture.[2] Furthermore, is has been shown that sialic acid is detectable in the supernatant of treated lymphocytes and that the electrophoretic mobility of the cells is reduced.[2] Additional evidence of a cell surface alteration comes from the observation that neuraminidase-treated lymphocytes agglutinate in calf serum, a reaction not observed with untreated cells. It seems unlikely, however, that agglutination could account for the alterations in the distribution of cells in vivo, since agglutination does not occur in autologous serum and since clumping would be expected to lead to increased trapping in the lungs, which was not observed. The effect of trypsin is probably also due to removal of cell surface constituents; in this connection it is of interest that trypsin has been shown to split glycopeptide fragments from erythrocyte membranes.[6] Whatever the mechanisms, in both instances the effects appeared to be reversible, since by 24 hr the treated cells displayed normal migration patterns.

The finding that trypsin-altered lymphocytes accumulate in the white pulp of the spleen at a time when they fail completely to accumulate in lymph nodes indicates that the properties of lymphocytes which are required for homing to these different lymphoid tissues are not identical. Recently, Goldschneider and McGregor [7] observed that lymphocytes enter the white pulp of the spleen by passing between the endothelial cells of the marginal sinus. In contrast, Marchesi and Gowans [8] previously found that lymphocytes enter lymph nodes by passing through the endothelial cells of the postcapillary venules; the lymphocytes become engulfed in a vacuole of the endothelial cell and emerge on the parenchymal side of the endothelium. The present findings indicate that the properties of lymphocytes required for their migration through the endothelial cells of the postcapillary venules in lymph nodes are sensitive to the action of trypsin while those required for migration in between the endothelial cells of the marginal sinus in route to the white pulp of the spleen are not. Migration to both sites appears to be depressed by neuraminidase treatment.

Summary

Using H^3-adenosine-labeled thoracic duct lymphocytes, an autoradiographic study was performed on the effects of the neuraminidase or trypsin treatment of lymphocytes on their distribution in allogeneic or syngeneic recipient rats following intravenous injection. Recipients of neuraminidase-treated cells showed a moderate reduction in the number of labeled cells in lymph nodes and in the white pulp of spleen at 30 min and at 2 hr, but essentially normal numbers of cells were found in these sites at 24 hr. The effect of trypsin treatment of lymphocytes was to eliminate almost completely "homing" to lymph nodes in the early period after injection, although homing to the white pulp of the spleen was not interfered with. At 24 hr, recipients of trypsin-treated cells showed normal numbers of lymphocytes in lymph nodes. These findings are interpreted to mean that the homing of lymphocytes to lymphoid tissue depends upon cell surface constituents. The fact that trypsin treatment prevented homing to lymph nodes at a time when homing to the white pulp of the spleen was normal indicates that these processes depend upon different mechanisms.

References

1. WOODRUFF, J., and GESNER, B. M. Lymphocytes: circulation altered by trypsin. *Science 161*:176–178, 1968.
2. WOODRUFF, J. J., and GESNER, B. M. The effect of neuraminidase on the fate of transfused lymphocytes. *J Exp Med 129*:551–567, 1969.
3. GOWANS, J. L. The effect of continuous re-infusion of lymph and lymphocytes on the output of lymphocytes from the thoracic duct of unanesthetized rats. *Brit J Exp Path 38*:67–78, 1957.
4. GOWANS, J. L., and KNIGHT, E. J. The route of re-circulation of lymphocytes in the rat. *Proc Roy Soc (Biol) 159*:257–282, 1964.
5. GESNER, B. M., and WOODRUFF, J. J. "Factors Affecting the Distribution of Lymphocytes." In *Cellular Recognition Mechanisms*, SMITH, R. T., and GOOD, R. A., Eds. Appleton-Century-Crofts, New York. In press.
6. WINZLER, R. J. The structure of a glycoprotein from erythrocyte membranes (abstr). Seventh International Congress of Biochemistry, 1967.
7. GOLDSCHNEIDER, I., and MCGREGOR, D. D. Migration of lymphocytes and thymocytes in the rat. I. The route of migration from blood to spleen and lymph nodes. *J Exp Med 127*:155–168, 1968.
8. MARCHESI, V. T., and GOWANS, J. L. The migration of lymphocytes through the endothelium of venules in lymph nodes: an electron microscope study. *Proc Roy Soc (Biol) 159*:283–290, 1964.

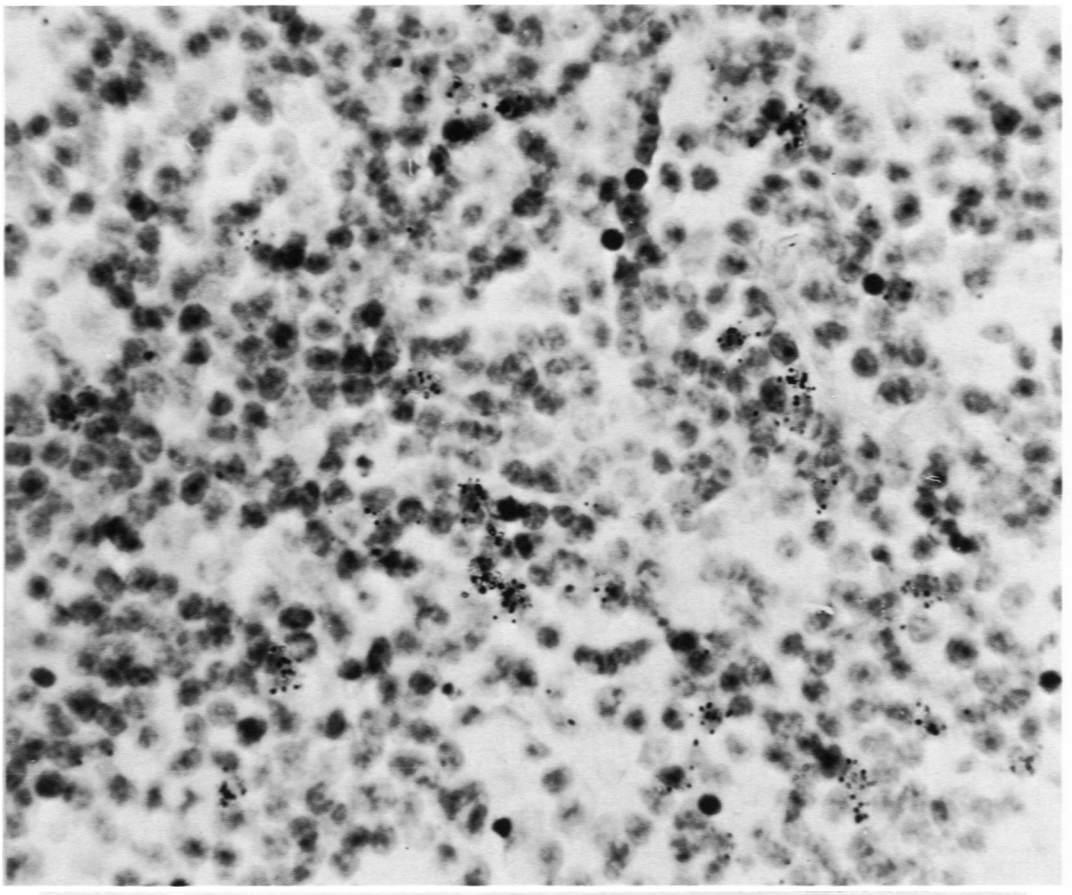
[*Illustrations follow*]

Legends for Figures

All photomicrographs are of autoradiographs, stained with hematoxylin and eosin. All magnifications are approximately × 700 except for Fig 4 which is about × 1200.

Fig 1. Lymph node from recipient of untreated lymphocytes killed 2 hr after injection. Many labeled cells are seen.

Fig 2. Spleen from recipient of untreated lymphocytes killed at 2 hr. Many labeled cells are present in white pulp.



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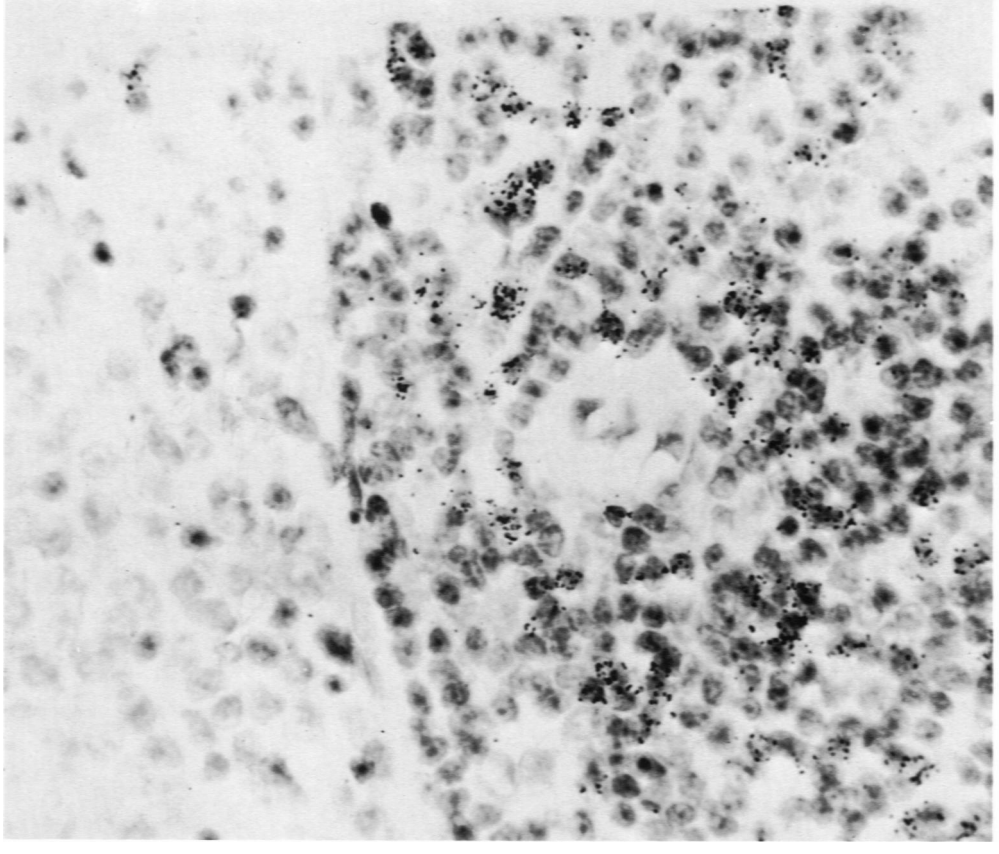

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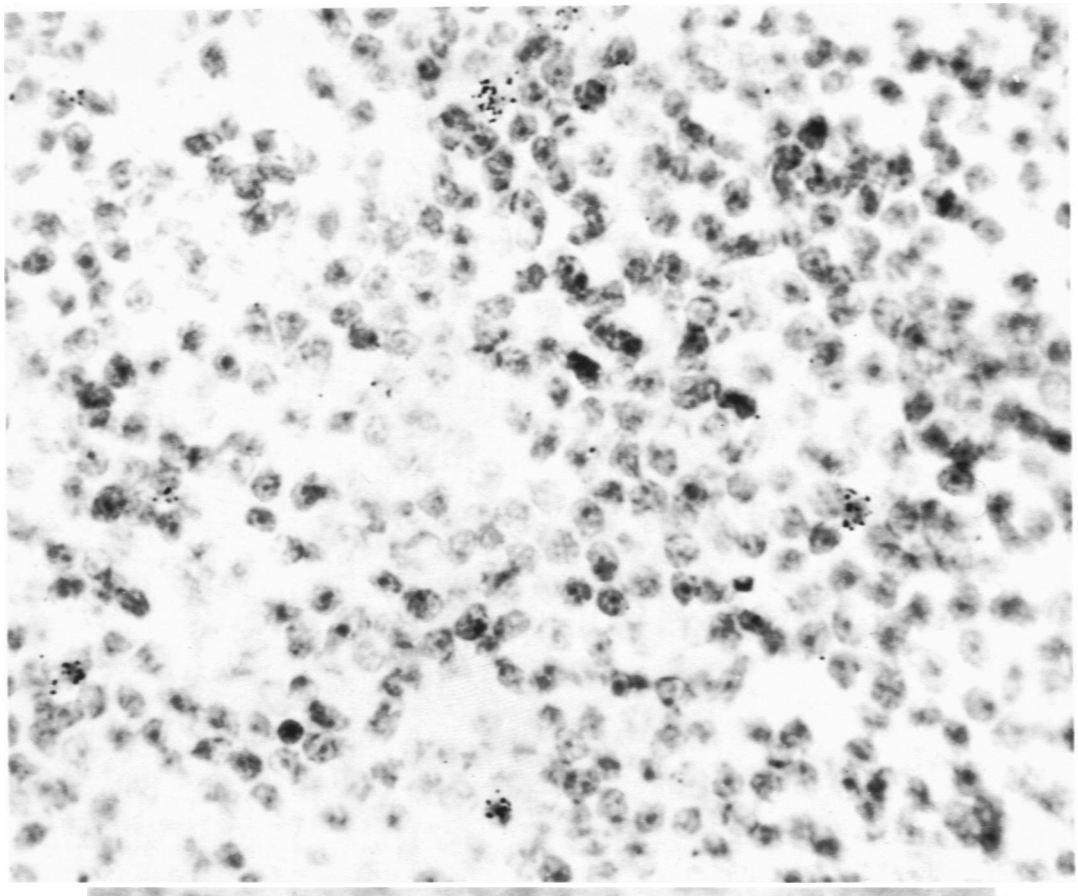
Fig 3. Lymph node from recipient of neuraminidase-treated lymphocytes killed 2 hr after injection. Substantially fewer labeled cells are found than in recipients of untreated cells.

Fig 4. Liver from recipient of neuraminidase-treated lymphocytes killed at 2 hr. Labeled cell is shown in sinusoid.



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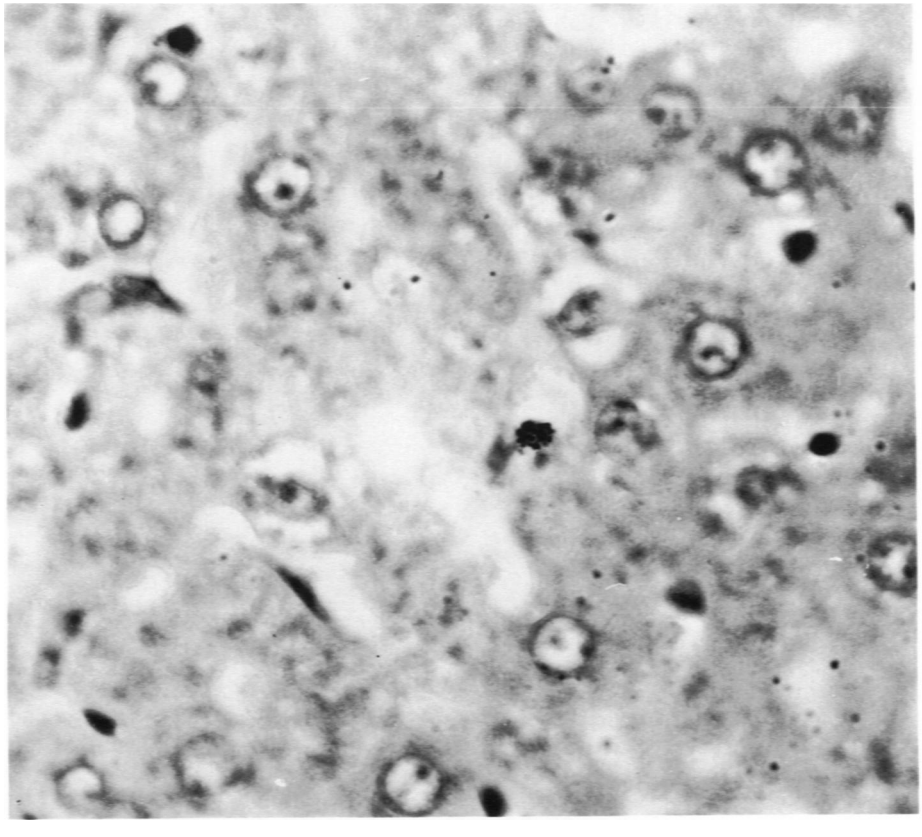

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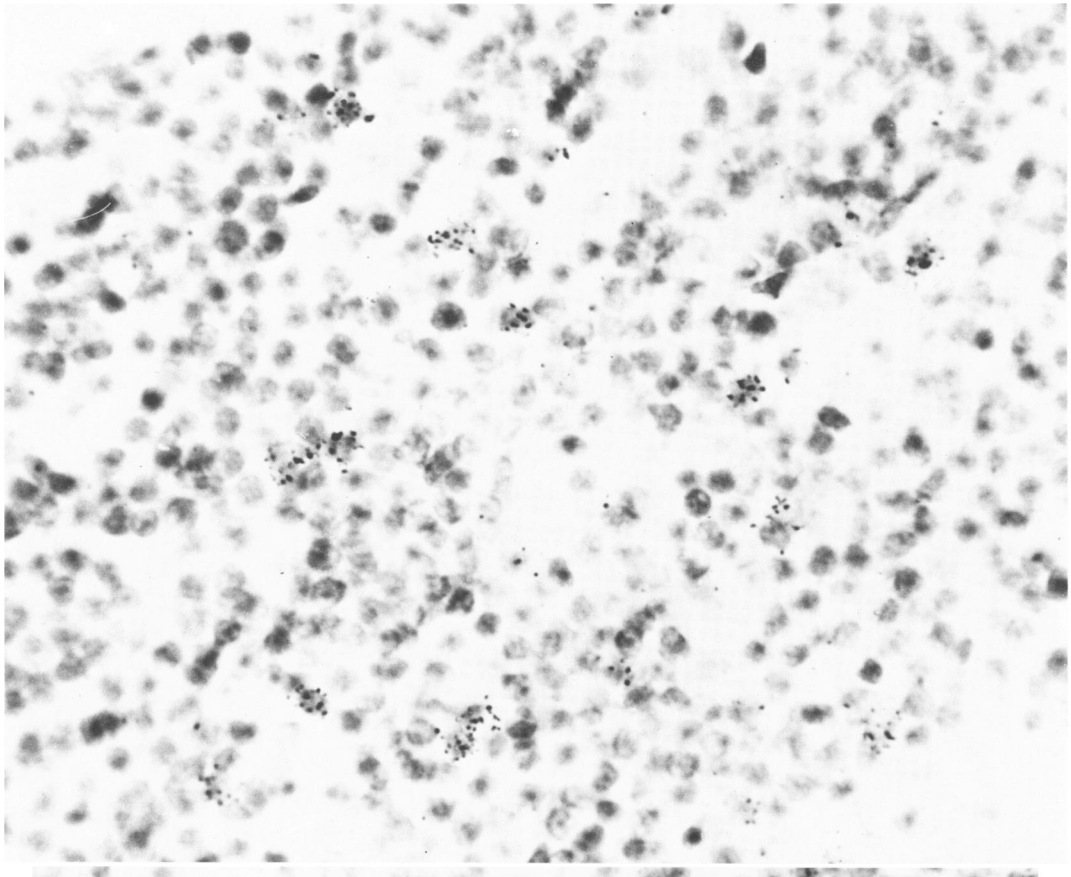
Fig 5. Lymph nodes from recipient of neuraminidase-treated cells 24 hr after injection. Many labeled cells are found, comparable to that seen in recipients of untreated cells.

Fig 6. Spleen from recipient of trypsin-treated cells at 2 hr. Many labeled lymphocytes are present in white pulp.



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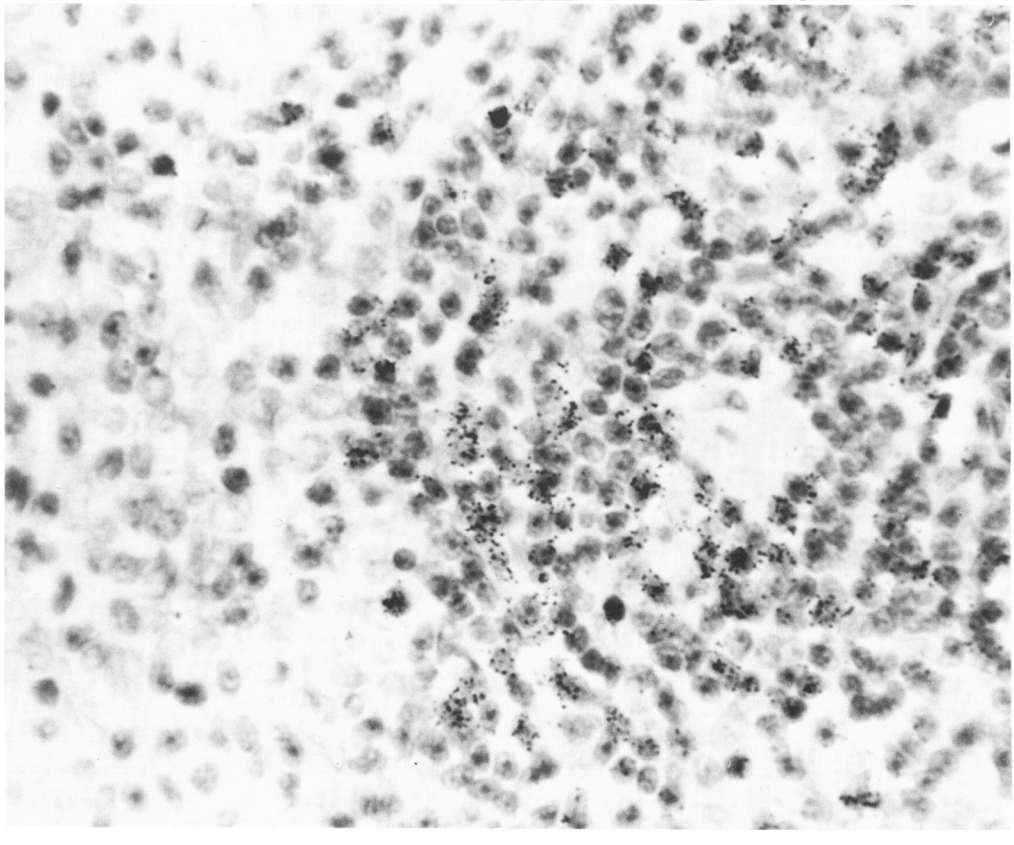

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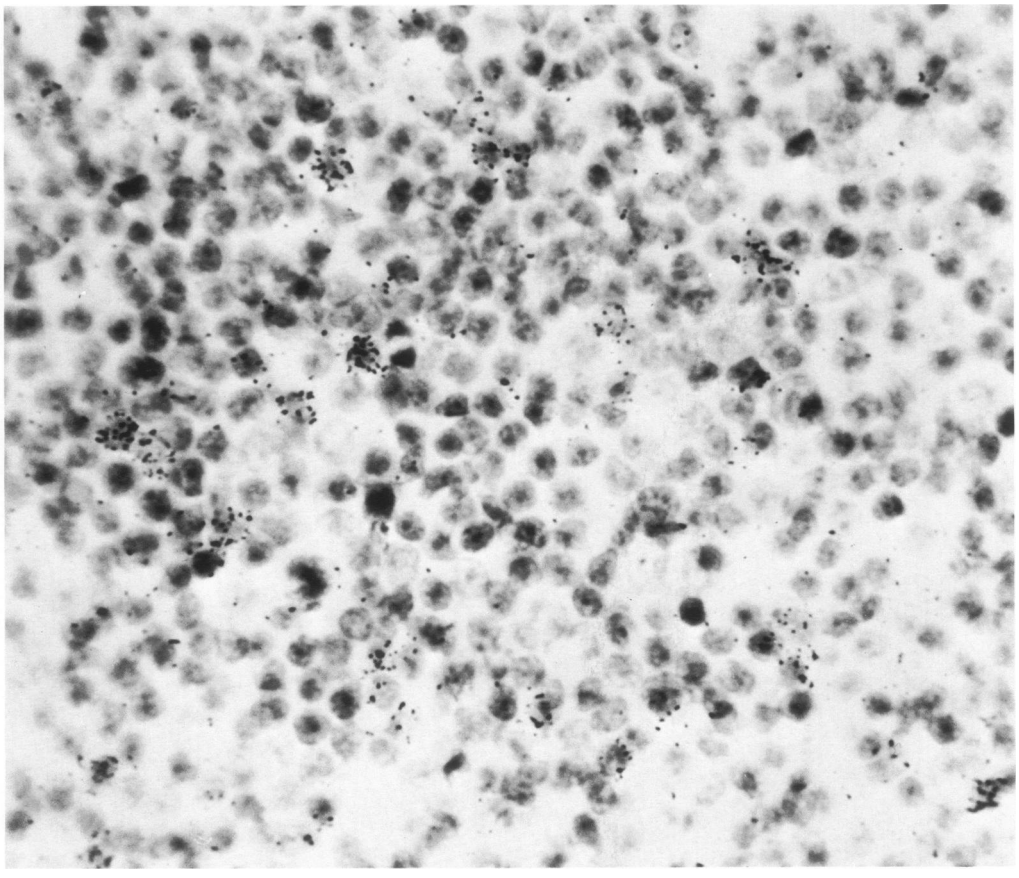

Fig 7. Lymph node from recipient of trypsin-treated cells at 24 hr. Many labeled cells are found.