projections from ray parenchyma cells often adjoin similar projections from adjacent parenchyma or tracheid cells (Fig. 2). These projections represent simple pit pairs, the only type of pit pairs occurring between parenchyma cells (*4*). Some xylem vessels and tracheids have large umbrella-shaped projections which probably represent bordered pits and pit connection replication (Fig. 3). Light-microscopic examination of silica replicas shows the pit pair projections, but the accompanying chromatic aberration caused by the silica renders the images indistinct. Consequently, carbon replicas of the silica replicas were prepared for electron-microscopic examination as previously described for sponge spicules (*5*). Carbon replicas worked well for masses of silica replica of one to ten cells, but with larger pieces the replicas collapsed or were confusingly superimposed.

This technique seems to have great potential for the three-dimensional examination of cellular spaces, intercellular connections, and the morphology of woody cells. More rigorous control of experimental conditions will provide information about siliceous petrifaction of plant tissues and possibly the biogenesis of siliceous structures in living plants and animals.

Cornus, Smilax, Morus, Osmunda, Rosa, Quercus, Acer, and *Pinus,* as well as *Betula* wood, have been silicified. To date, I have been unable effectively to silicify leaf tissue or various nonmineralized animal tissues (human hair, feathers, and insects).

RYAN W. DRUM

Botany Department,
University of Massachusetts, Amherst

References and Notes

1. J. G. Vail, *Soluble Silicates* (Reinhold, New York, 1951), vol. 1.
2. D. W. Parry and F. Smithson, *Ann. Bot.* **30**, 525 (1966).
3. R. W. Drum, *J. Ultrastruct. Res.*, in press.
4. K. Esau, *Plant Anatomy* (Wiley, New York, 1953).
5. R. W. Drum, *Science* **157**, 581 (1967).

27 March 1968

Lymphocytes: Circulation Altered by Trypsin

Abstract. *Rat thoracic duct lymphocytes altered by trypsin in vitro do not circulate normally. At early intervals after transfusion of lymphocytes labeled with chromium-51 selective accumulation of radioactivity in the lymph nodes is abolished, while uptake in the spleen is not reduced. Later, the cells appear to "home" to lymph nodes and recirculate to the lymph.*

Small lymphocytes circulate through the body by a unique route—they selectively emigrate from the blood stream into lymphoid tissue (except the thymus) and then recirculate to the blood via the lymphatics (*1–5*). The properties of lymphocytes which control their fate in the body are poorly understood. There is evidence, however, that lymphocytes must be viable in order to recirculate to the lymph (*1*). Also, it is known that lymphocytes need not be identical genetically with their host in order to "home" to lymphoid tissue and recirculate to the lymph (*1, 2, 4, 6, 7*). More recently it has been found that incubation of lymphocytes with a mixture of glycosidases (*7*) or with neuraminidase (*8*) before transfusion into recipients markedly altered the fate of the cells, although the cells appeared to be viable at the time they were transfused. In the present investigation lymphocytes were incubated in vitro with trypsin and the effect of this treatment on their fate in recipients was studied.

Lymphocytes obtained during the first 24 hours after cannulation of the main lymphatic duct of a donor rat (*1, 9*) were separated from the lymph by centrifugation at 4°C for 5 minutes at 150*g*. The cell pellet, containing 350 to 600 $\times$ 10^6 lymphocytes, was resuspended in 5 to 8 ml of saline, 100 μc of radioactive chromium (*10*) was added, and the mixture was incubated for 1 hour at room temperature. At the end of the incubation period the cells were washed three times with 40 ml of saline, counted, asasyed for radioactivity, and resuspended in saline so as to provide 100 $\times$ 10^6 lymphocytes per milliliter.

Lymphocytes labeled with Cr^{51} were dispensed in 0.5-ml samples, containing 50 $\times$ 10^6 lymphocytes, in separate tubes. Immediately after incubation for 5 minutes, with or without trypsin (*11*), in a 37°C water bath without shaking, each cell suspension was drawn into a syringe and transfused into the tail vein of a recipient rat lightly anesthetized with ether. Each injection was completed within 30 seconds. All transfusions were completed within 30 minutes after the labeled cells had been washed and resuspended in saline. Recipients were killed by cervical dislocation, and various organs were removed, weighed, and assayed for radioactivity. The superficial and deep cervical nodes and the mesenteric nodes from each recipient were carefully dissected free of fat, pooled, and assayed together. Usually a total of about 0.4 to 0.8 g of nodal tissue was obtained from one recipient and the counts per minute per gram were calculated. The spleens usually weighed 0.4 to 1.2 g. For this mixed lymphoid and reticuloendothelial organ, values are presented as counts per minute per organ since among recipients of untreated lymphocytes values more closely coincided by this measure than by comparing counts per minute per gram. Sprague-Dawley rats, a closed but not inbred stock, were used as donors and recipients unless otherwise indicated. In some experiments Fisher rats, members of a highly inbred strain, were used. All donors and recipients were males and they weighed from 250 to 300 g.

During the 5-minute incubation in vitro, prior to transfusion into recipients, there was no greater release of radioactivity from lymphocytes incubated with trypsin (50 $\times$ 10^6 lymphocytes and 0.01 mg of trypsin in 0.5 ml of saline) than from suspensions incubated without the enzyme; 96 percent of the total radioactivity in the samples was found in the cell pellet. Even after 30 minutes of incubation with trypsin, about 95 percent of the total radioactivity remained associated with the cells.

Lymphocytes incubated with trypsin for 5 minutes under these conditions showed no decrease in the percentage of motile cells (when resuspended in medium 199 containing 15 percent calf serum and observed for 3 hours by means of phase-contrast microscopy at 37°C) and no decrease in the percentage of cells excluding trypan blue (when resuspended in saline and incubated for 1 hour at 37°C); 90 to 98 percent of the lymphocytes incubated with or without the enzyme showed these characteristics of viability. During the 5-minute incubation period in vitro with trypsin, a fine mucinous material was released from the cells. This material coalesced to form a larger pre-

cipitate if the tubes were shaken, and, therefore, agitation of the tubes was avoided. Examination of the suspension under the light microscope showed some lymphocytes enmeshed in the material, but the overwhelming majority of the cells appeared to be free and unagglutinated. No decrease in the number of free cells was detected by ordinary counting techniques. The entire suspension was transfused into recipients; spontaneous deaths were extremely rare after the injection.

In recipients killed 30 minutes after transfusion of lymphocytes incubated with trypsin, there was a striking decrease in accumulation of radioactivity in the lymph nodes. In this tissue, the selective accumulation of radioactivity was virtually abolished when 0.0025 to 0.5 mg of trypsin was used. In contrast to the almost complete elimination of accumulation of radioactivity in the nodes, there was no decrease in accumulation of isotope in the spleen.

Livers and lungs of these animals variably contained somewhat more radioactivity than those of recipients of lymphocytes incubated without the enzyme (Table 1). Similar effects were found by using syngeneic donors and recipients. Injection of enzyme separately, immediately before transfusion of untreated lymphocytes, had no effect, which indicates that the effect of the enzyme was due to its action on donor cells rather than on the recipient animal (Table 1). The effect of trypsin in abolishing selective accumulation of radioactivity in the lymph nodes could be prevented by addition of a trypsin inhibitor from soybean to the in vitro reaction mixture. Addition of this inhibitor alone had no substantial effect.

Recipients killed at 1 hour after transfusion also showed almost complete inhibition of uptake of radioactivity in the nodes while other tissues, including spleen, liver, lung, bone marrow, kidney, thymus, muscle, and blood, remained relatively unaffected. Injection of unlabeled trypsin-treated lymphocytes 5 minutes before an injection of labeled untreated lymphocytes had no effect on the selective uptake of radioactivity in the nodes.

Tissues removed at 1, 4, 8, 22, and 48 hours after transfusion showed that, after a delay of 4 hours, the accumulation of radioactivity in the lymph nodes of recipients of trypsin-treated lymphocytes rose rapidly, and, by 22 hours, approached values obtained in recipients of untreated lymphocytes. The recovery of radioactivity in the spleens showed no decrease in recipients of trypsin-treated lymphocytes in the early intervals (1 to 4 hours) after transfusion. Recovery of radioactivity in the lungs and livers of recipients of enzyme-treated lymphocytes did not differ greatly from that in recipients of untreated lymphocytes (Fig. 1).

When heat-killed, labeled lymphocytes were transfused virtually no radioactivity accumulated in lymph nodes at any interval after transfusion, and the counts in the liver remained high (50 to 35 percent of total injected radioactivity) throughout the 48-hour test period. When free Cr^{51} was transfused the percentage of accumulation of radioactivity in the lymph nodes was also negligible.

The extent of recovery of radioactivity associated with lymphocytes from the main lymphatic duct of recipients of trypsin-treated lymphocytes coincided closely with that obtained in recipients of untreated lymphocytes. In both situations, when the recipients were observed for 48 hours after transfusion of the cells, about 24 percent of the total radioactivity that had been injected was recovered. It appeared, however, that there was a slight delay in recovery of labeled lymphocytes from recipients of trypsin-treated cells. When unlabeled lymphocytes and free Na_2-

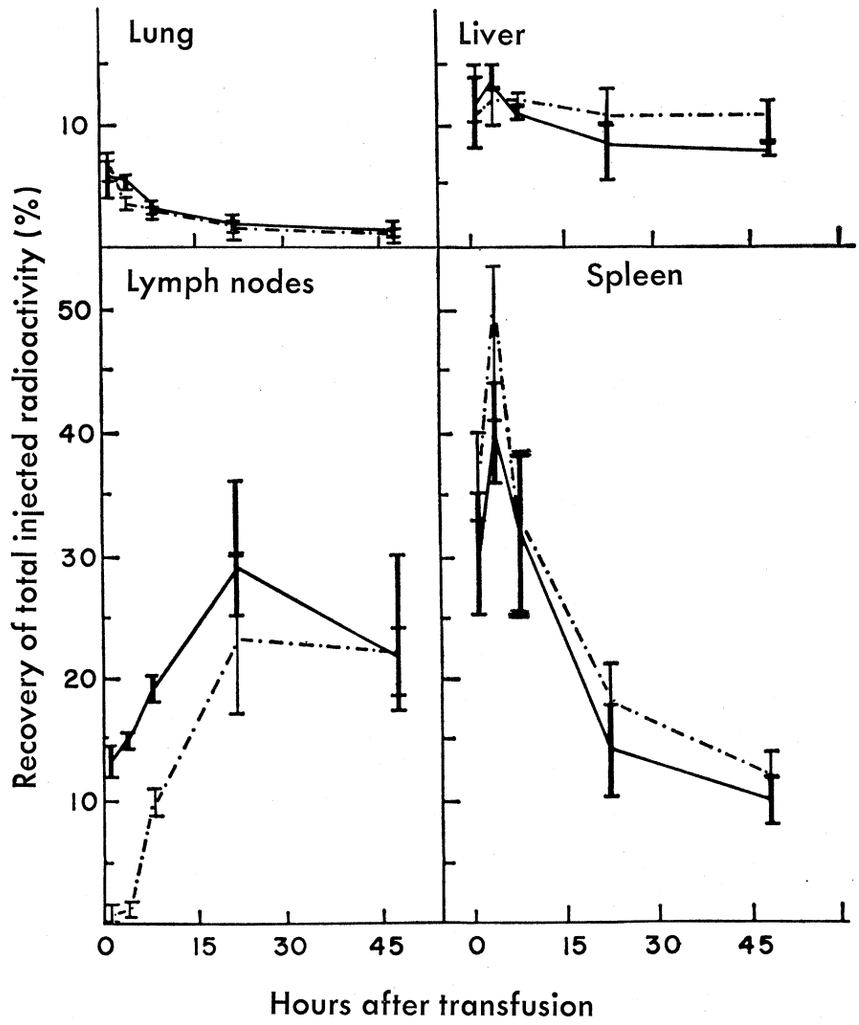

Fig. 1. Percentage of the total injected radioactivity that was recovered from lymph nodes, spleen, liver, and lung of recipients of untreated or trypsin-treated Cr^{51}-labeled lymphocytes; the animals were killed at intervals from 1 to 48 hours after transfusion. Each recipient received 50×10^6 Cr^{51}-labeled lymphocytes in 0.5 ml of saline, incubated for 5 minutes at 37°C either with 0.01 mg of trypsin (broken line) or without trypsin (solid line). For lung, spleen, and liver, the values represent the percentage of recovery in the entire organ; for lymph nodes, the percentage of recovery per gram of lymph node. Each interval represents the average value for four recipients.

Table 1. Effect of trypsin on the distribution of radioactivity in recipients killed 30 minutes after transfusion. Each recipient was transfused with 50 × 10^6 lymphocytes containing 155,791 counts per minute (cpm) after incubation with the indicated amount of trypsin at 37°C for 5 minutes. Values shown are those of a typical experiment. Essentially the same results were obtained in 20 similar experiments.

Trypsin (mg)	Lymph nodes (cpm per gram)	Spleen (cpm per organ)	Liver		Lung	
			(cpm per gram)	(cpm per organ)	(cpm per gram)	(cpm per organ)
0	14,822	32,241	1,425	16,331	7,951	15,833
0.0005	5,336	31,341	1,485	16,950	8,050	19,142
.0025	186	43,999	1,416	18,280	12,989	22,731
.005	235	46,846	1,256	15,486	8,271	15,610
.025	309	26,846	1,505	21,175	17,948	32,127
.05	272	37,473	1,632	23,713	10,575	18,709
.5	311	44,548	1,377	18,342	17,207	19,444
.05*	15,150	34,521	1,590	18,046	5,167	17,256

* Trypsin and lymphocytes incubated separately and injected separately into recipient.

$Cr^{51}O_4$ were transfused, a negligible percentage of radioactivity that had been injected (less than 1 percent) was recovered in association with the lymphocytes obtained from the duct.

The studies indicate that alteration of lymphocytes by trypsin markedly affects their fate in the body; during the first 4 hours after transfusion, there was a complete failure of trypsin-treated cells to selectively accumulate in lymph nodes, whereas other organs, including the spleen, showed no evidence of decrease in accumulation of cells. At later intervals after transfusion, the cells evidently regain their ability to selectively accumulate in lymph nodes and recirculate to the lymph. This interpretation seems highly probable because the most likely way by which the observed selective accumulation of radioactivity could have occurred in lymphoid tissue and the lymph is by being carried to these sites associated with the donor lymphocytes that were transfused rather than the recipient's own lymphocytes since (i) selective accumulation of radioactivity in lymph nodes does not occur after transfusion of Cr^{51}-labeled heat-killed cells or when free Cr^{51} is injected, and (ii) there is evidence that Cr^{51} that has been incorporated into lymphocytes is very poorly reincorporated into other lymphocytes or other cell types (*12*). The evidence that trypsin-treated lymphocytes "home" to the nodes at later intervals after transfusion and recirculate to the lymph indicates that the alterations in the fate of the cells induced by trypsin were not due to killing the cells because these activities of lymphocytes require that the cells be viable. Apparently the alterations in cells, which are responsible for their inability to "home" to lymph nodes at early intervals after transfusion, are repaired in vivo.

During the first 4 hours after transfusion of trypsin-treated lymphocytes, large numbers of these cells entered the systemic circuit, since there was no reduction in the accumulation of radioactivity in the liver and spleen. Also, during this interval, blood flow to the nodes was apparently normal since transfusion of unlabeled trypsin-treated lymphocytes before an injection of labeled untreated lymphocytes did not impair the accumulation of radioactivity in the nodes. These circumstances make it likely that the complete failure of enzyme-treated lymphocytes to selectively accumulate in lymph nodes was not due solely or primarily to the failure of cells to reach the nodes during this interval. The apparent explanation for the effect is that trypsin-treated lymphocytes circulated through the nodes during the first 4 hours after transfusion but did not selectively emigrate into the nodes because alterations in the lymphocytes prevented them from doing so.

The changes in lymphocytes induced by trypsin, which cause them to circulate abnormally, are unknown, but these changes are selective in the sense that lymphocytes altered by another enzyme (neuraminidase) circulate differently from trypsin-treated cells. Presumably, alterations in the surface or metabolism of the cells, or both, induced by trypsin were responsible for the effect. The most apparent explanation is that trypsin cleaves constituents of the lymphocyte surface required for the selective emigration through the endothelial cells of the post-capillary venules in lymph nodes. Lymphocytes emigrate into lymph nodes by becoming engulfed in a vacuole of the endothelial cell of post-capillary venule and emerging on the parenchymal side of the vessel (*13*), whereas they selectively emigrate into the white pulp of the spleen by passing in between the endothelial cells of the marginal sinus (*14*). The present evidence that selective accumulation of trypsin-treated lymphocytes in the spleen is not diminished during the interval when they fail to "home" to lymph nodes raises the possibility that the properties of lymphocytes required for their selective emigration through the endothelial cells of the post-capillary venules in lymph nodes are not identical to the properties required for their migration between the endothelial cells of the marginal sinus en route to the white pulp of the spleen. Further studies utilizing radioautographic techniques indicate that trypsin-treated lymphocytes do localize in the white pulp of the spleen during the time when they fail to enter lymph nodes (*15*).

JUDITH WOODRUFF
BERTRAM M. GESNER
Department of Medicine, New York University School of Medicine, New York 10016

References and Notes

1. J. L. Gowans, *Brit. J. Exp. Pathol.* **38**, 67 (1957).
2. ———, *J. Physiol.* **146**, 54 (1959).
3. ———, in *Kinetics of Cellular Proliferation*, F. Stohlman, Jr., Ed. (Grune and Stratton, New York, 1958), p. 64.
4. R. G. Shorter and J. L. Bollman, *Amer. J. Physiol.* **198**, 1014 (1960).
5. J. L. Gowans and J. Knight, *Proc. Roy. Soc. London Ser. B* **159**, 257 (1964); R. W. Caffrey, W. D. Reicke, N. B. Everett, *Acta Haematol.* **28**, 145 (1962).
6. J. L. Gowans, *Ann. N.Y. Acad. Sci.* **99**, 432 (1962).
7. B. M. Gesner and V. Ginsburg, *Proc. Nat. Acad. Sci. U.S.* **52**, 750 (1964).
8. J. Woodruff and B. M. Gesner, *J. Clin. Invest.* (abstr.) **46**, 1134 (1967).
9. J. L. Bollman, J. C. Cain, J. H. Grindley, *J. Lab. Clin. Med.* **33**, 1349 (1948); J. L. Bollman, *ibid.*, p. 1348.
10. Obtained from Isoserve as $Na_2Cr^{51}O_4$ (*p*H 7.0) in isotonic saline without preservative added; specific activity, 100 to 200 μc per microgram of Cr.
11. Obtained from General Biochemicals; salt-free, twice recrystallized.
12. W. L. Bunting, J. M. Kiely, C. A. Owen, Jr., *Proc. Soc. Exp. Biol.* **113**, 370 (1963); G. Holm and P. Perlman, *Immunology* **12**, 525 (1967).
13. V. T. Marchesi and J. L. Gowans, *Proc. Roy. Soc. London Ser. B* **159**, 283 (1964).
14. I. Goldschneider and D. D. McGregor, *J. Exp. Med.* **127**, 155 (1968).
15. B. M. Gesner, J. Woodruff, R. McCluskey, *Arthritis Rheum.* (abstr.), in press.
16. Supported by PHS grant No. 5 RO1 HE-09239-02, by PHS training grant 5T1-A1-5-10 to J.W., and by a career scientist award of the New York City Health Research Council to B.M.G. We thank Mrs. Sandra Teitelbaum for technical assistance.

10 May 1968