

## THE EFFECT OF NEURAMINIDASE ON THE FATE OF TRANSFUSED LYMPHOCYTES\*

BY JUDITH J. WOODRUFF,† M.D., AND BERTRAM M. GESNER,§ M.D.  
(From The Department of Medicine, New York University School of Medicine,  
New York, New York 10016)

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Small lymphocytes circulate in blood and lymph and form the major cellular constituents of lymphoid tissues. These cells have a unique route of circulation in the body. Large numbers of small lymphocytes, entering the blood via lymphatics, selectively emigrate from the blood stream in lymphoid tissues where they are aggregated and subsequently released into the lymph (1-7). The properties of lymphocytes which control their traffic and distribution in the body are poorly understood. It is known that lymphocytes killed by heat or ultraviolet radiation do not recirculate from blood to lymph (2). Also, it is known that the selective emigration of lymphocytes from blood into lymphoid tissues and their recirculation to the lymph does not require that the lymphocytes be genetically identical with the host (1-5, 8). Furthermore, it has been reported that treatment of lymphocytes in vitro with a crude glycosidase preparation (8) or with trypsin (9) prior to intravenous transfusion prevents the cells from circulating normally, though the lymphocytes appeared to be viable at the time they were transfused. These latter findings suggested that structures at the surface of lymphocytes might play a role in determining the distribution of these cells. It was, therefore, of interest to determine the fate of lymphocytes in vivo after they had been incubated with neuraminidase, a glycosidase which cleaves terminal sialic acid from heterosaccharides or glycoproteins (10) and which is known to be capable of releasing sialic acid from cell surfaces (11-14). The present report describes such studies and provides evidence that treatment of lymphocytes with neuraminidase in vitro drastically alters the fate and distribution of the cells when they are subsequently transfused intravenously into recipients.

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† Training Fellow, United States Public Health Service Training Grant 5T1-A1-5-10. Present address: Department of Medicine, Cornell University Medical College, Room F-231, New York, N.Y. 10021.

§ Career Scientist of the Health Research Council of the City of New York. Deceased, August 1968.

### Materials and Methods

*Animals.*—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 weighed 250–300 g.

*Reagents.*—Neuraminidase, derived from *Vibrio cholerae* and prepared to contain 500 units/ml, was obtained from General Biochemicals (Chagrin Falls, Ohio) and used in each experiment except where indicated. Another neuraminidase preparation (Behringwerke Ag., Marburg-Lahn, West Germany, and Hoechst Pharmaceutical Co., Inc., Cincinnati, Ohio) was used for comparison in several experiments; this preparation, described by the manufacturer as having no detectable proteinase, aldolase, or lecithinase C activity, was also derived from *Vibrio cholerae* and prepared to contain 500 units/ml. One unit of neuraminidase activity is defined by both manufacturers as being equivalent to the release of 1  $\mu\text{g}$  of *N*-acetyl neuraminic acid from orosomucoid at 37°C in 15 min at pH 5.5. Sialic acid, i.e. *N*-acetyl neuraminic acid, was obtained from General Biochemicals; heparin sodium from Fisher Scientific Co., Pittsburgh, Pa.; and tissue culture medium 199 (M 199) from Grand Island Biological Co., Grand Island, N.Y.  $^{51}\text{Cr}$  was purchased from Iso-Serve, Cambridge, Mass., and supplied as  $\text{Na}_2^{51}\text{Cr-O}_4$  in isotonic saline at pH 7.0 with no preservative added; the specific activity varied from 100–200  $\mu\text{C}/\mu\text{g}$  of chromium. Reagents used in determination of electrophoretic mobility (15) and of free sialic acid (16) were prepared as described elsewhere.

*Collection of Lymph.*—The main lymphatic duct of a donor rat was cannulated below the diaphragm by usual techniques (17, 2). After surgery, the unanesthetized animal was kept in a Bollman cage (18) with free access to commercial rat cake and 5% dextrose in saline. Lymph was collected at room temperature in 125 ml flasks containing 10–15 ml of isotonic saline and 20 units of heparin/ml. The cells for injection were obtained during an 8–12 hr period after free flow had been established. All collection periods were completed within the first 24 hr after cannulation. The collections used for experiments contained 350–500  $\times 10^6$  lymphocytes and only few erythrocytes; 90–95% of the lymphocytes obtained were typical “small” lymphocytes.

*Radioactive Labeling of Lymphocytes.*—Lymphocytes were separated from the lymph by centrifugation at 4°C for 5 min at 150 g. The cell pellet was resuspended in 5–8 ml of saline, 100  $\mu\text{C}$  of radioactive chromium was added, and the mixture was then incubated for 1 hr at room temperature. At the end of the incubation period the cells were washed three times with 40 ml of saline, counted, assayed for radioactivity, and finally resuspended in M 199 so as to provide 100  $\times 10^6$  lymphocytes/ml.

*Determination of Motility and Exclusion of Trypan Blue by Untreated and Neuraminidase-Treated Labeled Lymphocytes.*—50  $\times 10^6$   $^{51}\text{Cr}$ -labeled lymphocytes in 0.5 ml M 199 were incubated in a 37°C water bath with either no enzyme or 12.5 units of neuraminidase for 15 min. The cells were then diluted to 2 ml with M 199 and centrifuged at 150 g for 5 min. For studies of cell motility, the cell pellet was resuspended in M 199 with 15% calf serum so as to provide 5  $\times 10^6$  cells/ml. The cells were then observed for motility under phase-contrast microscopy at 37°C during a 3 hr period. For studies of exclusion of vital dye, the cell pellet was resuspended in 0.5 ml saline and then incubated at 37°C. Aliquots were removed at 0, 15, 30, and 60 min, mixed with an equal volume of 0.25% trypan blue in saline, and examined at a magnification of 100.

*Determination of Agglutinability of Untreated and Neuraminidase-Treated  $^{51}\text{Cr}$ -Labeled Lymphocytes.*—50  $\times 10^6$   $^{51}\text{Cr}$ -labeled lymphocytes in 0.5 ml M 199 were incubated with either no enzyme or 12.5 units of neuraminidase at 37°C for 15 min. The cells were then centrifuged and resuspended in either autologous rat serum, calf serum, or M 199 so as to provide 10<sup>7</sup> lymphocytes/ml. The cell suspensions were then incubated at 37°C and samples were streaked on slides and examined for agglutination at a magnification of 100 at intervals between 15 min and 2 hr. The degree of agglutination was recorded as 0–4+, each 1+ representing agglutination of approximately 25% of the cells.

*Determination of Changes in Electrophoretic Mobility of Heat-Killed and of Neuraminidase-Treated Lymphocytes.*—The electrophoretic mobility of untreated, heat-killed, and neuraminidase-treated lymphocytes was determined in a rectangular type apparatus by the method of Angers and Rottino (15). "Untreated" lymphocytes were prepared for electrophoresis by incubating  $50 \times 10^6$  washed lymphocytes in 0.5 ml saline or M 199 for 15 min at 37°C. Heat-killed lymphocytes were prepared by incubating  $50 \times 10^6$  washed lymphocytes in 0.5 ml saline for 10 min at 45°C. Neuraminidase-treated lymphocytes were prepared by incubating  $50 \times 10^6$  lymphocytes in 0.5 ml M 199 with 12.5 units of neuraminidase for 15 min at 37°C. At the end of each incubation period, 5 ml of M 199 was added to each suspension, the samples were then centrifuged, and the cell pellet resuspended in 5 ml saline. Immediately before making the electrophoretic determination, the samples were centrifuged and the cell pellets resuspended in Michaelis buffer at pH 7.0 so as to provide  $2-4 \times 10^6$  cells/ml. No more than 30 min elapsed between the last saline wash and suspension of the cells in Michaelis buffer. All measurements of electrophoretic mobility were performed at 25°C. In each suspension the mobility of 10 randomly selected lymphocytes was determined and the average value obtained.

*Determination of Release of Sialic Acid from Lymphocytes by Neuraminidase or by Acid Hydrolysis.*—Lymphocytes were separated from lymph and the cell pellet was resuspended in water for 30 sec to lyse the few red cells present. The suspension was then made isotonic with an appropriate volume of five times isotonic saline and the lymphocytes washed twice in isotonic saline. The samples were then centrifuged and resuspended in M 199 so as to provide  $500 \times 10^6$  cells in a final volume of 0.5 ml. Neuraminidase (50 units) was then added to suspensions and the samples incubated for 30 min. After incubation, the cells were sedimented by centrifugation at 150 g for 5 min and the supernatants were assayed for free sialic acid. Supernatants obtained from lymphocytes handled in the same manner but incubated without neuraminidase were used for comparison. Total nonlipid sialic acid content of lymphocytes was determined by suspending a washed, red cell-free, lymphocyte cell pellet containing  $500 \times 10^6$  lymphocytes in 2 ml of 0.1 N  $H_2SO_4$ . The suspension was then heated for 1 hr at 90°C. After centrifugation the supernatants were extracted with two volumes of isoamyl alcohol and duplicate aliquots of the aqueous phase were assayed for free sialic by the thiobarbituric acid method of Warren (16). Optical densities were measured both at 532 and 549  $m\mu$  to correct for absorbancy at 549  $m\mu$  not due to sialic acid. *N*-acetyl neuraminic acid was used as a standard.

*Assay for Leakage of  $^{51}Cr$  from Labeled Lymphocytes.*—Aliquots containing  $50 \times 10^6$   $^{51}Cr$ -labeled lymphocytes were suspended in 0.5 ml M 199 and incubated at 37°C with either no enzyme or 25 units of neuraminidase for 5–60 min. At the end of the incubation periods, the lymphocyte suspensions were centrifuged at 4°C for 5 min at 150 g, and the amounts of radioactivity in the cell pellet and in the supernatant were assayed. For all radioactive assays a Nuclear-Chicago (Des Plaines, Ill.) single sample deep well counter, model No. 0547, was used.

*Experimental Design for Transfusion of Lymphocytes into Recipients.*— $50 \times 10^6$   $^{51}Cr$ -labeled lymphocytes in 0.5 ml M 199 were dispensed in separate tubes. Immediately after incubation at 37°C for 15 min with or without neuraminidase, each cell suspension was transfused into the tail vein of a rat lightly anesthetized with ether. Each injection took no longer than 30 sec. Variations in distribution of radioactivity were minimized by completing all transfusions within 30 min after the labeled lymphocytes had been washed and resuspended in M 199. Recipients were killed by cervical dislocation and various organs removed. The superficial and deep cervical nodes and the mesenteric nodes from each recipient were carefully dissected free of fat and pooled. The lymph nodes and other organs were weighed and assayed for radioactivity. Usually a total of 0.4–0.8 g nodal tissue was obtained from one recipient. The spleens usually weighed 0.4–1.2 g. For this mixed lymphoid and reticuloendothelial organ, values are reported as cpm/organ since, from recipient to recipient of untreated lymphocytes, values more closely coincided by this measure than by comparing cpm/unit of weight.

In some experiments, recipients were partially hepatectomized by excision of the right and

left hepatic lobes 3 hr after transfusion of labeled lymphocytes. By this method, approximately 2/3 of the liver was removed (19). At 48 hr after the transfusion of cells, these partially hepatectomized recipients were sacrificed and the organs removed and assayed for radioactivity.

In another group of experiments, the main lymphatic duct of a recipient was cannulated 24 hr prior to intravenous transfusion of labeled lymphocytes. Recipients prepared in this manner were transfused intravenously with either neuraminidase-treated or untreated lymphocytes ( $200\text{--}300 \times 10^6$  labeled lymphocytes in 1.5 ml M 199, incubated for 15 min at  $37^\circ\text{C}$  with or without neuraminidase). The concentration of neuraminidase was adjusted to provide 12.5 units/ $50 \times 10^6$  cells. After transfusion, the recipient rats were returned to Bollman cages and lymph collected continuously from them for 72 hr. At intervals of 4–16 hr, the lymph was centrifuged and the cell pellets and supernatants were assayed for radioactivity. The percentage of total injected radioactivity recovered in the cell pellet per hour for each collection period was calculated and the value plotted on a graph at the midpoint of the collection period.

TABLE I  
*Release of Radioactivity from  $^{51}\text{Cr}$ -Labeled Lymphocytes after Incubation with and without Neuraminidase*

Time of incubation <i>min</i>	Units of neuraminidase			
	None		25	
	<i>cpm/cell pellet</i>	<i>cpm/supernatant</i>	<i>cpm/cell pellet</i>	<i>cpm/supernatant</i>
5	268,531	7,175	262,078	7,784
15	260,756	8,136	275,495	7,243
30	259,358	9,052	260,496	10,014
60	272,984	12,514	265,865	10,927

Each sample contained  $50 \times 10^6$   $^{51}\text{Cr}$ -labeled lymphocytes in 0.5 ml M 199.

#### RESULTS

*Condition of  $^{51}\text{Cr}$ -Labeled Lymphocytes at the Time of Transfusion into Recipients.*—After incubation for 15 min at  $37^\circ\text{C}$  with neuraminidase, lymphocytes appeared morphologically intact and unagglutinated under the light microscope. When preparations of lymphocytes incubated with neuraminidase were examined by phase-contrast microscopy at  $37^\circ\text{C}$ , 87–92% of the cells exhibited typical lymphocyte motility during the 3 hr observation period. In the populations incubated without neuraminidase, 88–93% of the lymphocytes were motile. Similarly, no difference was found in the percentage of lymphocytes excluding trypan blue; 95–98% of the lymphocytes in both enzyme-treated and untreated populations excluded the dye. The effect of the neuraminidase preparation on the release of radioactivity from labeled cells is shown in Table I. The data indicate that there was no significant difference in the amount of radioactivity associated with untreated and neuraminidase-treated lymphocytes even after 1 hr of incubation.

The above findings indicate that, at the time of transfusion, lymphocytes

incubated with neuraminidase were unagglutinated, possessed several characteristics of "viability," and retained the radioactive label as well as untreated lymphocytes. Evidence was obtained, however, that neuraminidase did alter the lymphocytes. When neuraminidase-treated lymphocytes were resuspended and incubated in undiluted calf serum for 2 hr at 37°C, some agglutination of the cells occurred (2+). When autologous serum was used as the suspending medium, minimal, if any, agglutination was found, and, using M 199, no agglutination was seen. Lymphocytes not exposed to neuraminidase, but otherwise handled in the same manner, remained unagglutinated when resuspended in

TABLE II  
*Effect of Neuraminidase on the Distribution of Radioactivity in Tissues of Recipients of <sup>51</sup>Cr-Labeled Lymphocytes*

Neuraminidase units	Lymph Nodes cpm/500 mg	Liver		Lung		Spleen	Thymus	Kidney
		cpm/500 mg	cpm/organ	cpm/500 mg	cpm/organ	cpm/organ	cpm/500 mg	cpm/500 mg
0	18,720	917	23,279	4,703	13,168	30,294	90	30
2.5	12,642	2,091	47,675	3,841	12,288	26,240	26	45
5.0	7,890	2,826	65,156	2,661	9,631	26,859	2	50
12.5	4,503	3,830	104,263	1,178	3,465	14,336	0	35
25.0	4,743	3,675	95,182	1,744	8,857	11,574	0	43
25.0*	18,420	812	25,089	3,823	14,082	31,321	40	37

Each recipient was transfused with  $50 \times 10^6$  labeled lymphocytes containing 169,640 cpm after incubation with the indicated amount of neuraminidase at 37°C for 15 min; recipients sacrificed 30 min after transfusion.

\* Neuraminidase and lymphocytes incubated separately and injected separately into recipient.

calf serum as well as in autologous serum or M 199. In addition to the altered agglutinability of neuraminidase-treated lymphocytes, it was found that the electrophoretic mobility of the enzyme-treated cells was substantially reduced; by the method used, the mean electrophoretic mobility of neuraminidase-treated lymphocytes (for five separate experiments) was  $1.09 \pm 0.05 \mu/\text{sec}$  per v/cm compared to a mean value (for eight separate experiments) of  $1.59 \pm 0.17 \mu/\text{sec}$  per v/cm for untreated lymphocytes. The electrophoretic mobility of lymphocytes killed by heat was essentially the same as untreated lymphocytes; i.e.,  $1.54 \pm 0.16 \mu/\text{sec}$  per v/cm (mean for five separate experiments). Incubation of lymphocytes with neuraminidase caused the release of free sialic acid from the cells.  $0.034 \pm 0.003 \mu\text{moles}$  sialic acid were released from  $500 \times 10^6$  lymphocytes incubated for 30 min at 37°C with 50 units of neuraminidase (mean of four separate determinations). This value represented 27% of the total nonlipid sialic acid found in comparable samples of lymphocytes ( $0.127 \pm$

0.003  $\mu$ moles sialic acid/500  $\times 10^6$  lymphocytes.) No free sialic acid was detected in supernatants of lymphocytes incubated without neuraminidase.

*The Effect of Neuraminidase on the Distribution of Radioactivity in Tissue of Recipients Sacrificed 30 Min after Transfusion of  $^{51}\text{Cr}$ -Labeled Lymphocytes.*—The distribution of radioactivity in tissues of recipients transfused with neuraminidase-treated lymphocytes differed greatly from that found in recipients of untreated lymphocytes. In recipients of  $^{51}\text{Cr}$ -labeled lymphocytes incubated without enzyme, there was a relatively high concentration of radioactivity in the lymph nodes and spleen and low concentration of label in the liver. However, in recipients of enzyme-treated  $^{51}\text{Cr}$ -labeled cells, much less radioactivity concentrated in the lymph nodes and spleen and substantially more in the liver.

TABLE III  
*Effect of Time of Incubation of Lymphocytes with the Neuraminidase Preparation on Accumulation of Radioactivity in Lymph Nodes and Liver*

Neuraminidase	Time in vitro	Lymph nodes	Liver
units	min	cpm/500 mg	cpm/500 mg
0	5	2,851	330
0	15	2,400	325
12.5	5	1,363	1,059
12.5	10	1,252	1,271
12.5	15	772	1,282

Each recipient received  $50 \times 10^6$  lymphocytes containing 30,200 cpm; recipients sacrificed 30 min after transfusion.

These alterations in the distribution of radioactivity depended on the amount of enzyme added to the in vitro reaction mixture (Table II). A clear-cut effect was observed using as little as 2.5 units of neuraminidase and maximal effect occurred with 12.5 units. In 25 similar experiments, a 50–75% reduction in accumulation of radioactivity/unit weight of lymph node occurred using 12.5 units of the neuraminidase preparation. In all such experiments, the fall in radioactivity in the lymph nodes and spleen was accompanied by a rise in the liver. The same alteration in the pattern of distribution of radioactivity was found when donors and recipients were members of the same highly inbred strain of rats. Also, the same effects were found when the Behringwerke neuraminidase was used instead of the neuraminidase preparation obtained from General Biochemicals.

When neuraminidase and lymphocytes incubated without enzyme were injected separately into one recipient, the distribution of radioactivity was the same as that observed in rats receiving untreated labeled lymphocytes alone (Table II). Thus, the alteration in the distribution of radioactivity caused by

incubating lymphocytes with neuraminidase was due to the effect of the enzyme preparation on the transfused lymphocytes rather than on the recipient rats.

The effect of varying the time of incubation of lymphocytes with the neuraminidase preparation from 5–15 min is shown in Table III. Using 12.5 units of neuraminidase, increasing the time of incubation in vitro increased the effect. However, the extent of the effect was not proportional to the time of incubation; a large proportion of the effect induced by incubating for 15 min occurred after only 5 min of incubation.

Neuraminidase is largely destroyed by heat at 65°C of 10 min (20). In four separate experiments, when the neuraminidase preparation was exposed to these conditions before incubation with lymphocytes, its ability to alter the accumulation of radioactivity in lymph nodes and liver of recipients was essentially abolished. A representative experiment is shown in Table IV.

TABLE IV  
*Effect of Heated Neuraminidase on Accumulation of Radioactivity in Lymph Nodes and Liver*

Neuraminidase	Lymph nodes	Liver
<i>units</i>	<i>cpm/500 mg</i>	<i>cpm/500 mg</i>
0	11,630	751
12.5	3,978	1,871
12.5*	11,231	700

Each recipient transfused with  $50 \times 10^6$  lymphocytes containing 126,656 cpm; animals sacrificed at 30 min after transfusion.

\* Neuraminidase heated at 65°C for 10 min prior to incubation with lymphocytes.

Since it has been shown that the release of sialic acid from sialylactose by neuraminidase can be inhibited by addition of free sialic acid to the reaction mixture (21), it was of interest to determine the effect of adding neutralized sialic acid to the lymphocyte-enzyme mixture. The results of one of three similar experiments is shown in Table V and demonstrate that the effectiveness of the neuraminidase preparation in altering the distribution of radioactivity in recipients could be inhibited by adding sialic acid to the in vitro reaction mixture. This inhibition by sialic acid occurred using the Behringwerke neuraminidase as well as the neuraminidase obtained from General Biochemicals. Neutralized sialic acid did not itself substantially alter the distribution of radioactivity in recipients.

*The Distribution of Radioactivity in Recipients Sacrificed 1–48 Hr After Transfusion.*—The accumulation of radioactivity in the lymph nodes, spleen, liver, and lungs of recipients sacrificed at various intervals after transfusion of untreated or neuraminidase-treated allogeneic lymphocytes is shown in Table VI.

*Recipients of Lymphocytes Incubated Without Neuraminidase.*—Recipients of untreated lymphocytes sacrificed at 1, 8, or 21 hr after transfusion, accumulated an average of 18%, 26%, and 37%, respectively, of the total radioactivity injected per gram lymph node. In recipients sacrificed at either 28 or 48 hr after transfusion, the average recovery per gram lymph node was 20%. The concentration of radioactivity in the spleen was highest in the first few hours after transfusion. The liver contained a relatively low and constant percentage of the total radioactivity injected at each interval tested. The concentration of radioactivity in the lungs was greatest in recipients sacrificed soon after transfusion; at later intervals after transfusion, very little radioactivity was recovered in that organ.

TABLE V  
*Alteration of Effect of Neuraminidase by Addition of Sialic Acid to the Incubation Mixture*

Neuraminidase	Sialic acid	Lymph nodes	Liver
<i>units</i>	<i>mg</i>	<i>cpm/500 mg</i>	<i>cpm/500 mg</i>
0	0	3,590	318
0	0.5	3,895	274
0	1.0	3,650	322
12.5	0	1,764	964
12.5	0.5	3,473	410
12.5	1.0	3,225	330

Each recipient was transfused with  $50 \times 10^6$  lymphocytes containing 65,095 cpm after incubation at 37°C for 5 min with the indicated amounts of neuraminidase and/or sialic acid; recipients were sacrificed at 30 min after transfusion.

Sialic acid, 10 mg/ml saline, was neutralized to pH 6.8 with 1 N NaOH prior to use.

*Recipients of Lymphocytes Incubated with Neuraminidase.*—At early intervals after transfusion, a substantially lower concentration of radioactivity was found in the lymph nodes of recipients of neuraminidase-treated lymphocytes as compared with that found in recipients of untreated lymphocytes. At 1, 3, and 8 hr after transfusion, only 6–10% of the injected radioactivity was recovered per gram of lymph node. However, as the interval between the time of injection and the time of sacrifice increased, the percentage of recovery of radioactivity in the lymph nodes increased. Thus, at 28 and 48 hr after transfusion, the percentage recovery of radioactivity in lymph nodes in recipients of neuraminidase-treated cells was about equal to that obtained in recipients of untreated lymphocytes. The percentage recovery of radioactivity in the livers of these recipients also differed markedly from that found in recipients of untreated lymphocytes. Much greater concentrations of radioactivity were found in the livers of these animals at 1 to 8 hr after transfusion. However, at later intervals, the percentage of total radioactivity recovered in the livers fell markedly. At each interval tested, less radioactivity was found in the spleens of these re-

TABLE VI  
*Effect of Neuraminidase on Distribution of Radioactivity in Tissue of Recipients Sacrificed at Intervals of 1 to 48 Hr after Transfusion of <sup>51</sup>Cr-Labeled Lymphocytes*

Time*	Exp. 1		Exp. 2		Exp. 3		Exp. 4		Exp. 5		Exp. 6	
	Treatment of cells†											
	O	N	O	N	O	N	O	N	O	N	O	N
hr	Percentage recovery of total injected radioactivity per gram lymph node											
1	24	6	18	8	12	8						
3	17	10	12	7								
8	28	7	24	6								
21	44	16	28	15	32	9	40,42	19,10				
28					25	20	20,16	19,18				
48					20	17			13,20,21, 24	16	20,27	21,26
	Percentage recovery of total injected radioactivity per total spleen											
1	12	7	26	8	28	20						
3	28	12	39	10								
8	28	11	36	13								
21	18	18	24	15	16	16	15,25	18,14				
28					14	12	18,22	13,12				
48					17	7			15,16,10, 11	11	13,10	10,9
	Percentage recovery of total injected radioactivity per total liver											
1	12	56	11	61	10	29						
3	8	49	9	57								
8	9	49	11	54								
21	8	16	11	31	8	20	12,16	39,43				
28					9	18	10,25	25,40				
48					8	20			7,8,6,8	18	6,7	13,16
	Percentage recovery of total injected radioactivity per total lung											
1	5	8	17	6	6	6						
3	2	1	20	10								
8	2	2	13	9								
21	2	2	4	6	2	2	2,2	2,2				
28					3	2	2,0.6	2,4				
48					1.2	0.6			2,0.8,0.6 0.6	0.3	0.6 0.6	0.2 0.2

\* Time (hr) recipients sacrificed after transfusion of cells. Each recipient was transfused with  $50 \times 10^6$  lymphocytes in 0.5 ml M 199.

† O, recipients of <sup>51</sup>Cr-labeled lymphocytes incubated without neuraminidase. N, recipients of <sup>51</sup>Cr-labeled lymphocytes incubated with 12.5 units neuraminidase.

recipients as compared to recipients of untreated cells. The pattern of accumulation of radioactivity in the lungs of these animals was similar to that observed in recipients of untreated lymphocytes.

When syngeneic, instead of allogeneic, donors and recipients were used, similar results were obtained (Fig. 1).

*Recipients of Heat-Killed Labeled Lymphocytes.*—Two recipients of heat-

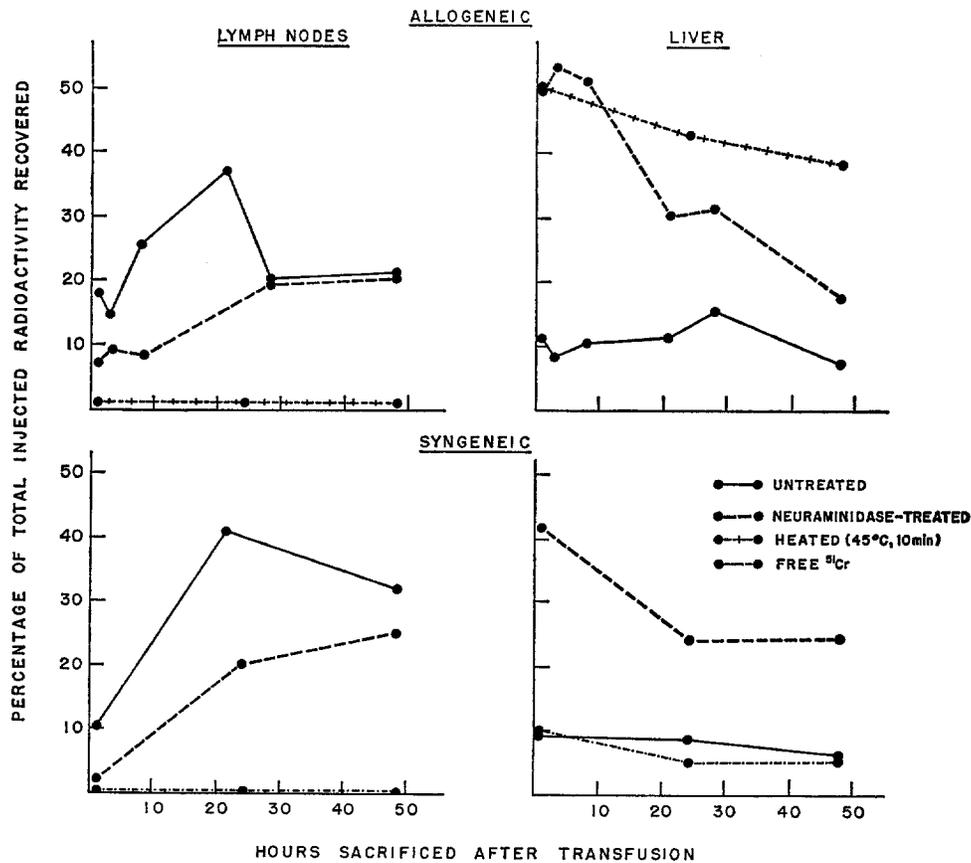


FIG. 1. Uptake of radioactivity in the lymph nodes and liver in recipients of <sup>51</sup>Cr-labeled untreated or neuraminidase-treated or heat-killed lymphocytes or of free <sup>51</sup>Cr.

For recipients of untreated and neuraminidase-treated allogeneic lymphocytes, each point represents the average value for animals in experiments shown in Table VI. Each point for recipients of syngeneic lymphocytes, heat-killed lymphocytes, and free Na<sub>2</sub><sup>51</sup>Cr-O<sub>4</sub> is the average value for two animals. For lymph nodes, the values represent percentages per gram lymph node, whereas for liver the values represent percentages for the total liver. The weights of the livers in recipients ranged from 10–15 g. Each recipient of lymphocytes received 50 × 10<sup>6</sup> cells.

killed labeled lymphocytes were sacrificed 1, 24, and 48 hr after transfusion. At 1 hr virtually no radioactivity accumulated in lymph nodes and an average of 55% of the total injected radioactivity was recovered in the liver. Unlike recipients of neuraminidase-treated cells, the concentration of radioactivity in lymph nodes did not rise in these recipients sacrificed at later intervals. Furthermore, although the recovery of radioactivity in the liver did fall somewhat in animals sacrificed 24 and 48 hr after transfusion, the extent of the loss of label from this organ was less than that observed in recipients of neuraminidase-treated cells sacrificed 21 or more hr after transfusion (Fig. 1).

TABLE VII

*Effect of Partial Hepatectomy 3 Hr after Transfusion of Neuraminidase-Treated or Untreated Lymphocytes on the Concentration of Radioactivity in Lymph Nodes of Recipients Sacrificed at 48 Hr*

Treatment of recipients	Treatment of transfused cells	Lymph nodes	
		Exp. 1	Exp. 2
None	None	2,784	2,747
	12.5 units neuraminidase	2,595	2,256
Partial hepatectomy	None	3,532	2,924
		3,901	3,334
	12.5 units neuraminidase	1,089	1,428
		1,349	1,049

Each recipient transfused with  $50 \times 10^6$  lymphocytes containing, in Exp. 1, 26,825 cpm and, in Exp. 2, 28,012 cpm.

Each value shown is that obtained in one recipient.

*Recipients of Free  $^{51}\text{Cr}$ .*—Two recipients of free  $\text{Na}_2^{51}\text{CrO}_4$  were sacrificed at 1, 24, 48 hr after injection. The pattern of distribution of radioactivity and the percentage of total injected radioactivity recovered in the tissues was unlike that found in any of the preceding groups. Essentially no radioactivity was recovered in lymph nodes and spleens at each interval tested. The liver contained about 10% of the injected dose 1 hr after transfusion, and this fell to about 5% in recipients sacrificed at 24–48 hr (Fig. 1).

*Accumulation of Radioactivity in Lymph Nodes of Recipients Partially Hepatectomized after Transfusion of Neuraminidase-Treated or Untreated Lymphocytes.*—In recipients of neuraminidase-treated lymphocytes, the timing of the fall in percentage recovery of radioactivity in the liver coincided with the rise in percentage recovery in lymph nodes. To determine whether radioactivity initially found in the liver contributed to the late rise of radioactivity in lymph

nodes, recipients were partially hepatectomized 3 hr after transfusion of labeled cells. The animals were then sacrificed 48 hr after transfusion and the radioactivity in lymph nodes assayed. As shown in Table VII, partially hepatectomized recipients of neuraminidase-treated lymphocytes had less radioactivity in lymph nodes than was found in nonhepatectomized recipients of enzyme-treated cells. Partial hepatectomy after transfusion of untreated lymphocytes did not substantially reduce the accumulation of label in lymph nodes.

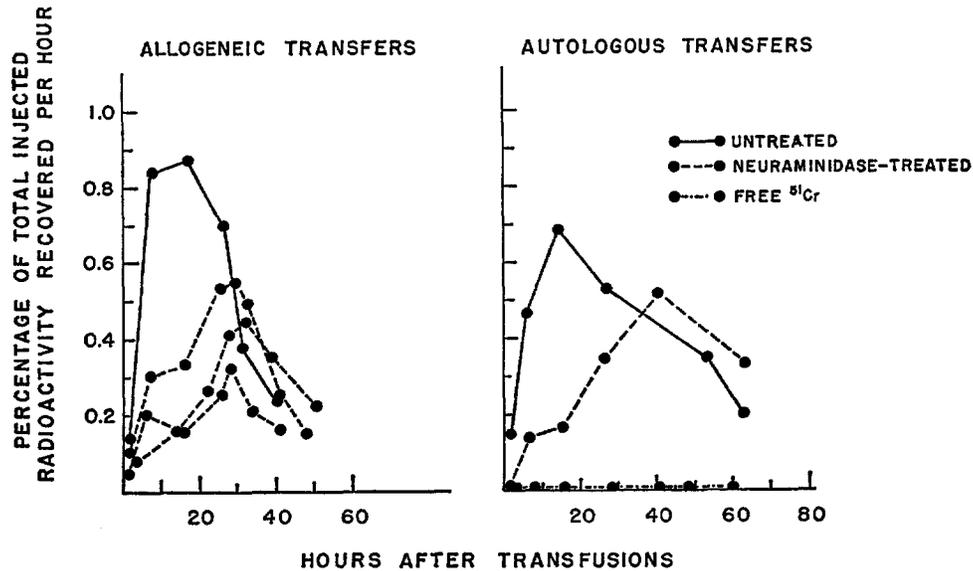


FIG. 2. Recovery of labeled lymphocytes from the main lymphatic duct of recipients of  $^{51}\text{Cr}$ -labeled untreated or neuraminidase-treated lymphocytes or of free  $^{51}\text{Cr}$ .

Recipients of labeled lymphocytes received  $200\text{--}300 \times 10^6$  lymphocytes containing 194,000–586,000 cpm. The recipient of free  $\text{Na}_2\text{-}^{51}\text{Cr-O}_4$  received 500,000 cpm  $^{51}\text{Cr}$  and  $300 \times 10^6$  unlabeled cells in separate sequential injections.

*Recovery of Labeled Cells in the Lymph of Recipients of Neuraminidase-Treated or Untreated Lymphocytes.*—The pattern of recovery of labeled cells in the lymph of animals transfused with neuraminidase-treated lymphocytes was markedly different from that found in recipients of untreated lymphocytes. As illustrated in Fig. 2, the peak recovery of radioactivity associated with cells occurred later in recipients transfused with neuraminidase-treated lymphocytes as compared to that found in recipients of untreated lymphocytes. A similar delay in the peak recovery of radioactivity from recipients of neuraminidase-treated cells was observed when either autologous or allogeneic lymphocytes were transfused. The time of the peak recovery of labeled cells in the lymph of

recipients of neuraminidase-treated lymphocytes roughly coincided with the late rise in accumulation of radioactivity in lymph nodes of animals transfused with enzyme-treated cells. Less of the total injected radioactivity was recovered from recipients of neuraminidase-treated cells compared with recipients of untreated cells. However, when autologous cells were used, the total percentage of radioactivity recovered from recipients of neuraminidase-treated lymphocytes more closely approximated the total recovered from recipients of untreated lymphocytes; 22% of the injected radioactivity was recovered associated with cells when enzyme-treated autologous lymphocytes were transfused compared to 29% in recipients of untreated autologous lymphocytes. When free  $^{51}\text{Cr}$  and unlabeled, untreated autologous lymphocytes were transfused separately, less than 1% of the total radioactivity injected was recovered in the cell pellets from lymph during the entire collection period.

#### DISCUSSION

These results demonstrate that the distribution of radioactivity in the tissues of recipients of  $^{51}\text{Cr}$ -labeled lymphocytes incubated with neuraminidase differs from that found in recipients of untreated lymphocytes. It is likely that the distribution of radioactivity in these experiments, especially that recovered in the lymph nodes and lymph, largely reflects the fate of the donor lymphocytes. In the case of untreated lymphocytes, the distribution of radioactivity generally coincides with the distribution of transfused donor lymphocytes demonstrated by others using various isotopes assayed by radioautographic as well as by counting techniques (1-7). The distribution of radioactivity in recipients of neuraminidase-treated cells is also thought to reflect generally the distribution of transfused lymphocytes since there was a selective accumulation of radioactivity in the lymph nodes of recipients of neuraminidase-treated cells at 28 hr and even as late as 48 hr after transfusion. The most likely way by which radioactivity could selectively accumulate in lymph nodes under the conditions of these experiments is by being associated with donor lymphocytes that enter the nodes. The failure to recover radioactivity in lymph nodes when free  $\text{Na}_2^{51}\text{CrO}_4$  or labeled heat-killed lymphocytes were transfused supports this view. Also, it has been found that  $^{51}\text{Cr}$  which has been incorporated into lymphocytes is very inefficiently reincorporated into other lymphocytes or other cell types (22, 23). Furthermore, in radioautographic studies using untreated and neuraminidase-treated lymphocytes labeled with  $^3\text{H}$ -adenosine, the distribution of labeled lymphocytes coincided with the distribution of  $^{51}\text{Cr}$  in these experiments.<sup>1</sup>

Our interpretation of these results is, therefore, that lymphocytes incubated with the neuraminidase preparation circulate differently than do untreated

<sup>1</sup> Gesner, B. M., J. J. Woodruff, and R. McCluskey. Manuscript in preparation.

lymphocytes. The results imply that, at early intervals after transfusion, many neuraminidase-treated lymphocytes become trapped in the liver while there is a reduction in accumulation of the cells in the lymph nodes and spleen. At later intervals after transfusion, at least some neuraminidase-treated cells emigrate from the liver, selectively accumulate in lymph nodes, and recirculate to the lymph. These effects occur when the relationship between donor and recipient is either allogeneic or syngeneic. And, they are due to the action of the enzyme preparation on the transfused lymphocytes rather than on the recipient animal since no changes in the distribution of radioactivity occurred in recipients when the enzyme preparation and labeled lymphocytes were transfused separately. It is likely that the neuraminidase content of the enzyme preparations was responsible for the effects since: (a) two different neuraminidase preparations with different degrees of purification yielded essentially the same results; (b) the ability of the enzyme preparation to produce the effects was abolished by heating the preparation at 65°C for 10 min before use; and (c) the effects were inhibited by addition of neutralized sialic acid to the *in vitro* reaction mixture.

The alterations in lymphocytes induced by neuraminidase which cause the cells to circulate abnormally are unknown. However, it is likely that the effect was mediated by the interaction between neuraminidase and sialic acid components of the lymphocyte surface. Presumably, changes in the cell surface and/or metabolism of the cell ensuing from this reaction led to the changes in the distribution of the cells. There is little doubt that the surfaces of the cells were altered since they agglutinated in sera which did not cause the agglutination of untreated lymphocytes and since the electrophoretic mobility of the cells was reduced. The later finding indicates that neuraminidase reduced the charge density of the lymphocyte surface and implies that sialic acid was cleaved from the peripheral zone of the cell (11-14); this view is further supported by the finding that free sialic acid could be detected in the supernatants of intact lymphocytes incubated with neuraminidase. It is not likely that the effect is due to "killing" the cells since the evidence indicates that neuraminidase-treated lymphocytes which initially become trapped in the liver subsequently emigrate from this organ and selectively concentrate in lymph nodes. These latter processes do not occur when heat-killed lymphocytes are transfused. In addition to this evidence that the cells remain viable *in vivo*, it has been found that neuraminidase treatment does not abolish the ability of rat lymphocytes to produce lethal graft-against-host reactions when transfused into X-irradiated mice given syngeneic bone marrow cells (24).

The association between neuraminidase-treated cells and the liver appears to be rather selective since the liver was the only organ in which increased radioactivity was found. The finding that heat-killed cells (or remnants thereof) were also trapped in the liver though they had no discernable change in their

electrophoretic mobility indicated that the charge density at the cell surface does not necessarily forecast the *in vivo* fate of these cells. Since all of the transfusions were made via the tail vein, which communicates with the inferior vena cava, presumably all of the transfused cells passed through the lungs before reaching the liver. Yet the accumulation of radioactivity in the lungs of recipients of neuraminidase-treated lymphocytes was not increased. In fact, when changes in the lung did occur, less radioactivity was often recovered in this organ in recipients of neuraminidase-treated cells. One possible explanation for the selective association of neuraminidase-treated lymphocytes with the liver is that changes induced in the cell surface by this enzyme (directly or indirectly) caused the lymphocytes to specifically bind to structures in the liver. Conversely, regeneration of a more normal lymphocyte surface may be necessary before these cells are released from the liver.

One probable cause for the reduced accumulation of neuraminidase-treated lymphocytes in lymphoid tissues was their rapid removal from the circulation in the liver. The finding that selective accumulation of radioactivity in the lymph nodes and spleen was not completely abolished with excessive amount of neuraminidase suggests that at least some neuraminidase-treated lymphocytes which reach these sites at early intervals after transfusion are capable of entering into them. These observations, however, do not decide whether removal of the cells in the liver is the only cause for the decreased accumulation of the cells in these lymphoid tissues. In order to investigate this problem further, several attempts were made to compare the distribution of radioactivity in recipients which were totally hepatectomized and enterectomized before transfusion of neuraminidase-treated or untreated lymphocytes. In all of these attempts, the recipients were extremely ill, and most experiments were unsuccessful because animals died very soon after the procedure. In only one experiment, a hepatectomized recipient of neuraminidase-treated lymphocytes and a comparable recipient of untreated cells survived for 30 min after transfusion. In that study, the accumulation of radioactivity in the cervical nodes in the recipient of enzyme-treated lymphocytes was reduced by 35% and the radioactivity in the lungs was not increased (cpm/unit of weight). This single experiment, in very sick animals, should of course be viewed with great caution. It is, however, consistent with the view that removal of neuraminidase-treated lymphocytes in the liver is not the entire explanation for the reduction in accumulation of the cells in lymph nodes. It is apparent that the relationships between neuraminidase-treated cells and the lymph nodes and spleen remain to be clarified.

The evidence that neuraminidase can alter the fate of lymphocytes suggests that sialic acid constituents of the cell surface play an important role in ensuring the normal fate and distribution of these cells in the body. The findings also imply that reactions involving surface sialic acid can influence the dis-

tribution of lymphocytes without "killing" the cells. Similar effects might be caused not only by neuraminidase but also by other enzymes which by their action alter the integrity of surface sialic acid. Perhaps such processes could account in part for previous, similar results obtained using a crude glycosidase preparation derived from *Clostridium perfringens* (8). In those studies, neuraminidase present in the crude enzyme preparation might also have contributed to the effect. Since sialic acid is one of several saccharide constituents of the cell surface, the present findings are consistent with the hypothesis that cell surface sugars play a physiological role in ensuring the normal distribution of these cells and might act as sites of cellular reactions which influence their fate and behavior in the body (25).

#### SUMMARY

Evidence has been obtained that incubation of rat lymphocytes with neuraminidase, prior to intravenous transfusion into allogeneic or syngeneic recipients, alters the distribution of the cells. Many enzyme-treated lymphocytes initially become trapped in the liver, and there is a decrease in the selective accumulation of these cells in the lymph nodes and spleen. Subsequently, many enzyme-altered cells emigrate from the liver, concentrate in lymph nodes, and recirculate to the lymph. The results suggest that sialic acid constituents of the lymphocyte surface play a critical role in ensuring the normal distribution of these cells in the body. The findings also imply that reactions involving surface sialic acid can markedly alter the fate of lymphocytes without "killing" the cells.

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#### BIBLIOGRAPHY

1. Gowans, J. L., and E. J. Knight. 1964. The route of recirculation of lymphocytes in the rat. *Proc. Roy. Soc. Ser. B. Biol. Sci.* **159**:257.
2. Gowans, J. L. 1957. 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. Pathol.* **38**:67.
3. Gowans, J. L. 1959. The recirculation of lymphocytes from blood to lymph in the rat. *J. Physiol.* **146**:54.
4. Gowans, J. L. 1962. The fate of parental strain small lymphocytes in F<sub>1</sub> hybrid rats. *Ann. N.Y. Acad. Sci.* **99**:432.
5. Shorter, R. G., and J. L. Bollman. 1960. Experimental transfusion of lymphocytes. *Amer. J. Physiol.* **198**:1014.
6. Gowans, J. L. 1959. The transfusion of lymphocytes in experimental animals. *In* The Kinetics of Cellular Proliferation. F. Stohlmann, Jr., editor. Grune and Stratton, New York. 64.
7. Caffrey, R. W., W. D. Reike, and N. B. Everett. 1962. Radioautographic studies of small lymphocytes in the thoracic duct of the rat. *Acta. Haematol.* **28**:145.

8. Gesner, B. M., and V. Ginsburg. 1964. Effect of glycosidases on the fate of transfused lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* **52**:750.
9. Woodruff, J., and B. M. Gesner. 1968. Lymphocytes: Circulation altered by trypsin. *Science*. **161**:176.
10. Gottschalk, A. 1957. Neuraminidase: The specific enzyme of influenza virus and *Vibrio cholerae*. *Biochim. Biophys. Acta*. **23**:645.
11. Wallach, D. F. H., and E. H. Eylar. 1961. Sialic acid in the cellular membranes of Ehrlich ascites carcinoma cells. *Biochim. Biophys. Acta*. **52**:594.
12. Ruhstroth-Bauer, G., G. F. Fuhrmann, E. Gzanzer, W. Kubler, and F. Rueff. 1962. Electrophoretische Untersuchungen an normalen und malignen Zellen. *Naturwissenschaften*. **49**:363.
13. Eylar, E. H., M. A. Madoff, O. V. Brody, and J. L. Oncley. 1962. The contribution of sialic acid to the surface charge of the erythrocyte. *J. Biol. Chem.* **237**:1992.
14. Seaman, G. V. F., and G. Uhlenbruck. 1963. The surface structure of erythrocytes from some animal sources. *Arch. Biochem. Biophys.* **100**:493.
15. Angers, J., and A. Rottino. 1961. The electrophoretic mobility of red blood cells of normal human beings. *Blood* **17**:119.
16. Warren, L. 1959. The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* **234**:1971.
17. Bollman, J. L., J. C. Cain, and J. H. Grindley. 1948. Techniques for collection of lymph from liver, small intestine or thoracic duct of the rat. *J. Lab. Clin. Med.* **33**:1349.
18. Bollman, J. L. 1948. A cage which limits the activity of rats. *J. Lab. Clin. Med.* **33**:1348.
19. Higgens, G. M., and R. M. Anderson. 1931. Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. *Arch. Pathol.* **12**:186.
20. Burnett, F. M., and J. D. Stone. 1947. The receptor destroying enzyme of *V. cholerae*. *Aust. J. Exp. Biol. Med. Sci.* **25**:227.
21. Walop, J. N., Th. A. C. Boschman, and J. Jacobs. 1960. Affinity of N-acetylneuraminic acid for influenza virus neuraminidase. *Biochim. Biophys. Acta*. **44**:185.
22. Bunting, W. L., J. M. Kiely, and C. A. Owen, Jr. 1963. Radiochromium-labeled lymphocytes in the rat. *Proc. Soc. Exp. Biol. Med.* **113**:370.
23. Holm, G., and P. Perlman. 1967. Quantitative studies on phytohemagglutinin induced cytotoxicity by human lymphocytes against homologous cells in tissue culture. *Immunology*. **12**:525.
24. Gesner, B. M., and J. J. Woodruff. 1969. Factors affecting the distribution of lymphocytes. In *Cellular Recognition*. R. T. Smith and R. A. Good, editors. Appleton-Century-Crofts, New York. In press.
25. Gesner, B. M. 1966. Cell surface sugars as sites of cellular reactions; possible role in physiological processes. *Ann. N. Y. Acad. Sci.* **129**:768.