EFFECT OF GLYCOSIDASES ON THE FATE OF TRANSFUSED LYMPHOCYTES

By B. M. Gesner* and V. Ginsburg

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

Communicated by W. Z. Hassid, July 6, 1964

Small lymphocytes circulate through the body by a unique route; they selectively emerge from the bloodstream in lymphoid tissue and recirculate to the blood via lymphatics. Thus, after radioactively labeled thoracic duct lymphocytes (syngeneic or allogeneic) are transfused intravenously, a relatively large percentage of the radioactivity soon accumulates in all lymphoid tissue of the recipient (except the thymus), and by radioautography, labeled small lymphocytes can be seen concentrated in the white pulp of the spleen, Peyer's patches, and lymph nodes. Subsequently, labeled lymphocytes can be recovered from the thoracic duct of the recipient.[1-7] In the present investigation thoracic duct lymphocytes were incubated *in vitro* with glycosidases prepared from *Clostridium perfringens* and the effect of this treatment on their fate in recipients was studied.

Materials and Methods.—Animals: Transfusions were performed between male Osborne-Mendel rats except where noted. These animals are kept as a closed (but not highly inbred) stock. Donor rats weighed 250–300 gm, and recipients 125–175 gm.

Collection and handling of P^{32}-labeled lymphocytes: A donor rat was injected subcutaneously with about 50 μmoles of $H_3P^{32}O_4$ (300 μc) in two equal doses at 12-hr intervals. Twelve hours after the second injection, the thoracic duct was cannulated by usual techniques.[8, 1] The animal was then placed in a Bollman cage and allowed free access to commercial rat cake and 5% dextrose in saline. The lymph was collected for 8–12-hr periods in 5 ml of saline, containing 20 units of heparin per ml, and kept at 0°. Clots, when present, were removed by passing the lymph through a thin layer of cotton wool.

Lymphocytes were separated from the lymph by centrifugation at 4° for 5 min at 150 $\times$ *g*. The supernatant fluid was discarded, and the sedimented cells were gently resuspended in 4.0 ml cold isotonic saline; the suspension was divided into 0.5-ml aliquots, and kept at 0° until used. After appropriate treatment, each sample was injected rapidly into the tail vein of a recipient lightly anesthetized with ether. Recipients in each experiment received the same number of cells from a single 8–12-hr collection period, but from experiment to experiment this number varied from 10 to 75 $\times$ 10^6 cells. The injections were completed within an hour after the cells were separated from the lymph, at which time at least 90% of the P^{32} activity was associated with the lymphocytes, and about 95% of the cells were viable as determined by motility under phase contrast microscopy or by uptake of nigrosin dye.[9]

Assay of radioactivity in organs of recipient rats: At various intervals after injection, the recipient rats were killed by cervical dislocation, and the spleens (and in some instances other organs) were removed. The organs were minced, spread on planchets, and dried on a hot plate. Radioactivity was then assayed with an end-window gas-flow counter. The dried samples weighed approximately 0.2 gm.

Preparation of glycosidases: The Clostridial enzyme fraction used in this study is similar to the glycosidases used by Morgan and his co-workers[10, 11] to release sugars from blood group substances. In a typical preparation, 4 liters of thioglycollate media (Difco) were inoculated with a culture of *Clostridium perfringens* (ATCC 10873) and growth continued for 72 hr at 37°. Bacteria were removed by centrifugation, and to the supernatant solution was slowly added an equal volume of acetone while it was cooled to $-10°$. The precipitate was collected by centrifugation, dissolved in 50 ml of water, and $(NH_4)_2SO_4$ was then added to 70% saturation. The protein precipitate was redissolved in 8 ml of distilled water, and the resulting solution dialyzed overnight against distilled water. After removal of insoluble material by centrifugation, the dialyzed prep-

aration which contained 3.7 mg protein per ml,[12] was heated at 45° for 10 min and used for the experiments described in this paper. This fraction, like the preparations described previously by Morgan and his co-workers,[10, 11] contains many glycosidases and liberates fucose, galactose, and *N*-acetylhexosamine from red cell stroma. It also removes the surface sugars of tumor cells as shown histochemically by Gasic and Gasic.[13] The activities of various preparations were estimated from their ability to liberate *N*-acetylhexosamine from red cell stroma under the following conditions: enzyme protein, 0.4 mg, was incubated at 37° with 3 mg (dry weight) of red cell stroma[14] in 0.4 ml of 0.03 *M* phosphate buffer, pH 7.1. At various times, aliquots were deproteinized[15] and *N*-acetylhexosamine (measured as *N*-acetyl-D-glucosamine) in the supernatant solutions determined.[16] The enzyme preparation used in this paper liberated 0.08 μmole of *N*-acetylhexosamine/hour/mg of enzyme protein. Other preparations liberated 0.02–0.1 μmole under the same conditions.

Results.—Effect of the enzyme preparation on the distribution of transfused lymphocytes: For several hours after transfusion of P^{32}-labeled lymphocytes, a relatively large percentage of the total radioactivity injected was found in the spleen. Assay of this organ in recipients served as a convenient method for determining whether treatment of donor lymphocytes with the enzyme preparation affected their fate in recipients. With the techniques used in this study, 30 min after receiving P^{32}-labeled lymphocytes, about 20–25 per cent of the total radioactivity injected was found in the spleen, in contrast to less than 5 per cent with heat-killed lymphocytes (45° for 10 min), or less than 2 per cent with $H_3P^{32}O_4$.

When P^{32}-labeled lymphocytes were incubated briefly *in vitro* with the enzyme and then transfused intravenously, less radioactivity appeared in the spleen of the recipient than when enzyme was not in the incubation mixture. Increasing amounts of enzyme caused greater reductions in the label recovered from the spleens as shown in Table 1. Injection of enzyme separately, immediately before transfusion of untreated lymphocytes, had no effect. In none of 15 similar experiments was the uptake of radioactivity in the spleen reduced to less than 15–25 per cent of the control value regardless of how much enzyme was used. Similar results were obtained when syngeneic transfers were performed.

Spleens removed at 1, 3, 8, and 12 hr after transfusion of enzyme-treated lymphocytes contained less radioactivity at each time interval than spleens of recipients transfused with untreated lymphocytes. Also, incubation of labeled lymphocytes with enzyme before transfusion resulted in a reduction in the amount of radioactive lymphocytes which subsequently emerged from the thoracic duct of recipients.

TABLE 1

EFFECT OF ENZYME ON THE ACCUMULATION OF RADIOACTIVITY IN THE SPLEEN

Enzyme (μg protein) added to incubation mixture	Cpm in spleen
0	504
10	454
20	300
60	202
120	154
240	130
240*	490

* Lymphocytes and enzyme were incubated separately and injected separately into recipient.

Lymphocyte samples containing 2700 cpm were incubated with the indicated amounts of enzyme for 5 min at 24°. Thirty min after injection, spleens of recipients were removed and assayed for radioactivity.

Increasing the time of incubation of lymphocytes with enzyme led to decreasing amounts of label in the spleen as shown in Table 2. Increasing incubation time beyond 15 min did not result in greater reduction, and the maximum decrease was to about 20–25 per cent of the untreated controls.

The effectiveness of the enzyme preparation in inhibiting lymphocyte accumulation in the spleen was decreased by heating, as shown in Table 3. This decrease

TABLE 2

EFFECT OF INCREASING THE TIME OF INCUBATION OF LYMPHOCYTES WITH ENZYME

Incubation time (min)	Enzyme Added to Incubation Mixture (cpm in spleen) None	15 μg	30 μg
5	484	344	240
10	430	182	90
15	490	108	—

Lymphocyte samples containing 2100 cpm were incubated for the indicated times at 24° with either no enzyme, 15 μg enzyme, or 30 μg enzyme. Thirty min after injection of the sample the spleens of the recipients were removed and assayed for radioactivity.

TABLE 3

EFFECT OF HEATING ON ACTIVITY OF ENZYME

Heat treatment of enzyme	Enzyme added to incubation mixture (μg)	Specific activity	Cpm in spleen
—	—	—	219
None	50	0.090	30
45°C	50	0.080	93
55°C	50	0.030	175
65°C	50	0.015	225

Samples of the crude enzyme preparation were heated at the indicated temperatures for 10 min. Subsequently each sample of the enzyme preparation was tested for its ability to inhibit the uptake of radioactively labeled lymphocytes in the spleen of recipients and to liberate acetylhexosamine from red cell stroma. Each enzyme sample was incubated with a lymphocyte suspension containing 1200 cpm for 5 min at 24°. The spleens were removed from the recipients 30 min after injection of lymphocytes. The specific activity of the enzyme (μmoles *N*-acetylhexosamine liberated/hour/mg protein) was determined as described in the text.

TABLE 4

EFFECT OF SUGARS ON ACTIVITY OF ENZYME

Treatment of Lymphocytes: Sugar	Enzyme, μg	Expt. 1 (cpm in spleen)	Expt. 2 (cpm in spleen)
—	0	628	455
—	20	360	270
D-Glucose	20	300	186
D-Galactose	20	270	236
D-Mannose	20	362	236
N-Acetyl-D-glucosamine	20	238	220
L-Fucose	20	436	372
N-Acetyl-D-galactosamine	20	536	450

To each 0.5 ml suspension of lymphocytes was added 6 mg of the indicated sugar in 25 μl water immediately before addition of enzyme. The mixtures were then incubated for 5 min at 24° and injected into recipients. There were 4100 cpm per sample in Expt. 1 and 2700 cpm per sample in Expt. 2. The spleen was removed from each recipient 30 min after injection of lymphocytes.

TABLE 5

REVERSAL OF EFFECT OF ENZYME BY EQUAL AMOUNTS OF L-FUCOSE AND *N*-ACETYL-D-GALACTOSAMINE

Total sugar (mg)	Enzyme (μg)	Cpm in spleen
—	—	1130
6	—	1024
—	20	620
1	20	600
3	20	922
6	20	858
12	20	904

The conditions of this experiment were the same as those described in Table 4. There were 4500 cpm in each sample.

was roughly paralleled by a loss in ability to liberate *N*-acetylhexosamine from red cell stroma.

The effect of the enzyme was specifically inhibited by L-fucose and *N*-acetyl-D-galactosamine but not by other sugars tested. Two typical experiments are shown in Table 4. Other sugars, including D-glucose, D-galactose, and *N*-acetyl-D-glucosamine, had no effect or actually reduced the accumulation of radioactivity in the spleen. The two active sugars in combination were no more effective than each singly. Maximum protective effect was obtained at a concentration of 6 mg/ml, as shown in Table 5. Sugars injected along with untreated lymphocytes or added to incubation mixtures of lymphocytes

with enzyme at the end of incubation had no effect on the accumulation of lymphocytes in the spleen.

Enzyme treatment not only led to decreased lymphocyte accumulation in the spleen but in lymph nodes as well; also, increased radioactivity was found in the liver (Table 6). *N*-Acetyl-D-galactosamine partially reversed these effects.

TABLE 6

REVERSAL OF THE EFFECT OF ENZYME ON ORGAN DISTRIBUTION OF RADIOACTIVITY BY *N*-ACETYL-D-GALACTOSAMINE

Organ	Untreated lymphocytes		Lymphocytes incubated with 25 μg enzyme		Lymphocytes incubated with 25 μg enzyme and 6 mg *N*-acetyl-D-galactosamine	
	Wt (gm)	Cpm	Wt (gm)	Cpm	Wt (gm)	Cpm
Spleen	0.85	1864	0.92	762	1.00	1384
Lymph nodes*	0.40	590	0.42	124	0.43	350
Liver	7.20	566	7.70	2706	6.90	1340
Lung	1.00	606	1.00	548	1.10	488
Thymus	0.45	12	0.40	20	0.35	4

* The submandibular, cervical, and mesenteric lymph nodes were dissected out free of fat and pooled. The conditions of incubation were the same as described in Table 4, except that in this experiment the recipients were killed 1 hr after injection. Each lymphocyte sample contained 8800 cpm.

Effect of the enzyme preparation on lymphocytes in vitro: The previous results show that treatment with enzyme drastically changes the distribution of transfused lymphocytes in the organs of recipients. After incubation of lymphocytes in 0.5 ml saline with 50 μg enzyme at 24° for 5 min, the cells appeared unchanged morphologically under the light microscope, and there was no substantial increase in leakage of P^{32} from the cells. A centrifuged sample of the treated cells had 8 per cent of its total radioactivity in the supernate solution as compared to 7 per cent for a comparable sample incubated without enzyme. Treated and untreated saline suspensions of lymphocytes, after 5 min incubation, were diluted to 2 ml with TC medium 199 (Difco) containing 15 per cent calf serum and viewed by phase contrast microscopy for 3 hr at 37°. During this time, about 90 per cent of the cells in both samples showed typical lymphocyte motility. Also, at the end of 3 hr there were no differences in the percentage of cells that took up nigrosin dye in the enzyme-treated and untreated samples. Apparently at the time the enzyme-treated cells were injected into recipients (i.e., after 5 min incubation in most experiments), they were not so severely damaged as to prevent them from exhibiting characteristics of "viability" three hr later. However, the lymphocytes were altered by treatment with enzyme. When treated cells were centrifuged after 5 min incubation and then resuspended in fresh syngeneic or allogeneic rat serum, microscopic examination showed the cells in most samples to be loosely agglutinated after 30 min at 37°. This loose agglutination was not observed in control samples.

Discussion.—The mechanisms which control the circulation of lymphocytes are of special interest because these cells circulate through the body by a unique route. Unlike other cell types, lymphocytes emerge from the blood in lymphoid tissue and recirculate to the blood via lymphatics. There is evidence that this process depends, in part, on the unique association between lymphocytes and the endothelial cells of the postcapillary venules in certain lymphoid tissue, for it is through these cells that lymphocytes, and only lymphocytes, emigrate from the blood and enter the parenchyma.[1-7]

The present work indicates that alteration of lymphocytes by glycosidases pro-

foundly affects their fate in the body. This conclusion is based primarily on the finding that the effect of the Clostridial enzyme could be specifically inhibited by the presence of certain sugars in the incubation mixture. Watkins and Morgan found that hydrolysis of A, B, and H blood group substances by enzymes from *Clostridium perfringens* or *Trichomonas foetus* can be prevented by different sugars and that these sugars act by inhibiting their respective glycosidases.[17] Similar mechanisms presumably are responsible for the protective effect of *N*-acetyl-D-galactosamine and L-fucose in our experiments. This view is further supported by the finding of Gasic and Gasic[13] that removal of surface sugars of tumor cells by our enzyme preparation could be completely prevented by the presence of *N*-acetyl-D-galactosamine or D-galactose and partially by L-fucose.

The evidence that glycosidases were responsible for altering the fate of transfused lymphocytes makes it likely that the effect was mediated through a change in the surface sugars of these cells. However, it is not clear from the present experiments how such changes contributed to the observed effect. It is possible, for example, that cleavage of sugars from lymphocytes exposes new sites which lead to their removal from the circulation by reticuloendothelial cells. The loose agglutination of enzyme-treated cells incubated with syngeneic serum *in vitro*, and the fact that there is an increase in radioactivity found in the liver after intravenous injection of these cells, would favor such an interpretation. When erythrocytes are treated with similar enzymes, new antigens develop on their surfaces ("T" antigens) which renders them panagglutinable.[18, 19]

However, it is also possible that the integrity of the sugars on lymphocytes is necessary for these cells to traverse their unique route through the body by acting as sites recognized by complementary structures on the surface of endothelial cells in the postcapillary venules of lymphoid tissue. This interaction could be a critical event which controls the selective emigration of lymphocytes from the blood into lymphoid tissue. Surface sugars are the attachment site of influenza virus to red blood cells,[20, 21] and have been suggested to play a role in the mating of bacteria[22] and of yeast,[23] and in the reaggregation of sponge cells[24] and mammalian cells in culture.[25, 26] The hypothesis that these sugars serve a physiological function by acting as sites of cellular interactions seems attractive for an additional reason. The sugars commonly found in the heterosaccharides of mammalian cell surfaces and glycoproteins include L-fucose, D-mannose, D-galactose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, and sialic acid. D-Glucose is absent.[27] If these heterosaccharides serve as recognition sites, an explanation can be advanced for the curious absence of D-glucose. The efficiency of a recognition surface based on D-glucosyl components would be impaired by free D-glucose much like haptenes interfere with antigen-antibody interactions. Evolutionary selection against this impairment, however slight, would tend to eliminate D-glucose as a component of these surfaces.

* Present address: Department of Medicine, New York University School of Medicine.

[1] Gowans, J. L., *Brit. J. Exptl. Pathol.*, **38**, 67 (1957).

[2] Gowans, J. L., *J. Physiol.*, **146**, 54 (1959).

[3] Gowans, J. L., in *Kinetics of Cellular Proliferation*, ed. F. Stohlman, Jr. (New York: Grune and Stratton, 1958), p. 64.

[4] Shorter, R. G., and J. L. Bollman, *Am. J. Physiol.*, **198**, 1014 (1960).

[5] Everett, N. B., W. O. Rieke, W. O. Reinhardt, and J. M. Yoffey, in *Ciba Foundation Sym-*

posium on Haemopoiesis, ed. G. E. W. Wolstenholme and M. O'Conner (London: Churchill, 1960), p. 43.

[6] Gowans, J. L., and J. Knight, *Proc. Roy. Soc. (London)*, **B159**, 257 (1964).

[7] Marchesi, V. T., and J. L. Gowans, *Proc. Roy. Soc. (London)*, **B159**, 283 (1964).

[8] Bollman, J. L., J. C. Cain, and J. H. Grindley, *J. Lab. Clin. Med.*, **33**, 1349 (1948).

[9] Kaltenbach, J. P., M. H. Kaltenbach, and W. B. Lyons, *Exptl. Cell Res.*, **15**, 112 (1958).

[10] Stack, M. V., and W. T. J. Morgan, *Brit. J. Exptl. Pathol.*, **30**, 470 (1949).

[11] Buchanan, D. J., M. J. Crumpton, and W. T. J. Morgan, *Biochem. J.*, **65**, 186 (1957).

[12] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. I. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[13] Gasic, G., and T. Gasic, *Proc. Soc. Exptl. Biol. Med.*, **114**, 660 (1963).

[14] Dodge, J. T., C. Mitchell, and D. J. Hanahan, *Arch. Biochem. Biophys.*, **100**, 119 (1963).

[15] Somogyi, M., *J. Biol. Chem.*, **160**, 69 (1945).

[16] Reissig, J. L., J. L. Strominger, and L. F. Leloir, *J. Biol. Chem.*, **217**, 959 (1955).

[17] Watkins, W. M., and W. T. J. Morgan, *Nature*, **175**, 676 (1955).

[18] McCrea, J. F., *Australian J. Exptl. Biol. Med. Sci.*, **25**, 127 (1947).

[19] Burnet, F. M., and S. G. Anderson, *Australian J. Exptl. Biol. Med. Sci.*, **25**, 213 (1947).

[20] Burnet, F. M., *Physiol. Rev.*, **31**, 131 (1951).

[21] Gottschalk, A., *Biochim. Biophys. Acta*, **23**, 645 (1957).

[22] Sneath, P. H. A., and J. Lederberg, these PROCEEDINGS, **47**, 86 (1961).

[23] Brock, T. D., *Science*, **129**, 960 (1959).

[24] Moscona, A. A., these PROCEEDINGS, **49**, 742 (1963).

[25] Moscona, A. A., in *Biological Interactions in Normal and Neoplastic Growth*, ed. M. J. Brennan and W. L. Simpson (Boston: Little, Brown and Co., 1962), p. 113.

[26] Weiss, L., in *The Structure and Function of Membranes and Surfaces of Cells, Biochemical Society Symposium No. 22*, ed. D. J. Bell and J. K. Grant (London: Cambridge University Press, 1963), p. 32.

[27] Pigman, W., and D. Platt, in *The Carbohydrates*, ed. W. Pigman (New York: Academic Press, 1957), p. 709.

GENETIC TRANSCRIPTION DURING MORPHOGENESIS

BY ROY H. DOI AND RICHARD T. IGARASHI

DEPARTMENT OF BACTERIOLOGY AND BOTANY, BIOLOGICAL RESEARCH LABORATORIES, SYRACUSE UNIVERSITY

Communicated by Severo Ochoa, July 13, 1964

Studies on genetic transcription indicate that an RNA[1] complementary to DNA is synthesized.[2-4] This "messenger RNA" is believed to serve as the template for protein synthesis.[5] One would expect then to find differences in messenger RNA populations during morphogenesis when obvious changes in structure and function are occurring. Investigations on sporulating bacteria have revealed that morphological changes are accompanied by the appearance of new enzymes,[6] by changes in pathways of electron transport,[7] and by the occurrence of spore-specific substances, e.g., dipicolinic acid.[8] The relative simplicity of the bacterial system has prompted us to use sporulating bacilli as a model system for investigation of genetic transcription during morphogenesis. This paper will describe studies on messenger RNA fractions from three growth phases of *Bacillus subtilis:* sporulation, germination, and step-down transition,[3, 9] i.e., passage from rapid to slow growth. Our data indicate that RNA's are transcribed from unique genetic loci during morphogenesis.