Journal of Supramolecular Structure 8:67–78 (1978)
Cell Surface Carbohydrates and Biological Recognition 503–514

Sialic Acid: A Specific Role in Hematopoietic Spleen Colony Formation

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Vibrio cholerae neuraminidase (VCN) treatment of donor bone marrow cells results in a reduction in the number of hematopoietic colonies (CFUs) formed in the spleens of lethally irradiated mice. Treatment of marrow cells with sodium periodate under mild conditions, known to preferentially oxidize sialic acid, also reduced CFUs while subsequent potassium borohydride reduction restored CFUs to 80% of control levels. Innoculum viability as measured by in vitro incorporation of tritiated precursors into proteins, nucleic acids, and oligosaccharides was unaffected by VCN treatment. The ability of bone marrow cells in culture to respond to the hormone erythropoietin, as measured by the incorporation of ^{59}Fe into cyclohexanone-extractable heme, was also not affected by neuraminidase, making a cytotoxic effect of the VCN preparation unlikely. Incubation of VCN-treated marrow with either β-galactosidase or trypsin had no effect on the VCN-induced reduction in CFUs. These results are consistent with the idea that membrane sialic acid plays a direct and specific role in the implantation and development of CFUs.

Key words: borohydride reduction, spleen colonies, neuraminidase (vibrio cholerae), periodate oxidation, N-acetyl-neuraminic acid, hematopoietic stem cell, erythropoietin

We have been interested in the characterization of the surface properties of the pluripotent hematopoietic stem cell, the precursor of all circulating blood cells. The discrete areas of proliferating hematopoietic tissue found in the spleens of lethally irradiated mice 7–10 days after injection of donor bone marrow (1) are clones derived from a single cell (2, 3), the colony-forming unit (CFUs), believed to represent the hematopoietic stem cell.

Although both bone marrow and spleen will support the proliferation and differentiation of CFUs (4), only a fraction of the total CFUs in the innoculum implant and proliferate in the spleen (5–7). In addition, the spleen has been shown to be a predominantly erythropoietic microenvironment, while bone marrow supports mainly granulopoietic differentiation (4). The inductive events in hematopoietic differentiation are believed to be mediated

Received March 29, 1977; accepted August 2, 1977

Abbreviations: NANA – N-acetylneuraminic acid; VCN – Vibrio cholerae neuraminidase; EPO – erythropoietin; CFU – colony-forming unit; GFH – glucose-free Hanks salt solution

0091-7419/78/0801-0067$02.30 © 1978 Alan R. Liss, Inc

by short-range cellular interactions (8). This differential implantation and subsequent induction of stem cells suggests the existence of a mechanism for CFU site recognition and adhesion, the specificity conferred by cell surface constituents.

Sialic acids occur as the terminal sugar in the oligosaccharide portion of numerous glycoproteins, mucins, and gangliosides (9). Enzymatic removal of sialic acids with neuraminidase (E.C. 1.2.3.18.) has been used to demonstrate an important function for these sugars in numerous biological events. Cleavage of sialic acid destroyed virus receptors (10), and altered both the circulation pattern of lymphocytes (11) and rosette formation (12). Changes in membrane sialic acids have also been shown to alter the velocity of platelet aggregation (13). In addition desialation of serum proteins (14) and erythrocytes (15, 16) results in their clearance from circulation

In an earlier study (17), we examined the role of sialic acid in stem cell function. Treatment of donor marrow with Vibrio cholerae neuraminidase prior to transplantation into lethally-irradiated hosts reduced the number of macroscopic spleen colonies, whereas pretreatment with a variety of proteases and glycosidases had no effect on CFUs. The reduction was independent of enzyme concentration at maximal levels and was lost after heat inactivation of VCN. These results are consistant with an enzymatic rather than cytotoxic effect of VCN. We have therefore proposed a role for sialic acid in the formation of at least some spleen colonies. This study confirms and extends our earlier findings. The VCN-induced reduction in spleen colonies could not be attributed to a decrease in viability of the innoculum, since the ability of the marrow to incorporate precursors of macromolecular synthesis is unaffected after VCN treatment. The response of VCN-treated marrow to the hormone erythropoietin was also unchanged.

Mild periodate oxidation of marrow effected a similar decrease in CFUs as VCN treatment. Periodate oxidation of VCN-treated marrow caused no further reduction in spleen colonies, indicating that both treatments were affecting the same moiety. Borohydride reduction of periodate-treated marrow restored CFU levels to 74–85% that of controls. Sequential incubation of neuraminidase-treated marrow with either β-galactosidase or trypsin did not alter the reduction in spleen colonies. These results suggest a direct and specific role for intact surface sialic acid residues in the formation of at least some hematopoietic spleen colonies.

MATERIALS AND METHODS

Female CF1 mice, 6–10 weeks old were used throughout the study. Cell suspensions were prepared by flushing marrow from tibiofibulae and femora by injection of glucose-free Hanks salt solution (GFH), pH 7.4, containing 0.1 mg/ml deoxyribonuclease I with a syringe and 23 gauge needle. Clumps of cells were dispersed by gentle passage through a pasteur pipette and the suspension filtered through a 200-mesh stainless steel screen. After 10 min at room temperature, cells were washed in a 30-fold dilution of GFH, pelleted by centrifugation, resuspended in GFH, pH 7.4, at 4×10^7 cells/ml and incubated with or without 20 U/ml VCN. GFH contains 1.3 mM $CaCl_2$. One unit of VCN is the enzyme activity required to release 1 μg of sialic acid from its substrate in 15 min at 37°C. VCN was assayed using N-acetylneuraminelactose as a substrate and the released sialic acid estimated by the method of Warren (18), using crystalline N-acetylneuraminic acid (NANA) as the standard.

The culture system has been extensively described elsewhere (19). Briefly, the medium at pH 6.9 contained 65% NCTC 109 made 30 mM with morpholinopropane sul-

fonic acid, 1 mM with L-glutamine. 30% fetal calf serum, and 5% mouse serum containing 2 μg/ml ferric nitrate. The complete medium was supplemented to 0.05 mg/ml gentamicin. All sera were heat inactivated for 30 min at 56°C. Cells were suspended at 15×10^6 nucleate cells/ml in complete medium and 0.2 ml of suspension were plated in 15 × 10 mm plastic tissue culture dishes (Linbro FB-16-24-TC). The plates were sealed and incubated at 37°C.

Five replicate cultures were established for each group. For heme synthesis experiments sheep plasma erythropoietin, 50 mU/ml (Connaught, step III, 2.2 units/mg protein, lot 3005-1) in NCTC 109 was added to the appropriate cultures at the start of incubation. After 20 h, 10 μl of ^{59}Fe-labeled mouse serum (0.4 μCi) were added to each plate and incubation continued for an additional 4 h. Cultures were stopped by the addition of 2 ml ice-cold 0.15 M NaCl/5 mM sodium phosphate, pH 7.4, (PBS) and transferred to test tubes. Plates were washed with an additional 1 ml PBS and the cells pelleted by centrifugation. Heme synthesis was measured as previously described (19).

In the remaining cultures, 10 μl of the appropriate isotope were added at the start of incubation. [5-^{3}H] Uridine (29 Ci/mM, 0.2 μCi) containing cultures were incubated for 0.5 h; whereas [methyl-^{3}H] thymidine (41 Ci/mM, 0.2 μCi), L-[4,5-^{3}H] leucine (60 Ci/mM, 0.2 μCi), and D-[1-^{3}H] glucosamine (3 Ci/mM, 1.0 μCi) containing cultures were incubated for 4 h. Cultures were terminated as described above. The cell pellet was washed twice in ice-cold PBS, made to 5% ice-cold trichloroacetic acid (TCA), and allowed to precipitate overnight. The precipitate was centrifuged at 1,000 × g, washed twice in 5% TCA, and solubilized in 0.5 ml NCS Tissue Solubilizer. After the addition of 10 ml toluene-based scintillation cocktail and transfer to vials, the samples were counted.

Mice which served as recipients in the CFU assay received 850 rads of ^{60}Co irradiation by rotation in the field of a ^{60}Co therapy source (Picker model, V9M/60). After 24 h, mice received 10^5 nucleate marrow cells via tail vein injection. Seven days later mice were sacrificed and the spleens removed and fixed in alcohol-formalin-acetic acid (750 ml 70% ethanol, 250 ml formaldehyde, 50 ml glacial acetic acid). Following at least 24 h of fixation colonies were counted under a dissecting microscope at 15 ×.

Marrow cells at 4×10^7 cells/ml which had been incubated previously in either VCN or buffer were incubated with β-galactosidase (20 U/ml, E. coli) or trypsin (Type III, 0.25%) in GFH, pH 7.4, for 1 h at 37°C. After trypsinization, cells were treated with excess soybean trypsin inhibitor. After one wash in a 30-fold dilution of GFH, cells were resuspended in GFH and 10^5 nucleate cells injected into lethally irradiated mice.

The release of material from tritium-labeled cells was used to assay the action of β-galactosidase and trypsin on marrow cells. Galactose and its derivitives were labeled with tritium at the C6 position (20, 21) after galactose oxidase treatment with sodium [^{3}H]-borohydride reduction (22). Galactose oxidase was purified by column chromatography. Two hundred and fifty units of galactose oxidase (Polyporus circinatus) dissolved in 0.5 ml 50 mM sodium phosphate, pH 7.0, was applied to a 1 × 13 cm Sepharose 6B column and eluted with 50 mM sodium phosphate buffer, pH 7.0. Galactose oxidase activity was measured by the Galactostat procedure (Worthington Biochemicals). The retarded material containing galactose oxidase activity was pooled. Marrow cells in GFH (2×10^7/ml) were incubated for 1 h at 37°C with 1.25 U/ml galactose oxidase. The reaction was terminated by the addition of 30 ml ice-cold PBS. After 2 washes with PBS, cells were treated for 15 min at 25°C with 1 mM freshly-prepared sodium [^{3}H] borohydride (661 mCi/mM), The reaction was terminated, and the cells washed 3 times with ice-cold PBS. Cells were resuspended in GFH, and treated with β-galactosidase or trypsin as described above.

To label sialic acid residues, marrow cell suspensions (2×10^7 cells/ml) were prepared in PBS, pH 7.4. Periodate oxidation was performed in 0.4 mM sodium periodate/PBS for 15 min at 25°C. The reaction was terminated by the addition of 30 ml ice-cold PBS, and the cells centrifuged. The washing procedure was repeated twice. Marrow cells (2×10^7 cells/ml) were reduced in either 1 mM freshly-prepared potassium borohydride or sodium [^{3}H] borohydride (178 mCi/mM)/PBS. After 15 min at 25°, the reaction was terminated and the cells washed as described above.

The products released by VCN hydrolysis of periodate oxidized/[^{3}H] borohydride reduced marrow were chromatographed on a Sephadex G-75 column (1×25 cm) with distilled water as eluant. One milliliter fractions were collected and monitored by liquid scintillation counting. The tritium-labeled, VCN-released material (98%) was present in the retarded peak. These retained fractions were pooled and lyophilized. This material was dissolved in a small quantity of water, and applied to Whatman No. 1 paper.

Descending paper chromatography was run for 18 h at room temperature. The solvent system employed was n-butyl alcohol/acetic acid/water (5/2/2). The paper was dried; a vertical strip containing the standards cut and dipped in $AgNO_3$ (23) or sprayed with thiobarbituric acid (24). The remaining chromatogram was cut into 1-cm horizontal strips, which were placed in a counting vial with 0.1 ml water. After 4 h, 0.5 ml NCS solubilizer was added and incubation continued for an additional 4 h. Ten milliliters of scintillation cocktail was added and radioactivity determined.

VCN was purchased from Calbiochem, La Jolla, California. Trypsin Type III, soybean trypsin inhibitor, DNase I, galactose oxidase, synthetic NANA, and N-acetylneuraminelactose were from Sigma Chemical Company, St. Louis, Missouri. NCTC 109 was from Microbiological Associates, Bethesda, Maryland, fetal calf serum from North American Biologicals, Miami, Florida, NCS and radioisotopes from Amersham/Searle, Arlington Heights, Illinois. CF-1 mice were purchased from Blue Spruce Farms, Altamont, New York.

RESULTS

Viability of Neuraminidase-Treated Marrow

Table I shows the effect of VCN treatment on the ability of bone marrow to incorporate precursors of nucleic acids, protein, and carbohydrate into acid insoluble material. Enzyme treatment of marrow had no effect on the 4-h incorporation of tritiated thymidine, leucine, or glucosamine. In addition, the 0.5-h incorporation of uridine was also unaffected. These results indicate that the reduction in spleen colonies caused by VCN is not mediated through a general decrease in innoculum viability.

The ability of marrow to respond to the hormone erythropoietin (EPO), the specific inducer of erythroid differentiation, was also tested after VCN treatment (Table II). VCN-treated marrow responded to EPO stimulation with the same level of ^{59}Fe incorporation into heme as untreated marrow.

Treatment of Neuraminidase-Treated Marrow With β-Galactosidase or Trypsin

Sialic acid may play a direct or an indirect role in spleen colony formation. A requirement for sialic acid in CFU adhesion to spleen sites would indicate a direct role for NANA; whereas if NANA functions by masking binding sites, an indirect role is indicated. Serum glycoprotein survival times are markedly shortened after their desialation (14). In that system, NANA has been shown to have an indirect role, since cleavage of sialic acid exposes galactose residues, which results in the binding of the glycoprotein to hepatic sites (25, 26). We therefore tested for a similar indirect mechanism to account for the reduction in

TABLE I. Incorporation of Precursors of Macromolecules Following Neuraminidase Treatment*

Treatment	cpm[a]	N[b]	p[c]
	Thymidine[d]		
Control	7,217 ± 434	5	–
Neuraminidase	7,460 ± 237	5	NS[f]
	Uridine[e]		
Control	8,039 ± 829	5	–
Neuraminidase	8,055 ± 75	5	NS
	Leucine[d]		
Control	10,446 ± 705	5	–
Neuraminidase	10,527 ± 820	5	NS
	Glucosamine[d]		
Control	2,214 ± 84	5	–
Neuraminidase	2,171 ± 264	5	NS

*Marrow cell suspensions at 4×10^7 cells/ml were prepared in glucose-free Hanks salt solution (GFH), pH 7.4, and incubated with or without 20 U/ml Vibrio cholerae neuraminidase for 1 h at 37°C. Cells were washed once with a 30-fold dilution of GFH and resuspended at 15×10^6 nucleate cells/ml in 65% NCTC 109, 30% fetal calf serum, and 5% mouse serum; 0.2 ml of the suspension was plated in 15 × 10-mm plastic tissue culture plates, sealed, and incubated at 37°C. Isotope (10 μl) in NCTC 109 was added at the start of the culture period. [5-^{3}H]Uridine (29 Ci/mM, 0.2 μCi) containing cultures were incubated for 0.5 h; whereas [methyl-^{3}H] thymidine (41 Ci/mM, 0.2 μCi), L-[4,5-^{3}H] leucine (60 Ci/mM, 0.2 μCi), and D-[1-^{3}H] glucosamine (3 Ci/mmol, 1.0 μCi) were incubated for 4 h. Cultures were terminated by the addition of 1 ml ice-cold phosphate buffered saline (PBS). Cells were washed twice in PBS and precipitated overnight in cold 5% trichloroacetic acid (TCA). After 2 washes in 5% TCA, the precipitates were prepared for liquid scintillation counting.

[a]Counts per minute/10^6 nucleate cells ± standard deviation.

[b]Number of cultures.

[c]Probability determined from Student's t test, significance level $p < 0.05$.

[d]4 h pulse, 4 h culture.

[e]0.5 h pulse, 0.5 h culture.

[f]NS: not significant.

colony-forming ability after VCN treatment. Incubation of neuraminidase-treated marrow with β-galactosidase (Table III) had no effect on the reduction in spleen colonies. Similarly, treatment of neuraminidase-treated marrow with trypsin had no effect on the reduction in spleen colonies (Table IV).

To test if β-galactosidase and trypsin were removing material from marrow cells, galactose and its derivitives were labeled with galactose oxidase followed by sodium [^{3}H]-borohydride reduction (Table V). Appreciable incorporation of tritium occurred when marrow cells were treated with VCN and the newly exposed galactosyl residues labeled with tritium. Both β-galactosidase and trypsin released a portion of this radioactivity (Table VI). Treatment of labeled cells with β-galactosidase released only 26% of the total radioactivity, whereas trypsin hydrolysis released 59% of the total radioactivity.

Effect of Periodate Oxidation on Spleen Colonies

Periodate oxidation, under conditions mild enough to be specific for carbohydrates (27, 28) was used to test for a specific role of sialic acid in spleen colony formation (Table VII). In all experiments, periodate oxidation decreased spleen colonies from un-

TABLE II. Incorporation of ^{59}Fe Into Heme Following Neuraminidase (VCN) Treatment*

Group	Percent uptake[1]
Control	0.07 ± 0.04[a]
Control + EPO	0.22 ± 0.05[b]
VCN	0.12 ± 0.03[c]
VCN + EPO	0.21 ± 0.02[b]

*Marrow cell suspensions at 4 × 10^7 cells/ml were prepared in glucose-free Hanks salt solution (GFH), pH 7.4, and incubated with or without 20 units Vibrio cholerae neuraminidase for 1 h at 37°C. Cells were washed once with a 30-fold dilution of GFH, and resuspended at 15 × 10^6 nucleate cells/ml in 65% NCTC 109, 30% fetal calf serum, 5% mouse serum. The suspension (0.2 ml) was plated in 15 × 10-mm plastic tissue culture plates, and 50 mU/ml erythropoietin (EPO) added to appropriate cultures. Cultures were sealed and incubated at 37°C. After 20 h, 10 μl of ^{59}Fe-labeled mouse sera (0.4 μCi) were added to each plate, and incubation continued for an additional 4 h. Cultures were terminated and the cells washed twice in ice-cold phosphate buffered saline and the incorporation of ^{59}Fe into cyclohexanone-extractable heme measured. Results are expressed as the average percent uptake of 5 replicate cultures ± standard deviation.

[1]Percent uptake of ^{59}Fe per 10^6 nucleate cells ± standard deviation.

Values followed by different letters differ from other experimental values at the significance level of $p < 0.05$, determined from Student's t test.

TABLE III. Treatment of Neuraminidase-Treated Marrow With β-Galactosidase: Effect of Spleen Colonies*

Treatment	Colonies[1]	N[2]
Control	9.0 ± 2.4[a]	15
β-Galactosidase	10.4 ± 0.6[a]	8
Neuraminidase	4.3 ± 0.9[b]	8
Neuraminidase + β-Galactosidase	3.0 ± 1.1[b]	8

*Marrow cell suspensions at 4 × 10^7 cells/ml were prepared in glucose-free Hanks salt solution (GFH), pH 7.4, and incubated with or without 20 U/ml Vibrio cholerae neuraminidase for 1 h at 37°C. Cells were washed once with a 30-fold dilution of GFH and incubated with or without 20 U/ml β-galactosidase (E. coli). After one wash in GFH, 10^5 nucleate marrow cells were transfused via tail vein injection into female CFl mice which had received 850 rads of ^{60}Co irradiation 24 h previously. After 7 days, mice were sacrificed and spleens removed and fixed for at least 24 h in alcohol-formalin-acetic acid. Macroscopic spleen colonies were counted under a dissecting microscope at 15 × magnification.

[1]Colonies/10^5 nucleate marrow cells injected, average ± standard error.

[2]Number of spleens.

Values followed by different letters differ from other experimental values at the significance level of $p < 0.05$, determined from Student's t test.

treated controls (average 51%, range 41–58%), whereas subsequent borohydride reduction restored spleen colonies to 80% of controls (range 74–81%).

Treatment of marrow with VCN, followed by periodate oxidation effected no further reduction in spleen colonies. To test if both treatments were affecting the same surface constituents, periodate-oxidized marrow was reduced with tritiated borohydride, and then incubated with VCN (Table VIII). All of the radioactivity except that which could be attributed to the borohydride treatment alone was released by VCN. The products of VCN cleavage were chromatographically identified (Table IX). The postenzyme incubation

TABLE IV. Treatment of Neuraminidase-Treated Marrow With Trypsin: Effect of Spleen Colonies

Treatment	Colonies[1]	N[2]
Control	16.7 ± 1.3[a]	8
Trypsin	16.9 ± 1.5[a]	8
Neuraminidase	8.8 ± 1.8[b]	8
Neuraminidase + Trypsin	8.5 ± 2.0[b]	8

*Cell suspensions at 4 × 10^7 marrow cells/ml were prepared in glucose-free Hanks salt solution (GFH), pH 7.4, and incubated with or without 20 U/ml Vibrio cholerae neuraminidase for 1 h at 37°C. Cells were washed once with a 30-fold dilution of GFH and incubated in either 0.25% trypsin/GFH or GFH for 1 h at 37°C. After the addition of excess soybean trypsin inhibitor and one wash in GFH, 10^5 nucleate cells were transfused via tail vein injection into female CFI mice which had received 850 rads of ^{60}Co irradiation 24 h previously. After 7 days, mice were sacrificed and spleens removed and fixed for at least 24 h in alcohol-formalin-acetic acid. Macroscopic spleen colonies were counted under a dissecting microscope at 15 × magnification.

[1]Colonies/10^5 nucleate marrow cells injected, average ± standard error.

[2]Number of spleens.

Values followed by different letters differ from other experimental values at the significance level of $p < 0.05$, determined from Student's t test.

TABLE V. Products of Galactose Oxidase Treatment and Sodium [^{3}H] Borohydride Reduction of Marrow Cells

Cells reduced with sodium [^{3}H] borohydride	cpm[a]
Cells (untreated)	1,713
Cells incubated with neuraminidase	2,214
Cells incubated with galactose oxidase	2,180
Cells incubated with neuraminidase and galactose oxidase	9,831

*Marrow cell suspensions at 4 × 10^7 cells/ml were prepared in glucose-free Hanks salt solution (GFH), pH 7.4, and incubated with or without 20 U/ml Vibrio cholerae neuraminidase for 1 h at 37°C. Cells were washed once with a 30-fold dilution of GFH. Cell suspensions (2 × 10^7 cells/ml) were incubated with or without 1.25 U/ml purified galactose oxidase (Polyporus circinatus) for 1 h at 37°C. Cells were washed 3 times with phosphate buffered saline (PBS). Cell suspensions (2 × 10^7/ml) in PBS were treated with 1 mm freshly prepared sodium [^{3}H] borohydride for 15 min at 25°C. The reaction was terminated by the addition of a 30-fold dilution of ice-cold PBS. After 2 additional washes with PBS, aliquots were counted for radioactivity.

[a]Counts per minute per 10^6 cells.

supernatant was applied to a 1 × 25-cm Sephadex G-75 column and eluted with distilled water. Ninety-seven percent of the radioactivity was found in the peak of material of < 1,000 molecular weight. This retained material was subjected to descending paper chromatography using n-butyl alcohol/acetic acid/water (5:2:2) as the solvent. Essentially all the radioactivity migrated with either the C^7 analog of sialic acid ($NANA_7$), or N-glycolylneuraminic acid.

Both treatments are therefore affecting the same moiety. These results indicate a direct function for sialic acid in colony formation. The effects of cleavage or modification

TABLE VI. Release of Tritium-Labeled Material From Asialo Marrow Cells by β-Galactosidase and Trypsin

Treatment	cpm[a]
Labeled cells	7,118
Cells after β-galactosidase	5,532
Supernatant after β-galactosidase	1,933
Cells after trypsin	3,074
Supernatant after trypsin	4,491

*Desialated mouse marrow was labeled with tritium by galactose oxidase/sodium [^{3}H] borohydride treatment. Cell suspensions (2 × 10^7 cell/ml) in glucose-free Hanks salt solution (GFH), pH 7.4, were treated with either 20 U/ml β-galactosidase or 0.25% trypsin for 1 h at 37°C. Cells were pelleted by centrifugation and an aliquot of the supernatant counted for radioactivity. After 3 washes in GFH, cells were counted for radioactivity.
[a]Counts per minute per 10^6 cells.

of NANA are not additive, but result in parallel reductions in spleen colonies. They therefore appear to be exerting their effect through sialic acid.

DISCUSSION

In this study we confirm and extend our earlier findings (17) that VCN treatment of donor bone marrow reduced the number of hematopoietic spleen colonies. The reduction in spleen colonies could not be attributed to a general decrease in the viability of the innoculum, such as that reported by Yuhas (29), since enzyme-treated marrow cells incorporated macromolecular precursors to the same extent as control cells. In addition, VCN treatment has been shown to have no effect on bone marrow oxygen consumption (30). Longer term culture viability was also unaffected since the ability of marrow to respond to erythropoietin was not inhibited after VCN treatment. These results argue against a cytotoxic effect of VCN on mouse bone marrow. Since the action of VCN is directed mainly towards the glycoprotein-bound NANA in intact cells (31), we feel it safe to assume that the reduction in spleen colonies we find after VCN treatment is a result of the cleavage of NANA from cell surface glycoproteins.

It is interesting that only a portion of the total CFUs are lost after VCN treatment. Although in a previous paper (17) we reported that maximal reduction in spleen colonies occurred when marrow cells were incubated with 1 U/ml VCN and that addition of a 50-fold excess of enzyme caused no further reduction, it still is possible that the partial reduction reflects an incomplete accessibility of sialic acid sites on marrow cells to VCN. On the other hand this differential susceptibility to VCN may indicate a heterogeneity within the CFUs pool. For example, VCN lability may reflect the age structure within the CFUs compartment, or it may be indicative of the stage of CFUs within the cell cycle. Edelman (32) has suggested that resting cells in G_1 may actually represent cohorts of cells temporally distributed in a random manner. Since CFUs are normally in a nonproliferative state (33), CFUs may only be susceptible to VCN for a limited period.

The possible indirect role of sialic acid in spleen colony formation was examined. Subsequent incubation of VCN-treated marrow with β-galactosidase or trypsin released a portion of the surface galactosyl residues, but did not alter the reduction in spleen colonies.

TABLE VII. Effect of Periodate Oxidation on Spleen Colonies*

Treatment	Colonies[1]	N[2]
	Experiment 1	
Control	17.9 ± 1.5[a]	8
Borohydride	16.0 ± 1.4[a]	8
Periodate	9.6 ± 1.4[b]	8
Periodate + Borohydride	13.2 ± 1.4[c]	8
	Experiment 2	
Control	15.4 ± 1.9[a]	6
Neuraminidase	5.9 ± 1.8[b]	6
Borohydride	14.2 ± 1.6[a]	6
Periodate	9.0 ± 1.8[b]	8
Neuraminidase + Periodate	7.2 ± 2.3[b]	7
Periodate + Borohydride	13.1 ± 2.3[a]	7
	Experiment 3	
Control	14.4 ± 1.1[a]	9
Neuraminidase	3.6 ± 1.5[b]	10
Borohydride	14.0 ± 1.8[a]	10
Periodate	6.0 ± 1.6[c]	10
Neuraminidase + Periodate	3.2 ± 0.7[b]	10
Periodate + Borohydride	11.8 ± 2.2[a]	10

*Marrow cell suspensions at 4×10^7 cells/ml were prepared in glucose-free Hanks salt solution (GFH), pH 7.4, and incubated with or without 20 U/ml Vibrio cholerae neuraminidase for 1 h at 37°C. Cells were washed once with a 30-fold dilution of 0.15 M NaCl/0.005 M sodium phosphate, pH 7.4 (PBS). Cell suspensions (2×10^7 cells/ml) were oxidized in 0.4 mM sodium periodate/PBS for 15 min at 25°C and washed 3 times in PBS. Reduction was performed in freshly prepared 1 mM potassium borohydride/PBS for 15 min at 23°C, and cells washed 3 times in PBS. Marrow cells in GFH (10^5 nucleate cells) were transfused via tail vein injection into female CFl mice which had received 850 rads of ^{60}Co irradiation 24 h previously. After 7 days, mice were sacrificed and spleens removed and fixed for at least 24 h in alcohol-formalin-acetic acid. Macroscopic spleen colonies were counted under a dissecting microscope at 15 × magnification.

[1]Colonies/10^5 nucleate marrow cells injected ± standard error.

[2]Number of spleens.

Values followed by different letters differ from other experimental values at the significance level of $p < 0.05$, determined from Student's t test.

It seems unlikely that the loss of colony forming ability is mediated through the exposure of penultimate galactose residues following the cleavage of NANA. It is possible, however, that β-galactosidase did not remove all the terminal galactose due to steric hindrance or to different positional specificity. In addition, since sialic acid is often linked to N-acetyl-galactosamine, it is possible that this sugar may play a role in colony formation. However, the experiments in which sialic acid was modified in situ suggest that sialic acids play a direct rather than a indirect role in colony formation.

Periodate oxidation of surface sialic acids also reduced spleen colonies. Although it is known that periodate may act at various locations in a carbohydrate or protein polymer, the mild conditions employed in this study suggest that sialic acid residues at the cell surface are preferentially attacked (27, 28).

TABLE VIII. Products of Vibrio Cholerae Neuraminidase Treatment of Modified Marrows Cells

	cpm[a]
Experiment 1	
[^{3}H] Borohydride reduced cells	1,197
Periodate oxidized, [^{3}H] borohydride reduced	
Cells	3,630
Cells after neuraminidase treatment	992
Supernatant after neuraminidase treatment	2,686
Experiment 2	
[^{3}H] Borohydride reduced cells	730
Periodate oxidized, [^{3}H] borohydride reduced	
Cells	3,210
Cells after neuraminidase treatment	763
Supernatant after neuraminidase treatment	2,098

*Marrow cell suspensions were prepared in glucose-free Hanks salt solution (GFH), pH 7.4, and washed twice in PBS. Cell suspensions (2×10^7 cells/ml) were oxidized in 0.4 mM sodium periodate in phosphate buffered saline, pH 7.4 (PBS) for 15 min at 25°C, and washed 3 times in PBS. Reduction was performed in 1 mM freshly prepared sodium [^{3}H] borohydride (178 mCi/mM)/PBS for 15 min at 25°C. After 3 washes in PBS, cells were suspended in GFH, and an aliquot counted for radioactivity, then 20 U/ml Vibrio cholerae neuraminidase was added, and the suspension incubated for 1 h at 37°C. Cells were pelleted by centrifugation and an aliquot of the supernatant counted for radioactivity. After one wash in GFH, cells were counted for radioactivity.

[a]Counts per minute per 10^6 cells.

TABLE IX. Chromatographic Behavior of Neuraminidase-Released Material From Tritium-Labeled Marrow Cells*

Substance applied	Mobility in solvent[a]
N-acetylneuraminic acid[c,d]	1.00
$NANA_7$[c,d]	1.40
N-glycolylneuraminic acid[e]	0.54
Neuraminidase cleavage product[b]	1.38, 0.60

*Mouse marrow cells (2×10^7 cells/ml) labeled with tritium by periodate oxidation/[^{3}H] borohydride reduction were incubated with 20 U/ml VCN in GFH, pH 7.4, for 1 h at 37°C. Cells were pelleted by centrifugation and the supernatant chromatographed on Sephadex G-75. The radioactive fractions were pooled and lyophilized. A sample of this material, along with standards, was applied to Whatman No. 1 paper. Descending chromatography was run for 18 h at room temperature. The paper was dried and cut into 2 vertical strips. One was sprayed with thiobarbituric acid, or dipped in $AgNO_3$; the other was cut into 1-cm horizontal pieces and radioactivity determined.

[a]Mobility relative to N-acetylneuraminic acid, solvent system n-butyl alcohol/acetic acid/water (5:2:2).

[b]These bands contained tritium.

[c]Positive reaction with $AgNO_3$.

[d]Positive reaction with thiobarbituric acid.

[e]Value reported for the mobility of N-glycolylneuraminic acid.

In addition, we have shown that periodate oxidation selectively modified sialic acid residues (Table VI) and that neuraminidase released all of these modified residues (Table V). It has been proposed that the generation of an aldehyde at C^7 of sialic acid by periodate oxidation may result in its interaction with other membrane components through a Schiff's base reaction (34). Presant and Parker (28) have shown that mild periodate oxidation of lymphocytes, at conditions similar to those used in this study, results in specific surface conformational changes. A similar interaction on CFUs could result in a rearrangement of membrane sialic acid-containing components, thereby removing them from participation in colony formation. Such a mechanism has been proposed to account for the reduced adhesion of mycoplasma to periodate-treated epithelial tissue (35). Since periodate oxidation of VCN-treated marrow effected no further reduction in spleen colonies, both treatments appear to exert their reduction through sialic acid.

An interesting finding of this study is the return of colony-forming ability to periodate-treated marrow following subsequent reduction with borohydride, suggesting the importance of the polyhydroxylated side chain of sialic acids in colony formation, Since the anionic sites of sialic acid are not affected by periodate, a charge related role of NANA in colony formation is not supported. Jeanloz and Codington (36) point out the polyhydroxylated side chain at C^7–C^9 of sialic acid appears to be unique among surface components and they suggest that the complex biochemical pathway of its synthesis points to its importance in biological events.

ACKNOWLEDGMENTS

This work was supported by N.I.H. grant AM-174-34-03 and University of Nebraska Research Council NIH Biomedical Support Grant RR-07055. Special thanks to Dr. John McGreer for the generous use of his radiation facilities, and to his staff for their valuable assistance. Thanks to Dr. Gilbert Ashwell for the gift of $NANA_7$.

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