Failure of Neuraminidase to Unmask Allogeneic Antigens on Cell Surfaces[1] (35950)

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Vibrio cholerae neuraminidase (VCN) cleaves the 2–3′ and 2–6′ glycosidic linkages between sialic acid residues and the mucopolysaccharides on the cell surfaces (1) without decreasing cell viability (2, 3). A number of alterations result from VCN treatment of cells. These include a reduction in negative charge (4), enhanced phagocytosis of treated cells (5, 6), inhibition of viral (7) or mycoplasma (8) induced hemagglutination, inhibition of cell aggregation (9), increased cell deformability (10), alterations in patterns of cellular migration (2), and interference with amino acid transport across treated cell membranes (11).

Two additional properties of VCN treated cells have received attention: (a) the apparent increased immunogenicity of such cells *in vivo* (12–19); and (b) the increased susceptibility of VCN-treated cells to lysis by antibody and complement (3, 20). Both phenomena have been interpreted to suggest that sialomucins act to "mask" antigens on cell surfaces. Apffel and Peters (21) have broadened this concept to suggest that sialoglycoproteins on the cell surface may act as a distinct system of immunoregulation.

Our previous studies with VCN indicated that mouse lymphoid cells were made extremely susceptible to the cytolytic action of alloantibody and complement by VCN (3). We have also shown that the *in vivo* immunogenicity of mouse lymphoid cells and mouse fetal tissue is increased by VCN treatment (3, 17). Furthermore the stimulatory effect of human lymphoid cells in MLC is markedly increased by prior VCN treatment (22). The present studies were designed to determine if these effects on lymphoid cells were due to an increased ability of VCN-treated cells to absorb specific alloantibody, *i.e.*, are an increased number of strong allogeneic antigens unmasked by the action of VCN on cell surfaces?

Materials and Methods. Animals. Adult C3H/HeJ (H-2^k) male mice (Jackson Laboratory, Bar Harbor, Maine) were used for lymphoid cells. C57 BL/6J (H-2^b) male mice were used as the source of anti-C3H antiserum. New Zealand white rabbits were used for the preparation of complement.

Antiserum. Antisera to C3H/HeJ were prepared in C57BL/6J mice by grafting C57 BL/6J animals twice sequentially with C3H/HeJ skin and injecting 25×10^6 C3H mouse spleen cells ip into the C57 BL/6J recipients weekly for 8 to 12 weeks (23). Bleeding was carried out 4 days after the last spleen injection. The blood was allowed to clot and was centrifuged at 2500*g* for 20 min at 0°. Complement was inactivated by heating to 56° for 30 min. The serum obtained was stored at —20° until used.

Complement. Rabbit serum was used as the source of complement. Blood was obtained from normal adult rabbit by ear vein puncture, allowed to clot at room temperature (25°) for 30 min and then at 0° for 1 hr. The serum was obtained by centrifugation at 2500*g* for 20 min at 0°. The rabbit serum was repeatedly absorbed with mouse liver powder (BBL, Division of Bioquist, Cockeysville, Maryland) at 0° (1 part mouse liver powder: 4 parts serum) to absorb out lytic factors directed against mouse cells. The serum was stored at —20° until used. When necessary the serum complement was inactivated by heating at 56° for 30 min.

Cell suspensions. C3H/HeJ lymph nodes were excised and teased apart in medium

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199-IX (M199) (Grand Island, New York) containing 0.1% bovine serum albumin (BSA) (Sigma). The cell suspensions were centrifuged at 200*g* for 5 min at 4° and washed three times in BSA + M199. After the last wash the cells were suspended in M199.

Labeling of cells with ^{51}Cr. The suspensions of cells (50×10^6/1.5 ml of M199) were mixed with 0.2 ml of $Na_2{}^{51}CrO_4$ (1.1 mCi/ml), (Amersham/Searle) and incubated at 37° for 45 min. After incubation, the excess $Na_2{}^{51}CrO_4$ solution was removed by repeated washing with large volumes of M199. After labeling, the viability of the cells were measured by their ability to exclude trypan blue (Grand Island Biological Company). Only suspensions with greater than 90% viability were used. The cell suspension was diluted with M199 + BSA to have a concentration of 10×10^6 cells/ml.

Incubation with neuraminidase. Vibrio cholerae neuraminidase (VCN) was obtained either from General Biochemicals, Chagrin Falls, OH or from Behringwerke AG Marburg, Lahn, West Germany. No differences in the two products could be detected in these experiments, so the former material was used for most of the latter experiments. The VCN was diluted with M199 as necessary. One milliliter of the enzyme solution was mixed with 1 ml (10×10^6 cells) of cell suspension and incubated at 37° for 1 hr. All the tubes were mixed well every 15 min. After incubation, the excess enzyme was removed by repeated washing with M199 + BSA. Inactivation of VCN for control experiments was carried out by heating at 65° for 30 min (2).

Cytotoxicity assay. Serial dilutions of C57 anti-C3H serum were made with M199 + BSA. To 0.05 ml of antiserum, 0.05 ml of labeled cells (5×10^5) were added and incubated at 37° for 15 min. After incubation, rabbit serum complement (0.05 ml) was added and again incubated for a total of 1 hr at 37°. At the end of incubation, all tubes were diluted with 1.5 ml of phosphate buffered saline (without Ca^{2+}, Mg^{2+}), pH 7.2. All the tubes were centrifuged at 800*g* for 6 min and 1 ml supernatant portions were taken out for counting in a gamma-radiation counter (Gamma/guard, Tracer Lab, Division of Laboratory for Electronics, Inc.). The percentage of ^{51}Cr release from the cells was calculated (24).

Antibody absorption studies. Unlabeled C3H/HeJ cells (0.05 ml) were incubated with various dilutions of C57 anti-C3H antiserum (0.05 ml) for 1 hr at 37°. The supernatant solutions were then removed for cytotoxicity assay using fresh, labeled C3H/HeJ lymphoid cells.

Results. Effect of neuraminidase on mouse lymphoid cells. ^{51}Cr-labeled C3H/HeJ mouse lymphoid cells were exposed to varying concentrations of VCN (1 unit/5×10^6 cells/ml to 250 units/5×10^6 cells/ml), incubated for 1 hr at 37°, and the amount of ^{51}Cr released from the cells was determined. Less than 5% of the total cellular ^{51}Cr was released from the cells during VCN incubation indicating that VCN does not lyse cells. Trypan blue exclusion tests confirmed that VCN has no effect on cell viability.

Effect of neuraminidase on the lysis of C3H/HeJ mouse lymphoid cells by C57 anti-C3H antibody and rabbit complement. ^{51}Cr-labeled C3H/HeJ lymphoid cells were treated with VCN (25 units/ml/5×10^6 cells) or heat-inactivated VCN. These cells were exposed to C57 anti-C3H serum and rabbit complement; and the amount of lysis was determined. The results are presented in Fig. 1. VCN rendered C3H/HeJ lymphoid cells markedly sensitive to cytolysis by specific antiserum and rabbit complement. No lysis occurred when the rabbit serum complement had been inactivated by heat. When the enzyme had been inactivated by heat, the degree of lysis did not exceed that of cells never exposed to the enzyme.

Effect of neuraminidase on the antibody absorption capacity of C3H/HeJ mouse lymphoid cells. VCN-treated, inactivated VCN-treated, and untreated C3H cells were added to serially diluted C57 anti-C3H sera. After 1 hr incubation at 37°, the supernatant solutions were used to determine the residual amount of C57 anti-C3H antibody by the cytotoxicity test using fresh ^{51}Cr-labeled C3H/HeJ mouse lymphoid cells and rabbit complement. The results are presented in

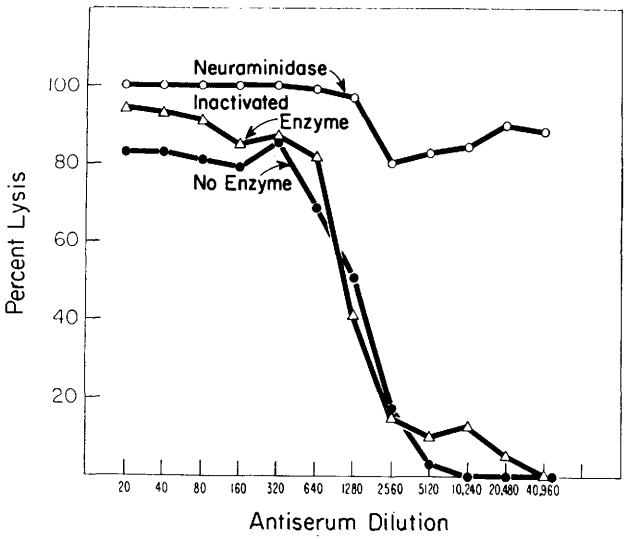

FIG. 1. Effect of *V. cholerae* neuraminidase (VCN) on the lysis of C3H/HeJ mouse lymphoid cells by C57 anti-C3H serum and rabbit complement.

Fig. 2. VCN-treated C3H cells did not absorb more C57 anti-C3H antibody than did untreated cells or cells treated with heat-inactivated VCN. Thus, VCN did not expose more complement-fixing allogeneic antibody sites on the surface of mouse lymphoid cells.

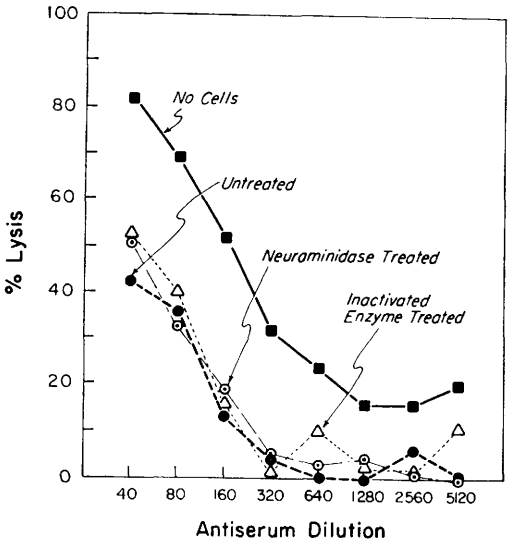

FIG. 2. Residual lytic activity of C57 BL/6J anti-C3H/HeJ antiserum after absorption of the antiserum with VCN-treated or untreated C3H/HeJ lymphoid cells. Heated (56°, 30 min) C57 anti-C3H serum (0.05 ml) was allowed to react with 0.05 ml of treated or untreated cells (5×10^5). After absorption, the residual antisera were reacted against radiochromated C3H cells (5×10^5) in presence of diluted rabbit serum complement.

The experiment was repeated utilizing a single dilution of C57 anti-C3H antiserum (1:20) and varying the number of unlabeled VCN-treated, or untreated C3H cells used for the absorption. Figure 3 demonstrates that VCN-treated C3H cells did not absorb more C57 anti-C3H antibody than did untreated cells or cells exposed to heat inactivated VCN.

Discussion. The present experiments clearly demonstrate that the increased susceptibility of cells treated with VCN to antibody-mediated lysis (3, 20) is not the result of an increased number of allogeneic antibody-binding sites on the cell surface. Similarly the increased *in vivo* immunogenicity of allogeneic antigens on VCN-treated lymphoid cells (17) cannot be the result of antigen "unmasking." Sanford and Codington (25) have reached similar conclusions. They found that the immunogenicity of TA-3 tumors with respect to allogeneic hosts was increased by VCN (12); the TA-3 cells, however, were not capable of absorbing more anti-H-2 antibody after VCN treatment (25).

VCN apparently increases the immunogenicity of other tumors: Landschütz acites

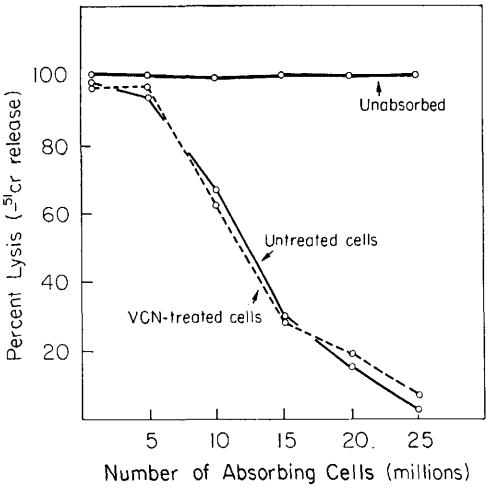

FIG. 3. Lysis of labeled C3H/HeJ lymphoid cells by the residual antibody left after absorption with varying number of VCN-treated or untreated C3H/HeJ lymphoid cells and rabbit complement.

tumor (16), L1210 leukemia (13), methylcholanthrene induced fibrosarcomas (15, 18, 19), and Ehrlich's ascites tumor (16) all grow less well in normally susceptible recipients. Antibody absorption studies designed to detect "unmasking" of tumor antigens have not been carried out in these cases. However, increased immunogenicity can be related to several properties of cells after treatment with VCN without invoking the "unmasking" hypothesis. (a) VCN removes sialic acid from the cell surface (2, 12, 25), the sialic acid may offer a steric hindrance to the perception of the antigen itself, or may sterically interfere with contact (9) between antigen-bearing and antigen-processing cells. (b) VCN also reduces the negative charge on the cell surface (4); and a negatively charged antigen-processing or antigen-responsive cell might be more attracted to a less highly charged cell. (c) Reducing the negative charge on the cell surface also reduces the rigidity of the cell surface (10) since the mutually repellent negative charges on the cell surface are removed (26). Since the T cells recognize a rather large antigenic determinant (in contrast to the small antigenic determinants recognized by the B cells), the increased deformability (10) of the antigenic cell itself might increase the area of contact between it and the antigen-responsive cell. (d) Finally, treatment of cells with neuraminidase renders them more easily phagocytized (5, 6), and phagocytosis of antigens seems to facilitate antigen processing and the development of immunity. Any or all of these processes may be at work in increasing the immunogenicity of normal cells or tumor cells.

It is also possible to explain the increased susceptibility of VCN-treated cells to antibody-mediated cytolysis without postulating antigen "unmasking." We have recently demonstrated that VCN-treated lymphoid cells are remarkably sensitive to complement (3, 27). Activation of autologous complement by cobra venom factor results in the lysis of autologous lymphoid cells only if they have been pretreated with VCN (3). It is likely that the potentiation of complement sensitivity by VCN treatment is also responsible for the increased susceptibility of VCN-treated cells to antibody-mediated cytolysis demonstrated here.

Thus both the increased immunogenicity of cells and their increased cytolytic tendencies could be explained without the antigen unmasking hypothesis. On the other hand, Schlesinger and Amos (20) have demonstrated that VCN unmasks an antigen on mouse lymphoid cells which is capable of absorbing a guinea pig serum antibody. Similarly, they showed that the theta (θ) antigen was "unmasked" on peripheral lymphoid tissues by VCN (20). It is likely that the "stronger" antigens are not masked by the sialic acid residues, but that the weaker antigens might well be. Thus, humoral antibody molecules might bind easily to prominent antigen determinant sites which are not either electrically or sterically masked by the sialic acid residues. "Weaker" antigens, *i.e.*, those with less prominent antigenic determinants might well be made available to antibody molecules by removal of sialic acid residues.

Three immunologic properties of VCN-treated cells can, therefore, be defined: increased immunogenicity, increased susceptibility to complement lysis, and the increased availability of weak or masked antigens on their cell surfaces. At times these properties

may correlate with one another—for example, it is possible that some of the increased immunogenicity of VCN-treated tumor cells is due to an "unmasking" of tumor specific antigens, or even to an increase in complement sensitivity. However, it is equally apparent that the increase in immunogenicity and susceptibility to lysis is not *always* due to an increase in antibody-binding sites.

Summary. Vibrio cholerae neuraminidase (VCN) does not lyse mouse lymphoid cells at any concentration up to 250 units/ml/5 $\times$ 10^6 cells. VCN treatment of C3H/HeJ lymphoid cells increases their susceptibility to lysis with C57 anti-C3H serum and complement. It does not, however, increase the capacity of these cells to absorb C57 anti-C3H complement-fixing antibody. Thus, neither the increased susceptibility to lysis nor the increased allogeneic immunogenicity of VCN-treated lymphoid cells is due to an unmasking of more allogeneic antigens on the cell surface.

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