Unmasking of Xenogeneic Neo-antigens on Mouse Lymphoid Cell Surfaces by *Vibrio cholerae* Neuraminidase[1] (36992)

PRASANTA K. RAY AND RICHARD L. SIMMONS[2]
Department of Surgery, University of Minnesota, Minneapolis, Minnesota 55455

The immunogenicity of certain cellular antigens can be increased by treatment of the cells with *Vibrio cholerae* neuraminidase (VCN). Allogeneic (1–5) and syngeneic (6–8) tumors fail to grow in normally susceptible recipients if the cells are first treated with VCN *in vitro*. Furthermore, firmly established solid syngeneic tumors can be made to regress if the tumor-bearing mice are injected with VCN treated tumor (8). The latter effect is totally immunospecific and regression cannot be induced with an immunologically distinct tumor. VCN treated mouse lymphoid cells (9) and embryonic tissues (10) have also been found to be more immunogenic in allogeneic recipients. *In vitro* studies have further demonstrated that VCN treated stimulatory cells will elicit a much greater degree of transformation from allogeneic responder cells in mixed lymphocyte culture (11).

It is possible that this increased immunogenicity of VCN treated cells is due to an unmasking of antigenic determinants on the cell surfaces. In this regard, numerous investigators (12–17) have shown that VCN renders cells increasingly sensitive to lysis by specific antibody and complement; but attempts to detect unmasking of H-2 antigens (12, 18–20) and HL-A antigens (21, 22) which correlate with increased immunogenicity *in vitro* have thus far been unsuccessful. Kassulke, Stutman and Yunis (23) have, however, demonstrated an increased number of blood group isoantigens on human leukemic cells treated with VCN, and Schlesinger and Amos (15) and Schlesinger and Gottesfeld (16) have reported unmasking of antigens on mouse cells to which guinea pigs had preformed antibody. Rosenberg, Plocinik and Rogentine (21) have reported that VCN unmasks an antigen on human lymphoid cells against which normal rabbit serum has preformed antibodies. Reisner and Amos (22) have reported that human sera showing no detectable anti-HL-A activity gave strong cytotoxic reactions with almost all VCN treated human lymphocytes. The present studies were designed to determine if VCN unmasks new xenoantigens on mouse lymphoid cell surfaces.

Materials and Methods. Animals. Adult C3H/HeJ (H-2^k) male mice (Jackson Laboratory, Bar Harbor, ME) were used for lymph node cells. Normal adult New Zealand white rabbits were used for the preparation of normal rabbit serum (NRS). Adult male guinea pigs and adult men were utilized for the preparation of normal guinea pig serum (NGPS) and normal human serum (NHS).

Preparation of normal rabbit, guinea pig, and human sera. Venous or cardiac blood was obtained from normal adult rabbits, guinea pigs or men and allowed to clot at room temperature (25°) for 30 min and then at 0° for 1 hr. It was then centrifuged at 2500*g* for 20 min at 0°, the supernatant sera were stored at −20° until used. Complement was inactivated for selected experiments by heating the sera to 56° for 30 min.

Cell suspensions. C3H/HeJ lymph nodes were excised and teased apart in Medium 199-IX (M199) (Grand Island, NY) containing 0.1% bovine serum albumin (BSA) (Sigma). The cell suspensions were centrifuged at 200*g* for 5 min at 4° and washed

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[2] John and Mary R. Markle Foundation Scholar in Academic Medicine. Address reprint requests to Doctor Simmons, Box No. 185, Mayo Memorial Building, University of Minnesota, Minneapolis, MN 55455.

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 was measured by their ability to exclude trypan blue (24). 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 the previous (7–14) experiments, so the former material was used throughout this study. The VCN was diluted with M199 (pH 7.2). One milliliter of the enzyme solution containing 50 units of enzyme was mixed with 1 ml (10 × 10^6 cells) of cell suspension such that the final enzyme concentration was 25 units VCN/5 × 10^6 cells/ml and incubated at 37° for 1 hr. A number of investigators have previously shown that VCN will catalyze the release of sialic acids from the surface of normal and malignant cells under these conditions without affecting cell viability (3, 4, 12–14, 18, 25, 26). After incubation, the excess enzyme was removed by repeating washing with M199 + BSA. Inactivation of VCN for control experiments was carried out by heating at 100° for 10 min (27).

Cytotoxicity assay. VCN treated, heat-inactivated VCN treated, or untreated cells (0.05 ml containing 5 × 10^5 cells) were incubated with serially diluted sera (0.05 ml) in tubes for 1 hr at 37°. After incubation all the tubes were diluted with 1.5 ml of Ca^{2+} and Mg^{2+} deficient phosphate buffered saline (pH 7.2) and centrifuged at 800*g* for 5 min. One milliliter of supernatant was taken out from each tube and counted in a gamma-radiation counter (Gamma/Guard, Tracer Labs, Division of Laboratory for Electronics Inc.) and the degree of cell lysis was determined (28).

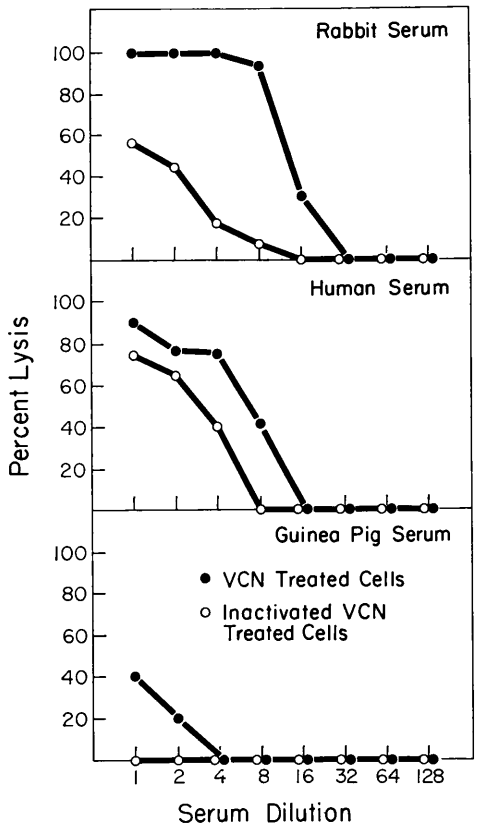

FIG. 1. Effect of xenogeneic sera on ^{51}Cr release (lysis) from *V. cholerae* neuraminidase (VCN) treated and untreated murine lymph node cells (mean of three determinations).

Serum absorption studies. Unlabeled C3H/HeJ cells (0.05 ml) were incubated with NRS, NGPS, or NHS (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 xenogeneic sera on neuraminidase treated cells. Radiolabeled C3H/HeJ lymphoid cells which had been treated with VCN or heat-inactivated VCN were incubated with serially diluted normal rabbit serum (NRS), normal guinea pig serum (NGPS) or normal human serum (NHS). Cytotoxicity was measured in terms of radioactive chromium release. The results are shown in Fig. 1. VCN treated C3H/HeJ lymph node cells were more easily lysed by

normal xenogeneic sera than those exposed to heat-inactivated VCN. Mouse lymph node cells are particularly susceptible to lysis by normal rabbit or human sera, and less susceptible to normal guinea pig sera. Heat-inactivation of the serum complement (56°, 30 min) in each serum totally abrogated its ability to lyse both VCN treated or inactivated VCN treated normal mouse lymph node cells.

The increased lysis of VCN treated mouse lymph node cells by xenogeneic sera could be due to; (a) their known increased susceptibility to complement mediated lysis (13, 14, 29) and/or (b) an unmasking of antigenic determinants on their cell surfaces for which antibodies were present in NRS, NGPS, or NHS.

Comparative ability of VCN treated cells and untreated cells to absorb complement fixing natural antibodies from xenogeneic sera. C3H/HeJ lymph node cells were incubated with VCN or heat-inactivated VCN. Washed, treated cells were then incubated with undiluted NRS, NGPS, or NHS, and the supernatant sera were tested for their cytotoxic effect against normal, untreated, ^{51}Cr labeled C3H/HeJ lymph node cells. The results are shown in Fig. 2. VCN treated cells did not absorb more xenogeneic complement-fixing antibody than did normal cells or those exposed to heat-inactivated VCN. These findings suggest that the number of antigenic determinants on cell surfaces which normally bind complement-fixing natural xenogeneic antibody were not increased on the cell surface by VCN treatment, *i.e.*, there was no unmasking of additional antigenic sites previously available on the cell surface. Furthermore, absorption with VCN treated cells did not lead to a greater loss of complement from the reactive sera than did absorption with normal cells.

It was possible, however, that VCN treatment led to the "unmasking" of new antigenic determinants for which xenogeneic antibodies were present; such antigens may not be available on normal cell surfaces and are only exposed after treatment with VCN. To test this hypothesis, VCN treated or untreated cells were incubated with undiluted NRS, NGPS, or NHS and the supernatant sera were

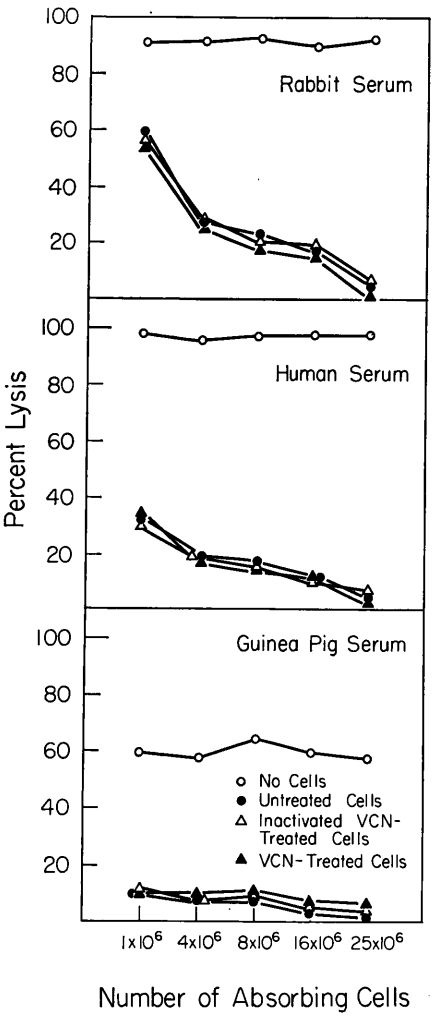

FIG. 2. Residual lytic activity against C3H/HeJ lymphoid cells of normal rabbit serum, human serum and guinea pig serum after absorption with *V. cholerae* neuraminidase (VCN) treated or untreated C3H/HeJ lymphoid cells. The sera were allowed to react with cells. After absorption, the sera were reacted against normal radiochromated C3H/HeJ cells.

tested for cytotoxic effect against VCN treated, ^{51}Cr labeled C3H/HeJ normal lymph node cells. The results are shown in Fig. 3. Sera absorbed with VCN treated cells lost much of their capacity to lyse VCN treated cells whereas sera absorbed with untreated and heat-inactivated VCN treated cells retained much of their ability to lyse VCN treated cells.

Discussion. Our results suggest that cells possess antigens not normally available for

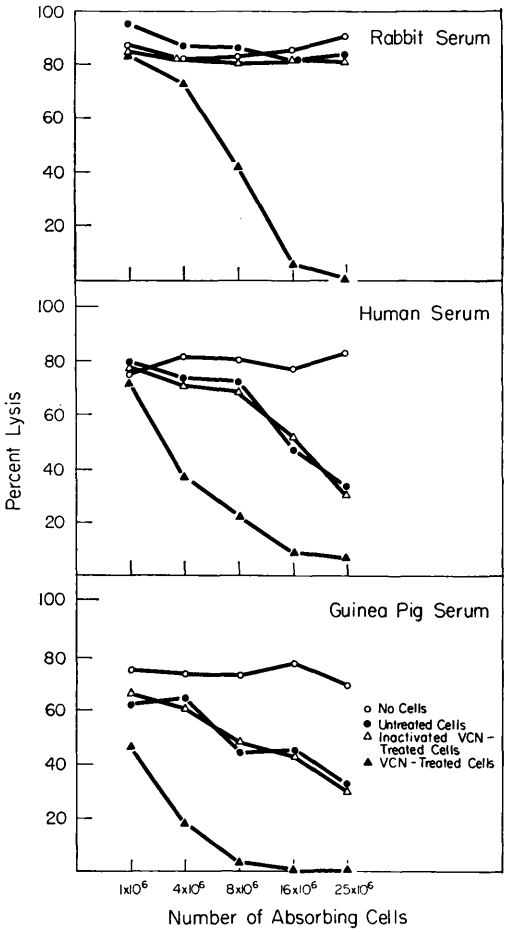

FIG. 3. Residual lytic activity against *V. cholerae* neuraminidase (VCN) treated C3H/HeJ lymphoid cells of normal rabbit serum, normal human serum, or normal guinea pig serum after absorption with VCN treated or untreated C3H/HeJ cells. The sera were allowed to react with cells. After absorption, the sera were reacted against VCN treated radiochromated cells.

antibody reaction. The lysis by VCN of the 2-3′, 2-6′ and 2-8′ α-glycosidic linkages between the terminal sialic acids and other carbohydrates on the lymphoid cell membrane (13) apparently unmasks those sites permitting the binding of xenogeneic antibodies. Though preformed xenogeneic antibodies have been frequently described (30–34), the antibodies which react specifically with VCN treated cells appear to be reactive with antigenic components not normally exposed on the cell surface.

These conclusions are consistent with independent observations of other investigators. VCN treated cells are highly susceptible to lysis by antibody and complement (12–17). The increased susceptibility to alloantibody does not seem to be due to the unmasking of previously defined strong alloantigens either in mice (12, 18–20) or in man (21, 22). However, Schlesinger and Amos (15) and Schlesinger and Gottesfeld (16) reported an increased capacity of VCN treated cells to absorb the cytolytic component of guinea pig serum for mouse thymocytes. Most recently Rosenberg, Plocinik and Rogentine (21) and Reisner and Amos (22) have independently reported that human lymphocytes treated with VCN are capable of absorbing antibodies from both xenogeneic (21) and allogeneic sera (22) which react primarly with VCN treated cells and less well, or not at all, with normal cells. Reisner's studies suggest that the human leukocyte antigens exposed by VCN are not related to HL-A determinants and that the antigens may be pan-reactive, in that both VCN treated monkey and human erythrocytes can absorb them (22). This interpretation may also help explain the lysis of VCN treated cells by autologous serum (14, 20, 29) since autologous sera may contain antibodies against cellular antigens on their own cells which are not normally available for reaction. The relationship of this kind of phenomenon to post-infectious "autoimmune" hemolytic anemias (35) is a matter for further investigation.

These experiments do not completely resolve the question regarding the mechanism of the increased lysis of VCN treated cells (12–17) by antibody and complement. The complement sensitivity of these cells may be increased (13, 14, 29). On the other hand, it is possible that the increased complement sensitivity of VCN treated cells in autologous plasma (13, 14, 29) could be at least partly due to the increased ability of VCN treated cells to bind autologous antibody to unmasked components on cell surfaces. The early report of Dalmasso and Müller-Eberhard (36) showing that VCN facilitates the binding of complement to cell membranes may merely reflect the binding of complement to cells

which have already bound antibody to unmasked neo-antigens. It is not known, therefore, if VCN treated cells have an inherently greater complement sensitivity unrelated to the antibody content of serum. Experiments are in progress in this regard.

The question remains whether the serologic behavior of VCN treated cells helps explain the increased immunogenicity of such cells *in vivo* (1–10, 37, 38). Increased immunogenicity of embryonic (10), lymphoid (9), and tumor cells (1–8, 37, 38) appears to be specific for antigens preexisting on the cell surface. It is possible that the unmasking of neo-antigens to which the recipient has preexisting antibody would augment his immune response to other antigens on the cell surface. For example, VCN treated cells are more easily opsonized for phagocytosis than normal cells, and macrophages appear to be important in antigen handling and/or presentation (39). On the other hand, the mechanisms involved in antigen unmasking, increased complement sensitivity, and increased immunogenicity may be totally independent functions of the enzyme altered cell surfaces.

Summary. C3H/HeJ mouse lymphoid cells treated with *Vibrio cholerae* neuraminidase (VCN) are more easily lysed by normal rabbit, normal guinea pig, and normal human sera than are untreated cells or cells exposed to heat-inactivated VCN.

Absorption studies suggest that VCN does not unmask a greater number of previously available xenogeneic specificities on the cell surface. Instead, VCN appears to unmask new antigens for which preformed antibodies also exist in xenogeneic sera.

1. Sanford, B. H., Transplantation **5**, 1273 (1967).

2. Currie, G. A., vanDoornick, W., and Bagshawe, K. D., Nature (London) **219**, 191 (1968).

3. Currie, G. A., and Bagshawe, K. D., Brit. J. Cancer **22**, 843 (1968).

4. Bagshawe, K. D., and Currie, G. A., Nature (London) **218**, 1254 (1968).

5. Lindenmann, J., and Klein, P. A., Recent Results Cancer Res. **9**, 66 (1967).

6. Currie, G. A., and Bagshawe, K. D., Brit. J. Cancer **23**, 141 (1969).

7. Simmons, R. L., Rios, A., Ray, P. K., and Lundgren, G., Nat. Cancer Inst. **47**, 1087 (1971).

8. Simmons, R. L., Rios, A., Lundgren, G., Ray, P. K., McKhann, C., and Haywood, G., Surgery **70**, 38 (1971).

9. Simmons, R. L., Rios, A., and Ray, P. K., Nature (London) New Biol. **231**, 179 (1971).

10. Simmons, R. L., Lipschultz, M. L., Rios, A., and Ray, P. K., Nature (London) New Biol. **231**, 111 (1971).

11. Lundgren, G., and Simmons, R. L., Clin. Exp. Immunol. **9**, 915 (1971).

12. Ray, P. K., and Simmons, R. L., Proc Soc. Exp. Biol. Med. **138**, 600 (1971).

13. Ray, P. K., and Simmons, R. L., Clin. Exp. Immunol. **10**, 139 (1972).

14. Ray, P. K., Gewurz, H., and Simmons, R. L., Clin. Exp. Immunol. **11**, 441 (1972).

15. Schlesinger, M., and Amos, D. B., Transplant. Proc. **3**, 895 (1971).

16. Schlesinger, M., and Gottesfeld, S., Transplant. Proc. **3**, 1151 (1971).

17. Grothaus, E. A., Flye, M. W., Yunis, E., and Amos, D. B., Science **173**, 542 (1971).

18. Sanford, B. H., and Codington, J. F., Tissue Antigens **1**, 153 (1971).

19. Shimada, A., and Nathenson, S. G., J. Immunol. **107**, 1197 (1971).

20. Hauschka, T. S., Weiss, L., Holdridge, B. A., Cudney, T. L., Zumft, M., and Planinsek, J. A., J. Nat. Cancer Inst. **47**, 343 (1971).

21. Rosenberg, S. A., Plocinik, B. A., and Rogentine, G. N., J. Nat. Cancer Inst. **48**, 1271 (1972).

22. Reisner, E. G., and Amos, D. B., Transplantation **14**, 455 (1972).

23. Kassulke, J. T., Stutman, O., and Yunis, E. J., J. Nat. Cancer Inst. **46**, 1207 (1971).

24. Boyse, E. A., Old, L. J., and Chowronlinkov, I., Methods Med. Res. **10**, 39 (1964).

25. Ray, P. K., and Simmons, R. L., Surg. Forum **22**, 101 (1971).

26. Woodruff, J. J., and Gesner, B. M., J. Exp. Med. **129**, 551 (1969).

27. Codington, J. F., Sanford, B. H., and Jeanloz, R. W., J. Nat. Cancer Inst. **45**, 637 (1970).

28. Wigzell, H., Transplantation **3**, 423 (1965).

29. Ray, P. K., Gewurz, H., and Simmons, R. L., Transplantation **12**, 327 (1971).

30. Bolande, R. P., and Meclain, J. P., Proc. Soc. Exp. Biol. Med. **103**, 345 (1960).

31. Landy, M., Michael, J. G., Trapani, R. J., Achinstein, B., Woods, M. W., and Shear, M. J., Cancer Res. **20**, 1279 (1960).

32. Terasaki, R. I., Esail, M. L., Cannon, J. A., and Longmire, W. P., J. Immunol. **87**, 383 (1961).

33. Federoff, S., and Weble, S. J., Nature (London) **193**, 80 (1962).

34. Herberman, R. B., Transplantation **8**, 813 (1969).

35. Jacob, H., *in* "Current Therapy" (H. Conn, ed.), p. 211. Saunders, Philadelphia (1970).

36. Dalmasso, A. P., and Müller-Eberhard, H. J., Proc. Soc. Exp. Biol. Med. **117**, 643 (1964).

37. Bekesi, J. G., St.-Arneault, G., and Holland, J. F., Cancer Res. **31**, 2130 (1971).

38. Bekesi, J. G., St.-Arneault, G., and Holland, J. F., J. Nat. Cancer Inst. **49**, 107 (1972).

39. Lee, A., Proc. Soc. Exp. Biol. Med. **128**, 891 (1968).

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