THE SEROLOGIC BEHAVIOUR OF NEURAMINIDASE-TREATED LYMPHOID CELLS

ALLOANTIGENICITY AND COMPLEMENT SENSITIVITY

P. K. RAY, H. GEWURZ* AND R. L. SIMMONS

Department of Surgery, University of Minnesota, Minneapolis, Minnesota, U.S.A.

SUMMARY

Vibrio cholerae neuraminidase (VCN) renders mouse lymphoid cells highly susceptible to the cytolytic effects of alloantibody and complement (C). This increased susceptibility to lysis is not due to unmasking of alloantigens since no increase in alloantibody-binding capacity of VCN-treated cells could be detected. However, VCN-treated cells can be lysed by normal rabbit serum, human serum, and guinea-pig serum even if specific antibody is not added to the incubation mixture. VCN, therefore, while not unmasking strong H-2 histocompatibility antigens, may be capable of unmasking other antigens with which heterologous sera can react.

The increased susceptibility to immune cytolysis of VCN-treated cells appears to be related, at least partially, to its extreme susceptibility to C. Complement inactivation by heat totally abrogated the lytic effect, as did C inactivation by ammonium hydroxide, viscarin, and zymosan. In addition, activation of the autologous serum C within the fluid phase by cobra venom factor produced cytolysis only in VCN-treated cells. Thus, VCN renders nucleated cells highly susceptible to lysis by C.

INTRODUCTION

Sialic acid residues are major constituents on the surface of plasma membranes (Benedetti & Emmelot, 1967) and make the major contribution to the negative charge of the cell surface (Ambrose, 1966). The enzyme *Vibrio cholerae* neuraminidase (VCN) is known to cleave terminal sialic acid from heterosaccharides or glycoproteins (Gottschalk, 1957) and is capable of releasing sialic acid from cell surfaces (Wallach & Eyler, 1961; Ruhenstroth-Bauer *et al.*, 1962; Eyler *et al.*, 1962; Seaman & Uhlenbruck, 1963) without altering cellular viability (Woodruff & Gesner, 1969). A number of functional alterations of cells may be induced by removal of sialic acid from surface membrane utilizing VCN. These include enhanced phagocytosis (Weiss, Mayhew & Ulrich, 1966; Lee, 1968) inhibition of

* Present address: Professor and Chairman, Department of Immunology, Presbyterian-St. Luke's Hospital, 1753 West Congress Parkway, Chicago, Illinois 60612, U.S.A.

Correspondence: Dr R. L. Simmons, Box No. 185, Mayo Memorial Building, University of Minnesota, Minneapolis, Minnesota, U.S.A.

viral (Bayer, 1964) or mycoplasma (Gesner & Thomas, 1966) induced haemagglutination, inhibition of cell aggregation (Kemp, 1968), increased cell deformability (Weiss, 1965), alteration of lymphoid cell migratory patterns (Woodruff & Gesner, 1969), and modification of amino acid transport across cell membranes (Brown & Michael, 1969).

Recent evidence suggests that neuraminidase may increase the immunogenicity of both normal and malignant cells. Gasic & Gasic (1962), originally pointed out that VCN interfered with the metastatic spread of tumours. Sanford (1967), then showed that TA-3 ascites tumour failed to grow in allogeneic recipients if the tumour cells had been treated with VCN *in vitro*. Similar results have been obtained utilizing L 1210 leukaemia (Bagshawe & Currie, 1968). Landschutz ascites tumour (Currie & Bagshawe, 1968b), methylocholanthrene-induced fibrosarcomas (Currie & Bagshawe, 1969), the Walker 256 tumour (Cormack, 1970), and Ehrlich's ascites tumour (Lindenmann & Klein, 1967). Simmons *et al.* (1971b,c,d) have shown that total immunospecific rejection of firmly established methylcholanthrene fibrosarcomas can be induced by challenging the tumour-bearing animals with syngeneic tumour cells treated *in vitro* with VCN. In a similar way, *in vitro* VCN treatment has been shown to increase the immunogenicity of transplanted allogeneic lymphoid cells (Simmons, Rios & Ray, 1970, 1971a) and foetal tissue (Simmons *et al.*, 1971e). Currie, vanDoorninck & Bagshawe's demonstration (1968c) that VCN unmasked histocompatibility antigens on the mouse trophoblast, however, has not been confirmed (Simmons *et al.*, 1971e).

The removal of sialic acid residues from mucopolysaccharides seems to increase their immunogenicity. Currie & Bagshawe (1967) postulated that the sialic acid content of the periphery of cells may act in some way as a barrier to the detection of antigens by the host organism. Apffel & Peters (1970) have even broadened this concept to suggest that sialoglycoproteins on the cell surface may act as a distinct system of immunoregulation in biology.

Schlesinger & Amos (1971) have recently shown that lymphoid cells which have been exposed to VCN are easily lysed when incubated with specific antibody and complement (C). They have demonstrated that peripheral lymphoid cells are capable of absorbing somewhat more anti-theta (θ) antibody after incubation with VCN. Similarly, the ability of mouse lymphoid cells to absorb a natural antibody from guinea-pig serum was increased by exposure of the cells to VCN. The increased absorptive capacity of VCN treated cells, however, did not seem to account for the marked increase in cytolytic tendency of cells treated with VCN.

The present report describes results of the studies on the effect of VCN on the serologic behaviour of lymphoid cells *in vitro*. It provides evidence that treatment of lymphoid cells with VCN renders those cells extremely susceptible to the lytic action of serum complement (C) and does not 'unmask' a greater number of allogeneic antibody binding sites on the cell surface. A part of the results described in this paper has been presented earlier in the form of two abstracts (Ray, Gewurz & Simmons, 1970; Ray & Simmons, 1971).

MATERIALS AND METHODS

Animals

Adult C3H/HeJ, C57 BL/6J, CBA/J and BALB/c male mice obtained from the Jackson Laboratory, Bar Harbor, Maine, were used for lymphoid cells. C57 BL/6J male mice were

used to prepare anti-C3H antisera. Normal adult New Zealand white rabbits were used for the preparation of serum complement (C) and lymphoid cells. A few experiments utilized normal adult guinea-pigs or normal adult humans for serum C sources.

Preparation of antisera

Antisera to C3H/HeJ male mice were prepared in C57 BL/6J male mice by grafting C57 BL/6J animals twice sequentially with C3H/HeJ skin and injecting 25×10^6 C3H mouse spleen cells i.p. into the C57 BL/6J recipients weekly for 8–12 weeks (Kinne & Simmons, 1967). Bleeding was carried out 4 days after the last spleen cell injection. The blood was allowed to clot at room temperature (25°C) and centrifuged at 2500 g for 20 min at 0°C. The sera obtained were stored at -20°C until used.

Antibody absorption studies

0·05 ml of unlabelled C3H/HeJ cells were incubated with various dilutions of C57 anti-C3H antisera (0·05 ml) for 1 hr at 37°C. The supernatant solutions were then removed for cytotoxicity assay using fresh, radio-labelled C3H/HeJ lymphoid cells and C.

Complement

Normal rabbit sera (NRS) were usually used as the source of C. Blood was obtained from normal adult rabbits by ear vein puncture, allowed to clot at room temperature (25°C) for 30 min and then at 0°C for 1 hr. NRS obtained by centrifugation at 2500 g for 20 min at 0°C were repeatedly absorbed with mouse liver powder (BBL, Division of Bioquest, Cockeysville, Maryland) at 0°C (one part mouse liver powder: four parts serum) to absorb out lytic factors directed against mouse cells. NRS was either used fresh or was frozen and stored at -20°C until used.

In some experiments pooled, fresh-frozen, ether-free guinea-pig serum C (Texas Biological Lab, Inc., Texas) was used.

Human serum C was prepared from freshly drawn venous blood from a normal adult male. The blood was allowed to clot at room temperature (25°C) for 30 min and the serum C was prepared in the same way as described above.

When necessary sera were heated at 56°C for 30 min to inactivate C activity. Other, inhibitors of C activity included: Zymosan, 1·35 mg/ml (National Biochemicals Corp., Cleveland, Ohio); Ammonium hydroxide, 0·15 N; Viscarin, 5 mg/ml (Marine Colloids, Inc., New Jersey). NRS and Viscarin solutions were mixed in equal volumes and incubated at 37°C for 60 min. Zymosan and NH_4OH were incubated with NRS for 90 min. After incubation the mixed solution was tested for the residual cytolytic-C activity by using neuraminidase-treated mouse lymphoid cells. The residual haemolytic C activity was measured using sheep red blood cells and rabbit anti-sheep antibody (Mayer, 1961). In some experiments cobra venom factor (CVF) was prepared (Shin, Gewurz & Snyderman, 1969) and used as an activator of the late-acting C-cascade (Shin, Gewurz & Snyderman, 1969; Götze & Müller-Eberhard, 1970).

Collection and preparation of cell suspension

Mouse or rabbit lymph nodes were excised from animals killed by cervical compression or etherization. The nodes were teased apart in Medium 199-IX (M199) (Grand Island, New York) containing 0·1% Bovine serum albumin (BSA) (Sigma Chemical Co., St Louis,

P. K. Ray, H. Gewurz and R. L. Simmons

Mo.). Hereafter we call this media MBSA. The cell suspensions in 2 ml of MBSA were centrifuged at 250 *g* for 5 min at 4°C and washed three times with large volumes of MBSA. After the last wash the cell pellet was suspended in M199.

Human erythrocyte suspension was prepared from freshly drawn venous blood from a normal adult male. Ten millilitres of blood was mixed with 0·25 ml of Na-heparin (1000 units) and then 2·5 ml of plasma gel (Laboratoire, Roger Bellow, France) was added and mixed. The tube is kept at 45°C angle for 20 min at room temperature (25°C) and then the leucocyte rich supernatant was removed from the top. The erythrocyte rich sediment was suspended in normal saline and the above procedure repeated at least three times and finally the cells were resuspended in MBSA and their viability measured by trypan blue exclusion method.

Radioactive labelling of cells

The suspensions of lymphoid cells (80–100 × 10⁶ cells/1·5 ml) and when necessary human erythrocytes in M199 were mixed with 0·2–0·3 ml of $Na_2^{51}CrO_4$ (1·1 mCi/ml). (Amersham/ Searle Corp., Arlington Heights, Illinois) and incubated at 37°C for 45 min. After incubation the excess $Na_2^{51}CrO_4$ solution was removed by repeated washing with large volumes of M199 (4 × 2 ml) and the cells suspended in MBSA. After labelling, the viability of the cells was 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 finally with MBSA so as to provide 10 × 10⁶ cells/ml. The uptake of label corresponded to 50,000–100,000 cpm/10⁷ cells.

Incubation of cells with neuraminidase

Neuraminidase from *Vibrio cholerae* was obtained either from General Biochemicals, Chagrin Falls, Ohio or from Behringwerke AG Marburg, Lahn, West Germany. The latter preparation is described by the manufacturer as having no detectable proteinase, aldolase, or lecithinase C activity. Both contained 500 units of enzyme/ml. One unit of neuraminidase activity is defined by both manufacturers as being equivalent to the release of 1 μg of *N*-acetyl neuraminic acid from a glycoprotein substrate at 37°C in 15 min at pH 5·5. Since no differences in activity in the two products could be detected by us (Ray *et al.*, 1970; Ray & Simmons, 1971) or by others (Woodruff & Gesner, 1969) the former material was used throughout the experiments. Enzyme dilution was made with M199 and diluted enzyme containing the desired amount in 1 ml was mixed with 1 ml (10 × 10⁶ cells) cell suspension and incubated at 37°C for 1 hr. All the tubes were mixed well every 15 min. After incubation the excess enzyme was removed by repeated washing with large volumes of MBSA and finally resuspended in MBSA. Inactivation of VCN was carried out by heating at 100°C for 10 min (Codington, Sanford & Jeanloz, 1970).

In experiments where the effect of the pH of the media on VCN activity was studied, MBSA (pH 7·2) was adjusted to acid and alkaline pHs by 0·1 N HCl and 0·1 N NaOH solution respectively. 50 units of VCN and 10 × 10⁶ ⁵¹Cr-labelled C3H lymphoid cells were added to a total volume of 2 ml of media adjusted prior to varying pHs in a Beckman expandomatic pH meter, model 76. After incubation at 37°C for 1 hr, all the cells were washed three times with MBSA (pH 7·2). After washing they were resuspended in 1 ml of MBSA. Cytotoxicity was determined in the presence of C57 anti-C3H sera and NRSC.

In experiments to find out the optimum time of enzymatic incubation, C3H lymphoid

cells were incubated with VCN (25 units enzyme/5×10^6 cells/ml) for varying periods of time (0–60 min). After incubation, the cells were washed and resuspended in 1 ml of MBSA. The cytotoxicity was measured in the presence of C57 anti-C3H sera and NRSC as described above.

In experiments where the negative feedback inhibition of the activity of VCN was studied, *N*-acetyl neuraminic acid (sialic acid) in normal saline and neutralized with NaOH solution was used as the inhibitor.

Fifty units of VCN were mixed with 1 ml of neutralized *N*-acetyl neuraminic acid (5 mg) solution in normal saline. Then 1 ml of ^{51}Cr-labelled C3H lymphoid cells (10×10^6) in M199 were added, mixed well and incubated at 37°C for 1 hr. In three separate groups, normal lymphoid cells were incubated with VCN (50 units), neutralized *N*-acetyl neuraminic acid (5 mg), and media. After incubation the cells were washed three times with MBSA and finally suspended in 1 ml of MBSA. These cells were used in the cytotoxicity assay in presence of C57 anti-C3H sera and NRSC.

Cytotoxicity assay

The cytotoxicity assay was essentially that of Wigzell (1965) with a little modification. Serial dilutions of C57 anti-C3H sera were made with MBSA. To 0·05 ml of antisera. 0·05 ml of VCN treated cells (5×10^5) were added and incubated at 37°C for 15 min. Control tubes with untreated and heat-inactivated VCN-treated cells were always run. After incubation 0·05 ml of C (1/6 dilution of NRS in M199) was added and again incubated for a total of 1 hr at 37°C. At the end of incubation all the tubes were diluted with 1·5 ml of phosphate buffered saline (PBS) (without Ca + + or Mg + +) pH 7·2. All the tubes were centrifuged at 800 *g* for 5 min and 1 ml supernatant portions were taken out from each tube for counting in a gamma-radiation counter (Gamma/Guard, Tracer Lab, Division of Laboratory for Electronics, Inc.). The release of radioactivity (counts/minute) was calculated and expressed as the percent of total radioactivity in 1 ml cell suspension from a tube containing an equal number of radiochromated cells. No antiserum or C was added in this tube and it was diluted with 1·5 ml of distilled water instead of PBS. The amount of radioactivity liberated spontaneously from the cells not exposed to antiserum or C but otherwise treated in the same way was subtracted from all other values. Tubes for C control were always run. Percent release of radioactivity was considered as a measure of the percent lysis of cells (Wigzell, 1965).

Fluid phase activation of complement using cobra venom factor

The effect of the fluid phase activation of autologous serum C on rabbit lymphoid cells in the absence of any antibody was also carried out. In this experiment the three components of the incubation mixture, labelled rabbit cells (0·05 ml) containing 5×10^5 cells, autologous rabbit serum (0·05 ml), and CVF (0·29 μg in 0·05 ml) were added to one another in varying sequences. Two of the components were mixed, incubated at 37°C for 30 min prior to the addition of the third component. After a second 30-min period of incubation at 37°C the reaction was stopped by the addition of 1·5 ml of PBS. After centrifugation the radioactivity in 1 ml supernatant was measured in the usual way. Controls containing only cells plus M199, or cells plus serum, or cells plus CVF were also run simultaneously.

Measurement of sialic acid

Sialic acid released by the action of VCN was assayed by the method of Aminoff (1961)

with a little modification. The reagents used were (a) 25 mM periodic acid in 0·125 N-H_2SO_4 (pH 1·2), (b) 2% solution of sodium arsenite in 0·5 N-HCl, (c) 0·1 M solution of 2-thiobarbituric acid (Sigma) in water, adjusted to pH 9·0 with NaOH, (d) butan-1-ol containing 5% (V/V) of 12 N-HCl. Since this is a colorimetric assay, M199 containing phenol red indicator was not used for the enzyme incubation anticipating that it might interfere in the colour production. We used balanced salt solution (BSS) of pH 7·0 prepared according to the method of Earle (1943) but without any indicator.

Lymphoid cells from C3H/HeJ mouse or from New Zealand white rabbits were prepared from teasing the respective lymph nodes apart in BSS. The cells were washed three times and finally suspended in BSS. Their viability was measured by trypan blue exclusion technique and finally diluted with BSS as necessary.

Fifty units of VCN in 1 ml BSS was added in 1 ml of cell suspension containing 100–1000 million cells and incubated at 37°C for 1 hr. After incubation the cells were spun down (800 *g*, 5 min) and the supernatant taken out for the assay of liberated sialic acid. Supernatants handled in the same manner but incubated without VCN were used for comparison. Total non-lipid sialic acid content of the lymphoid cells was determined by suspending an equal number of lymphoid cells in 2 ml of 0·1 N-H_2SO_4 and heating for 1 hr at 90°C in a water bath. It was then centrifuged, and the supernatant saved for sialic acid assay. To all the supernatants 1 ml of periodate reagent was added and incubated for 30 min at 37°C in a water bath. The excess of periodate was then reduced with 0·8 ml of sodium arsenite. After the colour of iodine had disappeared (30 sec to 1 min), 8 ml of thiobarbituric acid reagent was added, mixed well in a covered test tube and heated in a boiling water bath for 10 min. The coloured solutions were then cooled in ice water and shaken with 5 ml of acid butanol. The separation of the two phases was facilitated by centrifugation at 800 *g* for 5 min and then colours in the butanol layer were compared at 549 mμ in a Beckman spectrophotometer. *N*-acetyl neuraminic acid (Sigma, Type IV) dissolved in BSS was used as standard.

RESULTS

Condition of ^{51}Cr-labelled lymphoid cells after treatment with neuraminidase

After incubation with varying amounts of VCN (1 unit/5×10^6 cells/ml to 250 units/5×10^6 cells/ml) at 37°C for 1 hr, the lymphoid cells appeared morphologically intact and unagglutinated when viewed through a light microscope. Cells were not dead as revealed by their ability to exclude trypan blue and also by the failure of VCN to induce the release of ^{51}Cr from the labelled cells. 95–98% of the lymphoid cells survived incubation with VCN, heat-inactivated VCN, or incubation in M199 as previously noted by other investigators (Woodruff & Gesner, 1969).

Ability of Vibrio cholerae neuraminidase to release cell surface sialic acid

To see if the commercial preparation of VCN was active enough to release cell surface sialic acid, varying numbers of washed, red cell free, rabbit lymphoid cells were incubated for 60 min at 37°C with 50 units of VCN in a total volume of 2 ml of BSS. This experiment was repeated varying the amount of VCN and keeping the number of cells constant.

Total non-lipid sialic content of equal number of lymphoid cells was determined by acid digestion. The results are presented in Table 1. The data indicate that VCN is active

TABLE 1. Release of sialic acid from lymphoid cells by the action of *Vibrio cholerae* neuraminidase

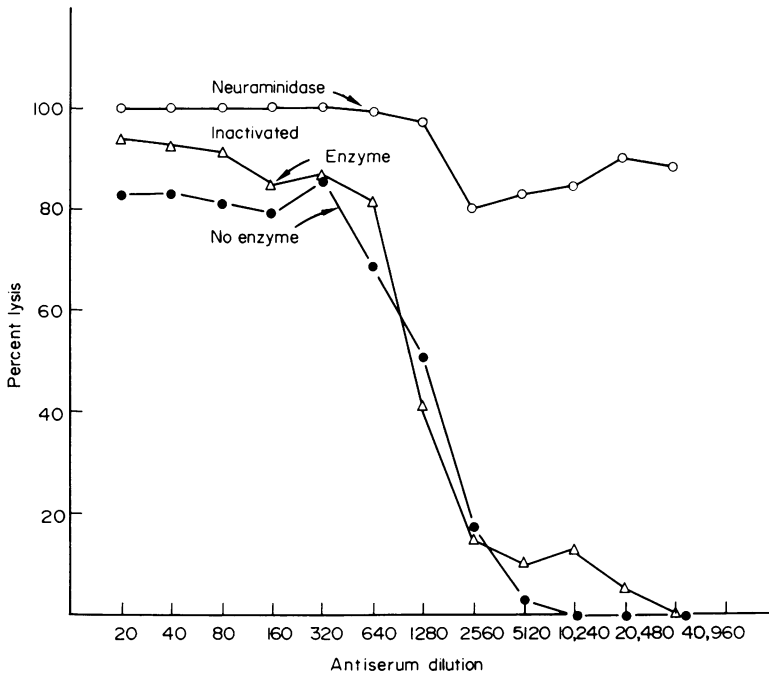| No. of Experiments	Source of Lymphoid cells	No. of cells used × 10⁶	Amount of enzyme used (units)	Amount of sialic acid released (nmoles)*	Total non-lipid sialic acid liberated by acid digestion (nmoles)*	Total sialic acid released by the enzyme* (%)
I	Rabbit	100	50	6·4	321	1·99
		200	50	14·1	698	2·02
		500	50	33·5	1746·9	1·91
		800	50	56·0	2994·5	1·87
		1000	50	68·1	3655·9	1·87
II	Rabbit	200	12·5	5·4	—	0·93
		200	25	6·0	—	1·03
		200	50	7·6	—	1·31
		200	125	8·3	—	1·43
		200	250	9·2	—	1·58
		200	500	10·4	—	1·79
		200	1000	8·7	—	1·50
		200	—	—	580	—
III	C3H/HeJ mouse	300	50	16·9	—	2·4
		300	—	—	703·5	—
IV	C3H/HeJ mouse	200	250	11·8	—	2·5
		200	—	—	469	—

* Average of duplicate tubes.

Fig. 1. Effect of *Vibrio cholerae* neuraminidase (VCN) on the lysis of C3H/HeJ mouse lymphoid cells by C57 anti-C3H antibody and rabbit complement. The figure shows results with 25 units of VCN and heat-inactivated VCN. Results with the other concentrations of VCN have not been shown here since they followed the same cytolytic profile.

TABLE 2. Effect of pH of the media on the activity of *Vibrio cholerae* neuraminidase (VCN)‡ to render mouse cells increasingly susceptible to cytolysis

pH of media	Cell treatment	Lysis (%) (^{51}Cr release)*
5·5	VCN	90
6·5	VCN	84
7·2†	VCN	84
8·5§	VCN	75
7·2†	Inactivated VCN	24
7·2†	None	22

* Average of 2 experiments.

† pH of the media.

‡ The experiments were conducted using 1 : 2560 dilution of antisera and diluted non-toxic normal rabbit serum complement.

§ During incubation the cells looked like viscous gel.

enough to release sialic acid from the cell surface. Our results correspond well with that of Woodruff & Gesner (1969) who reported an amount of 0·034 μmoles sialic acid released from 500×10^6 lymphocytes incubated for 30 min at 37°C with 50 units of neuraminidase. Though we used rabbits to get large numbers of lymphoid cells, comparable amounts of sialic acid is also released from mouse lymphoid cells by VCN (Table 1).

TABLE 3. Effect of time of incubation on the activity of *Vibrio cholerae* neuraminidase (VCN)‡ to render mouse cells increasingly susceptible to cytolysis

Time of incubation (minutes)	Cell treatment	Lysis (%) (^{51}Cr release)†
0	VCN	54*
15	VCN	66
30	VCN	81
45	VCN	86
60	VCN	92
60	Inactivated VCN	27
60	None	26

   * Enzyme was added to the cell suspension, mixed quickly and thereafter centrifuged down and washed with large volume of media. This was considered to be zero minute treatment.
   † Average of 3 experiments.
   ‡ Experiments were done using 1 : 2560 dilution of antisera and diluted non-toxic normal rabbit serum complement.

TABLE 4. Inhibition of the activity of *Vibrio cholerae* neuraminidase by *N*-acetyl neuraminic acid (sialic acid)*

Amount of sialic acid used (mg)	Neuraminidase (units)	Lysis (%) (^{51}Cr release)†
0	50	86
5	50	50
0	0	40
5	0	40

   * Highly reactive antisera (1 : 81920 dilution) and NRSC (1 : 4 dilution) were used in this experiment.
   † Average of three titrations.

Only about 2% of total non-lipid sialic acid is released by VCN under the conditions of this experiment. Increasing the relative concentration of enzyme ten-fold did not increase the amount of sialic acid released by VCN. However, these results show that VCN preparation used in the present study is fully active in its enzymatic action.

P. K. Ray, H. Gewurz and R. L. Simmons

Effect of neuraminidase on the lysis of C3H/HeJ mouse lymphoid cells by C57 anti-C3H antibody and normal rabbit serum complement

^{51}Cr-labelled C3H/HeJ lymphoid cells were treated with VCN or inactivated VCN at concentrations ranging from 5 units/5×10^6 cells/ml to 100 units/5×10^6 cells/ml. These

TABLE 5. Effect of absorption of C57 anti-C3H antisera with VCN-treated or untreated cells on their ability to lyse C3H lymphoid cells added after absorption

Antisera dilution	Lysis (%) by sera previously absorbed with cells*			
	VCN-treated	Inactivated VCN-treated	Untreated	No absorption
1:40	55	50	55	82
1:80	34	40	35	68
1:160	18	16	14	52
1:320	2	2	4	30
1:640	2	8	2	24
1:1280	—	—	—	15
1:2560	—	—	—	15
1:5120	—	—	—	20

* Average of duplicate tubes.

TABLE 6. Effect of absorption of C57 anti-C3H antisera with increasing number of VCN-treated or untreated cells on their ability to lyse C3H lymphoid cells added after absorption

Number of cells absorbed (millions)	Lysis (%) by sera previously absorbed with cells*		
	VCN-treated	Untreated	No absorption
1	97	98	100
5	99	97	100
10	62	64	98
15	29	30	98
20	20	18	97
25	8	4	98

* Average of duplicate experiments.

cells were then exposed to C57 anti-C3H serum and normal rabbit serum complement (NRSC) and the amount of cell lysis determined by the cytotoxicity assay. The results are presented in Fig. 1. All concentrations of VCN rendered C3H/HeJ lymphoid cells markedly sensitive to cytolysis by specific antisera and NRSC. Except when otherwise stated, in our subsequent experiments, we used an incubation system having 25 units of VCN/5×10^6 cells/ml.

To see if pH of the media has any effect, enzymatic incubation was performed at different pH's of the media previously adjusted with dil HCl and/or dil NaOH solution. The results are presented in Table 2. pHs in the range of 5·5 to 8·5 have no appreciable effect on the activity of VCN to render mouse lymphoid cells increasingly susceptible to cytolysis.
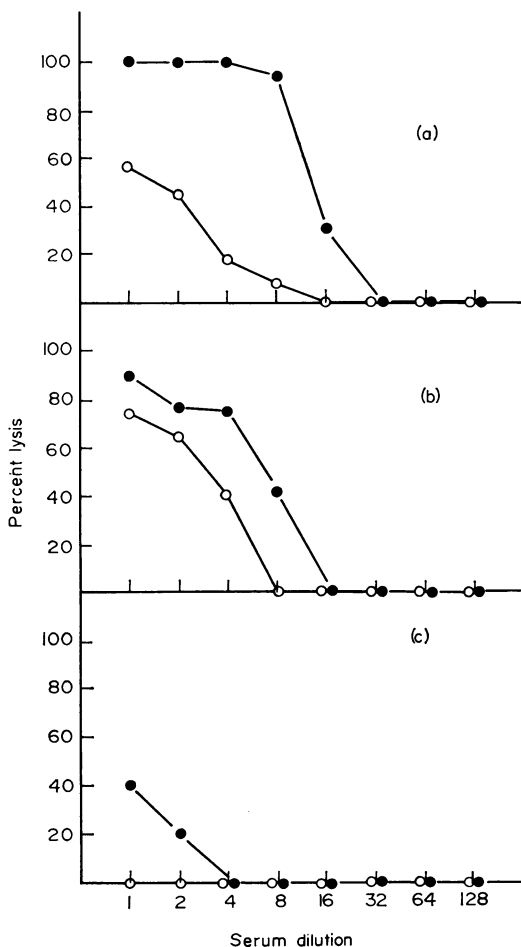

FIG. 2. Effect of *Vibrio cholerae* neuraminidase (VCN) on the lysis of C3H/HeJ lymphoid cells by normal rabbit (a), human (b), or guinea-pig (c) sera. ●, VCN treated cells; ○, inactivated VCN treated cells.

However, at pH 8·5 the cells appeared to form a gel. Hence, the M199 appeared to be adequate for enzyme action and was used throughout this study.

Another experiment was conducted varying the time of enzymatic incubation at 37°C. Results in Table 3 illustrate that even momentary contact with the enzyme renders cells more susceptible to cytolysis than prolonged incubation with the heat-inactivated enzyme. The effect, however, increased with time of incubation reaching a maximum in 45–60 min. In all the subsequent experiments enzyme incubation was performed for 60 min.

Since it has been shown that release of free sialic acid from sialyl-lactose by VCN can be inhibited by the addition of free sialic acid to the reaction mixture (Walop, Boschman & Jacobs, 1960), experiments were carried out to see the effect of adding neutralized sialic acid to the lymphoid cell-VCN reaction mixture.

TABLE 7. Effect of rabbit serum* on neuraminidase treated human erythrocytes and mouse lymphoid cells

	Lysis (%)								
Cells	VCN Treated†			Inactivated VCN Treated			Untreated		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
Human RBC	13	46	30	—	—	—	—	—	—
Mouse lymphoid cells									
(a) C3H	76	100	80	—	—	—	—	—	—
(b) CBA	70	100	85	—	—	—	—	—	—
(c) BALB	62	100	89	—	—	—	—	—	—
(d) C57	67	90	81	—	—	—	—	—	—

* Dilution of rabbit serum in media 199 used is 1:4.
† Results are average of duplicate tubes.

TABLE 8. Effect of C-inhibitors on the cytolytic ability of rabbit serum towards the neuraminidase treated C3H/HeJ mouse lymphoid cells

Treatment of normal rabbit serum	Residual cytolytic C activity in terms of maximum release of ^{51}Cr from neuraminidase treated cells (%)	Residual haemolytic C activity against SRBC and anti-sheep serum* (%)
No pretreatment	100	100
Heat (56°C, 30 min)	0	0
NH$_4$OH, 0·15 N (37°C, 90 min)	0	0
Viscarin, 5 mg/ml (37°C, 60 min)	0	0
Zymosan, 1·35 mg/ml (37°C, 90 min)	0	10

* Values were calculated considering CH$_{50}$ as 100%.
Figures in parentheses represent temperature and time of incubation.

The results of the mean of three similar experiments are presented in Table 4. The results demonstrate that the activity of VCN preparation can be largely inhibited by the addition of neutralized sialic acid, the feed-back inhibitor of neuraminidase, to the *in vitro* reaction mixture. There remains no doubt, therefore, that the effect observed with the Commercial preparation of VCN was due to the enzyme neuraminidase present in it.

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°C the supernatant solutions were used to determine the residual amount of C57 anti-C3H antibody by the cytotoxicity test using fresh ^{51}Cr-labelled C3H/HeJ mouse lymphoids cell and rabbit complement. The

TABLE 9. The effect of fluid-phase activation of autologous complement on the lysis of neuraminidase treated rabbit lymphoid cells

Treatment of cells	Complement activation	Lysis (%)*
A. CVF incubated with rabbit serum followed by additon of cells		
None	CVF	0
Inactivated VCN	CVF	0
VCN	CVF	0
B. Cells incubated with heat-inactivated rabbit serum followed by addition of CVF	CVF	
None	CVF	0
Inactivated VCN	CVF	0
VCN	CVF	0
C. Cells incubated with rabbit serum followed by addition of CVF		
None	CVF	0
Inactivated VCN	CVF	0
VCN	CVF	29†
VCN	—	5‡

* Mean of three titrations.

† In a separate experiment using different neuraminidases and 1×10^5 radiochromated rabbit lymphoid cells and autologous serum complement activated by CVF, 42% cell lysis was observed with VCN treated cells, 23% with *Clostridium perfringens* neuraminidase, but no lysis could be seen with influenza virus neuraminidase (unpublished data).

‡ Lysis is very inconsistent. Some experiments showed lysis almost equal to that observed with CVF activated C-lysis of VCN treated cells, other experiments showed no lysis.

results are presented in Table 5. 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 allogeneic antibody-binding sites on the surface of mouse lymphoid cells.

The experiment was repeated utilizing a single dilution of C57 anti-C3H antiserum (1:20) and varying the number of unlabelled VCN treated, or untreated C3H cells used for the absorption. Table 6 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 and thus do not expose more allogeneic antibody-binding sites on mouse lymphoid cell surfaces.

K

Effect of neuraminidase on the lysis of C3H/HeJ lymphoid cells by normal rabbit serum (NRS), normal human serum (NHS), and normal guinea-pig serum (NGPS)

Labelled C3H/HeJ mouse lymphoid cells were exposed to VCN or inactivated VCN or no enzyme and incubated at 37°C for 1 hr. Washed cells (5×10^5) were added to serially diluted NRS, NHS, and NGPS and incubated for 1 hr at 37°C. Cytotoxicity was measured by the release of radioactive chromium (^{51}Cr). The results are presented in Fig. 2. All the heterologous sera produced greater degrees of lysis of VCN treated cells than untreated lymphoid cells. NGPS, however, was the least potent in producing lysis of VCN treated cells. Some amount of lysis in the case of inactivated VCN treated and untreated cells might be due to the presence of natural antibodies against the mouse lymphoid cells (Colley & Waksman, 1970), but these again are far less than what was observed with VCN treated cells.

Effect of NRS on neuraminidase treated erythrocytes and lymphoid cells

Human erythrocytes (HRBC), and mouse lymphoid cells (C3H/HeJ, CBA/J, BALB/c, and C57 BL/6J) were washed and labelled with ^{51}Cr and exposed to VCN or inactivated VCN in the same way as described above. The cells were incubated with 1 : 4 dilution of NRS (non-cytotoxic to inactivated VCN treated and untreated cells) at 37°C for 60 min. The cytotoxicity was measured in terms of radioactive chromium released. Percent lysis was calculated as described earlier. The results are presented in Table 7. It appears that VCN treated mouse lymphoid cells and HRBC are increasingly lysed by NRS. So the effect of VCN to render (a) C3H mouse lymphoid cells increasingly susceptible to cytolysis by different heterologous sera (Fig. 2), and (b) HRBC, and different strain mouse lymphoid cells increasingly susceptible to cytolysis by NRS, appeared to be non-specific in terms of what kind of cells and sera are used.

Effect of various complement inhibitors on the lytic ability of NRS

NRS was exposed to various C inhibitors (Table 8) and the residual cytolytic activity of NRS against radio-chromated VCN-treated C3H/HeJ mouse lymphoid cells was compared to the residual haemolytic C activity as measured in a SRBC-rabbit-antisheep antibody haemolytic system. The C-inhibitors interfered with the cytolysis of the VCN treated cells (Table 8). The degree of inhibition correlated fairly well with the degree of C depletion as measured by the haemolytic C assay (Mayer, 1961).

Lysis of rabbit lymphoid cells by autologous serum complement activated by cobra venom factor (CVF)

0·29 μg of CVF was added to an incubation mixture of 0·05 ml of NRS (1 : 1) and 0·05 ml of radiochromated autologous rabbit lymphoid cells (5×10^5). The amount of lysis was measured in terms of percent ^{51}Cr release. The results are presented in Table 9. Addition of CVF to rabbit cells incubated with heat inactivated NRS (56°C for 30 min) caused no lysis. Incubation of CVF with NRS prior to the addition of rabbit cells caused no lysis. The addition of CVF to the incubation mixture of rabbit lymphoid cells and NRS caused lysis only when the cells had been treated with VCN. These results prove that VCN treated cells are lysable by autologous serum C if the C-cascade is activated in the fluid phase. However, some lysis was seen when autologous serum alone was incubated with VCN treated lymphoid cells which perhaps indicates that VCN treated cells are lysable by

serum C-components and more readily if C-cascade is activated. Whether this involves the participation of any other factor(s) besides C-components remains unclear.

DISCUSSION

Currie & Bagshawe (1968b) hypothesized that the sialomucins on cells by virtue of their high negative charge acted to interfere with the perception of cellular immunogens by lymphocytes. They further hypothesized that the trophoblast (Currie, vanDoorninck & Bagshawe, 1968c) and tumours (Currie & Bagshawe, 1968b) with thick sialomucin coats would fail to express their antigens due to this electronegative force. This hypothesis gained some support from the findings of Sanford (1967) that TA-3 tumour, a mouse ascites tumour which has lost strain specificity, would not kill recipient mice if the cells were treated *in vitro* with neuraminidase prior to intraperitoneal inoculation. Independently, Currie & Bagshawe showed that neuraminidase treated Landschutz ascites tumour cells (Currie & Bagshawe, 1968b), L 1210 leukaemia cells (Bagshawe & Currie, 1968) and methylcholan-threne induced sarcoma cells (Currie & Bagshawe, 1969) failed to grow in normally susceptible mice. Recipients were subsequently shown to be immune to the tumour. Linden-mann & Klein (1967) showed that Ehrlich's ascites tumour was rendered more immunogenic when treated with neuraminidase prior to inoculation. Similarly, we have recently shown that mouse lymphoid cells treated with VCN are rendered more immunogenic upon transfusion to allogeneic recipients (Simmons, Rios & Ray, 1970; 1971a). Subsequent skin grafts are more rapidly rejected after such inoculations if the inoculum had been treated with neuraminidase.

The present experiments clearly demonstrate that mouse lymphoid cells are rendered highly susceptible to the cytolytic effects of alloantibody and C by prior exposure to VCN (Fig. 1). The effect is due to the enzymatic action of VCN itself; since heat inactivation of the VCN or incubation of VCN with sialic acid, a feedback inhibitor of neuraminidase, interferes with the action of VCN (Table 4). Our results are not in conflict with those of Schlesinger & Amos (1971) who also described increased lysis of mouse peripheral lymphoid cells by anti-θ serum and guinea-pig C, as well as increased lysis of mouse lymphoid cells by guinea-pig sera themselves. It is likely that the increased lysis of mouse cells de-scribed by Schlesinger & Amos (1971) as well as that noted in the present studies (Fig. 2) in response to NRS, NHS, and NGPS is due to the presence of preformed antibodies against VCN-exposed heterophile antigenic site(s).

Despite the increased susceptibility to lysis, mouse lymphoid cells appear not to be damaged or destroyed by the VCN itself. Trypan blue exclusion, and chromium release studies demonstrate no loss of viability after incubation with VCN. Woodruff & Gesner (1969) have noted similarly, that the viability of mouse lymphoid cells is not altered by VCN, and no loss of viability after injection of those mouse lymphoid cells intravenously into syn-geneic recipients. Lundgren & Simmons (1971) have shown that human lymphoid cells exposed to VCN are fully capable of responding both to PHA and to allogeneic stimulation in mixed lymphocyte culture. Neither Sanford (1967) nor Currie & Bagshawe (1968b) have noted any interference with viability of tumour cells exposed to VCN and Simmons *et al.* (1971d) have described normal growth of VCN treated tumour cells in immunosuppressed recipients. It has been reported that the sialic acid residues are reconstituted on the surface of VCN treated cells within 48 hr to 4 days (Yarnell & Ambrose, 1969).

Although neuraminidase did not injure mouse lymphoid cells at any concentratoin tested, and did cause marked increased lysis by specific alloantibodies and C (Fig. 1), no increase in alloantibody-binding capacity of VCN treated cells could be detected (Tables 5 and 6). Similarly, Currie & Bagshawe (1968a) demonstrated that neuraminidase treatment did not modify the capacity of Landschutz cells to bind agglutinins directed against tumour cells, and Sanford & Codington (1971) noted that the number of H-2 antigens on TA-3 tumour cells was not increased after VCN treatment.

Schlesinger & Amos (1971) have recently shown that θ-antigen and another antigen on mouse lymphoid cells to which there is a preformed antibody in guinea-pig serum, are unmasked by treatment with neuraminidase. Kassulke, Stutman & Yunis (1971) have shown that blood group isoantigens on leukaemic cells are unmasked by VCN treatment. It is possible that neuraminidase acts to unmask some antigens and not others. It apparently does not unmask strong histocompatibility antigens on lymphoid cells (Tables 5 and 6).

Further experiments reported here have shown that NRS, NHS, and NGPS by itself could show increased cytolysis of VCN-treated cells even if specific antibody was not added to the incubation mixture (Fig. 2). Inactivation of C prevents the cytotoxic effect (Table 8). It is probable that heterophile antibody besides the normal cytotoxic antibodies (Colley & Waksman, 1970) was present in different normal sera and that it was directed against the VCN-exposed cell surface antigens. It is possible that VCN, while not unmasking strong H-2 histocompatibility antigens, is capable of unmasking other antigens with which different normal sera could react. Our subsequent studies have shown that heterologous sera contain antibody or antibody-like molecules absorbable by VCN-treated cells (Ray & Simmons, in preparation). This is, however, quite consistent with the findings of Schlesinger & Amos (1971). However, the increased susceptibility to immune cytolysis of VCN treated lymphoid cells appears to be primarily related to their extreme susceptibility to C (Table 9). Complement inactivation by heat totally abrogated the lysis as did C inactivation by ammonium hydroxide, viscarin, and zymosan (Table 8). In addition, activation of the terminal components of the C-cascade (C3–C9) within the fluid phase by CVF (Shin, Gewurz & Snyderman, 1969; Götze & Müller-Eberhard, 1970; Pickering et al., 1969) produced cytolysis only in VCN treated cells (Table 9). In this experiment, autologous cells were exposed to autologous serum where no antibody was present.

Lymphoid cells are normally not susceptible to cytolysis by serum C probably because of the presence of C1 esterase inactivator in normal serum (Ratnoff & Lepow, 1957). The inactivator has recently been identified as α_2-sialoglycoprotein (Pensky & Schwick, 1969) and thus may resemble the sialoglycoprotein surface of normal cells. The sialocomplex may behave as an inhibitor of C-action and when surface sialic acid is removed by VCN, the C-cascade gets better access to react upon the cell. That VCN treated erythrocytes bind more C-components was pointed out by Dalmasso & Müller-Eberhard (1964) and that surface sialic acid can act as C inhibitor has also been suggested by Yachnin, Laforet & Gardner (1961), and Arquilla, Hamashige & Miller (1964).

Several possibilities to explain the increased C sensitivity of VCN treated cells exist: (a) Sialic acid residues are primarily responsible for the negative charge on the cell surface (Weiss, 1965). VCN treatment reduces this negative charge and may thereby lessen the normal repulsion between the negatively charged C components and the cell surface. (b) Alternatively, VCN may 'unmask' or sensitize C-binding sites on cell surfaces by altering

molecular configurations (Gottschalk, 1960) of the cell surface. (c) Finally, VCN may damage, but does not lyse cells in such a way that binding fewer C molecules than normal will be sufficient to lyse the cell.

As discussed earlier, the present experiments also demonstrate that the increased susceptibility of cells treated with VCN to antibody mediated lysis is *not* the result of an increased number of allogeneic antibody-binding sites on the cell surface. Similarly, the increased *in vivo* immunogenicity of VCN treated cells, we reported earlier (Simmons, Rios & Ray, 1970 and 1971a), cannot be the result of allogeneic antigen 'unmasking'. VCN apparently increased the immunogenicity of a number of tumours (Bagshawe & Currie, 1968; Currie & Bagshawe, 1968b; Currie & Bagshawe, 1969; Cormack, 1970; Lindenmann & Klein, 1967; Simmons *et al.*, 1971b, c, d). All grow less well in normally susceptible recipients. Antibody absorption studies to detect unmasking of tumour antigens have not been carried out in these cases. Increased immunogenicity can however, 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 (Table 1); the sialic acid may offer a stearic hindrance to the perception of the antigen itself (Currie & Bagshawe, 1968a) or may stearically interfere with contact between the antigen-bearing and the antigen-processing cells; (b) VCN also reduces the negative charge on the cell surface (Weiss, 1965) 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 (Weiss, 1965) since the mutually repellent charges on the cell surface are removed. Since the T cell recognize rather large antigenic determinants in contrast to the small antigenic determinants recognized by the B cells and the increased deformability of the antigenic cell itself might increase the area of contact between it and the antigen-responsive cell; and (d) finally, treatment of cells with VCN renders them more easily phagocytosed (Weiss, Mayhew & Ulrich, 1966; Lee, 1968) and phagocytosis of antigen 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 (Simmons, Rios & Ray, 1970; 1971a) or tumour cells (Bagshawe & Currie, 1968; Currie & Bagshawe, 1968b; Currie & Bagshawe, 1969; Cormack, 1970; Lindenmann & Klein, 1967; Simmons *et al.*, 1971b, c, d). Thus, both increased cytolytic tendencies and increased immunogenicity of cells treated with VCN can be explained without the antigen 'unmasking' hypothesis.

Three immunologic properties of VCN treated cells can therefore be defined: their increased immunogenicity, their increased susceptibility to C 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 tumour cells as has been reported by us (Simmons *et al.*, 1971b, c, d) and others (Bagshawe & Currie, 1968; Currie & Bagshawe, 1968b; Currie & Bagshawe, 1969; Cormack, 1970; Lindenmann & Klein, 1967) might be due to an unmasking of tumour specific antigens or even to their increased sensitivity to C. 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.

All these experiments suggest that the sialic acid constitutents of the cell surface play a significant role in ensuring the normal fate of the cells in the body. It appears that the integrity of cell surface sialic acid is extremely important in immunochemical phenomena

and surface sugars containing sialic acid might be an important immunochemical site of reaction.

The action of VCN to sensitize the cell to C-mediated lysis may have some bearing on the action of bacterial neuraminidases during bacterial infections. For example, the *Vibrio cholerae* organism probably releases its neuraminidase and sensitizes the cells of the infected host to the action of the autologous serum C which might be activated by the endotoxin of the organism itself (Gewurz, 1971). Thus, host cell death may be mediated by fluid phase activation of C. The role of neuraminidase in the pathogenesis of infection may, thus, depend on this double interaction of cell with neuraminidase and C-activation.

REFERENCES

AMBROSE, E.J. (1966) Electrophoretic behavior of cells. *Prog. Biophys. molec. Biol.* **16,** 241.

AMINOFF, D. (1961) Methods for the quantitative estimation of *N*-acetyl neuraminic acid and their application to hydrolysates of sialomucoids. *Biochem. J.* **81,** 384.

APFFEL, C.A. & PETERS, J.H. (1970) Regulation of antigenic expression. *J. Theoret. Biol.* **26,** 47.

ARQUILLA, E.R., HAMASHIGE, S. & MILLER, A. (1964) Inhibition of immune hemolysis by glycoprotein antigens. *Fed. Proc.* **23,** 506.

BAGSHAWE, K.D. & CURRIE, G.A. (1968) Immunogenicity of L1210 murine leukaemia cells after treatment with neuraminidase. *Nature (Lond.),* **218,** 1254.

BAYER, M.E. (1964) The reaction of receptor glycoprotein with influenza virus and neuraminidase: An electron microscopic study. *Trans. N.Y. Acad. Sci.* **26,** 1103.

BENEDETTI, E.L. & EMMELOT, P. (1967) Studies on plasma membranes. IV. The ultrastructural localization and content of sialic acid in plasma membranes isolated from rat liver and hepatoma. *J. Cell Sci.* **2,** 499.

BROWN, D.M. & MICHAEL, A.F. (1969) Effect of neuraminidase on the accumulation of alpha-aminoisobutyric acid in HeLa cells. *Proc. Soc. exp. Biol. (N.Y.)* **131,** 568.

CODINGTON, J.F., SANFORD, B.H. & JEANLOZ, R.W. (1970) Glycoprotein coat of the TA3 cell. I. Removal of carbohydrate and protein material from viable cells. *J. nat. Cancer Inst.* **45,** 637.

COLLEY, D.G. & WAKSMAN, B.H. (1970) Cytotoxic effect of normal rabbit serum on rat lymphoid cells. *Transplantation,* **9,** 395.

CORMACK, D. (1970) Effect of enzymatic removal of cell surface sialic acid on the adherence of Walker 256 tumor cells to mesothelial membrane. *Cancer Res.* **30,** 1459.

CURRIE, G.A. & BAGSHAWE, K.D. (1967) The masking of antigens on trophoblast and cancer cells. *Lancet,* i, 708.

CURRIE, G.A. & BAGSHAWE, K.D. (1968a) The effect of neuraminidase on the immunogenicity of the Landschutz ascites tumour. Site and mode of action. *Brit. J. Cancer,* **22,** 588.

CURRIE, G.A. & BAGSHAWE, K.D. (1968b) The role of sialic acid in antigenic expressions: Further studies of the Landschutz ascites tumour. *Brit. J. Cancer,* **22,** 843.

CURRIE, G.A., VAN DOORNINCK, W. & BAGSHAWE, K.D. (1968c) Effect of neuraminidase on the immunogenicity of early mouse trophoblast. *Nature (Lond.),* **219,** 191.

CURRIE, G.A. & BAGSHAWE, K.D. (1969) Tumour specific immunogenicity of methylcholanthrene-induced sarcoma cells after incubation in neuraminidase. *Brit. J. Cancer,* **23,** 141.

DALMASSO, A.P., & MÜLLER-EBERHARD, H.J. (1964) Interaction of autologous complement with red cells in the absence of antibody. *Proc. Soc. exp. Biol. (N.Y.)* **117,** 643.

EARLE, W.R. (1943) Production of malignancy *in vitro.* IV. The mouse fibroblast cultures and changes seen in the living cells. *J. nat. Cancer Inst.* **4,** 165.

EYLER, E.H., MADOFF, M.A., BRODY, O.V. & ONCLEY, J.L. (1962) The contribution of sialic acid to the surface charge of the erythrocyte. *J. Biol. Chem.* **237**, 1992.

GASIC, G. & GASIC, T. (1962) Removal of sialic acid from the cell coat in tumor cells and vascular endothelium, and its effects on metastasis. *Proc. nat. Acad. Sci. (Wash.)* **48**, 1172.

GESNER, B. & THOMAS, L. (1966) Sialic acid binding sites: Role in hemagglutination by mycoplasma gallisepticum. *Science*, **151**, 590.

GEWURZ, H. (1971) Interaction between endotoxic lipopolysaccharides (LPS) and the complement (C) system: Solubilization of C-consuming substances during brief adsorptions at 0°. *Proc. Soc. exp. Biol. (N.Y.)* **136**, 561.

GOTTSCHALK, A. (1957) Neuraminidase: The specific enzyme of influenza virus and *Vibrio cholerae. Biochim. Biophys. Acta.* **23**, 645.

GOTTSCHALK, A. (1960) Correlation between composition, structure, shape and function of a salivary mucoprotein. *Nature (Lond.),* **186**, 949.

GÖTZE, O. & MÜLLER-EBERHARD, H.J. (1970) Lysis of erythrocytes by complement in the absence of antibody. *J. exp. Med.* **132**, 898.

KASSULKE, J.T., STUTMAN, O. & YUNIS, E.J. (1971) Blood-group isoantigens in leukemic cells: Reversibility of isoantigenic changes by neuraminidase. *J. nat. Cancer Inst.* **46**, 1201.

KEMP, R.B. (1968) Effect of the removal of cell surface sialic acids on cell aggregation *in vitro. Nature (Lond.),* **218**, 1255.

KINNE, D.W. & SIMMONS, R.L. (1967) Passive serologic transfer of allograft immunity in immunologically suppressed mice. I. Immunosuppression by heterologous antilymphocyte serum. *J. Immunol.* **98**, 251.

LEE, A. (1968) Effect of neuraminidase on the phagocytosis of heterologous red cells by mouse peritoneal macrophages. *Proc. Soc. exp. Biol. (N.Y.)* **128**, 891.

LINDENMANN, J. & KLEIN, P.A. (1967) *Recent Results in Cancer Research*, 9. Immunologic aspects of viral oncolysis, p. 66, Springer-Verlag, New York.

LUNDGREN, G. & SIMMONS, R.L. (1971) Effect of neuraminidase on the stimulatory capacity of cells in human mixed lymphocyte cultures. *Clin. exp. Immunol.* **9**, 915.

MAYER, M.D. (1961) *Experimental Immunochemistry.* (Ed. by E. A. Kabat). Charles C. Thomas Publishing Co., Springfield, Ill. p. 225.

PICKERING, R.J., WOLFSON, M.R., GOOD, R.A. & GEWURZ, H. (1969) Passive hemolysis by serum and cobra venom factor: A new mechanism inducing membrane damage by complement. *Proc. nat. Acad. Sci. (Wash.),* **62**, 521.

PENSKY, J. & SCHWICK, H.G. (1969) Human serum inhibitor of C1 esterase: Identity with alpha$_2$-neuramino glycoprotein. *Science*, **163**, 698.

RATNOFF, O.D. & LEPOW, I.H. (1957) Some properties of an esterase derived from preparations of the first component of complement. *J. exp. Med.* **106**, 327.

RAY, P.K., GEWURZ, H. & SIMMONS, R.L. (1970) The mechanism of increased sensitivity of neuraminidase treated cells to antibody induced cytolysis. *Fed. Proc.* **29**, 573.

RAY, P.K. & SIMMONS, R.L. (1971) Comparative effect of bacterial and viral neuraminidase in the sensitization of mouse lymphoid cells to complement. *Fed. Proc.* **30**, 571.

RUHENSTROTH-BAUER, G., FUHRMANN, G.F., GZANZER, E., KUBLER, W. & RUEFF, F. (1962) Electrophoretische untersuchungen an normalen und malignen zellen. *Naturwissenschaften*, **49**, 363.

SANFORD, B.H. (1967) An alteration in tumor histocompatibility induced by neuraminidase. *Transplantation*, **5**, 1273.

SANFORD, B.H. & CODINGTON, J.F. (1971) Further studies on the effect of neuraminidase on tumor cell transplantability. *Tissue Antigens*, **1**, 153.

SCHLESINGER, M. & AMOS, D.B. (1971) The effect of neuraminidase on the serological properties of murine lymphoid cells. *Transplant. Proc.* **3**, 895.

SEAMAN, G.V.F. & UHLENBRUCK, G. (1963) The surface structure of erythrocytes from some animal sources. *Arch. Biochem. Biophys.* **100**, 493.

SHIN, H.S., GEWURZ, H. & SNYDERMAN, R. (1969) Reaction of cobra venom factor with guinea pig complement and generation of an activity chemotactic for polymorphonuclear leukocytes. *Proc. Soc. exp. Biol. (N.Y.),* **131**, 203.

SIMMONS, R.L., RIOS, A. & RAY, P.K. (1970) Mechanism of neuraminidase induced antigen 'unmasking'. *Surg. Forum*, **21**, 272.

SIMMONS, R.L., RIOS, A. & RAY, P.K. (1971a) Immunogenicity and antigenicity of lymphoid cells treated with neuraminidase. *Nature (Lond.),* **231,** 179.

SIMMONS, R.L., RIOS, A., LUNDGREN, G., RAY, P.K., MCKHANN, C.F. & HAYWOOD, G.R. (1971b) Immunospecific regression of methylcholanthrene fibrosarcoma with the use of neuraminidase. *Surgery,* **70,** 38.

SIMMONS, R.L. & RIOS, A. (1971c) Immunotherapy of cancer: Immunospecific rejection of tumors in recipients of neuraminidase-treated tumor cells plus BCG. *Science,* **174,** 591.

SIMMONS, R.L., RIOS, A., RAY. P.K. & LUNDGREN, G. (1971d) Effect of neuraminidase on growth of a 3-methylcholanthrene induced fibrosarcoma in normal and immunosuppressed syngeneic mice. *J. nat. Cancer Inst.* **47,** 1087.

SIMMONS, R.L., LIPSCHULTZ, M.L., RIOS, A. & RAY, P.K. (1971e) Failure of neuraminidase to unmask histocompatibility antigens on trophoblast. *Nature (Lond.),* **231,** 111.

WALLACH, D.F.H. & EYLER, E.H. (1961) Sialic acid in the cellular membranes of Ehrlich ascites carcinoma cells. *Biochim. Biophys. Acta.* **52,** 594.

WALOP, H.N., BOSCHMAN, TH.A.C. & JACOBS, J. (1960) Affinity of N-acetyl neuraminic acid for influenza virus neuraminidase. *Biochim. Biophys. Acta.* **44,** 185.

WEISS, L. (1965) Studies on cell deformability. I. Effect of surface charge. *J. Cell Biol.* **26,** 735.

WEISS, L., MAYHEW, E. & ULRICH, K. (1966) The effect of neuraminidase on the phagocytic process in human monocytes. *Lab. Invest.* **15,** 1304.

WIGZELL, H. (1965) Quantitative titrations of mouse H-2 antibodies using Cr^{51}-labelled target cells. *Transplantation,* **3,** 423.

WOODRUFF, J.J. & GESNER, B.M. (1969) The effect of neuraminidase on the fate of transfused lymphocytes. *J. exp. Med.* **129,** 551.

YACHNIN, S., LAFORET, M.T. & GARDNER, F.H. (1961) pH dependent hemolytic systems. I. Their relationship to paroxysmal nocturnal hemoglobinuria. *Blood,* **17,** 83.

YARNELL, M.M. & AMBROSE, E.J. (1969) Studies of tumour invasion in organ culture. II. Effects of enzyme treatment. *Europ. J. Cancer,* **5,** 265.