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Table 2. Effect of Ca^{2+} and Mg^{2+} on the hexose transport stimulation induced by dichloroacetic acid and insulin

Additions	3-O-Methylglucose uptake (pmol/mg protein/min) None	Insulin (1 μg/ml)	Dichloroacetic acid (250 μM)
Treatment			
None	2.14 ± 0.27 (100)	5.36 ± 0.53* (250)	4.07 ± 0.50* (190)
EDTA (5 mM)	2.26 ± 0.30 (100)	4.53 ± 0.32* (200)	3.62 ± 0.11* (160)
EDTA (5 mM) + A23187 (50 μM)	2.20 ± 0.28 (100)	2.43 ± 0.35 (110)	2.20 ± 0.19 (100)
EDTA (5 mM) + A23187 (50 μM) + $MgSO_4$ (5 mM)	2.82 ± 0.18 (100)	5.21 ± 0.46* (185)	4.26 ± 0.30* (151)
EDTA (5 mM) + A23187 (50 μM) + $CaCl_2$ (5 mM)	3.53 ± 0.44 (100)	4.10 ± 0.37 (116)	3.74 ± 0.40 (106)

Cells were washed and the medium was replaced with Dulbecco's modified Eagle/medium containing EDTA and /or A23187 and/or $MgSO_4$ and/or $CaCl_2$. After 10 min, dichloroacetic acid or insulin was added and then incubated for 2 h. Then 3-O-methylglucose uptake was assayed as described in 'Materials and methods'. Data are expressed as the mean ± SE (n=3). The data in parentheses indicate % of activity: the value obtained in control experiment was defined as 100%. *$p < 0.05$ (vs control value).

cytoplasmic Mg^{2+} concentration[13]. Recently, Kono et al.[9] reported that Mg^{2+} supports the binding of insulin to its receptor and facilitates the insulin-sensitive hexose transport in adipocytes. Our results indicate that dichloroacetic acid has an insulin-like stimulatory effect on hexose transport in Swiss 3T3 cells, and also indicate an important role of intracellular Mg^{2+} in the regulation of hexose transport induced by dichloroacetic acid or insulin. However, the half-maximal stimulation occurred at 40 min after the treatment with dichloroacetic acid, while it was found only after 5 min with insulin. Since such a difference was also observed in their stimulatory effect on pyruvate dehydrogenase when measured in whole cells, the delay of the affect of dichloroacetic acid on hexose transport system may be caused by a slower transmission of the signal or lower permeability of plasma membrane to the compound. Thus, it was found that dichloroacetic acid has an insulin-like stimulatory effect on hexose transport, although its effect is less than that of insulin.

It has been reported that some membrane proteins are phosphorylated when cells are incubated with lipolytic hormones[14,15], and that insulin reverses the phosphorylation induced by these agents[15,16]. Dichloroacetic acid has also been known to inhibit protein phosphorylation[2,3]. These findings led us to propose that dichloroacetic acid-induced stimulation of hexose transport may be mediated by the inhibition of phosphorylation of some membrane proteins. Investigation of the action of dichloroacetic acid will provide further information on that of insulin.

1 This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, and the Ministry of Health and Welfare of Japan.

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0014-4754/87/0323-02$1.50 + 0.20/0

Cyclosporin A enhances Streptozocin-induced diabetes in CD-1 mice[1]

R. Iwakiri, S. Nagafuchi, E. Kounoue, S. Nakano, T. Koga, M. Nakayama, M. Nakamura and Y. Niho

First Department of Medicine, Faculty of Medicine, Kyushu University, Fukuoka 812 (Japan), 10 February 1986

Summary. Cyclosporin A (CYA), when administered to CD-1 mice treated with a subdiabetogenic dose of Streptozocin (STZ), exacerbated the STZ-induced insulitis and elevated the plasma glucose levels, parallel to a reduction of the insulin content of the pancreas. The possible mechanisms of CYA-mediated aggravation of STZ-induced diabetes are discussed.
Key words. Cyclosporin A; cellular immunity; Streptozocin; insulitis; diabetes mellitus.

There are data on autoimmunity to pancreatic B-cells in case of insulin dependent diabetes mellitus (IDDM)[2]. In autopsied patients, evidence of insulitis has been obtained[3], and islet cell antibody (ICA) and/or islet cells surface antibody (ICSA) are detectable in many newly diagnosed patients with IDDM[4,5]. Experimentally, Rossini et al. first reported the role of cellular immunity in spontaneously diabetic BB-rats[6], and accumulating data have suggested that autoimmune mechanisms play a significant role in the development of experimental diabetes mellitus. Consequently, Paik et al. and Nakamura et al. clearly showed the importance of thymic immunity and insulitis in the development of Streptozocin (STZ) induced DM in mice[7,8]. This clinical and experimental evidence suggests that autoimmune mechanisms might play a crucial role in the pathogenesis of IDDM. The possibility that patients with autoimmune related IDDM can be treated with immunosuppressive agents has to be given attention. Indeed, clinical trials using Cyclosporin A (CYA), a specific T-cell suppressant[9], have to some extent been

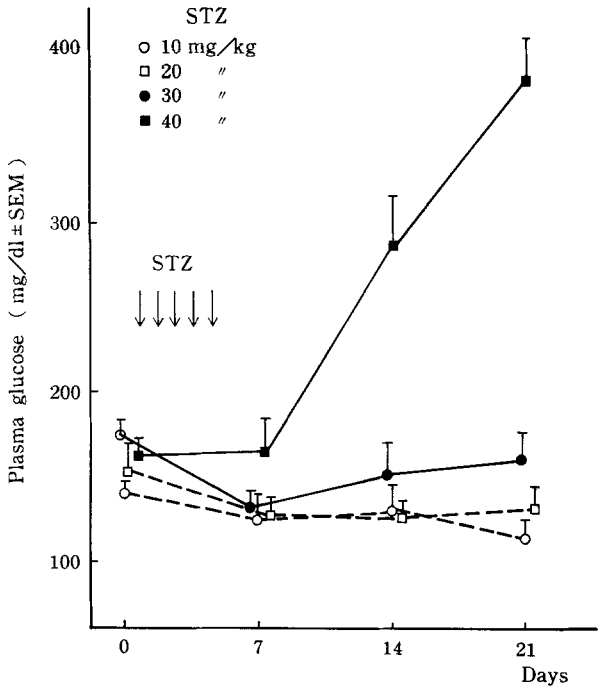

Figure 1. Plasma glucose levels following treatment with STZ. STZ was given to mice from days 1 to 5, and plasma glucose levels were determined over a 3-week period. Each point represents the mean plasma glucose level + SEM. CD-1 mice: 10 mg/kg b.wt/day (N = 5) (○—○), 20 mg/kg b.wt/day (N = 5) (□—□), 30 mg/kg b.wt/day (N = 5) (●—●), 40 mg/kg b.wt/day (N = 5) (■—■).

successful[10,11]. As little is known of the effect of CYA on the development of experimentally induced DM, we investigated in mice such effects of CYA on STZ-induced insulitis and hyperglycemia.

Materials and methods. Mice. Male CD-1 mice at 6 weeks of age were purchased from Charles River Japan Inc., Kanagawa Japan. *Streptozocin.* Streptozocin (STZ, Lot no. 1180k) purchased from the Upjohn Company (Kalamazoo, Michigan, USA) was dissolved in 0.01 M citrate buffer, pH 4.5, and used after passing through a 0.45-μm Millipore filter. The first day of STZ administration was designated as day 1 of the study. *Cyclosporin A.* Cyclosporin A (CYA, Lot no. 84101) purchased from Sandoz (Basel, Switzerland) was dissolved in Tween 80, ethanol and Dulbecco's modified Eagle's medium (D-MEM). A stock solution of 1 mg/ml was prepared by dissolving 100 mg CYA in 10 ml ethanol and 2 ml Tween 80, then D-MEM was added to a final volume of 100 ml.

Determination of plasma glucose level. Blood samples were obtained from the retro-orbital venous plexus of non-fasting mice, using heparinized hematocrit capillary tubes. After centrifugation, plasma was assayed for glucose concentration by the glucose oxidase method, using a GOD-PAPkit (Boehringer-Mannheim GmbH Diagnostica, FRG).

Histologic examination and evaluation of insulitis. For light microscopy, pancreases were fixed in 10% formalin and embedded in paraffin. 5-μm wide sections made from every 15 μm for each pancreas were stained with hematoxylin and eosin. All the islets were evaluated for insulitis as follows: negative (−); no mononuclear cell infiltration, mild (+); a few mononuclear cell infiltrations around the islet, moderate (++); obvious cell infiltrations in and around the islet, but the structure of the islet was not destroyed, severe (+++); massive cell infiltrations were observed as structure of the islet was destroyed.

Determination of insulin content of pancreas. Pancreases were mixed with a few milliliters of acid ethanol and disrupted by sonication. Following a 12-h incubation at −20 °C, the preparations were centrifuged at 4 °C, and supernatants obtained. The extraction procedures were repeated three times and all specimens were collected. IRI (immuno-reactive-insulin) was measured by radioimmunoassay.

Results. Plasma glucose levels following treatment with various doses of STZ are shown in figure 1. To determine the dose necessary to cause hyperglycemia, 10, 20, 30, and 40 mg/kg of STZ were injected into mice i.p. for five consecutive days. Plasma samples were obtained on days 0, 7, 14, and 21. At doses of 10, 20, 30 mg/kg b.wt/day, plasma glucose levels remained the same until day 21, and no mouse became diabetic. However, with a dose of 40 mg/kg b.wt/day, plasma glucose levels were elevated significantly on day 14 and all the mice became diabetic. To examine the effect of CYA on the STZ treated mice, 30 mg and 35 mg/kg b.wt/day of STZ were given.

The effects of CYA on STZ treated mice are shown in figures 2 and 3. To examine the effect of CYA on STZ treated mice, 20 mg/kg of CYA and 30 mg or 35 mg/kg of STZ were given i.p., simultaneously for 5 days, and CYA was given continuously for an additional 5 days. As shown in figure 2, the addition of CYA increased the plasma glucose levels. On day 14 in either the 30 mg/kg or 35 mg/kg b.wt of STZ treated groups, all the CYA treated mice became diabetic. In contrast, plasma glucose levels of 30 mg/kg b.wt of STZ only treated mice were slightly elevated and 2 out of 6 mice treated with 35 mg/kg b.wt of STZ became diabetic. Similar diabetogenic effects of CYA were observed in case of 10 mg/kg b.wt of CYA (data not shown).

On day 14, all the mice were killed and the histopathology of the islets was examined. In mice treated with 30 mg/kg of STZ, 374 islets were examined and evaluated for insulitis. The results were

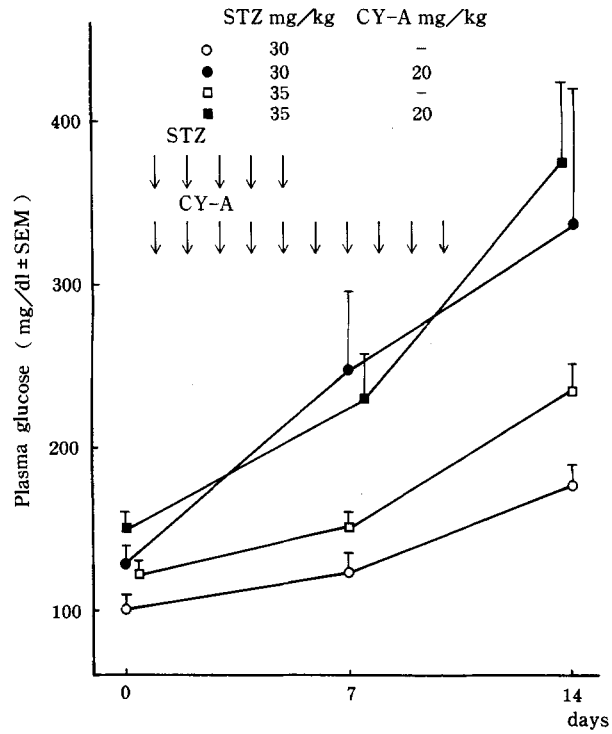

Figure 2. Plasma glucose levels following treatment with STZ only or with STZ + CYA. 20 mg/kg of CYA and/or 30 mg or 35 mg/kg of STZ were given from days 1 to 5, and CYA only was given for an additional 5 days. Plasma glucose levels were determined over a 2-week period. Each point represents the mean plasma glucose level + SEM. CD-1 mice: 30 mg/kg b.wt/day of STZ only (N = 6) (○—○), 30 mg/kg b.wt/day of STZ and 20 mg/kg b.wt/day of CYA (N = 6) (●—●), 35 mg/kg b.wt/day of STZ only (N = 6) (□—□), 35 mg/kg b.wt/day of STZ and 20 mg/kg b.wt/day of CYA (N = 6) (■—■).

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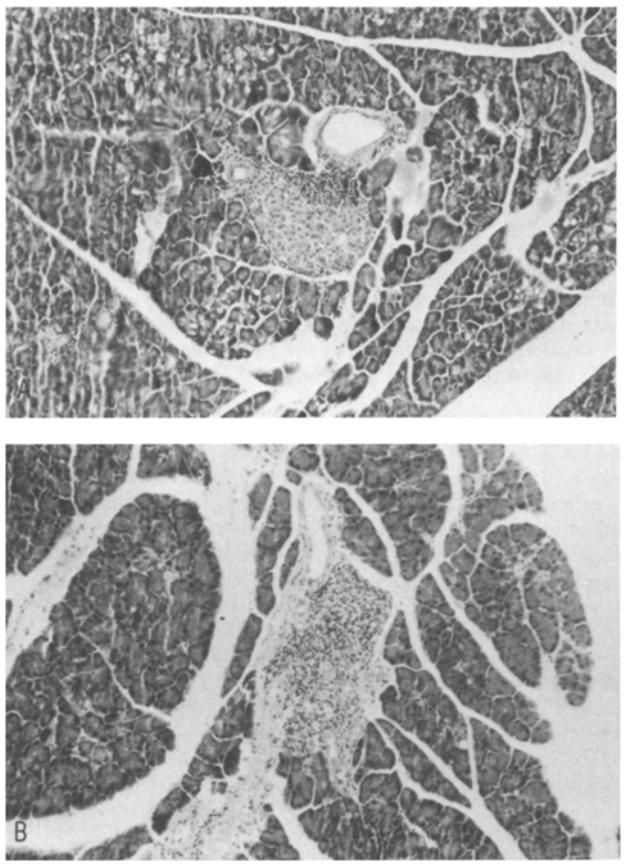

Figure 3. Histopathology of the islets on day 14. *A* STZ only treated CD-1 mice (×100). *B* STZ + CYA treated CD-1 mice (×100). Mononuclear cell infiltrations in and around the islets are apparent. We evaluated (A) as moderate insulitis, and (B) as severe insulitis.

as follows: negative, 62.3%; mild, 22.5%; moderate, 10.4%; severe, 4.8%. In mice treated with 30 mg/kg of STZ and 20 mg/kg of CYA, 315 islets were also examined and evaluated for insulitis. Results were: negative, 50.8%; mild, 30%; moderate, 14.9%; severe, 7.3%. Thus, insulitis was more frequent and more extensive in the STZ + CYA treated mice than in the the CYA only treated mice, in which neither insulitis nor degeneration of islet cell was observed. Figure 3 illustrates the extent of insulitis. We evaluated (A) as moderate insulitis, and (B) as severe insulitis.

To exclude the possible influence of the CYA solvent (ethanol + Tween 80 + D-MEM), STZ plus solvent controls were also studied. We found no differences between this STZ + solvent treated group and the group treated with STZ only. Thus, the influence of solvent could be disregarded (data not shown). We also examined mice given 20 mg/kg b.wt of CYA for 10 days and found no elevation in plasma glucose levels (data not shown).

Effect of CYA on STZ treated CD-1 mice. Pancreatic insulin contents after treatment of mice with STZ and/or CYA. STZ was given from days 1 to 5 and CYA was given from days 1 to 10. All mice were killed on day 14 and pancreatic IRI contents were examined

STZ (mg/kg)	CYA (mg/kg)	PG ± SEM (mg/dl)	IRI ± SEM ($\times 10^3$ μU/pancreas)
–	–	112.0± 2.3	158.5±12.7
–	20	115.8± 3.2	85.8± 1.9
30	–	178.0±12.3	33.5± 5.1
30	20	339.0±63.2	16.8± 3.8
40	–	344.0±17.0	13.4± 0.5

STZ: streptozocin; CYA: cyclosporin A; PG: plasma glucose; IRI: immuno-reactive-insulin.

The effects of CYA on pancreatic IRI contents of mice treated with STZ are shown in the table. The pancreatic insulin contents of STZ and/or CYA treated mice were determined. The mice were killed on day 14 and pancreatic IRI contents were examined. IRI contents of STZ + CYA treated mice were reduced to about 65% of that of STZ only treated mice, and to about 14% of that of the non-treated control mice. In the case of mice treated with CYA only, the pancreatic insulin contents were reduced to about 50%, compared to findings in the non-treated control.

Discussion. CYA has been postulated to interfere with the primary processes of lymphocyte activation such as inhibition of IL-2 production by T-lymphocytes or acceleration of PG-E production by macrophages. CYA mainly suppresses T-cell mediated immune responses[9, 12, 13]. As reported, thymic immunity enhances the STZ-induced DM by exacerbating the insulitis[7, 8]. Therefore, CYA may suppress the STZ-induced insulitis, a T-cell dependent autoimmunity[8]. Unexpectedly, CYA did not suppress the development of insulitis, rather it enhanced cellular infiltrations and exacerbated the hyperglycemia in the STZ treated CD-1 mice. Although the mechanisms involved are not clear, several explanations have to be considered; 1) Direct damage to pancreatic islets by CYA alone. 2) Interactions of STZ and CYA in the islet cells might induce a new antigenic expression on the cell surface, resulting in an enhanced antigenic stimulation followed by the induction of autoimmunity. 3) Interaction of STZ and CYA may modify the immune system to enhance the autoimmunity to islets. 4) Depression of suppressor T-cell activity towards cell surface antigens of pancreatic islets by CYA. 5) A mechanism other than (4) to enhance the autoimmunity to pancreatic islets by CYA.

As shown in our study, following daily, i.p. injections of 20 mg/kg of CYA to CD-1 mice for 10 days, pancreatic IRI contents were reduced to about 50% of the contents in the control mice, *thereby suggesting that pancreatic B-cell function deteriorated in the presence of CYA.* However, neither morphological changes in the pancreatic B-cells nor hyperglycemia occurred in the mice treated with CYA only. On the other hand, Helmchen et al. found degenerative changes in pancreatic B-cells and the induction of diabetes mellitus by oral administration of 50 mg/kg of CYA to Wistar rats for 7 days[14]. These discrepancies may be due to the different doses of CYA given or to the different animal species used. Decrease of insulin production and inhibition of DNA synthesis was found to be significant in CYA treated cultured mouse pancreatic islets[15], and Kojima et al. reported the CYA induced inhibition of islet cell replication in mice[16].

Therefore, it was concluded that CYA directly damages pancreatic islet cells. The insulitis was more frequent and more extensive in the STZ + CYA treated mice than the STZ-only treated mice in our study. Thus, CYA may possibly alter the immune response resulting in an enhanced impairment of islet function.

Although CYA has been widely used to treat patients with transplants[9], and clinical applications of CYA to treat IDDM are under way[10, 11], our data do suggest that islet cell function should be carefully monitored during CYA therapy.

1 The authors are grateful to M. Ohara for comments on the manuscript.

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0014-4754/87/0324-0451.50 + 0.20/0

Carbohydrate interference of complement-dependent cell lysis

J.J. Killion

School of Medicine, Department of Physiology, Oral Roberts University, Tulsa (Oklahoma 74171, USA), 6 June 1986

Summary. The antibody-mediated cytotoxicity of three autoreactive sera, an allogeneic hyperimmune serum and a xenogeneic hyperimmune serum was abrogated by the presence of either glucosamine, galactosamine, lactulose or lactose. This inhibition could be overcome in a dose-dependent fashion by increasing the amount of complement in the cytotoxicity assay, but not by increasing the amount of antibody. Furthermore, the inhibition was specific for these sugars in that isomers and N-acetylated derivatives were not inhibitory. The results suggest that these sugars directly blocked events of the complement cascade.
Key words. Complement; antibody; carbohydrate; cell-lysis.

Carbohydrates have been shown to inhibit a variety of biological phenomena such as antibody-mediated lysis[1], antigen-induced lymphocyte proliferation[2], natural killer cell reactions[3], mixed-lymphocyte reactivity[4], tumoricidal lymphocytes[5] and adhesion of macrophages[6]. I have reported the existence of autoreactive antibodies in the sera of tumor-bearing, genetically autoimmune and alloimmunized mice[7,8], which were detected by their cytotoxicity against neuraminidase-treated syngeneic spleen cells. Together, these observations motivated attempts to identify putative carbohydrate-like antigens at the surface of neuraminidase-treated cells by blocking techniques using carbohydrates in aqueous solution. However, during the course of the experiments, it became apparent that certain carbohydrates specifically blocked antibody-mediated lysis in a fashion that suggested carbohydrate-mediated block of the complement (C) cascade.

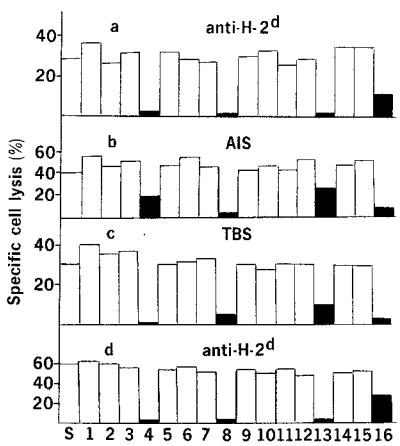

Figure 1. Percent specific cytotoxicity of antisera for murine spleen cells, in the presence of either saline or cabohydrates. *a–c* Three autoreactive sera incubated with neuraminidase (VCN)-treated spleen cells. Anti-H-2^d serum was a polyclonal, hyperimmune C57BL/6 anti-DBA/2. Autoimmune serum (AIS) was from NZBWF1 mice greater than 8 months of age. Tumor-bearing serum (TBS) was from L1210 lymphoma-bearing B6D2F1 mice. These 3 sera have complement-dependent cytolytic antibodies against VCN-treated syngeneic cells. This cytotoxicity was blocked by 75 mM solutions of carbohydrates 4, 8, 13 and 16. *d* Inhibition of cytotoxicity of the anti-H-2^d serum by the same carbohydrates as above, when the sera was reacted with normal DBA/2 spleen cells. Code for carbohydrate solution: Control, isotonic saline; 1 = D-(+)-glucose, 2 = βD-(+)-glucose, 3 = 2-deoxy-D-glucose, 4 = D-(+)-glucosamine, 5 = α-Methyl-D-glucoside, 6 = N-acetyl-D-glucosamine, 7 = D-(+)-galactose, 8 = D-(+)-galactosamine, 9 = α-Methyl-D-galactoside, 10 = N-Acetyl-D-galactosamine, 11 = α-D-(+)-fucose, 12 = α-L-(−)-fucose, 13 = β-D-lactulose, 14 = α-Methyl-D-mannoside, 15-N-Acetyl-β-D-mannosamine, 16 = α-lactose.

Materials and methods. Preparation of serum. The three autoreactive sera used in this study have been described[7,8]. Tumor-bearing serum (TBS) was defined as the sera obtained from B6D2F1 mice on day 5 following an injection of L1210 leukemia cells. Autoimmune sera (AIS) was obtained from NZBWF1 mice of greater than 8 months of age and known to contain autoreactive antibodies. Anti-H-2^d sera was prepared by 4 monthly injections of DBA/2 liver and spleen cells (H-2^d) into the peritoneal cavity of histoincompatible C57BL/6 mice (H-2^d). Sera were collected from clotted blood after bleeding the tail ventral artery into ice-cold plastic tubes. These sera were cytotoxic to a variety of neuraminidase-treated (*Vibrio cholera*, Grand Island Biological Co.) cells at a maximum titer of 1:8 with greater than 90% cell kill. Rabbit anti-mouse sera (Cappel Laboratories) was used at a 1:20 dilution (40–70% cytotoxicity against murine spleen cells). All mice, except for the NZBWF1 (ORU Biomedical Research Center breeding facilities) were obtained from the Jackson Laboratories.

Neuraminidase treatment. Murine spleen cells were washed twice in RPMI 1640 medium containing 1% heat-inactivated fetal calf serum (1% medium) and treated with VCN as previously described[7]. VCN treatment was 1 unit/10^6 cells, pH 5.6, 1 h, 37 °C.

Cytotoxicity assay. Washed, untreated or VCN-treated cells were labeled with ^{51}Cr (100 μCi per 10^7 cells, 3 h, 37 °C) and triplicate determinations of the complement-dependent cytotoxicity of the sera were made as follows: 50 μl of labeled cells were added to equal volumes of antisera (diluted 1:5) which had been preincubated with saline (positive control) or a panel of isotonic carbohydrate solutions used at a final concentration of 75 mM.