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Modification at C6 of the Terminal Galactosyl Residues of Cobra Venom Factor Abolishes Anti-α-Gal Antibody Immunoreactivity without Affecting Functional Activity

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The *N*-linked oligosaccharides of cobra venom factor (CVF) contain unique terminal α-galactosylated Lewis X structures. We have previously shown that CVF immobilized on nylon membranes binds naturally occurring human anti-α-Gal antibody. The present study shows that soluble CVF can effectively inhibit the binding of anti-α-Gal antibody to CVF-coated microtiter plates, indicating that the terminal α-galactosyl residues of the functionally active CVF are accessible to anti-α-Gal antibody binding. Modification of the terminal galactosyl residues of CVF by treatment with galactose oxidase and *in situ* derivatization of the generated aldehyde groups with hydrazides abolished the human anti-α-Gal antibody immunoreactivity without affecting the complement-activating activity.

Cobra venom factor (CVF)[2] is a 146-kDa complement-activating glycoprotein in cobra venom (1-3). The activity of CVF is due to the formation of CVF,Bb, a functional analog of C3b,Bb, the physiologic C3/C5 convertase of the alternative pathway of complement. Although CVF,Bb and C3b,Bb closely resemble each other in their formation and function, the former is completely resistant to the action of the complement regulatory factors H and I (1). Therefore, CVF can deplete animal complement with the formation of membrane attaching complexes, which can cause bystander lysis of cells. This property of CVF has been used for selective cell killing by cross-linking CVF to antibodies directed against cell surface antigens (1, 4-7). Recently, CVF has been shown to prevent the hyperactive rejection of a pig-to-baboon organ transplant by complement depletion, suggesting a potential application of CVF in pig-to-human organ transplantation (8, 9).

CVF contains three *N*-linked oligosaccharide chains, the majority of which terminate with unique α-galactosyl Lewis X structures (10). We have previously shown (10) that CVF immobilized onto PVDF membranes can bind naturally occurring human anti-α-Gal antibody (11). Therefore, this immunoreactivity precludes applications of CVF in man.

Recently, it was reported that intramuscular injection of CVF to baboons markedly reduces the levels of serum C3 and CH50, and serum-cytotoxicity to pig kidney PK cells (12). However, there was a rapid increase in the levels of anti-α-Gal antibodies due to the presence of terminal α-Gal residues in CVF. Thus, a second injection of CVF to the animals on day 14 was ineffective in reducing serum C3 and CH50 levels and serum-cytotoxicity to PK cells (12), presumably because the increased anti-α-Gal antibody level facilitates a rapid clearance of CVF from the circulation by immune complex formation.

Although our previous study demonstrated that nondenatured CVF immobilized on nylon membranes binds to human anti-α-Gal antibody, it was not clear whether soluble CVF is also immunoreactive to this antibody (10). In this study, we show that soluble, functionally active CVF can bind human anti-α-Gal antibody, and this immunoreactivity can be abolished without affecting the functional activity by selective modification of terminal galactosyl residues by oxidation with galactose oxidase in the presence of hydrazides.

MATERIALS AND METHODS

Materials. CVF was isolated from *Naja naja kaouthia* venom as described previously (13). 3-(2-Pyridyldithiol)propionic hydrazide

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[2] Abbreviations: CVF, cobra venom factor; HRP, horseradish peroxidase; PVDF, polyvinylidene difluoride; PDPH, 3-(2-pyridyldithio)-propionyl hydrazide; M_2C_2H, 4-(Maleimidomethyl)-cyclohexane-1-carboxyl-hydrazide; HABA, 4′-hyroxyazobenzene-2-benzoic acid; *p*-nitropheny phosphate; PDPH-CVF, PDPH derivative of CVF; M_2C_2H-CVF, M_2C_2H derivative of CVF.

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(PDPH) was synthesized as reported by Zara *et al.* (14). Coffee bean α-galactosidase (10 units/mg protein) and galactose oxidase (25 units/mg protein) were from Boehringer Mannheim (Indianapolis, IN). Centricon 30 microconcentrators from Amicon (Danvers, MA). 4-(Maleimidomethyl)-cyclohexane-1-carboxyl-hydrazide (M_2C_2H) and biotin hydrazide were from Pierce Chemical Co. (Rockford, IL). HRP (type VIA, 1000 U/mg), formic hydrazide, avidin-HABA reagent kit, melibiose-agarose and α-methyl galactoside were from Sigma Chemical Co. (St. Louis, MO). Protein A-Sepharose from Pharmacia (Piscataway, NJ). *p*-Nitrophenyl phosphate was from Kirkegaard Perry laboratories (Gaithersburg, MD). Alkaline phosphatase conjugated goat anti-human IgG (enzyme activity 41,800 units/ml) was from Tago, Inc. (Burlingame, CA). The natural anti-α-Gal IgG was purified from human serum as described by Li *et al.* (15). Guinea pig erythrocytes were obtained by femoral vein puncture.

De-α-Galactosylation of CVF and analysis of the released galactose. CVF (500 μg in 400 μl of 100 mM sodium phosphate, pH 6.5) was treated with coffee bean α-galactosidase (1.2 units, added in two equal aliquots at 0 and 8 h) (10). After 20 h, at 37°C, the enzyme-digest was filtered through Centricon 30 tubes. De-α-galactosylated CVF in the retentate was purified on a 2 ml DEAE-Sephacel column (10), concentrated and dialyzed against PBS, pH 7.2. Galactose in the Centricon 30-filtrate was analyzed by Dionex BioLC HPLC (Dionex, Sunnyvale, CA) on a CarboPac PA1 high-pH anion-exchange column (16). Elution was with 20 mM NaOH at a flow rate of 0.9 ml/min. The galactose peak was quantitated with reference to a standard solution.

Modification of terminal galactosyl residues. CVF (2.5 mg in 1 ml of 100 mM sodium phosphate, pH 6.5) was incubated with galactose oxidase (5 units) and HRP (30 units) in the presence of a hydrazide (PDPH, M_2C_2H, biotin hydrazide or formic hydrazide, 50-100 mol/mol of CVF) at 37°C for 30 h (17,18). Excess hydrazide was removed by filtration using Centricon 30 tubes.

To determine the levels of unmodified α-galactosyl residues, if any, aliquots of hydrazide-derivatized CVF (50 μg in 50 μl of 100 mM sodium phosphate, pH 6.5) were treated with α-galactosidase (0.2 units), filtered through Centricon 30 tubes, and the filtrates analyzed for galactose by Dionex HPLC as described above (16).

Estimation of hydrazide moiety coupled to CVF. The extent of hydrazide derivatization at C6 positions of the terminal galactose residues of CVF was estimated by measuring the amounts of PDPH and Biotin hydrazide coupled to galactose oxidase-oxidized CVF. To determine PDPH, an aliquot of PDPH-CVF (150 μg in 50 μl of PBS, pH 7.2) was reduced with 50 mM dithiothreitol at room temperature for 20 min to release pyridine-2-thione (19). The absorbance of the solution was measured at 343 nm. The pyridine-2-thione content was determined by using a molar absorptivity of $8.08 \times 10^3\ M^{-1}\ cm^{-1}$ at 343 nm (19). The amount of biotin hydrazide that was coupled to CVF was estimated by the HABA dye-displacement method using the HABA-avidin kit (20). It was assumed that similar amounts of M_2C_2H and formic hydrazides were coupled to the enzyme-oxidized CVF.

To determine the extent of hydrazide addition to the aldehyde groups formed at C6 positions of the terminal galactosyl residues, aliquots of the derivatized CVF (100 μg in 50 μl of 50 mM borate buffer, pH 9.0) were reduced with $NaBH_4$ (7.5 μg). After 2 h at room temperature, glycerol (10 μl) was added and incubated at room temperature for 1 h. The solutions were dialyzed against 100 mM sodium phosphate, pH 6.5, incubated with α-galactosidase, and then filtered on Centricon 30 tubes. The filtrates were analyzed for galactose by Dionex HPLC as described above.

Hemolytic assay for CVF activity. The activity of untreated CVF, galactose oxidase-treated CVF and hydrazide-derivativatized CVF was measured by the by-stander lysis of guinea pig erythrocytes in guinea pig serum (10). CVF or hydrazide-derivatized CVF (4 to 8,000 ng) in 20 μl of PBS, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% (w/v) gelatin, pH 7.2, was mixed with 20 μl of guinea pig serum and 20 μl of guinea pig erythrocytes (5×10^8 cells/ml in the above buffer). The tubes were vortexed and incubated at 37°C for 40 min with occasional shaking. The reaction was stopped by the addition of 1 ml of cold PBS, pH 7.2. The unlysed erythrocytes were pelleted by centrifugation, and the released hemoglobin in the supernatant was measured at 412 nm to determine the extent of hemolysis.

Binding of human anti-α-Gal antibody CVF-coated microtiter plates. Untreated CVF, de-α-galactosylated CVF, or hydrazide-derivatized CVF (10 μg/1 ml in 50 mM Tris-HCl, 150 mM NaCl, pH 8) was coated onto ninety six-well (Becton Dickinson Labware, San Jose, CA) microtiter plates (100 μl/well) overnight at 4°C. The wells were blocked with 1% bovine serum albumin (150 μl/well) in 50 mM Tris-HCl, 150 mM NaCl, pH 8.0, for 2 h at room temperature, incubated with serially diluted (1:2) human anti-α-Gal antibody (0.16 μg to 50 μg/ml, 100 μl per well) in 50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 8.0, at room temperature for 2 h, and then washed four-times with 50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 8.0, and incubated with alkaline phosphatase-conjugated goat anti-human IgG (1:2000 diluted) in the above buffer at room temperature for 1 h. The wells were washed and incubated with 100 μl solution of *p*-nitrophenyl phosphate (1 mg/1 ml in 1 M diethanolamine, pH 9.5) at room temperature for 40-60 min. The absorbance of the released *p*-nitrophenol was measured at 410 nm using a MR 600 Dynatech microplate reader (Dynatech Laboratories, Chantilly, VA).

Inhibition of human anti-α-Gal antibody binding to CVF-coated microtiter plates by soluble CVF. Ninety six-well microtiter plates were coated with 100 μl of untreated CVF (10 μg/ ml in 50 mM Tris-HCl, 150 mM NaCl, pH 8.0) and then blocked with 1% bovine serum albumin as described above. In separate Eppendorf tubes, aliquots of human anti-α-Gal antibody (25 μg/100 μl) in 50 mM Tris-HCl, 150 mM NaCl, pH 8.0, was mixed with varying amounts (0.8 μg to 2500 μg in 100 μl of the above buffer) of CVF, α-galactosidase-treated CVF or hydrazide-derivatized CVF at room temperature for 2 h. The antibody/CVF solutions (100 μl each) were then transferred to microtiter plate wells, incubated at room temperature for 2 h and then washed with 50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 8.0. The anti-α-Gal antibody bound to the wells was measured using alkaline phosphatase-conjugated goat anti-human IgG as described above. The percentage inhibition of anti-α-Gal antibody binding to CVF-coated microtiter plates by untreated CVF, α-galactosidase-treated CVF or hydrazide-derivatized CVF in solution was calculated from the formula [(OD_{410} without the addition of soluble CVF $-$ OD_{410} with the addition of soluble CVF) $\div$ OD_{410} without the addition of soluble CVF] $\times$ 100.

RESULTS

Nature of terminal galactosyl residues. We have previously shown that the oligosaccharide chains of CVF terminate with α-galactosyl residues. Treatment of CVF with coffee bean α-galactosidase and HPLC analysis using pulsed amperometric detection showed that the enzyme released 4.1 mol of galactose from 1 mol of CVF. This value agrees with the previously reported content of terminal α-galactosyl residues in CVF (29 nmol/1 mg of CVF) (10).

Modification of terminal galactosyl residues. To selectively modify the terminal galactosyl residues, CVF was treated with galactose oxidase in the presence of HRP. The aldehyde groups generated at C6 positions of the terminal galactosyl residues were derivatized *in situ* by the addition of PDPH, M_2C_2H, biotin hydrazide

or formic hydrazide to the enzyme incubation mixture. Treatment of the hydrazide-derivatized CVF with α-galactosidase did not release measurable amounts of galactose, suggesting that the primary hydroxyl groups of terminal α-galactosyl residues were quantitatively oxidized by galactose oxidase.

The extent of hydrazide (PDPH, M_2C_2H, biotin hydrazide, or formic hydrazide) that coupled to the aldehyde groups generated on the terminal α-galactosyl residues of CVF was measured by reducing the derivatized CVF with $NaBH_4$ and then treating with α-galactosidase. Galactose was not detected in the incubation mixtures, suggesting that the derivatization of the generated aldehyde groups with various hydrazides was quantitative.

The extent of derivatization at C6 positions of the terminal galactosyl residues of CVF was also determined by directly measuring the amounts of dithiopyridyl or biotin residues coupled to CVF. Treatment with DDT released about 5.6 mol of pyridine-2-thione from 1 mol of PDPH-CVF. In the case of biotin hydrazide, about 5.2 mol of biotin residues were coupled to CVF. These results indicate that CVF contains about 5.2-5.6 mol of terminal galactosyl residues; 4.1 mol of α-galactosyl residues and 1.1 to 1.5 mol of terminal β-galactosyl residues.

Upon incubation of CVF with galactose oxidase and PDPH without HRP, only 3.2 moles of PDPH was coupled to 1 mol of CVF. However, incubation with 3-fold more galactose oxidase than that usually used in the presence of HRP gave almost quantitative derivatization (~5.3 mol of PDPH per 1 mol of CVF) of the terminal galactosyl residues.

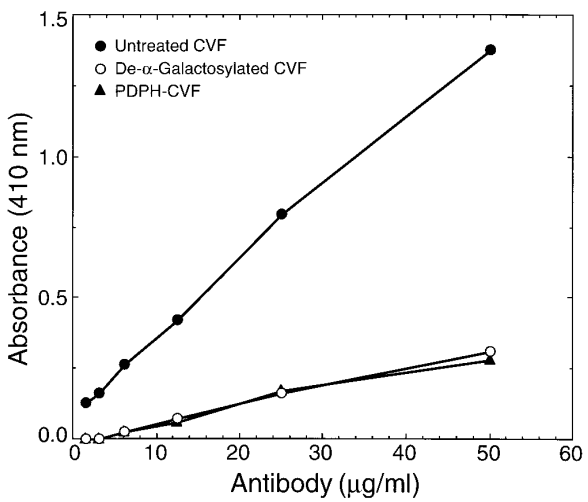

FIG. 1. Immunoreactivity of CVF with human anti-α-Gal antibody. Ninety six-well microtiter plates were coated with untreated CVF, α-galactosidase-treated CVF or hydrazide-derivatized CVF, blocked with 1% bovine serum albumin and then allowed to interact with the indicated amounts of human anti-α-Gal antibody. The bound anti-α-Gal antibody was measured using alkaline phosphatase-conjugated goat anti-human IgG and *p*-nitrophenyl phosphate. M_2C_2H-CVF and biotin hydrazide-CVF showed low levels of binding similar to that of PDPH-CVF.

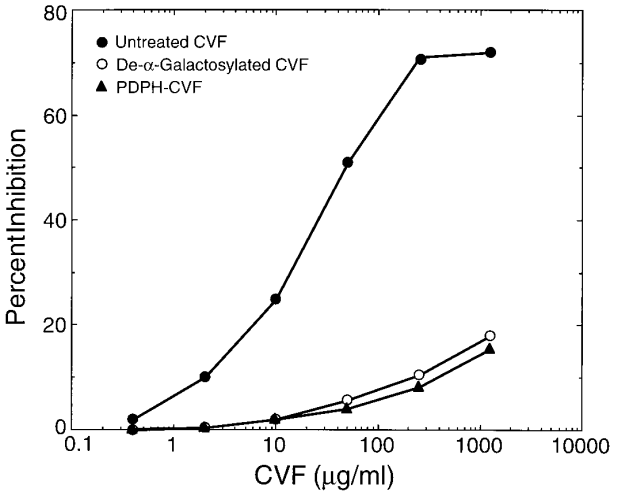

FIG. 2. Inhibition of human anti-α-Gal antibody to microtiter plate-coated CVF by soluble CVF. Ninety six-well microtiter plates were coated with untreated CVF (100 μl of 10 μg/ ml solution per well) and then blocked with 1% bovine serum albumin. Aliquots of human anti-α-Gal antibody (25 μg/100 μl) in Eppendorf tubes were mixed with the indicated amounts of CVF, α-galactosidase-treated CVF or hydrazide-derivatized CVF, and then added to the microtiter plate wells. The bound antibody was measured using alkaline phosphatase-conjugated goat anti-human IgG and *p*-nitrophenyl phosphate. M_2C_2H-CVF and biotin hydrazide-CVF showed low levels of inhibition similar to that of PDPH-CVF.

Immunoreactivity with human anti-α-Gal antibody. CVF-coated microtiter plates could bind human anti-α-Gal antibody in a dose dependent manner (Fig. 1). In contrast, only a background level of binding was observed for α-galactosidase treated-CVF or hydrazide derivatives of CVF (Fig. 1), suggesting that the antibody binding is specific to terminal α-galactosyl residues.

To determine whether the terminal α-galactosyl residues of non-denatured CVF are accessible to binding with anti-α-Gal antibody, the antibody was incubated with soluble CVF and then allowed to bind to CVF-coated microtiter plates. As shown in Fig. 2, prior incubation of CVF with the anti-α-Gal antibody markedly inhibited the antibody binding to CVF-coated microtiter plates. Under similar conditions, de-α-galactosylated or hydrazide derivatives of CVF were only marginally inhibited antibody binding to CVF-coated microtiter plates (Fig. 2).

Complement activating activity of CVF. The activity of CVF and hydrazide derivatives of CVF was measured by the bystander lysis of unsensitized guinea pig erythrocytes in guinea pig serum. The dose-response curves for hydrazide derivatives of CVF were similar to that of the unmodified CVF (Fig. 3), suggesting that

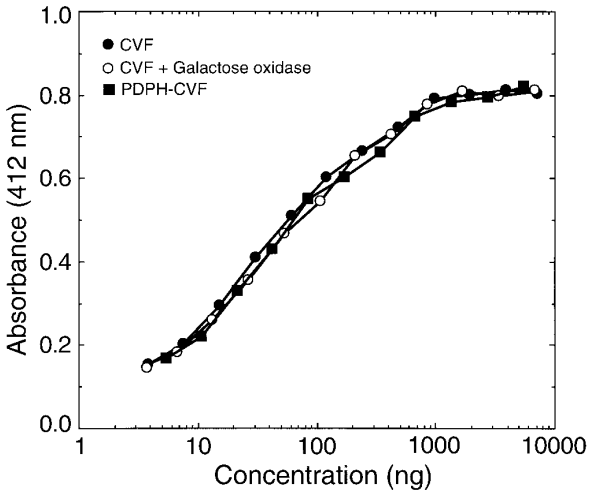

FIG. 3. Determination of CVF activity by measuring complement-mediated lysis of unsensitized guinea pig erythrocytes. Indicated amounts of CVF α-galactosidase-treated CVF, and hydrazide-derivatized CVF were incubated with unsensitized guinea pig erythrocytes in guinea pig serum. The absorbance of released hemoglobin was measured at 412 nm after pelleting the unlysed erythrocytes. Each point represents the average of duplicate determinations. M_2C_2H-CVF and biotin hydrazide-CVF gave activity curves similar to those of untreated CVF and PDPH-CVF.

the derivatization of the terminal galactosyl residues of CVF does not affect the CVF activity.

DISCUSSION

We have previously shown that CVF immobilized onto nylon membranes can bind naturally occurring human anti-α-Gal antibody in an α-galactosyl residue-dependent manner (10). Untreated CVF, de-α-galactosylated, and deglycosylated CVF all showed similar human serum complement-consumption activity (10). Based on these results, we suggested that the binding of the antibody to untreated CVF has no effect on the functional activity (10). However, the binding of anti-α-Gal antibody to soluble, functionally active CVF was not directly demonstrated. Since it is possible that the previously observed binding of anti-α-Gal antibody to immobilized CVF was due to the exposure of terminal α-galactosyl residues upon the denaturing of CVF, we sought to determine whether the soluble CVF also binds human anti-α-Gal antibody. The results presented here demonstrate that CVF coated on microtiter plates can bind human anti-α-Gal antibody in an α-galactosyl residue-dependent manner (Fig. 1). The antibody binding can be effectively inhibited by prior incubation of soluble CVF with human anti-α-Gal antibody (Fig. 2). Together, these data demonstrate that the terminal α-galactosyl residues of the functionally active CVF are accessible for human anti-α-Gal antibody binding.

CVF may have the potential for cancer therapy based on targeted cell killing (1, 4-7). Recently, CVF has been studied for use in prevention of hyperactive organ rejection in discordant organ transplantation (8, 9). However, in a model study, CVF was found to stimulate the production of anti-α-Gal antibody in baboons (12). Like humans, baboons are immunoreactive to anti-α-Gal antibody (11). Therefore, if CVF were to be used for medical applications man, it is desirable to abolish the anti-α-Gal antibody immunoreactivity. Although, this immunoreactivity can be abolished by treatment with α-galactosidase, we have recently shown that de-α-galactosylated CVF is cleared from the circulation in mice at a significantly higher rate compared to untreated CVF due to recognition of Lewis X structures by the liver cell lectins (21). In humans, however, both untreated CVF and de-α-galactosylated CVF are expected to be rapidly cleared from the circulation; the former due to formation of immune complexes with naturally occurring anti-α-Gal antibody and the latter due to recognition of Lewis X structures by liver cell lectins. The results of the present study demonstrate that modification of the terminal galactose residues of CVF by treatment with galactose oxidase and derivatization of aldehyde groups with hydrazides, abolishes anti-α-Gal antibody immunoreactivity without affecting the CVF activity. Since the hydrazide derivatives of CVF can not form immune complexes with naturally occurring anti-α-Gal antibody, it is likely that this modification does not affect the plasma half life of CVF in humans.

This study established that CVF contains about 5.6 residues of terminal galactosyl residues, 4.1 α-linked residues and the remainder β-linked. This agrees with the number of terminal galactose residues ($\sim$6 residues/mol CVF) predicted based on carbohydrate compositional and oligosaccharide structural analyses (unpublished results). These results also indicate that oxidation of the primary hydroxyl group of terminal galactosyl residues of CVF by galactose oxidase and coupling of hydrazides to the generated aldehyde group are almost quantitative.

Addition of horseradish peroxidase to the incubation mixture facilitated the oxidation of the terminal galactose residues by preventing inactivation of galactose oxidase by hydrogen peroxide formed during the oxidation of galactosyl residues. When horseradish peroxidase was not added, the amount of galactose oxidase that was used could oxidize only 60% of the terminal galactose residues of CVF. However, 3 times the amount of galactose oxidase could oxidize almost all the terminal galactosyl residues of CVF.

Galactose oxidase can further oxidize the aldehyde group at the C6 positions of galactosyl residues to a carboxyl group (22). However, we avoided this complication by incubating CVF with galactose oxidase in the presence of hydrazide so that the generated aldehyde

groups immediately form hydrazone bonds, and thus are not over oxidized by the enzyme.

The selective and facile procedure described here for the quantitative derivatization of terminal galactosyl residues of CVF can be used as a strategy for the carbohydrate site-directed conjugation of CVF to antibodies. The PDPH and M_2C_2H contain protected functional groups that can be used as sites in cross-linking of CVF to antibodies. Since the derivatization process does not affect the functional activity of CVF, this approach is likely to offer immunoconjugates with maximal retention of CVF activity. Moreover, such a strategy should yield immunoconjugates that are nonreactive to human anti-α-Gal antibody.

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REFERENCES

1. Vogel, C.-W. (1991) *in* Handbook of Natural Toxins, Vol. 5: Reptiles and Amphibian Venoms (Tu, A. T., Ed.), pp. 147–188, Marcel Dekker, New York.
2. Vogt, W. (1990) *in* Medical Use of Snake Venom Proteins (Stocker, K. F., Ed.),pp. 79–96, CRC Press, Boca Raton, FL.
3. Fritzinger, D. C., Bredehorst, R., and Vogel, C.-W. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 12775–12779.
4. Vogel, C.-W. (1991) *in* Immunology and Allergy Clinics of North America, Vol. 11: Human Cancer Immunology II (Oettgen, H. F., Ed.), pp. 277–299, W. B. Saunders Co.,Philadelphia, PA.
5. Juhl, H. L., Petrella, E. C., Cheung, N.-K. V., Bredehorst, R., and Vogel, C.-W. (1989) *Mol. Immunol.* **27,** 957–964
6. Petrella, E. C, Wilkie, S. D., Smith C. A., Morgan, Jr., A. C., and Vogel, C.-W. (1987) *J. Immunol. Methods.* **104,** 159–172
7. Juhl, H., Sievers, M., Baltzer, K., Helmig, F., Wolf, H., Brenner, W., and Holger, K. (1995) *Cancer Res.* **55,** 5749s-5755s.
8. Candinas, D., Lesnikoski, B. A., Robson, S. C., Scesney, S. M., Otsu, I., Myiatake, T., Marsh, H. C., Ryan, U. S., Hancock, W. W., and Bach, F. H. (1996) *Transplant. Proc.* **28,** 581.
9. Kobayashi, T., Taniguchi, S., Ye, Y., Niekrasz, M., Kosanke, S., Neethling, F. A., Wright, L. J., Rose, A. G., White, D. J. G., and Cooper, D. K. C. (1996) *Transplant. Proc.* **28,** 560.
10. Gowda, D. C., Petrella, E. C., Raj, T. T., Bredehorst, B., and Vogel, C.-W. (1993) *J. Immunol.* **152,** 2977–2986.
11. Galili, U., Clark, M. R., Shohet, S. B., Buehler, J., and Macher, B. A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1369–1373.
12. Taniguchi, S., Kobayashi, T., Neethling, F. A., Ye, Y., Niekrasz, M., White, D. J. G.,and Cooper, D. K. C. (1996) *Transplant.* **6,** 678–681.
13. Vogel, C.-W., and Müller-Eberhard, H. J. (1984) *J. Immunol. Methods* **73,** 203–220.
14. Zara, J. J., Wood, R. D., Pomato, N., Boon, P., Bredehorst, R., and Vogel, C.-W.(1991) *Anal. Biochem.* **104,** 156–162.
15 Li, S., Yeh, J-C., Cooper, D. K. C., and Cummings, R. D. (1995) *Xenotransplantation* **2,** 279–288.
16. Hardy, M. R., and Townsend, R. R. (1994) *Methods Enzymol.* **230,** 208–225.
17. Morell, A. G., and Ashwell, G. (1972) *Methods Enzymol.* **28,** 205–208.
18. Bayer, E. A., Ben-Hur, H., and Wilschek, M. (1988) *Anal. Biochem.* **170,** 271–281.
19. Carlsson, J., Drevin, H., and Axen, R. (1978) *Biochem. J.* **173,** 723–737.
20. Grenn, N. M. (1970) *Biochem. J.* **94,** 23c-24c.
21. Gowda, D. C., Satyaswaroop, P. G., and Fu, Q. (1997) *Biochem. Biophys. Res.Commun.* **231,** 316–320.
22. Kelleher, F. M., and Bhavanandan (1986) *J. Biol. Chem.* **261,** 11045–11048.