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A New Non-equilibrium Enzyme Linked Immunosorbent Assay for a Glycogen-derived Urinary Tetrasaccharide

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The tetrasaccharide, Glcα1-6Glcα1-4Glcα1-4Glc, denoted $(Glc)_4$, is a limit dextrin formed by amylolytic degradation of glycogen. In order to evaluate the possible clinical importance of $(Glc)_4$ excretion as an indicator of certain physiological and pathological conditions, we have developed a new rapid and inexpensive immunoassay using a monoclonal antibody raised against $(Glc)_4$ glycosidically-linked to a carrier protein. As the antibody is highly specific, it can be used to measure native $(Glc)_4$ directly, without the chemical reduction step required in previous methods. A new type of non-equilibrium ELISA inhibition test was developed based on the capacity of free $(Glc)_4$ to decrease initial rates of antibody binding to $(Glc)_4$-coated microtiter wells. The method is highly reproducible and is as sensitive and accurate as the gas chromatography method or radioimmunoassay used previously.

We have developed an assay [1] for rapid determination of a glucose-containing tetrasaccharide, Glcα1-6Glcα1-4Glcα1-4Glc, $(Glc)_4$ which is normally excreted in human urine. The oligosaccharide is formed by amylolytic degradation of glycogen [2, 3]. Increased urinary excretion has been reported in pregnancy [4, 5]; and in patients with glycogen storage diseases [1, 6, 7] and Duchenne muscular dystrophy [8]. Previous radioimmunoassays for $(Glc)_4$ employed a rabbit antiserum (R 895) [5] or a mouse monoclonal antibody (61.1) [9], both raised against $(Glc)_4$ coupled *via* a phenethylamine linker arm to keyhole limpet hemocyanin (KLH). Both monoclonal antibody 61.1 and rabbit antiserum

Abbreviations. $(Glc)_4$, Glcα1-6Glcα1-4Glcα1-4Glc; KLH, keyhole limpet hemacyanin; BSA, bovine serum albumin; PEG, polyethylene glycol.

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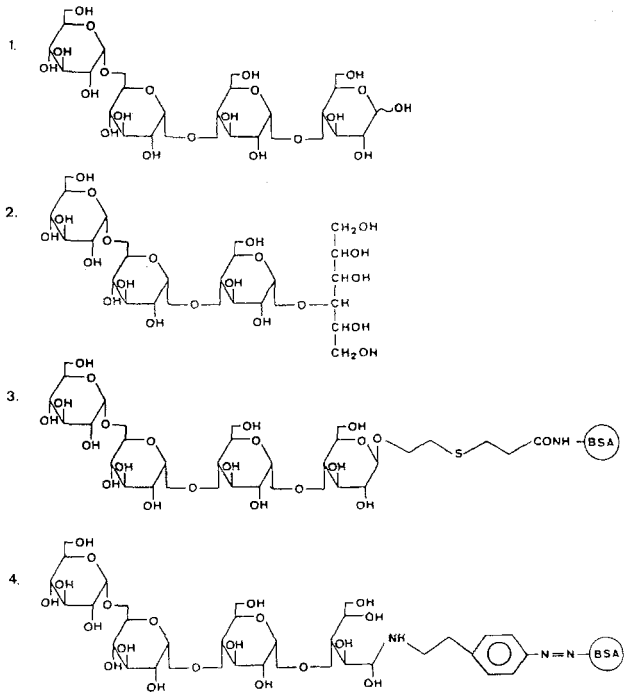

Figure 1. Native $(Glc)_4$ (1), $(Glc)_4$-OL (2), $(Glc)_4$ coupled glycosidically to BSA *via* a 2-(2-methoxycarbonyl-ethyl-thio)ethyl derivative (3) and $(Glc)_4$ coupled to BSA *via* a phenethylamine linker (4).

R895 bound the alditol derivative of $(Glc)_4$ with higher affinity than the native oligosaccharide, and hence, urine samples had to be reduced in order to maximize sensitivity of the radioimmunoassay method.

A new monoclonal antibody has now been developed against $(Glc)_4$ coupled glycosidically *via* a 2-(2-methoxycarbonylethylthio)ethyl linker arm to KLH [10]. This antibody reacts slightly better with native $(Glc)_4$ than with its alditol form and therefore is useful for an enzyme-linked immunosorbent assay (ELISA), allowing rapid determination of free $(Glc)_4$ in deionized urine samples without reduction. The assay, which is performed under non-equilibrium conditions, is as accurate and sensitive as previously described radioimmunoassays. The method offers the additional advantages of being less expensive and more convenient for analysis of large numbers of urine samples and avoids using radioactive materials.

Materials and Methods

Mice

Female mice of the strain Balb/cABom (Gl Bomholtgaard, Ry, Denmark) were used. At the time of the first immunization the mice were 5-9 weeks old.

Table 1. Comparison of inhibition of binding of [^{3}H]-$(Glc)_4$-OL to monoclonal antibodies 401/6 and 61.1, and to rabbit antiserum R 895 by various oligosaccharides and glycogen.

Inhibitor	Structure of oligosaccharide	nmoles required for 50% inhibition		
		401/6	61.1	R 895
$(Glc)_4$	Glcα1-6Glcα1-4Glcα1-4Glc	0.32	20.1	4.5
$(Glc)_4$-OL	Glcα1-6Glcα1-4Glcα1-4Glucitol	0.55	1.3	0.45
Panose	Glcα1-6Glcα1-4Glc	0.53	1.2	3
Panitol	Glcα1-6Glcα1-4Glucitol	>400	300	3
Isomaltose	Glcα1-6Glc	>200	>1000	850
Isomaltotriose	Glcα1-6Glcα1-6Glc	>300	>1000	30
Maltose	Glcα1-4Glc	6	15	>1000
Maltotriose	Glcα1-4Glcα1-4Glc	24	50	>1000
Maltotetraose	Glcα1-4Glcα1-4Glcα1-4Glc	7	30	600
Glycogen		>500*	>500*	200*
Glucose		>2000		

*Expressed as nmol glucose

Antigens

$(Glc)_4$, glycosidically coupled *via* a 2-(2-methoxycarbonylethylthio)ethyl group to KLH (400 mol $(Glc)_4$ per mol KLH) (Fig. 1) or to bovine serum albumin (BSA) (20 mol $(Glc)_4$ per mol BSA) were prepared. The synthesis of this spacer arm glycoside and its coupling to proteins has been described [10]. The antigens were designated $(Glc)_4$-KLH and $(Glc)_4$-BSA, respectively.

Antibodies

Rabbit antiserum (R 895) and a mouse monoclonal antibody (61.1), both directed against reduced $(Glc)_4$ coupled *via* a phenethylamine derivative to KLH, were previously described [5, 9]. Rabbit anti-mouse IgM, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgA were purchased from Miles Laboratories, Elkhart, ID, USA.

Inhibitors

Oligosaccharides and polysaccharides used as inhibitors for the immunochemical characterization of the antibody are the same as previously described [9]. They are listed in Table 1.

Urine

Urine specimens from normal individuals were collected and stored at -18°C until analyzed. The samples were deionized by passage through a mixed bed ion exchange column of AG 3-X4A (OH^-) and AG 50W-X8 (H^+) (Bio- Rad, Richmond, CA, USA), concentrated to dryness by rotary evaporation under vacuum, and redissolved to their original

volumes in 0.15 M NaCl, 0.02 M potassium phosphate buffer, pH 7.4, and 0.02% Tween 20 (PBS-Tween).

Immunization

Each of four mice was immunized (day 1) subcutaneously with 50 μg $(Glc)_4$-KLH emulsified with Freunds complete adjuvant (Difco Laboratories, Detroit, MI, USA). Fifty μg of antigen mixed with Freunds incomplete adjuvant (Difco) was given intraperitoneally (i.p.) on days 16 and 28. The mice were boosted with 400 μg of antigen given i.p. on four consecutive days immediately before fusion (day 52).

Hybridomas

Hybridomas were produced according to Kennett [11] using 50% polyethylene glycol (PEG 1500, Merck, Darmstadt, W. Germany). Spleen cells from the immunized mice were fused with Sp2/0 cells at a ratio of 10:1. Screening for antibody production was carried out using solid phase ELISA [12] with $(Glc)_4$-BSA (0.1 μg/well) as target antigen bound to microtiter plates (Dynatech, Cooke, Plochingen, W. Germany). Cloning was performed as described by Nowinski *et al.* [13]. Hybridomas were expanded in cell culture and grown as ascites tumours. Immunoglobulin classes and subclasses of monoclonal antibodies were determined by the immunodiffusion technique according to the method of Ouchterlony [14], using the rabbit anti-mouse antisera described above.

Antibody Purification

Partial purification of mouse monoclonal IgM antibodies from ascites was carried out in the following way: 3 ml of ascites was centrifuged (800 $\times$ *g*, 5 min) filtered (0.22μ filter, Leer Medical, Irigny, France) and applied to a Sephacryl S-300 (Pharmacia, Uppsala, Sweden) column (1.5 $\times$ 86 cm) equilibrated in 0.1 M Tris-HCl buffer pH 8.0. The elution was monitored with a UV 280 recorder (LKB, Stockholm, Sweden). Fractions corresponding to the void volume were pooled and concentrated about 10 times by dialysis against polyethylene glycol 20 000 (Kebo AB, Stockholm, Sweden).

Nitrocellulose Filter Assay

Antibody binding of $(Glc)_4$-OL-[^{3}H] and hapten inhibition of binding was performed using a nitrocellulose filter assay as previously described [5, 15]. Briefly, the assay is performed by incubating varying amounts of inhibitor with 38.5 μg antibody 401/6 (see later) in a final volume of 300 ml for 1 h at 4°C. $(Glc)_4$-OL-[^{3}H] (10^5 cpm) is then added in a volume of 100 μl and incubation is continued overnight at 4°C. The mixture is passed through a nitrocellulose filter and the filter is washed rapidly with 10 ml buffer and counted by liquid scintillation.

ELISA

ELISA tests were conducted using polystyrene microtiter plates (Dynatech, Cooke,) according to procedures described by Engvall and Perlmann [12] using rabbit anti-mouse immunoglobulin conjugated to either horseradish peroxidase or alkaline phosphatase

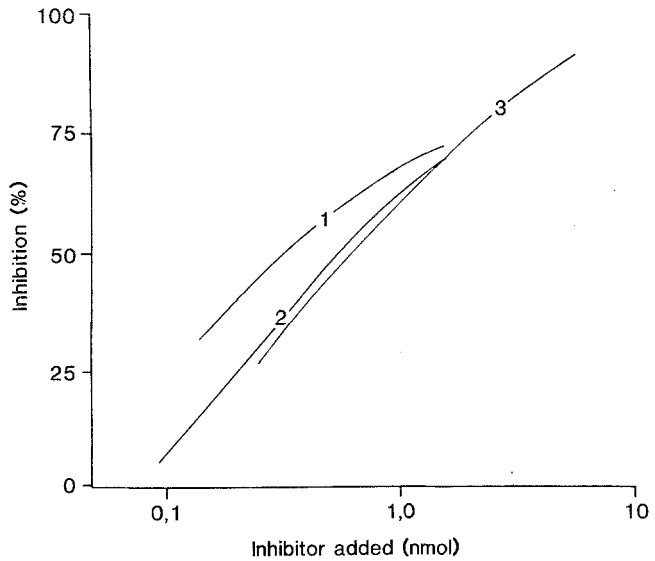

Figure 2. Inhibition by $(Glc)_4$ (1), Panose (2) and $(Glc)_4$-OL (3) of $(Glc)_4$-OL-[1-^{3}H] binding by monoclonal antibody 401/6. The assay was performed as described in [15] but at a temperature of 4°C.

(Dakopatts, Copenhagen, Denmark). Peroxidase reactions were developed using a solution containing 0.1% recrystallized 5-amino-salicylate in 0.01 M sodium phosphate buffer, pH 6.0, plus H_2O_2 freshly added to a concentration of 0.01%.

The substrate solution for developing the phosphatase reaction contained *p*-nitrophenylphosphate (10 mg/ml) freshly dissolved in a stock buffer containing 1 M diethanolamine plus 0.5×10^{-3} M $MgCl_2$ and 0.02% NaN_3 adjusted to pH 9.8 with 1 M HCl. All enzyme reactions were incubated at 22°C.

Results

Hybridizations

Antibodies from supernatant fluids of fifteen hybridomas (8% of growing colonies) bound to $(Glc)_4$-BSA. Of these, one designated 401/6 bound $(Glc)_4$-OL-[1-^{3}H] with affinity high enough to permit detection of binding of the hapten in the nitrocellulose filter assay. This IgM antibody was partially purified from hybridoma ascites fluid as described above.

Specificity

Hapten inhibition of $(Glc)_4$-OL-[^{3}H] binding to antibody 401/6 was studied using the nitrocellulose filter assay (Fig. 2) and the results were compared with those previously

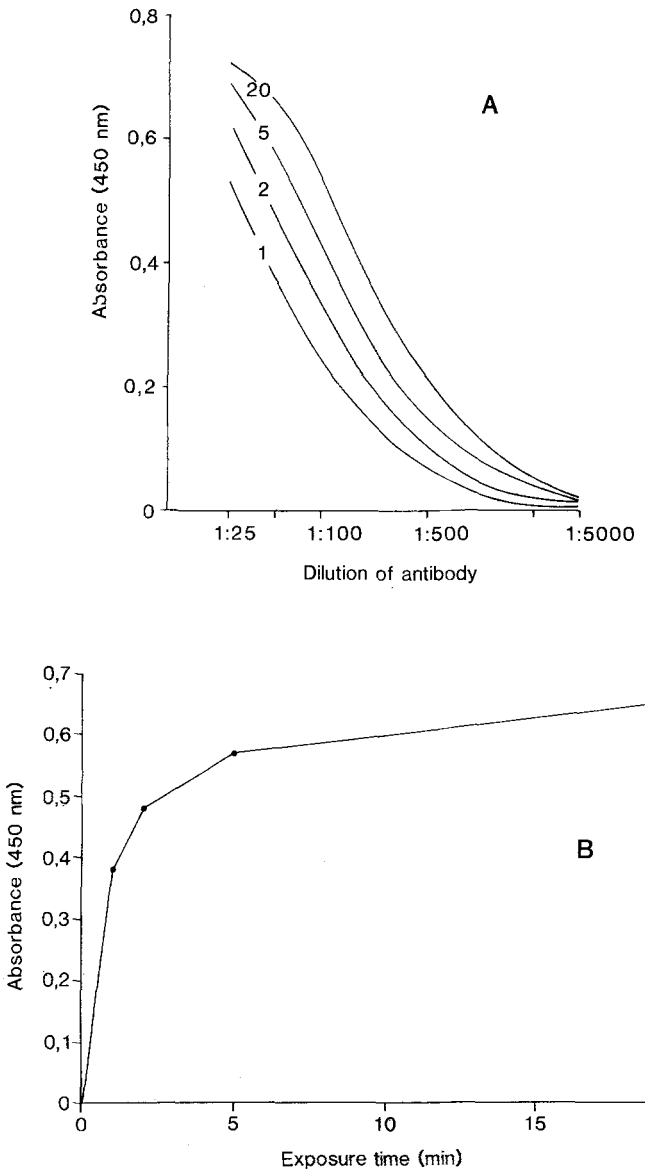

Figure 3. A. Binding of 401/6 to $(Glc)_4$-BSA-coated microtiter plates. Microtiter plates were coated overnight with 0.1 μg $(Glc)_4$-BSA per well and the wells were then saturated by incubation with 200 μl 1% BSA dissolved in PBS-Tween for 2 h. The wells were emptied and washed three times with PBS-Tween. Aliquots (50 μl) of purified 401/6 antibody solution containing 770 μg/ml (or serial dilutions) were added to the microtiter wells and incubated for 1, 2, 5, or 20 min at 4°C. The wells were then washed as above and 100 μl of anti-mouse immunoglobulin-horseradish peroxidase or anti-mouse immunoglobulin-phosphatase (both reagents diluted 1:500 in PBS-Tween) was added to each well and incubated for 30 min. The wells were then washed as above and 100 μl substrate solution (see Materials and Methods section) was added. The peroxidase reaction was followed at 450 nm and the phosphatase reaction at 405 nm using a microtiter plate scanner.

B. Binding of antibody 401/6 at a dilution of 1:50 (15.4 μg/ml) to wells of a microtiter plate at 1, 2, 5, and 20 minutes. Data is replotted from Figure 3A.

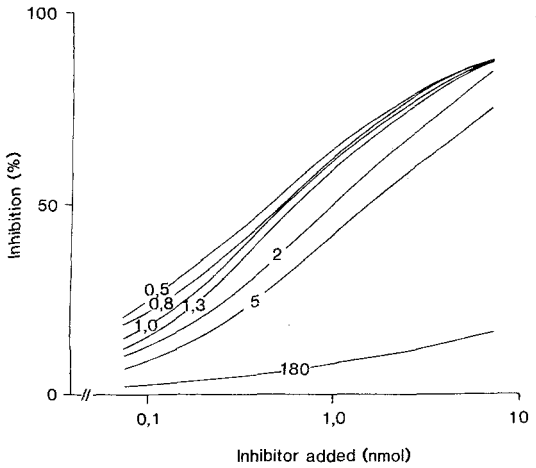

Figure 4. Inhibition of antibody 401/6 binding to $(Glc)_4$-BSA-coated microtiter plates. Antibody-hapten preincubations were performed for 2 h at 4°C. Incubations on the plates were done at 0.5, 0.8, 1.0, 1.3, 2.0, 5.0 and 180 min. The amounts of antibody bound to the plate were determined as described in the legend to Fig. 3A.

reported for rabbit antiserum R895 [5] and the monoclonal antibody 61.1 [9] (see Table 1). In preliminary experiments it was determined that antibody 401/6, like 61.1, binds hapten with higher affinity at 4°C than at room temperature, and therefore, all inhibition studies were performed at 4°C. For antibody 401/6, native $(Glc)_4$ inhibits 50% when 0.32 nmol is added to the assay under standard conditions (see Materials and Methods). Panose and $(Glc)_4$-OL are almost two-fold less active as inhibitors; oligosaccharides of the maltosyl series exhibit 20 to 80-fold lower inhibitory activity and those of the isomaltosyl series, like glycogen and glucose, failed to produce 50% inhibition at the concentrations tested.

Binding of 401/6 to $(Glc)_4$-BSA-coated Plates

As shown in Fig. 3A, the amount of antibody bound to the microtiter well at various dilution increases with increasing time of exposure to the plate from 1-20 min. At a dilution of 1:50 (15.4 μg/ml) the amount of antibody bound to the solid phase increased to about 80% of the final value after 5 min (Fig. 3B). Hapten inhibition studies as described below were carried out at an antibody dilution of 1:50 with exposure time of 1 min.

Inhibition of Antibody Binding to $(Glc)_4$-BSA-coated Microtiter Plates by $(Glc)_4$

Different amounts of $(Glc)_4$ dissolved in PBS-Tween were preincubated for 2 h with antibody 401/6 (15.4 μg/ml) at 4°C. Triplicate 50 μl aliquots of this antibody-hapten solution were then transferred to the $(Glc)_4$-BSA-coated microtiter plate and incubated for 0.5 to 180 min. The wells were then washed and the amount of bound antibody measured as described in the legend to Fig. 3. As shown in Fig. 4, the sensitivity of the assay is maximal when preincubated antibody-hapten mixtures are exposed to $(Glc)_4$-BSA-coated

microtiter wells for 0.5-1.3 min: during this time interval the quantity of $(Glc)_4$ required to produce 50% inhibition is 0.6-0.8 nmol. The amount of $(Glc)_4$ required for 50% inhibition increases for longer periods of exposure to the $(Glc)_4$-BSA coated plate.

When a standard exposure time of 1 min is used and all operations are carried out at precisely 4°C, the inhibition curve obtained is highly reproducible (±10%) for quantities of inhibitor ranging from 0.15-2.5 nmol. In the studies presented here, preincubation of antibody with hapten inhibitor was carried out for 2 h. Nearly identical results were obtained for antibody-hapten incubation times ranging from 15 min to 2 h (data not shown).

Quantitation of $(Glc)_4$ in Urine by ELISA Inhibition

To determine the precision of the ELISA inhibition method for measuring $(Glc)_4$ in urine, six samples were analyzed four to five times giving a variation coefficient of less than 10%. Samples giving higher than 80% inhibition were diluted appropriately and analyzed again. The accuracy was estimated by comparing the results of the nitrocellulose filter assay [5] and the ELISA inhibition method, giving a correlation coefficient of 0.96 for seven urines containing $(Glc)_4$ in concentrations ranging over 10-fold.

Discussion

Development of a rapid immunoassay for $(Glc)_4$ was made possible by production of a monoclonal antibody (401/6) with high affinity for the native form of the oligosaccharide. The immune response was elicited by immunization with $(Glc)_4$ glycosidically-linked *via* a 2-(2-methoxycarbonylethylthio)ethyl linker arm to KLH [10], and hybridoma supernatant fluids were screened for antibody binding to $(Glc)_4$ coupled *via* the same linker to BSA. In previous studies a mouse monoclonal antibody and rabbit antisera were produced using antigens containing $(Glc)_4$-phenethylamine coupled to carrier proteins [5, 9]. Affinities of these antibodies for $(Glc)_4$-OL were 10-15 times greater than for native $(Glc)_4$. To achieve maximum sensitivity and specificity of radioimmunoassays utilizing these reagents it was necessary for samples to be reduced with sodium borohydride prior to analysis. The selectivity of antibody 401/6 for native $(Glc)_4$ over $(Glc)_4$-OL is about 30 times that of the previously-described monoclonal antibody 61.1 (see Table 1). The improved affinity and specificity of antibody 401/6 for native $(Glc)_4$ permits immunoassay of the oligosaccharide at a level of sensitivity comparable to previous assays without prior reduction of samples.

In preliminary experiments, we found that the affinity of antibody 401/6 for $(Glc)_4$ is significantly greater at 4°C than at 22°C (data not shown). Similar temperature dependence was reported previously for binding of $(Glc)_4$-OL by monoclonal antibody 61.1 [9]. As the forward rate constants of antibody-hapten complex formation are usually much greater than reverse rate constants, it seems likely that the major effect of temperature is on the rate of antibody-hapten dissociation [16]. This notion is consistent with our observation that preincubation of antibody 401/6 with $(Glc)_4$ for only 15 min is sufficient to produce the maximal inhibitory effect at a given inhibitor concentration.

In the immunoassay we have constructed, a preincubated mixture of antibody plus $(Glc)_4$ inhibitor is added to a microtiter well coated with polyvalent $(Glc)_4$-BSA and after

a carefully timed exposure the well is washed and the amount of antibody bound to the well is determined. In the absence of $(Glc)_4$ inhibitor, the amount of antibody bound to the solid phase increases rapidly during the first 5 min of incubation and approaches a plateau after 20 min (Fig. 3B). When preincubated antibody-$(Glc)_4$ mixtures are exposed to the plate for 5 min or less, the final amount of antibody bound to the plate decreases 10-90% as amounts of inhibitor increase from 0.1 to 10 nmol (Fig. 4). As exposure time to the plate increases, the sensitivity (concentration of inhibitor required to inhibit antibody binding to the plate by 50%) decreases: at 180 min 10 nmol $(Glc)_4$ inhibits less than 20% (Fig. 4).

We have not determined the difference in specific molar activity between free $(Glc)_4$ and $(Glc)_4$ coupled to BSA as inhibitors in the experiments reported here. In previous studies, $(Glc)_4$-phenethylamine-BSA was up to 375-fold more active than $(Glc)_4$-OL as an inhibitor [5, 9]. Thus, it seems likely that antibody 401/6 binds to polyvalent $(Glc)_4$-BSA on a solid phase with avidity several orders of magnitude higher than its single site affinity for $(Glc)_4$ hapten in free solution, rendering attachment of antibody to the walls of the microtiter well virtually irreversible. Under these conditions, the amount of antibody bound during a brief exposure (in the order of 1 min) depends upon the initial rate of antibody binding to the plate. This rate varies inversely with the fraction of antibody combining-sites occupied by hapten inhibitor molecules and therefore can be used to determine the amount of hapten inhibitor present in unknown samples.

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References

1 Hallgren P, Hansson G, Henriksson KG, Häger A, Lundblad A, Svensson S (1974) Eur J Clin Invest 4:429-33.
2 Walker GJ, Whelan WJ (1960) Biochem J 76:257-63.
3 Ugorski M, Seder A, Lundblad A, Zopf D (1983) J Exp Pathol 1:27-38.
4 Hallgren P, Lindberg BS, Lundblad A (1977) J Biol Chem 252:1034-40.
5 Zopf DA, Levinson RE, Lundblad A (1982) J Immunol Methods 48:109-19.
6 Lennartson G, Lundblad A, Sjöblad S, Svensson S, Öckerman PA (1976) Biomed Mass Spectrom 3:51-54.
7 Lennartson G, Lundblad A, Lundsten J, Svensson S, Häger A (1978) Eur J Biochem 83:325-34.
8 Lundblad A, Svensson S, Yamashina I, Ohta M (1979) FEBS Lett 97:249-52.
9 Lundblad A, Schroer K, Zopf D (1984) J Immunol Methods 68:217-26.
10 Dahmén J, Frejd T, Magnusson G, Noori G, Carlström A-S (1984) Carbohydr Res 127:27-33.
11 Kennett RH (1980) Monoclonal Antibodies, Plenum Press, New York, p 365-67.
12 Engvall E, Perlmann P (1972) J Immunol 109:129-35.

13 Nowinski RC, Lostrom ME, Tam MR, Stone MR, Burnette WN (1979) Virology 93:111-26.
14 Ouchterlony Ö (1958) Prog Allergy 5:1-78.
15 Zopf D, Tsai CM, Ginsburg V (1978) Methods Enzymol 50:163-69.
16 Parker CW (1976) Radioimmunoassay of Biologically Active Compounds, Prentiss-Hall, Englewood Cliffs, NJ, USA, p 125-26.