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Cell Antigens Recognized by Rabbit Antibodies Specific for Oligomannosyl Determinants

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Rabbit antibodies to cell wall mannans of various microbial strains and their mutants were found to be cross-reactive to cell carbohydrates of mammalian sperm and 4–6-day-old blastocysts. Immunochemical studies indicate that oligomers of α1→2, α1→3, α1→6, and probably also α→4 linked mannose residues of sperm carbohydrates are available for antibody binding. At least 80% of binding activity of a yeast mannan antibody to sperm can be effectively inhibited by specific haptens or digestion with exo-α-D-mannosidase, an enzyme activity highest in testicular tissue. In order to determine the role of this enzyme in the metabolism of the cross-reactive mannan antigens of sperm, the relative amount of a specific α-linked oligomannosyl determinant of bovine sperm from homozygous normals was compared to that of heterozygous carriers of α-mannosidase deficiency.

Extensive cross-reactivity between the microbial and mammalian oligomannosyl determinants suggest that these are conserved structures in cell carbohydrates, although the organization of these units in the microbial cell wall lipopolysaccharide has very little similarity to the carbohydrate moieties of mammalian glycoproteins.

Key words: **microbial mannans, surface antigens, mannosidosis, concanavalin A, pea lectin, sperm, blastocyst**

The plant lectin concanavalin A (Con A), because of its affinity for α-linked oligomannosyl determinants, has been used as an agglutinin for a large variety of cells (1). Con A binding can be effectively inhibited by a relatively high concentration (200 mM) of α-methyl mannoside (α-methyl M) and by a much lower concentration (10 mM) of trioses such as αM1→2αM1→2αM or αM1→3αM1→2αM (M = D-mannopyranose), derived from yeast mannans (2). In contrast, carbohydrate specific antibodies provide greater definition of the immunodeterminants, with respect to the internal or terminal location of the mannosyl residues and their probable linkages. For example, the lowest concentration of Con A required for the microagglutination of various microbial strains and their mutants varies over a wide range, but unlike the strain-specific antibodies, Con A does not provide as

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many clues to their structure (see Results). Mannan antibodies have negligible affinity for α-methyl M and usually require 0.1–1.0 mM concentrations of specific haptens consisting of larger oligomers (tetra-hexasaccharide) for inhibition (3,4). On the basis of structural considerations, one would expect cross-reactivity of mannan antigens to mammalian carbohydrates, and this may give rise to a general difficulty in raising rabbit antibody. In this respect, lectins are unique reagents especially in substituting for rabbit antibody to polysaccharides which are not good immunogens either due to self-antigens which are too closely related to the carbohydrate determinants, or to the lack of structural features which determine the immunogenecity of a foreign antigen.

In order to avoid the problem of cross-reactivity of self-antigens, we have used sperm as the principal target cells in our study. The testicular tissue is considered to be an immunologically previledged site and antibody to sperm can be produced both in males and females (5, 6); moreover, earlier studies on lectin binding to sperm (7) could serve as a framework for our study of antibody binding to α-linked oligomannosyl antigens of cell carbohydrates (8).

METHODS

Sources of Sperm, Tissue Materials, and Extracts

Semen from 9 Angus bulls was purchased as frozen samples from the Genetic Division of the Carnation Company, Wisconsin. Angus semen from 4 genetically ascertained donors was sent as frozen samples from Massey University, New Zealand. Of these 4 samples, 2 were known heterozygous carriers (C-1 and C-2) of bovine mannosidosis (9) and the other 2 were homozygous normal (N-1 and N-2). Fresh human semen samples were obtained through the Urology Clinic, La Jolla (courtesy of Dr. Vincent J. Flynn), and fresh rhesus semen from the Oregon Primate Research Center, Beaverton; testis cross sections were prepared from mature mouse testis; fresh rabbit semen and rabbit blastocysts were prepared as described previously (10); the preparation of sperm eluate by treating sperm with lithium diiodosalicylate (11) and iodination of extracted material by the IC1 method has been described previously (8). Human fibroblasts from a heterozygous carrier of mannosidosis and his homozygous child, were obtained from Drs. R. Matalon and G. Dawson.

Microbial Mannans

Mannan chemotypes of representative microbial strains are listed in Table I.

Microagglutination Titers

Heat-killed bacteria or yeast cultures were washed and resuspended in saline solution to a concentration of 10^8 bacteria/ml or 10^7 yeast cells/ml. Purified pea lectin was a gift from Dr. I. Trowbridge (12); concanavalin A, twice crystallized, was purchased from Miles-Yada. Microtiter plates with a U-shaped base were used to determine the agglutination titers.

Sperm Agglutination and Immobilization Titers

The serological activities were determined according to standard procedures (13, 14).

TABLE I. Atlas of Carbohydrate Antigens

Antigen	Structure
Bacterial antigens	
Salmonella cholera[a] suis 5210	Glc ↓1,3 $\rightarrow M^{1}\rightarrow{}^{4}M^{1}\rightarrow{}^{4}M^{1}\rightarrow{}^{4}M^{1}\rightarrow{}^{3}\alpha GlcNAc\rightarrow$
Salmonella thompson[a]	Glc ↓1,3 $\rightarrow M^{1}\rightarrow{}^{2}M^{1}\rightarrow{}^{2}M^{1}\rightarrow{}^{2}M^{1}\rightarrow$
Salmonella newport[a]	Abe 2-OAc-Glc ↓1,3 ↓1,3 $\rightarrow{}^{4}\alpha Rha\ {}^{1}\rightarrow{}^{2}\alpha M^{1}\rightarrow{}^{2}\alpha M^{1}\rightarrow{}^{3}\alpha Gal\rightarrow$
Escherichia coli 08[b]	$\rightarrow\alpha M^{1}\rightarrow{}^{2}\alpha M^{1}\rightarrow{}^{2}\alpha M^{1}\rightarrow{}^{3}\alpha M^{1}\rightarrow{}^{2}\alpha M^{1}\rightarrow{}^{2}\alpha M^{1}\rightarrow{}^{3}\alpha M^{1}\rightarrow$
Yeast antigens	
Saccharomyces cerevisiae[c]	
Yeast mutant X2180-1A *mnnl4*	↓ $\alpha M^{1}\rightarrow{}^{3}\alpha M^{1}\rightarrow{}^{2}\alpha M^{1}\rightarrow{}^{2}\alpha M$ ↓
Yeast mutant X2180-1A-4 *mnnl, mnn4*	↓ $\alpha M^{1}\rightarrow{}^{2}\alpha M^{1}\rightarrow{}^{2}\alpha M$ ↓
Yeast mutant X2180-1A-5 *mnn2*	$\rightarrow{}^{6}\alpha M^{1}\rightarrow{}^{6}\alpha M^{1}\rightarrow{}^{6}\alpha M^{1}\rightarrow{}^{6}\alpha M^{1}\rightarrow$
Kluyveromyces lactis[c]	αGlcNAc ↓1,2 ↓ $\alpha M^{1}\rightarrow{}^{3}\alpha M^{1}\rightarrow{}^{3}\alpha M^{1}\rightarrow{}^{2}\alpha M^{1}\rightarrow{}^{2}\alpha M^{1}\rightarrow{}^{2}\alpha M$ ↓
Candida albicans[d] type B	↓ $\alpha M^{1}\rightarrow{}^{2}\alpha M^{1}\rightarrow{}^{2}\alpha M^{1}\rightarrow{}^{2}\alpha M^{1}\rightarrow{}^{2}\alpha M^{1}\rightarrow{}^{2}\alpha M^{1}$ ↓

[a] From Ref. 27
[b] From Ref. 23
[c] From Ref. 40
[d] From Ref. 3

Immunological Methods

Rabbit antibodies were cross-absorbed to make them strain specific. The strain-specific antibody was purified for immunoglobulin fraction. The purified immunoglobulin fraction was treated with pepsin to make the divalent $F(ab)_2$ fragments (15). The $F(ab)_2$ fragments were separated from other reaction products by gel filtration and fluoresceinated by conjugating with fluorescein isothiocyanate (FITC). An average ratio of 2 FITC molecules per $F(ab)_2$ molecule was obtained. The fluoresceinated $F(ab)_2$ preparation was reduced and alkylated (16) to make univalent $F(ab)_1$ fragments (Fl–Fab). The Fl-Fab specific to the side chain mannotetraose, M1→3αM1→2αM1→2αM was purified by affinity chromatography on mannan-conjugated Biogel. The specific absorbent was prepared by treating 5 ml of aminoethyl Biogel P-150 with an excess of 1-ethyl-3-(dimethylamino-propyl)carbodiimide (500 mg) at pH 4.7 in the presence of 250 mg of purified mannan. The Fl-Fab fragments bound to the absorbent were specifically eluted as a single peak with a 1 mM solution of the tetraose hapten (4). Isolation and purification of yeast mannan and side chains were prepared as described previously (17, 18). The antibody and polysaccharides of other yeast strains and bacterial lipopolysaccharides (LPS) were prepared in a manner similar to the described procedure (19).

Preliminary tests of antibody binding were carried out in the following manner: the crude antibody was cross-absorbed to make it strain specific. The strain-specific supernatant fraction was then absorbed on the strain that was initially used to immunize the animal. The absorbed fraction was eluted by acid pH, neutralized by dialysis, precipitated with ammonium sulfate, and passed through a gel filtration step to recover the immunoglubulin fraction. This fraction was used for microagglutination and immobilization tests on sperm.

Sperm cells were obtained as a pellet by centrifuging bull semen after it had been diluted tenfold with phosphate buffered (pH 7.2) saline. Washed sperm cells were fixed for 1 h in ice by adding 3 volumes of cold methanol to 1 volume of dilute cell suspension (20). After fixation, cells were washed 3 times, and the final suspension in cold phosphate buffered saline (PBS) was passed through a nonabsorbent filter (75 μm mesh) to remove cell clumps present in some preparations. The incubation mixtures for F1-Fab binding were made with 10^5 sperm cells, varying amounts of antibody, and PBS in 200 μl final volume. Haptens or relevant polysaccharides were added to a concentration of 100 μM for competitive inhibition of F1-Fab binding to sperm cells. The mixture was first incubated for 30 min at 37°C and then left overnight at 4°C.

Blastocysts recovered from the uterus on the fourth or fifth day of pregnancy were subjected to mechanical removal of the surrounding investments. After washing 3 changes of 0.05 M PBS, pH 7.5, the embryo tissues were treated with different samples of control and immune sera, uterine fluids, and uterine fluid immunoglobulins, for 30 min. The tissues were rinsed in PBS and incubated for 15 min in FITC-conjugated goat antirabbit-7S globulin IgG or antirabbit secretory uterine IgA (SIgA). The embryo tissues were rinsed in PBS and mounted in glycerine on glass microslides (10).

Fluorescent cells were visualized through Zeiss Fluorescent Illuminator No. II, lamp HG 200 W.4, Zeiss ultracondenser, interference exciter filter KP500 FITC, and barrier filter 53; microphotographs were taken with Kodak Tri-X (ASA 400) with 30-sec exposure time using a 40/1.0 oil planaphochromat (with iris) objective. Stained spermatozoa were passed through the flow microfluorometer (21) at a rate of 10,000 cells per min, for slightly less than 100 sec. The frequency distribution data, obtained as relative amounts of fluorescence in the abscissa (linear scale) in millivolt units and cell number (Y axis) were retrieved from the memory of the integrator onto punched tapes for statistical analysis. The frequency distribution was obtained for each assay point as shown in Fig. 5a. Fluorescent polystyrene microspheres (diameter 10 μm, PTL #305, a gift from Dr. M. M. Fulwyler of Particle Technology, Inc., Los Alamos, New Mexico) and sperm from a given donor stained with the same antibody preparation at a nearly saturating concentration, were used to calibrate the instrument and determine staining variations. The ratio of FITC: $F(ab)_1$ was adjusted so that the assay points in an entire titration curve fit into the total span of the linear intensity scale at a single gain setting. Staining variations of sperm from 10 Angus bull donors, measured under these conditions, were usually 5% and never larger than 10%.

Exo-α-D-Mannosidase Digestion of Cell-bound Antigen

Enzyme digestion by Arthrobacter exo-α-D-mannosidase (22) was carried out by mixing 10^7 methanol-fixed sperm cells in 0.5 ml buffer (pH 6.8, 0.05 M phosphate buffer, 100 μg/ml pure preheated bovine serum albumin) containing 5 units of enzyme (specific activity 88.7 units/mg of protein, 1 unit = 1 μmole of mannose equivalent released per hour under these assay conditions with S. cerevisiae X2180-1A mannan as substrate). Following digestion at 37°C for 2 h, the cells were washed 3 times and observed under phase microscopy for intactness prior to antibody binding. The partially purified enzyme preparation was a gift from Dr. W. C. Raschke.

RESULTS

A crude test of relative affinity of Con A to a variety of α-linked mannosylated structures was devised by determining the lowest concentration of Con A required to agglutinate a number of bacterial and yeast strains and their mutants. Since agglutinability depends on many factors besides relative affinity, we also used another lectin, derived from pea (1, 12), which has properties such as molecular weight, mitogenicity, and inhibition by α-methylmannoside which are very similar to those for Con A. Thus, by comparing the titers of 2 similar lectins on the same strain, we gained an appreciation of the range of α-linked mannosylated structures which could be recognized. An atlas of chemotypes of the strains is given in Table I, and the microagglutination titers are given in Table II.

Con A titers are the same for the terminal structures αM1→3αM1→ and αM1→2αM1→ (D, E, and G) whereas pea lectin titers prefer the former and, unlike Con A, have a distinct preference for the long side chains of yeast mannans (D, E, and G). The pea lectin titer of S. newport compared to those of S. cholera suis and S. thompson are also interesting because pea lectin apparently distinguishes between 2-OAc glucose and the glucose of the side chains. The titer on yeast mutant X2180 1A-5 is due to the αM1→6αM1→ linkage characteristic of this mutant (4).

General Properties of Rabbit Antibodies to Mannans

Rabbits respond poorly to the αM1→2αM1→2αM side chain of X2180 1A-4 and no response could be detected for the very similar internally repeated structure of E. coli:08 antigen (23). All other mannans give high titered antibodies in rabbits; in contrast, most mouse strains do not respond to these antigens. In some instances the lack of response may be explained on the basis of cross-reactivity to self-antigens, as is shown (Fig. 1) by

TABLE II. Relative Specificites of Concanavalin A and Pea Lectin (to be read with the Atlas of Carbohydrate Antigens, Table I)

	Minimum concentration of lectin required for the agglutination of bacteria and yeast strains						
Antigens[a]	(A) Salm. cholera suis	(B) Salm. thompson	(C) Salm. newport	(D) S. cerevisiae X2180-1A	(E) K. lactis	(F) Mutant X2180-1A-5	(G) Mutant X2180-1A-4
Lectins							
Concanavalin A (μg/ml)	125	250	125	62.5	125	125	62.5
Pea lectin (μg/ml)	625	no agg. with 2 mg/ml	75	10	10	150	40

[a]The sequences of oligosaccharide immunodeterminants are given in the Atlas, Table I. Agglutination by both lectins is inhibited by 0.2 M α-methylmannoside or α-methylglucoside.

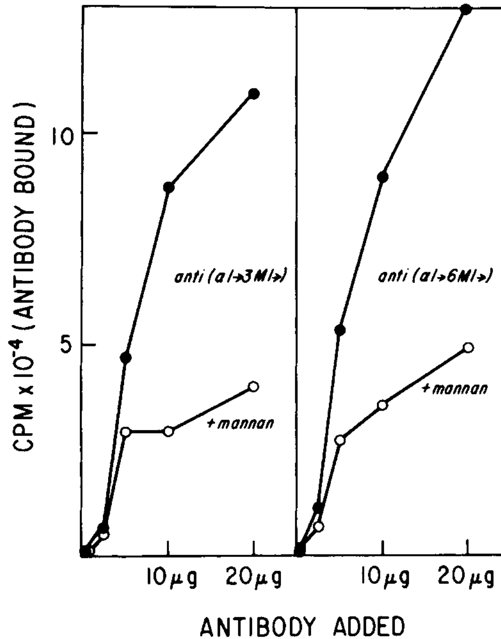

Fig. 1. Binding of antimannan antibodies, X2180-1A (α1→3→) and X2180-1A-5 (α1→6→), to mouse lymphoma line S49. Washed cells were incubated in 200 μl of phosphate buffered saline containing 5×10^5 cells with varying amounts of antibody (10^5 counts/μg protein) in the presence or absence of inhibiting concentrations of mannan (5 μg/tube). Tubes were incubated for 30 min at room temperature. At the end of incubation, cells were washed 3 times and the radioactivity was counted in a gamma counter.

the binding of mannan antibody to cultured mouse lymphocyte S49; however, mannan antibodies are not cytotoxic and bind poorly to viable mouse lymphocytes (Fig. 1) and to several other somatic cells screened by us. These antigens are available for antibody binding on fixed cells as is shown in the human fibroblast culture in Fig. 2.

The lack of response in rabbits to E. coli:08 mannan, is surprising because the internal →αM1→3αM→ linkage within the mannan, probably protrudes from the backbone structure and should have been recognized by the antigen-sensitive cells. In fact, bacteriophage Ω8 recognizes this structure on E. coli:F492 and selectively cleaves this bond upon absorbtion and the mannanless mutant strain F612 was isolated on the basis of phage resistance (24). Hence the F492 mannan may very well be a cross-reacting rabbit self-antigen. The poor immune response to the small side chain, αM1→2αM1→2Mα1→2αM, of mutant A-4 (Table I) in rabbits, however, should probably be explained on the basis of the small stacked structure of this side chain after the terminal αM1→3 linkage is delected in the mutant since rabbits respond well to the longer side chain of C. albicans (Table I). Thus the height of response in rabbits to microbial mannans appears to be a product of structural properties and the degree of cross-reactivity of mannans to self-antigens.

Mannan Antibodies Cross-reacting With Sperm Carbohydrates

The cross-reactivity of mannan antibodies to sperm surface antigens was first detected by sperm agglutination reaction (8). Some of these antibodies can immobilize sperm in the presence of complement (Table III). Although many species of mammalian

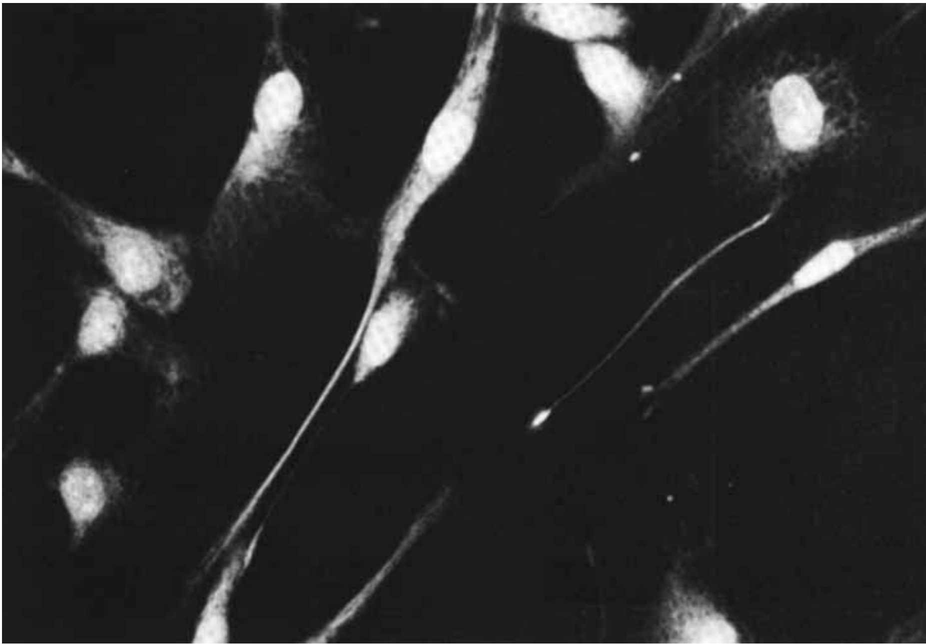

Fig. 2. Binding of affinity purified fluoresceinated anti-X2180-1A (α1→3→) antibody to fixed human fibroblast of a heterozygous carrier of mannosidosis. Cells were grown on coverslips and fixed with a 3:1 mixture of methanol and saline for 30 min on ice. The coverslip was washed 3 times and stained directly with fluoresceinated divalent antibody fragment. The intensity of immunofluorescence stain of cytoskeleton-like structures was similar to that of fibroblast culture from homozygous, affected (mannosidosis) child. The staining is inhibited by the presence of yeast mannan.

TABLE III. Sperm Immobilization by Mannan Antibody in Presence of Complement

Specificity of antiserum	Human[a] sperm	Rabbit[a] sperm
X2180-1A	1.0 (100%)	1.0 (100%)
X2180-1A-5	1.0 (100%)	5.0 (20%)
Salm. cholera suis	1.0 (100%)	2.5 (40%)
K. lactis	1.0 (100%)	2.0 (50%)

[a]Immobilization tests were performed in the presence of complement (5). A value of 1.0 indicates that the antibody has no effect on mobility, and 5.0 indicates 20% mobility. Note that rabbit antibody is ineffective as cytotoxic antibody to human sperm but can be effective on rabbit sperm at the same antibody concentrations. Both species of sperm are agglutinated by these antisera and give slightly different patterns of intensity distribution in immunofluorescent staining. Compare the sperm immobilization values with the results given in Table V.

sperm, such as human, rhesus, bovine, rabbit, and mouse, show various degrees of agglutinability, not all are subjected to complement-dependent killing. This is illustrated in the results presented in Table III; rabbit sperm is immobilized by X2180-1 A-5, S. cholera suis, and K. lactis antibodies whereas human sperm is not.

It is of interest that the cell surface oligomannosyl antigens of sperm are detected in the ejaculated sperm by mannan antibodies because the activity of exo-α-mannosidase is

high in testicular tissue (25), especially in epididymis, and one might have expected these antigens to be degraded by the enzyme during the passage of sperm through the epididymis unless they remain masked and are exposed at a subsequent stage. Thus, one may ask when do these antigens first appear in spermatogenesis? Are the residues terminally oriented or are they internal linkages protected from exo-α-mannosidase digestion? Does the amount of sperm antigens increase in partial deficiency of α-mannosidase?

The first appearance of these antigens on sperm head takes place at the end of the haploid phase of spermatid development. This could be particularly well illustrated in the immunofluorescence staining of the cross-reaction of mouse testis, shown in Fig. 3.

The homozygous condition of genetic deficiency in the activity of α-mannosidase in Angus cattle (9) and in man (26) causes the lysosomal accumulation of mannosylated derivatives of heterosaccharides of incompletely degraded glycoproteins. Although the heterozygotes are normally fertile, we had anticipated that the segregation of the mutant allele from the normal allele in the haploid phase of spermatogenesis might result in the accumulation of excess mannosylated carbohydrates in the sperm acrosome. Should this occur, antibody to mannans, especially that of X2180-1A yeast, would detect the excess antigen since the $\alpha 1 \rightarrow 3$ mannosylmannose structure is one of the most common linkages among the carbohydrate moieties of glycoproteins. The binding of the yeast univalent antibody to bovine sperm and the inhibition of binding by the tetraose hapten insolated from yeast mannan or by predigestion of sperm by exo-α-mannosidase are shown in Fig. 4. Note the relative absence of antibody fluorescence on the principal tail piece compared to deep staining of other structural segments, such as the acrosomal, postnuclear cap, and

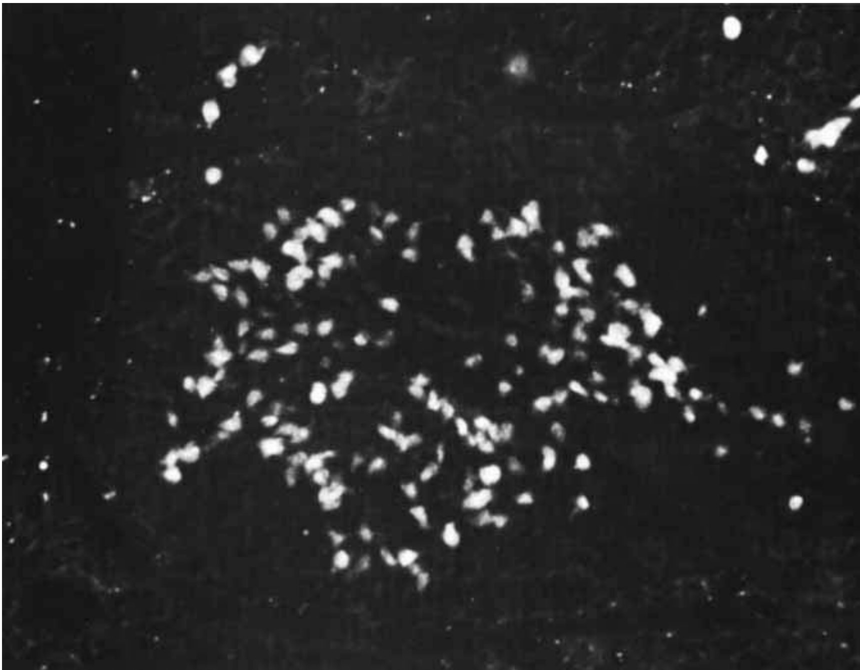

Fig. 3. Immunofluorescence staining of adult mouse testis cross section with anti-X2180-1A-5 fluoresceinated divalent antibody fragment. Staining is inhibited by specific mannan. Note the specific staining of sperm heads at late spermatid stage.

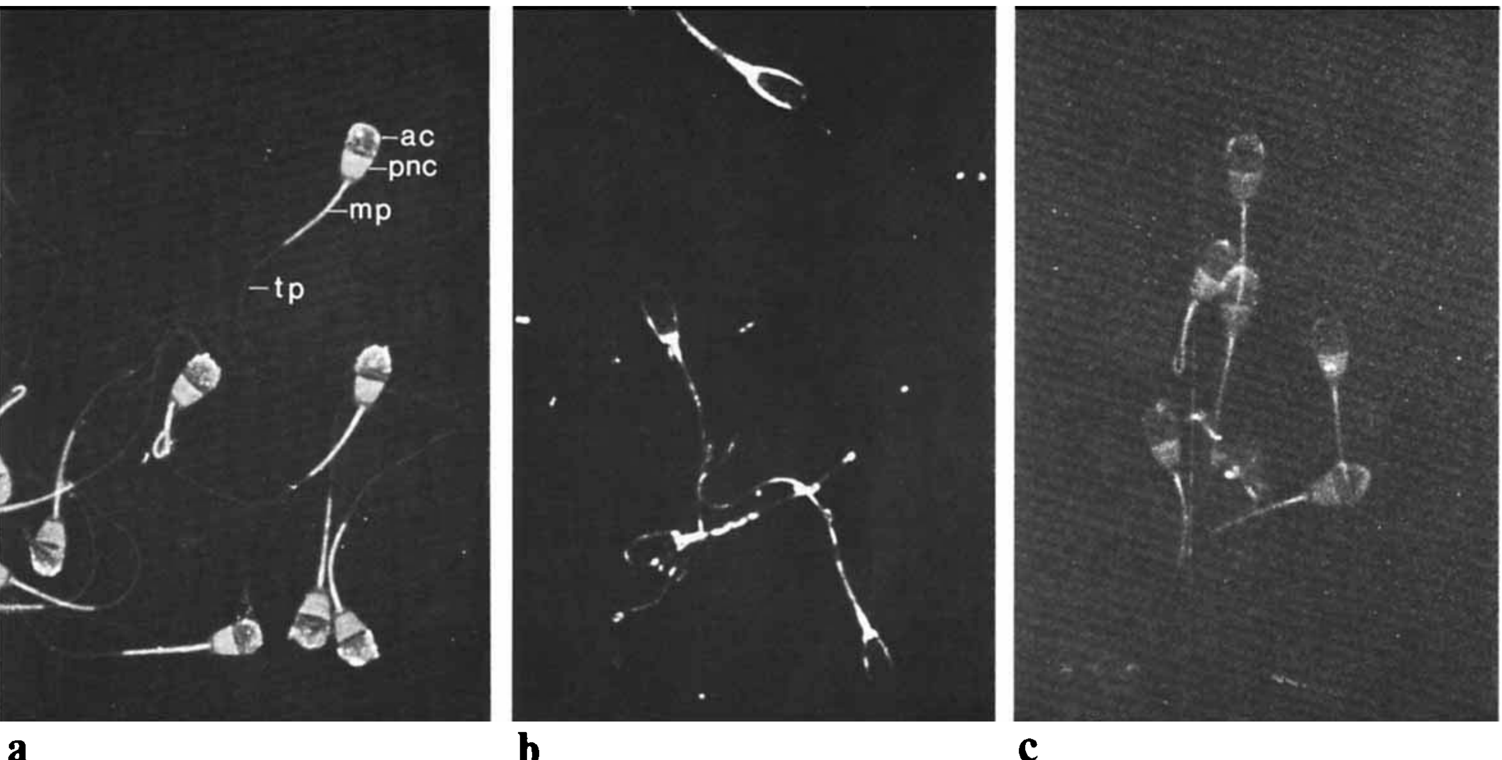

Fig. 4. Microphotograph of bull sperm stained with fluorescent univalent antibody fragment F1-Fab specific for yeast mannan X2180-1A. a) The following structures are labeled: acrosome (ac), midpiece (mp), postnuclear cap (pnc) and principal tail piece (tp). b) Bull sperm was predigested with α-mannosidase prior to immunofluorescence staining with saturating concentration of antibody. Note the inhibition of antibody binding. c) Bull sperm stained in the presence of specific tetraose hapten as inhibitor.

midpiece regions (Fig. 4a). The binding of most of this antibody, approximately 80%, is inhibited by predigestion of sperm with α-mannosidase (Fig. 4b) or by 100 μM concentration of tetraose hapten in the incubation mixture (Fig. 4c).

The fluorescence emitted by the fluoresceinated univalent antibody fragments bound to sperm was measured individually and recorded in a relative scale by the flow microfluorometer. The accumulated data was plotted to obtain the frequency distribution of antigen binding to sperm from the normal and the heterozygous donors. The plot in Fig. 5a shows a curve with a unimodal fit of the observed data, obtained at a nonsaturating concentration of antibody. At saturating concentrations of the antibody, the modal distribution of antibody-bound cells would move toward the right of the plot shown in Fig. 5a. The progressive saturation of antigen sites with increasing concentration of univalent antibody is shown in Fig. 5b (normal donor) and 5c (heterozygote for α-mannosidase deficiency), by plotting the mean value of the distribution found at each assay point. The ability of the mannotetraose hapten to inhibit antibody binding to sperm and the sensitivity of the binding sites on sperm to prior digestion with α-mannosidase suggest that a terminal α1→3 (or α1→2) mannosylmannose linkage in a side chain of a glycoprotein constitutes the structural basis of cross-reactivity between sperm carbohydrates and yeast mannan. However, the antigen content of sperm from 2 heterozygotes of mannosidosis C-1 and C-2 are very similar to that of the normal homozygous pair N-1 and N-2 (Fig. 5b and 5c). Since the homozygous condition of α-mannosidase deficiency leads to premature death (9, 26), we have not had the opportunity to examine sperm from these individuals.

The presence of cross-reactive mannan antigens was also detected in sperm eluates (8). The eluate was prepared by mild treatment of sperm with lithium diiodosalicylate and precipitated by a variety of antimannans. The specificity of the immunological reactions was determined by competing with individual mannans. The results are given in Table IV. A small percent of eluted material, approximately 10%, specifically cross-reacts with mannan antibodies.

Cross-reactive Oligomannosyl Antigens Common to Sperm and Blastocyst

Female rabbits, immunized with rabbit epididymal sperm mixed with complete Freund's adjuvant, produce serum IgG antibody and uterine IgG and secretory IgA antibodies against sperm (10). The serum antibody shows both sperm agglutinating and immobilizing activities. The uterine secretory IgA (SIgA) from immune uterine fluid (IUF) does not have those activities, but the binding to sperm can be detected by immunofluorescence assays; the SIgA also binds to blastocyst, and is believed to be one of the principal reasons for infertility in the immunized females (10). In preliminary tests, we found antimannan activities in the serum and IUF antibodies. The 2 sera were then absorbed with each of the microbial strains and the absorbed antibody was assayed on sperm and blastocyst for reduction in titer and change in the distribution of immunofluorescence staining on sperm structures. The results are summarized in Table V. For example, Salm. cholera suis 5210 used as an absorbent reduced the sperm agglutination titer from 128 to 8 and the sperm immobilization value from 12 to 2 of the serum antibody, but the immunofluorescence pattern given by this antibody remained unchanged. The IUF antibody activity against blastocyst, which was due to SIgA, was significantly reduced by absorbtion with the same strain. Thus, S. cholera suis 5210 and K. lactis antigens show cross-reactivities to antigens common to both sperm and blastocyst, as detected by the specificities present in the rabbit antisera raised by immunization of females with sperm. Similar results were

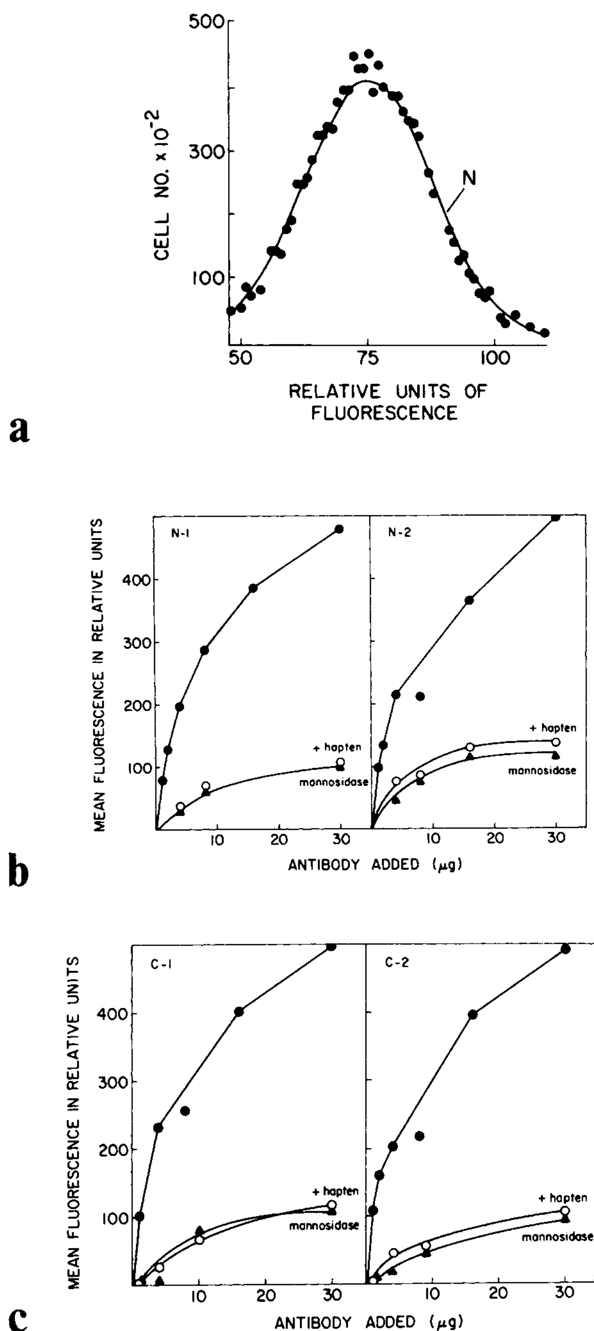

Fig. 5. Cytofluorometric assay of fluoresceinated univalent antibody binding to bovine sperm. Rabbit antibody was prepared against X2180-1A mannan and affinity purified with tetraose hapten. a) An example of the frequency distribution and a test for normality is presented to show how each data point in adjacent figures was obtained. The mean fluorescence calculated from distribution at each antibody concentration is plotted in the accompanying figures; b) normal homozygous donors N-1 and N-2; c) 2 heterozygous donors C-1 and C-2. a) Solid circles are actual data points retrieved from the flow microfluorometer. Smooth curve N represents a unimodal Gaussian fit. b, c) Antibody added to sperm alone (•); antibody added to sperm in the presence of mannotetraose hapten (○); antibody added to sperm pretreated with Arthrobacter exo-α-D-mannosidase (▲).

TABLE IV. Precipitation of ^{125}I-labelled Lithium Diiodosalicylate (LIS) Extract of Rabbit Sperm by Rabbit Antibody to Various Mannans

Antibody specificity	Amount (cpm) of LIS extract added (1.6×10^4 cpm/μg)	Amount of LIS extract precipitated[a]	
		Without inhibitor mannan	With inhibitor mannan
Salm. cholera suis	1.0×10^6	1.15×10^5	4.5×10^3
X2180-1A-5	5.0×10^5	5.8×10^4	5.1×10^3
Candida albicans type A	8.0×10^5	4.3×10^4	4.5×10^3
Candida albicans type B	3.2×10^5	2.7×10^4	4.5×10^3

[a]The LIS extract was labelled with ^{125}I (specific activity 1.6×10^4 cpm/μg) and precipitated in phosphate buffered saline containing 1% bovine serum albumin, by the antibody in presence or absence of 10 μg of specific mannan as indicated. Following incubation for 1 h at 37°C, goat immunoglobin prepared against that of rabbit was added in equivalent amount to precipitate the mannan sera. The double precipitate was incubated overnite at 4°C, washed 3 times and counted in a gamma counter.

TABLE V. Residual Activities of Sperm Antibodies on Sperm and Blastocyst After Absorption With Microbial Preparations

Antigens used for absorption	Anti-RES[a]		Indirect immunofluorescence			
			Sperm			Blastocyst
	SAT[a]	SIV[b]	Anti-RES AIgG*	IUF AIgG*	IUF AIgA*	IUF AIgA*
Saline	128	12	WS-4	WS-3	WS-3	3
RES	4	1	WS-1	WS-0	WS-0	0
X2180-1A-4	128	12	WS-4	T-2	WS-3	3
X2180-1A-5	128	3	WS-4	Ac-1	Ac-1	3
X2180-1A	128	6	WS-4	WS-3	WS-3	1
S. cholera suis	8	2	WS-4	T-1	WS-3	1
K. lactis	16	12	WS-4	T-2	WS-1	1
S. paratyphi	128	12	WS-4	T-2	WS-3	3
S. thompson	128	12	WS-4	T-2	T-2	2

[a]Sperm agglutining titer (SAT) of rabbit epididymal sperm (RES) antibody

[b]Sperm immobilizing value $= \frac{\text{Motile sperm (\%) – control serum}}{\text{Motile sperm (\%) – immune serum}}$

[c]Abbreviations:
WS – Whole sperm (acrosome and tail equal intensity); Ac – acrosome; T – main tail piece; Use of Ac or T indicates a reduced staining intensity for that structure only; AIgG* – goat antirabbit IgG globulin-FITC conjugate; AIgA* – goat antirabbit IgA globulin-FITC conjugate;
IUF – secretory IgA purified from uterine fluid of rabbits immunized with sperm.

Agglutination titers are expressed as reciprocals of endpoint dilutions of antibody. Immunofluorescence is expressed in an intensity scale 0–4.

obtained with SIgA of rabbits immunized with Salm. cholera suis 5210 (Fig. 6). Although the Salmonella and yeast serotype antibodies do not cross-react with each other, the sperm and blastocyst specific antibodies probably recognize the GlcNAc linked to mannose in their cell wall antigens.

DISCUSSION

The existence of many somatic antigens of yeast and bacteria with serotypic differences which are directly related to their mannan structures (4, 27) prompted us to undertake a study on the binding of rabbit antimannan antibodies to cell antigens. Unlike Con A, mannan antibodies are not inhibited by α-methyl M but require haptens of higher molecular weight, thus providing a greater definition of the antigenic determinants to which they bind (Tables I and II). Some of the mannan chemotype differences are produced by mutations affecting α-linked mannosyl transferases (Table I); thus, rabbit antibodies can be raised against new specificities. Besides mutations, genetic alteration of mannan structures can also be brought about by introduction of new genetic elements in the form of virus genome into bacteria. An elegant example of this structural transformation of cell surface determinants was demonstrated by Staub (19); changes similar to these are common in enteric bacteria (27) and also have been postulated to occur in virus transformation of mammalian cells, which may play an important role in tumor metastasis (28). In bacteria, specific α-linked mannose oligomers can give rise to specific binding sites for bacteriophages.

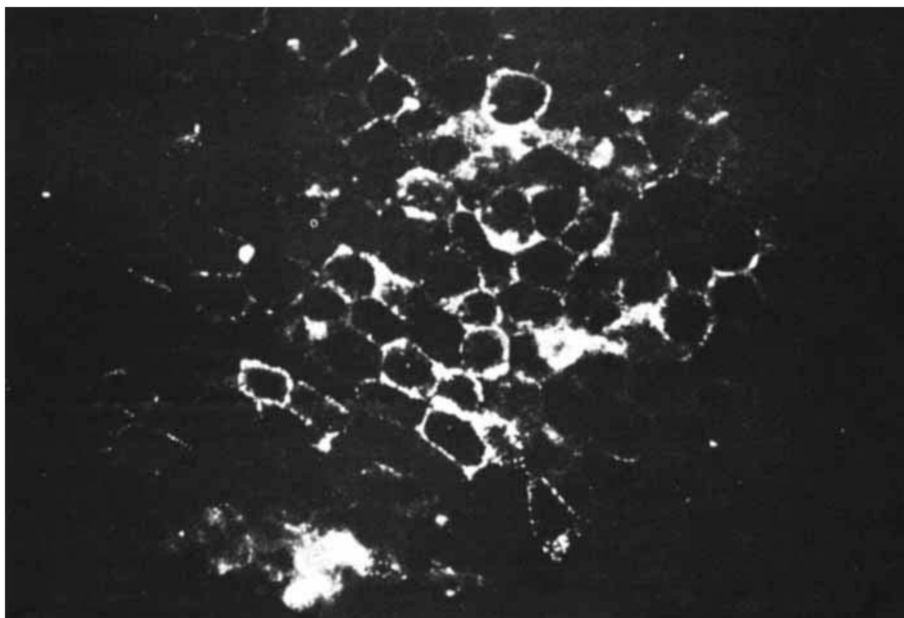

Fig. 6. Indirect immunofluorescence staining of a 6-day-old unfixed rabbit blastocyst to show the patchy localization of bound antibody on the top layer of cells. Uterine fluid secretory IgA from a rabbit immunized with S. cholera suis polysaccharide was added for 30 min at room temperature. After many washes, the embryo was stained with fluoresceinated goat immunoglobulin with specificity for rabbit secretory IgA. Absorbed antibody and normal uterine IgA were used as negative control (10).

For example, bacteriophage Ω8 binds to the internal α1→3 linkage of E. coli:08 cell wall mannan (24). In these respects the alteration of oligomannosyl glycopeptides and the appearance of a cell wall surface agglutinin during chick embryo development (29, 30) are of interest because expression of new genetic functions in embryogenesis may induce the synthesis of a glycoprotein with agglutinin activity and specific structural changes in cell surface carbohydrates to act as signals for cell recognition and interaction (31). However, no integral membrane proteins with binding activities like Con A or pea lectin have as yet been described. The α-linked oligomannosyl antigens can be considered as conserved structures in cell carbohydrates; their topographical location on cell surface was maintained in spite of the general organization of these oligomers in microbial cell wall polysaccharides, attached to core structure containing lipids (27) has very little similarity to carbohydrate moieties of mammalian glycoproteins (32).

Many of the linkages explored in this study, especially α1→3, are found in what appears to be internal cytoskeleton of fixed cell preparations (Fig. 2). As cell surface antigens they are found on lymphocyte and sperm (Table III). In sperm, they are also immunogenic so that antimannan activities can be detected in sperm-specific antibody (Table V).

Our first attempt to find a quantitative difference in the antigen in genetic deficiency of lysosomal enzyme such as α-mannosidase (Fig. 5) has not been fruitful in understanding the metabolism of the sperm antigen. However, mannan antibodies should provide an alternative diagnostic method for manosidosis, since some of the oligomers identified in the storage material of human patients (33, 34) are likely to cross-react with the antibodies described here. The stored material should be detectable in cytological preparations (Fig. 2) or by radioimmune assay of urinary glycopeptides (Table IV). A quantitative immunochemical assay of accumulated product is perhaps more desirable since the lowered enzyme levels are not always a clear indication of this disease (35).

The binding of X2180-1A (α1→3 or α1→2 terminal) antibody to bovine sperm is sensitive to exo-α-mannosidase digestion (Figs. 4 and 5), suggesting that these are terminal determinants. The α1→6→ determinant is another sperm antigen. In mouse, the antigen first appears on the sperm head at the late spermatid stage (Fig. 3), but it could not be detected in the epididymis (unpublished results). The loss of detectable reactivity in the epididymus could either be due to degradative activity of epididymal mannosidase or to an antigen masked so that it is no longer available for antibody binding. The antigen can also be detected on intact mouse cerebellar cells and antibody to α1→6 determinant inhibits the differentiation of fetal cerebellar cells in tissue culture (36). Thus, in mouse it is an antigenic determinant of cell surface carbohydrates and is common to at least sperm (Tables III and V), lymphocyte (Fig. 1), and cerebellar cells (36).

The absorption of antiblastocyst activity of sperm antibody by K. lactis and S. cholera suis, 5210 (Table V) and the appearance of specific fluorescence at the surface of adherence of adjacent cells (Fig. 6) are interesting because that particular distribution of staining pattern was not observed in any of the tissue staining. The follicular cells of mouse ovary are deeply stained by the K. lactis antibody, but the antigen appears to be intracellular. We are presently investigating the contribution of GlcNAc in the antibody specificity of the sperm antibody absorbed by the 2 antigens since the role of GlcNAc containing glycoproteins and lipids have been implicated in the adherence of fibroblast cells (37). The implantation of blastocysts in rabbit uterus can be inhibited by active immunization of females with S. cholera suis, 5210 antigen and to a lesser extent by the K. lactis antigen (unpublished results).

We have listed a variety of tissues which bind the rabbit antibody to mannans, and the structures to which they bind; it could be surface antigens of cells, internal glycoproteins, or what appears to be the cytoskeleton-like structure of the fibroblast cells. The tissues, which share a common antigen do not appear to have a common developmental origin and the biological role of these determinants is still obscure.

We have recently initiated a series of experiments with specific rabbit antibodies (38) to investigate the biological activity of the oligomannosyl antigens together with the sialic acid-containing cell antigens in development, since the role of sialic acids in specific recognition of cells and soluble proteins is established in many cases (39). In the accompanying paper (36) we describe the results of experiments which were designed to examine the possible role of the cross-reacting carbohydrate antigens on the surface of differentiating cerebellar cells of the mouse fetus.

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REFERENCES

1. Nicolson GL: Int Rev Cytol 39:89, 1974.
2. Suzuki S, Hatsukaiwa H, Synayama S, Honda N: Jpn J Microbiol 15:437, 1971.
3. Sunayama H, Suzuki S: Jpn J Microbiol 14:371, 1970.
4. Ballou CE: Adv Enzymol 40:239, 1974.
5. Menge AC: J Reprod Fertil (Suppl) 10:171, 1970.
6. Alexander NJ: Fed Proc Fed Am Soc Exp Biol 34:1692, 1975.
7. Nicolson GL, Yanagimachi R: Science 177:276, 1972.
8. Sarkar S: J Reprod Med 13:93, 1974.
9. Phillips NC, Robinson D, Winchester BG, Jolly RD: Biochem J 137:363, 1974.
10. Menge AC, Rozenberg A, Burkons DM: Proc Soc Exp Biol Med 145:371, 1974.
11. Marchesi VT, Andrews EP: Science 174:1247, 1971.
12. Trowbridge IS: Proc Natl Acad Sci USA 70:3650, 1973.
13. Kibrick S, Belding DL, Merrill B: Fertil Steril 3:430, 1952.
14. Isojima S, Li TS, Ashitaka Y: Am J Obstet Gynecol 101:677, 1968.
15. Nisonoff A, Wissler FC, Lipman LN: Science 132:1770, 1960.
16. Nisonoff A, Wissler FC, Woernley DL: Biochem Biophys Res Commun 1:318, 1959.
17. Kocourek J, Ballou CE: J Bacteriol 100:1175, 1969.
18. Raschke WC, Kern KA, Antalis C, Ballou CE: J Biol Chem 248:4660, 1973.
19. Fuller NA, Etievant M, Staub AM: Eur J Biochem 6:525, 1968.
20. Hjort T, Hansen KB: Clin Exp Immunol 8:9, 1971.
21. Sarkar S, Jones OW, Shioura N: Proc Natl Acad Sci USA 71:3512, 1974.
22. Jones GH, Ballou CE: J Biol Chem 244:1043, 1969.

23. Reske K, Jann K: Eur J Biochem 31:320, 1972.
24. Reske K, Wallenfels B, Jann K: Eur J Biochem 36:167, 1973.
25. Snaith SM, Levvy G: In Pigman W (ed): "The Carbohydrates." New York: Academic Press, 1957, p 401.
26. Ockermann PA: In Hers HG, Van Hoof F (eds): "Lysosomes and Storage Diseases." New York: Academic Press, 1973, p 291.
27. Luderitz OW, Westphal O, Staub AM, Nikaido H: In Weinbaum G, Kadis S, Ajl SJ (eds): "Microbial Toxins." New York: Academic Press, 1971, vol IV, p 145.
28. Nicolson GL: Biochem Biophys Acta 458:1, 1976.
29. Ceccarini C, Muramatsu T, Tsang J, Atkinson PH: Proc Natl Acad Sci USA 72:3139, 1975.
30. Yamada KM, Yamada SS, Pastan I: Proc Natl Acad Sci USA 72:3158, 1975.
31. Reitherman RW, Rosen SD, Frazier WA, Barondes SA: Proc Natl Acad Sci USA 72:3541, 1975.
32. Spiro RG: Adv Protein Chem 27:349, 1973.
33. Tsay GC, Dawson G, Matalon R: J Pediatr 84:865, 1974.
34. Norden NE, Lundblad A, Svensson S, Autio S: Biochemistry 13:871, 1974.
35. Desnick RJ, Sharp HL, Grabowski GA, Brunning RD, Quie PG, Sung JH, Gorlin RJ, Ikonne JV: Pediatr Res 10:985, 1976.
36. Trenkner E, Sarkar S: J Supramol Struct 6:465, 1977.
37. Pouyssegar J, Willingham M, Pastan I: Proc Natl Acad Sci USA 74:243, 1977.
38. Heidelberger M: In Kwapinski JB (ed): "Immunochemistry of Bacterial Polysaccharide in Research in Immunochemistry and Immunobiology." Baltimore: Baltimore University Press, 1973, vol III, p 1.
39. Ashwell G, Morrell AG: Adv Enzymol 41:99, 1974.
40. Ballou CE, Raschke WC: Science 184:127, 1974.