Ia ANTIGENIC SPECIFICITIES
ARE OLIGOSACCHARIDE IN NATURE:
HAPTEN-INHIBITION STUDIES*

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The Ia (*I* region associated) antigenic system in the mouse is coded for by genes located within the *H-2* complex and consists of at least four subregions (*IA*, *IB*, *IE*, AND *IC*) which code for a minimum of 21 (Ia.1 to 21) specificities (1–3), although the existence of *IB* and *IE* subregions is now being questioned. These antigens were first detected on the surface of B lymphocytes and were subsequently found on macrophages, epidermal cells, and sperm cells (4). It is also apparent that some Ia specificities are present on either resting and activated T-blast cells (2–8). In addition, we have detected considerable amounts of Ia antigenic material in mouse serum and other tissue fluids, particularly after antigenic stimulation (8, 9). The current interest in this system stems from the close involvement of the *I* region in immune responses (1–3), especially with the recent demonstration that soluble factors of both antigen-specific and nonspecific nature which are involved in T-B cell collaboration (10, 11) and suppression (12), also carry Ia antigens. In this context, we have found that although B cells have a higher density of Ia antigens on their surface than T cells, the Ia antigens in serum are derived from T cells (9).

Biochemical studies of the Ia specificities have proceeded along two lines. The cell surface Ia antigens of B cells have been extracted with detergents and were found to be glycoproteins with mol wt of approximately 30,000 (13, 14). By contrast, our chemical studies of the serum Ia material suggest that it is predominantly (but not exclusively) carbohydrate in nature as the material has the following properties: (*a*) It is rich in carbohydrate and almost devoid of protein (15); (*b*) The Ia antigenicity is destroyed by neuraminidase and periodate, but not by pronase (15); (*c*) The Ia antigen binds to concanavalin A and *Lotus* lectins (15); (*d*) It is extracted and purified from serum by methods identical to those used to prepare oligosaccharides from human milk (15–17); (*e*) Its behaviour on paper and thin layer chromatography is similar to that of other oligosaccharides (15), and after acid hydrolysis of the purified Ia product a number of simple sugars have been detected by gas-liquid chromatography (manuscript in preparation). In this paper, we describe hapten inhibition studies using a variety of sugars that show that for at least four Ia antigens (Ia.1, Ia.3, Ia.7, and Ia.15) the antigenic specificity is defined by sugars.

Materials and Methods

Mice. The mice used are shown in the tables. The various strains are maintained inbred at the Austin Hospital and were originally obtained from several sources (8).

Antisera. Inhibition studies were performed with both conventional mouse anti-Ia sera and also with our new rabbit anti-Ia sera. The alloantisera were prepared as described elsewhere (8, 9). The specificities detected by the A.TH anti-A.TL (AS 383) serum are shown in Table I. By selecting a variety of target cells, different specificities could be detected. The heterologous rabbit anti-I^k was prepared as reported previously (8). Briefly, rabbits were immunized with CBA/H whole mouse serum and the resultant rabbit antiserum extensively absorbed with dialyzed CBA/H serum. The rationale for this preparative method is that the bulk of the serum Ia material is of low molecular weight and is therefore dialyzable, and is thus removed from the CBA/H serum used for the absorption. As we shall subsequently report (manuscript in preparation) the rabbit anti-I^k serum contains all of the specificities found in the alloantiserum (shown by absorption and by reaction with different target cells). The anti-H-2 sera used were D4, D8, D28, and D31. These sera are described in the Catalogue of Mouse Alloantisera and were obtained from the Transplantation Division, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. The anti-H-2 antisera were used at concentrations giving 90% lysis of the target cells: D4 (1/125); D8 (1/64); D13 (1/250); D28 (1/64); D28b (1/32); and D31 (1/500), and any inhibition from this value was sought. It should be noted that these H-2 antisera could potentially contain anti-Ia antibodies relevant to the H-2^d (BALB/c) target. These should be Ia.6 (in D4, D13, D28b) which is present in very low titers and not of relevance to these studies. D31 contains anti-Ia.11, 16. The titer of anti-Ia.16 is 1/64, and we believe (from other studies) that the Ia.11 titer is similar. These anti-Ia antibodies are therefore probably not of relevance in these particular inhibition studies.

Sugars. The sugars used are listed in the tables and were obtained as follows: D-galactose and L-arabinose were from Merck Ag, Darmstadt, Germany. D-glucuronic acid, D-galacturonic acid, D-mannose, and L-fucose were from BDH Chemicals Ltd., Poole, England. D-fucose, D-glucosamine,[1] D-galactosamine, D-xylose, D-lyxose, cellobiose, and maltose were from Calbiochem, San Diego, Calif. N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, N-acetyl-D-mannosamine, D-glucose, D-talose, melibiose, raffinose, stachyose, lactose, lactulose, L-rhamnose, D-ribose, sucrose, sorbose, methyl-α-D-glucopyranoside, methyl-α-D-mannopyranoside, and N-acetylneuraminic acid were from Sigma Chemical Co., St. Louis, Mo. D-galactonolactone was from Kochlight Laboratories, Colnbrook, Buckshire, England. Phenyl-α-D-galactopyranoside and phenyl-β-D-galactopyranoside were obtained as a gift from Dr M. A. Germyn, CSIRO, Victoria, Australia. These sugars were dissolved in phosphate-buffered saline (PBS)[2] at a concentration of 20 mg/ml. In some cases the pH was readjusted to 7.2 with 0.1 M NaOH.

Hapten-Inhibition Assays. Two assays were used similar to those described in our earlier studies (8, 9).

INHIBITION OF CYTOTOXICITY OF ANTI-Ia ALLOANTIBODY. The anti-Ia serum was titered against the appropriate spleen cell target (Table I) and a suitable dilution selected for subsequent inhibition studies (8), i.e., one tube less than the highest dilution causing maximum cell lysis. Doubling dilutions of each sugar solution were made in L15 (Microbiological Associates, Baltimore, Md.) containing 0.5% bovine serum albumin (Miles Laboratories, Inc., Elkhart, Ind.), so that the first well contained 1 mg of the sugar in 50 μl of the L15 medium. 50 μl of anti-Ia antiserum at the appropriate dilution was then added to each well, and the test then left at room temperature for 2 h, the final concentration of the sugar in the first well being 10 mg/ml. Subsequently, spleen cells and complement were added and a cytotoxic test performed as described previously (8).

INHIBITION OF ROSETTING BY RABBIT ANTI-Ia. The binding of rabbit anti-Ia antibodies to spleen cells was detected by a rosetting technique using sheep red blood cells (SRBC) coated with sheep anti-rabbit IgG via chromic chloride (8). A dilution of the rabbit antiserum was chosen which was

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[1] The common names D-glucosamine, N-acetyl-D-glucosamine, D-galactosamine, N-acetyl-D-galactosamine, and N-acetyl-D-mannosamine refer to 2-amino-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-glucose, 2-amino-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-galactose, and 2-acetamido-2-deoxy-D-mannose, respectively.

[2] *Abbreviations used in this paper:* PBS, phosphate-buffered saline; RFC, rosette-forming cells.

TABLE I

Antisera and Target Cells Used in the Hapten Inhibition Studies

Antiserum	Target cell (Ia specificities)	Titer for inhibitions	Ia specificities detected
A.TH anti-A.TL (anti-Ia.1, 2, 3, 7, 15, 19)	B10.M (H-2^f) (Ia.1, 5, 14, 17, 18)	1/512	Ia.1
	C57BL/6 (H-2^b) (Ia.3, 8, 9, 15, W20)	1/1,000	Ia.3 (15)*
	BALB/c (H-2^d) (Ia.6, 7, 8, 11, 15, 16)	1/1,000	Ia.7 (15)*
	D2.GD (H-2^{g2}) (Ia.8, 11, 15, 16)	1/256	Ia.15
	C3H (H-2^k) (Ia.1, 2, 3, 7, 15, 17, 18, 19)	1/2,000	Ia.1, 2, 3, 7, 15, 19
Rabbit anti-I^k (anti-Ia.1, 2, 3, 7, 15, 17, 18, 19)	A.TFR2 (H-2^{ap2}) (Ia.1, 5, 14, 17, 18)	1/25	Ia.1 (17, 18)‡
	DBA/1 (H-2^q) (Ia.3, 5, 9, 10, 13, 16)	1/50	Ia.3
	BALB/c (H-2^d) (Ia.6, 7, 8, 11, 15, 16)	1/100	Ia.7 (15)*
	D2.GD (H-2^{g2}) (Ia.8, 11, 15, 16)	1/25	Ia.15

* Antiserum used at a dilution where specificity 15 is barely detected.
‡ Antiserum absorbed with A.TH spleen cells to remove anti-17, 18 antibodies.

one tube less than the highest dilution that produced maximum rosetting. 50 μl of this antibody dilution was added to serial dilutions (50 μl) of the various sugars, and the mixture was incubated at room temperature for 2 h. In these tests the first well contained 200 μg of sugar. 100 μl of spleen cells (2 × 10^7/ml in PBS/10% fetal calf serum) of the appropriate haplotype (see Table I) were then added to each tube and the rosetting test performed as previously described (8).

In both inhibition assays, inhibitory activity was expressed as the sugar concentration which caused a 50% reduction in cell lysis or a 20% reduction in the formation of rosette forming cells (RFC). The concentrations stated in the tables and figures represent the sugar concentration in the assays after the addition of anti-Ia serum.

Results

Inhibition of Different Anti-Ia Sera with Sugars. The ability of a range of monosaccharides and glycosides to inhibit the binding of allogeneic and xenogeneic anti-Ia antibodies to mouse lymphocytes was assessed. Target lymphocytes of the appropriate *I*-region haplotype were chosen so that Ia specificities 1, 3, 7, and 15 could be examined (Table I). The results are presented in Tables II-IV and in Figs. 1 and 2. The values shown in the tables represent the concentration of sugar which, in the case of the allogeneic anti-Ia sera, caused a 50% reduction in cell lysis or which produced, in the case of the xenogeneic anti-Ia sera, a 20% reduction in RFC. The results obtained with the different Ia specificities are considered separately below.

Inhibition of Anti-Ia.1 Antibodies. Inhibition of the Ia.1 reaction was examined using the A.TH anti-A.TL alloantiserum and B10.M target cells; or in the xenogeneic system using A.TFR2 target cells and the rabbit anti-I^k serum absorbed with A.TH spleen cells (Table I). In both assay systems the most
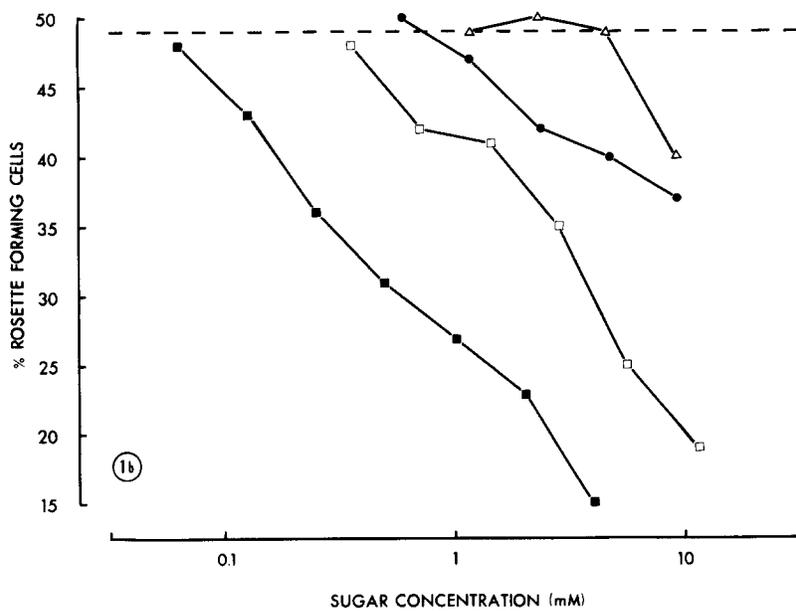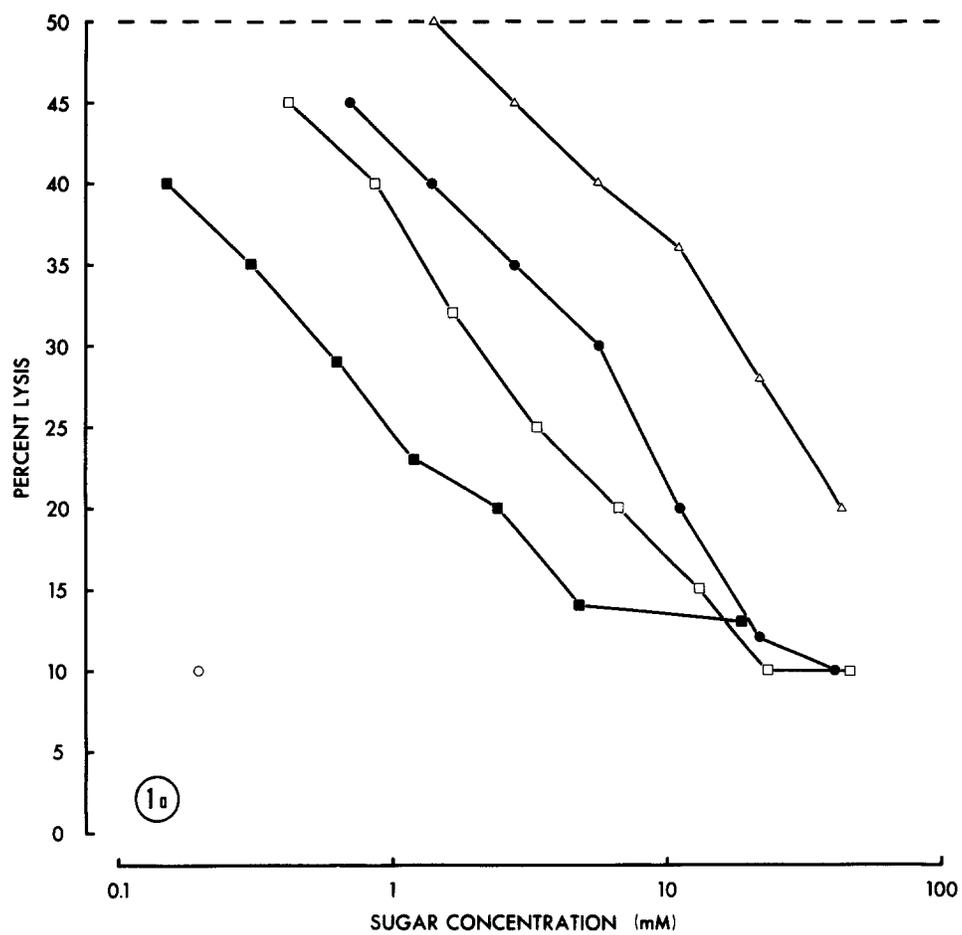
effective inhibition was obtained with N-acetyl-D-mannosamine (Table II). D-mannose and methyl-α-D-mannopyranoside were also effective inhibitors but at higher concentrations than N-acetyl-D-mannosamine. No inhibition was produced by any of the other sugars used even at concentrations as high as 55 mM (Table II). It should be noted that both assay systems were performed in different laboratories and gave the same results. The results suggest that N-acetyl-D-mannosamine is the immunodominant sugar for the Ia.1 specificity.

Inhibition of Anti-Ia.3 Antibodies. The Ia.3 reaction was measured by reacting the allogeneic A.TH anti-A.TL serum with C57BL/6 target cells. In this system Ia.3 and Ia.15 antigenic specificities could potentially be detected (Table I). However, the serum was used at a dilution of 1/2,000, which is well beyond the range of reaction for Ia.15 (1/200 on D2.GD target cells). In the RFC system, DBA/1 was used as the target cell as in this combination only Ia.3 is recognized by the rabbit antiserum. The most effective monosaccharide inhibitor was D-galactose (inhibition with 2.10 and 0.69 mM solutions in the cytotoxicity and RFC inhibition assays, respectively) (Table II). Other hexose sugars such as D-glucose, D-mannose, and L-fucose did not inhibit the reaction (at concentrations up to 55 mM), nor did any of a number of pentose sugars tested. However, sugars related to galactose in which only substitution at C-2 was involved, such as N-acetyl-D-galactosamine, D-galactosamine, or D-talose, which differs from galactose only in the orientation of the hydroxyl group at C-2, were also effective inhibitors. Some oligosaccharides with a terminal nonreducing D-galactose unit, namely melibiose, raffinose, and stachyose, were also effective inhibitors. Inhibition was also obtained with D-galacturonic acid and with the glycoside phenyl-α-D-galactopyranoside, but not with phenyl-β-D-galactopyranoside, nor with the galactose-containing disaccharides, lactose or lactulose. The immunodominant sugar for the Ia.3 antigenic specificity thus appears to be α-linked D-galactose.

Fig. 1 compares in more detail the ability of several galactose-containing sugars to inhibit the binding of either allogeneic (Fig. 1a) or xenogeneic (Fig. 1b) anti-Ia antibodies to mouse spleen cells. Although slightly higher concentrations of the various sugars were required to inhibit the binding of the alloantibodies than the xenoantibodies, the hierarchy of inhibition was the same in both assays, i.e., raffinose > D-galactose > D-galactosamine > N-acetyl-D-galactosamine.

Inhibition of Anti-Ia.7 Antibodies. The Ia.7 specificity was detected by the reaction of the A.TH anti-A.TL mouse serum or the rabbit anti-I^k serum with BALB/c (H-2^d) cells. In both cases Ia.15 could be potentially detected, but the antisera were used at dilutions above which Ia.15 is detected (Table I). The only sugar that produced complete inhibition was L-fucose which inhibited at compa-

Fig. 1. Comparison of the ability of several galactose containing sugars to inhibit the binding of anti-Ia. 3 antibodies to mouse spleen cells. The sugar inhibitors are N-acetyl-D-galactosamine (△), D-galactosamine (●), D-galactose (□), and raffinose (■). The dotted line represents the percent lysis or percent RFC in the absence of any inhibitor or in the presence of a noninhibitory sugar. The antisera and target cells used are presented in Table I. (a) Inhibition of cytotoxicity of allogeneic anti-Ia. 3 serum. The percent lysis of spleen cells incubated with complement alone is indicated (○). (b) Inhibition of rosetting of xenogeneic anti-Ia. 3 serum.
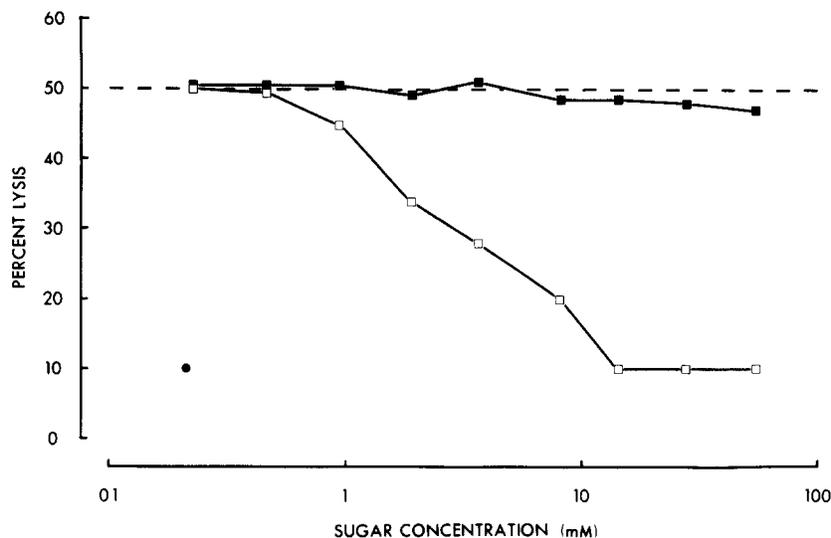
FIG. 2.   Comparison of the ability of L-fucose (□) or D-galactose (■) to inhibit the cytotoxicity of an allogeneic anti-Ia. 7 serum on BALB/c spleen cells. The nature of the antiserum and target cell used is described in detail in Table I. The percent lysis of spleen cells incubated with complement alone is indicated (●).

rable concentrations in both inhibition assay systems (Table III, Fig. 2). Apart from sialic acid (N-acetylneuraminic acid) which inhibited at a concentration of 16.17 mM in the cytotoxicity but not in the rosetting system, no other sugar gave any inhibition. In particular, there was no inhibition by D-fucose. Thus, L-fucose is implicated in the Ia.7 specificity.

Inhibition of Anti-Ia.15 Antibodies.   The Ia.15 specificity in both assay systems was detected by using D2.GD target cells (Table I). In both inhibitory assays, N-acetyl-D-glucosamine caused significant inhibition: 1.36 mM by cytotoxicity and 0.14 mM by RFC inhibition (Table III). However, no inhibition was detected by other sugars related to glucose. Apart from N-acetyl-D-galactosamine, which gave some inhibition in both assays, there was no inhibition by any of the other monosaccharides, disaccharides, or oligosaccharides tested. On the basis of this data, it was concluded that the Ia.15 specificity is defined by N-acetyl-D-glucosamine.

Inhibition of Anti-I^k Serum (Ia.1, 2, 3, 7, 15, 19).   When the alloantiserum A.TH anti-A.TL was reacted with H-2^k target cells at a concentration of 1/1,000 a composite picture was obtained as several Ia specificities were detected simultaneously. However, the antiserum was used at a concentration that detected mainly the Ia.1, Ia.3, and Ia.7 specificities. The Ia.15 reaction would not be detected, and the alloantiserum contains anti-Ia.2 antibody to a titer of 1/128 and would probably not have been detected; also we have no information as to whether the antiserum contains any anti-Ia.19 antibodies. We found inhibition with D-mannose, D-galactose, and L-fucose (Table IV) which is in accord with the detection of Ia specificities 1, 3, and 7, respectively, as described above. In addition, there was some inhibition by N-acetylneuraminic acid, but, as with other specificities, this was considerably less than the inhibition obtained with other sugars.

TABLE II

Ability of Different Sugars to Inhibit the Binding of Allogeneic and Xenogeneic Anti-Ia.1 and Anti-Ia.3 Antibodies to Mouse Spleen Cells*

Sugar inhibitor	Sugar concentration (mM) required for inhibition‡			
	Ia.1		Ia.3	
	Allogeneic anti-Ia	Xenogeneic anti-Ia	Allogeneic anti-Ia	Xenogeneic anti-Ia
Monosaccharides				
D-galactose	−§	−§	2.10	0.69
N-acetyl-D-galactosamine	−	−	29.0	9.04
D-galactosamine	−	−	5.80	2.32
D-galacturonic acid	−	−	6.44	NT
D-glucose	−	−	−	−
N-acetyl-D-glucosamine	−	−	−	−
D-glucosamine	−	−	−	−
D-glucuronic acid	−	−	−	−
D-mannose	13.87	5.55	−	−
N-acetyl-D-mannosamine	2.72	0.54	−	−
N-acetylneuraminic acid	−	−	−	−
D-talose	−	NT	6.94	NT
D-fucose	−	−	−	−
L-fucose	−	−	−	−
D-ribose	−	−	−	−
L-rhamnose	−	−	−	−
L-arabinose	−	−	−	−
D-lyxose	−	−	−	−
D-xylose	−	−	−	−
Glycosides				
Phenyl-α-D-galactopyranoside	−	NT	4.87	NT
Phenyl-β-D-galactopyranoside	−	NT	−	NT
Methyl-α-D-glucopyranoside	−	NT	−	NT
Methyl-α-D-mannopyranoside	12.87	5.15	−	−
Oligosaccharides				
Melibiose	−	NT	0.87	NT
Raffinose	−	−	0.52	0.12
Stachyose	−	NT	0.90	NT
Lactose	−	−	−	−
Lactulose	−	NT	−	NT
Maltose	−	−	−	−
Cellobiose	−	−	−	−
Sucrose	NT	−	NT	−

NT, not tested.

* Nature of the antisera and target cells presented in Table I.

‡ Values represent the concentration of sugar needed to inhibit either the cytotoxicity of the allogeneic anti-Ia by 50% or the rosetting of the xenogeneic anti-Ia by 20%.

§ −, no inhibition by sugar at 10 mg/ml (>55.5 mM for glucose) in the allogeneic anti-Ia assay and 2 mg/ml (>11.1 mM for glucose) in the xenogeneic anti-Ia assay.

Sugar Inhibition Studies With H-2 Antisera. The experiments described above suggest that many of the *I*-region-associated antigenic specificities are defined by sugars. We then carried out experiments to determine whether *H-2K* and *H-2D* region-defined antigens are also sugar in nature by measuring the

TABLE III

Ability of Different Sugars to Inhibit the Binding of Allogeneic and Xenogeneic Anti-Ia.7 and Anti-Ia.15 Antibodies to Mouse Spleen Cells*

	Sugar concentration (mM) required for inhibition			
Sugar inhibitor	Ia.7		Ia.15	
	Allogeneic anti-Ia	Xenogeneic anti-Ia	Allogeneic anti-Ia	Xenogeneic anti-Ia
Monosaccharides				
D-galactose	–	–	–	–
N-acetyl-D-galactosamine	–	–	5.65	2.26
D-galactosamine	–	–	–	–
D-galacturonic acid	–	–	–	–
D-glucose	–	–	–	–
N-acetyl-D-glucosamine	–	–	1.36	0.14
D-glucosamine	–	–	–	–
D-glucuronic acid	–	–	–	–
D-mannose	–	–	–	–
N-acetyl-D-mannosamine	–	–	–	–
N-acetylneuraminic acid	16.17	–	32.34	–
D-talose	–	NT	–	NT
D-fucose	–	–	–	–
L-fucose	1.87	0.75	–	–
D-ribose	–	–	–	–
L-rhamnose	–	–	–	–
L-arabinose	–	–	–	–
D-lyxose	–	–	–	–
D-xylose	–	–	–	–
Glycosides				
Phenyl-α-D-galactopyranoside	–	NT	–	NT
Phenyl-β-D-galactopyranoside	–	NT	–	NT
Methyl-α-D-glucopyranoside	–	NT	–	NT
Methyl-α-D-mannopyranoside	–	–	–	–
Oligosaccharides				
Melibiose	–	NT	–	NT
Raffinose	–	–	–	–
Stachyose	–	NT	–	NT
Lactose	–	–	–	–
Lactulose	–	NT	–	NT
Maltose	–	–	–	–
Cellobiose	–	–	–	–
Sucrose	NT	–	NT	–

* Footnotes as in Table II.

ability of a range of sugars to inhibit the cytotoxicity of anti-H-2 antisera for BALB/c spleen cells. the antisera used were able to detect the private H-2 specificities of H-2^d (i.e., H-2D.4 and H-2K.31), and the public H-2^d specificities (i.e., H-2.8, 13, 27, 28 and 29). The sera used were D4, D8, D13, D28, D28b, and D31, which were all obtained from the Catalogue of Mouse and Alloantisera. We were unable to find inhibition by any of the sugars used in the Ia antigen studies (Tables II and III) at concentrations up to 55 mM.

TABLE IV

Ability of Different Sugars to Inhibit the Binding of Allogeneic Anti-I^k Antibodies to C3H Spleen Cells

Sugar inhibitor	Sugar concentration (mM) required for inhibition‡
D-galactose	13.9
N-acetyl-D-galactosamine	11.3
D-glucose	>55.5
N-acetyl-D-glucosamine	>45.2
D-mannose	5.5
N-acetyl-D-mannosamine	4.5
N-acetylneuraminic acid	16.2
L-fucose	6.1
Cellobiose	>29.2
Maltose	>29.2

* A.TH anti-A.TL serum as in Table I used at 1/1,000 dilution.
‡ Values represent the concentration of sugar needed to inhibit the cytotoxicity of the antiserum by 50%.

Discussion

Using both allogenic and xenogenic antisera, our results show that sugar residues are involved in the Ia antigen specificities 1, 3, 7, and 15. The individual specificities involve N-acetyl-D-mannosamine, D-galactose, L-fucose, and N-acetyl-D-glucosamine, respectively (Table V). In contrast, several public and private H-2 antigenic specificities did not appear to be sugar defined, a finding consistent with other studies (18). The results, to be described separately, are interpreted in terms of Landsteiner's (19) observation that a substance with a structure related to the determinant group of an antigen can combine with the antibody and thus competitively inhibit the antibody-antigen reaction. As the structural relationship between the simple substance and the determinant group becomes closer, the concentration required to inhibit the antigen-antibody reaction becomes lower.

Inhibition of Anti-Ia.1 by D-Mannose and Related Sugars. The finding that mannose substituted at position two with an acetamide group is a more effective inhibitor than either mannose or methyl-α-D-mannopyranoside suggests that a two substituted mannose unit is involved in the determinant group. As the most effective inhibitor, N-acetyl-D-mannosamine is not a commonly found constituent of mammalian glycoproteins; it seems more likely that mannose substituted with another sugar at position two is involved, but resolution of this point must await further tests with mannose-containing oligosaccharides.

Inhibition of Anti-Ia.3 by D-Galactose and Related Sugars. Inasmuch as a range of sugars inhibited the Ia.3 reaction a more detailed analysis of the nature of this specificity was possible. For this specificity galactose was the most effective inhibitor of all the monosaccharides tested, and all the sugars that inhibited the reaction were related to galactose (see Table II).

On the basis of the monosaccharide inhibitors it appears that the nature of the substituent at C5 in the hexose sugar influences the efficiency of the sugar as an inhibitor, i.e., galactose, which has a primary alcohol at C5, is the most efficient

TABLE V

Immunodominant Sugar in Different Ia Antigenic Specificities as Judged by Hapten-Inhibition Studies

Specificity	Immunodominant sugar
Ia.1	N-acetyl-D-mannosamine
Ia.3	α-D-galactose
Ia.7	L-fucose
Ia.15	N-acetyl-D-glucosamine

inhibitor, while oxidation of this substituent as in galacturonic acid decreases its efficiency (Fig. 3). If the substituent is reduced as in D-fucose or absent as in L-arabinose, the ability of the sugar to inhibit the reaction is lost (Fig. 3). Furthermore, the orientation of the hydroxyl groups at C4 must be that of galactose for the sugar to be an effective inhibitor as neither glucose, mannose, nor their derivatives inhibited the reaction. Glucose differs from galactose only in the orientation of the C4 hydroxyl, while mannose differs from galactose in the orientation of both C2 and C4 hydroxyls (Fig. 4). However, sugars differing from galactose only in their C2 substituents (e.g., D-galactosamine and N-acetyl-D-galactosamine) are effective as inhibitors, e.g., D-talose which differs from galactose only in the orientation of the C2 hydroxyl (Fig. 4).

These results suggest that D-galactose or a closely related sugar is involved in the determinant group of Ia.3, the C4 configuration of galactose being essential for antigenicity, whereas the C2 and C5 configurations play less important roles. The predominance of C4 in antigenicity is consistent with the galactose being at the terminal nonreducing end of a sugar sidechain.

Tests with the phenyl-galactosides showed that only the α-linked galactoside was an inhibitor, and this suggested that the determinant would probably be in α-linkage. The α-linkage requirement was confirmed with tests on a series of oligosaccharides. These tests showed that oligosaccharides having a terminal nonreducing galactose unit in α-linkage, such as melibiose, raffinose, and stachyose, were effective inhibitors, while disaccharides having terminal nonreducing galactose units in β-linkage, such as lactose and lactulose, were not inhibitors (Table VI). The ineffectiveness of mannose and glucose as inhibitors was confirmed by the observation that neither methyl-α-glucoside nor methyl-α-mannoside were inhibitors. Similarly, the disaccharides maltose and cellobiose, which possess terminal nonreducing glucose units linked α and β, respectively, were not effective as inhibitors. Collectively, these results indicate that D-galactose or a closely related sugar is involved in the determinant group of Ia.3, and that the sugar is probably in α-linkage.

Inhibition of Anti-Ia.7 by L-Fucose. Apart from N-acetylneuraminic acid which inhibited at high concentrations, L-fucose was the only sugar tested which inhibited in either assay system. Both these sugars are known to be present as terminal groups in mammalian glycoproteins. The enantiomorph D-fucose, which does not occur naturally in mammalian sources and was included in the test primarily because of its structural relationship to D-galactose, was not an inhibitor.

Inhibition of Anti-Ia.15 by N-Acetyl-D-Glucosamine. Anti-Ia.15 was inhibited by low concentrations of N-acetyl-D-glucosamine but not by glucose,

X = -CH₂OH   D-Galactose
X = -H   L-Arabinose
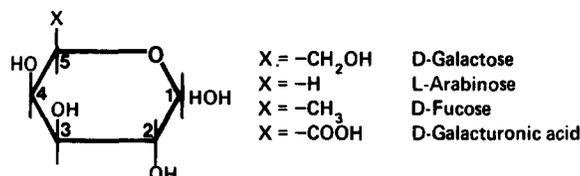X = -CH₃   D-Fucose
X = -COOH   D-Galacturonic acid

FIG. 3. The structure of some of the monosaccharides used in the hapten-inhibition studies and particularly relevant to an understanding of the structure of the Ia. 3 antigenic specificity (see Discussion).
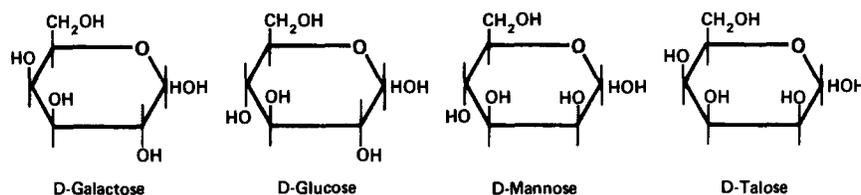

D-Galactose   D-Glucose   D-Mannose   D-Talose

FIG. 4. Structure of some additional monosaccharides used in the hapten-inhibition studies.

glucuronic acid, D-glucosamine, methyl-α-D-glucopyranoside, or either the α- or β- (1 → 4) linked glucose disaccharides, maltose or cellobiose (Table VI). However, N-acetyl-D-galactosamine was a moderate inhibitor, whereas N-acetyl-D-mannosamine showed no inhibition, a result that suggests that the orientation of the C2 acetyl group is important in determining antigenicity. Thus, these studies implicate N-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucose) or a related sugar in the determinant group. This is in keeping with the general finding that this sugar is present in mammalian cell membrane glycoproteins but that glucose itself is rarely present.

The four sugars that we have found to be involved in the Ia antigens of mice, namely D-mannose, D-galactose, L-fucose, and N-acetyl-D-glucosamine, are frequently found in the glycoproteins of mammalian cell membranes. L-fucose is always found as a terminal nonreducing group, and D-mannose, D-galactose, and N-acetyl-D-glucosamine can occur as either end groups or core groups in glycoproteins (20). In fact, on the basis of our findings there now appears to be a remarkable similarity between the Ia antigens and the human ABO blood group substance specificities, as both antigen systems can occur as cell membrane determinants as well as serum glycoproteins, and in both systems the antigenic determinants are defined by terminal sugar sequences. Furthermore, the experimental approach described in this paper is similar to the classical work of Morgan and Watkins (21) who showed that L-fucose specifically inhibited the hemagglutination of human O cells by extracts of *Lotus tetragonolobus* and of Kabat and Ginsberg et al. (22, 23) who (a) showed that N-acetyl-D-galactosamine inhibited the lectin-induced hemagglutination of blood group A cells and who (b) demonstrated that the reaction of blood group B with anti-B antibody could be inhibited by various α-linked D-galactosides. However, although we have shown that different monosaccharides are implicated in each Ia specificity studied, this does not necessarily imply that the total specificity resides in the terminal sugar. It would be anticipated that the Ia antigenic specificities are spread over several sugar units as has been shown for most antigenic determi-

TABLE VI

Structures of the Different Oligosaccharides Used in the Inhibition Studies

Oligosaccharide	Structure
Melibiose	O-α-D-Gal-(1→6)-D-Glc
Lactose	O-β-D-Gal-(1→4)-α-D-Glc
Lactulose	O-β-D-Gal-(1→4)-α-D-Fru
Maltose	O-α-D-Glc-(1→4)-α-D-Glc
Cellobiose	O-β-D-Glc-(1→4)-α-D-Glc
Sucrose	O-α-D-Glc-(1→2)-β-D-Fru
Raffinose	O-α-D-Gal-(1→6)-α-D-Glc-(1→2)-β-D-Fru
Stachyose	O-α-D-Gal-(1→6)-α-D-Gal-(1→6)-α-D-Glc-(1→2)-β-D-Fru

nants (24). Consistent with this interpretation are preliminary studies in our laboratories which have also implicated galactose in the Ia.8 and Ia.9 specificities.

Our studies therefore demonstrate that the specificity of at least four Ia antigens present on the surface of splenic B cells resides in the carbohydrate, rather than protein moiety of these cell surface glycoproteins. Presumably, the same conclusion holds for the Ia antigen-bearing oligosaccharide(s) which we have recently isolated from mouse serum (8, 9, 15) and which constitute >98% of the Ia antigenic activity detected in normal serum. In this context it should be noted that human ABO blood group substances can also exist in several molecular forms, such as low molecular weight oligosaccharides, glycoproteins, and glycolipids. In agreement with the ABO system, we have recently identified a high molecular weight serum glycoprotein in mouse serum, which also bears Ia antigenic determinants (25). Whether the Ia specificities reside in the carbohydrate or peptide fraction of the cell surface glycoproteins has been previously questioned, and there is some evidence, contrary to our data, of specificity residing in the protein moiety (26). However, these studies have not resolved whether the amino acid moiety participates directly in the serological specificity or whether it merely maintains the carbohydrate side chains in the correct conformation. Our studies clearly implicate carbohydrate as playing an immunodominant role in the Ia specificities. It is of interest that the relative roles played by carbohydrate or protein in defining the HLA specificities in man does not as yet appear to have been settled (27).

Our demonstration that several of the Ia antigens are defined by sugars has several important theoretical implications. First, it strongly suggests that at least some of the genes in the *I* region of the *H-2* complex code for glycosyl transferases. Furthermore, based on the Ia specificities examined, both the *I-A* (Ia.1, 3, 15) and *I-C* (Ia.7) subregions (2) should contain glycosyl transferase genes. Second, the close relationship between Ia antigens and many T-cell-derived immunoregulatory substances (10-12) suggests that carbohydrates may play a role in T-B cell collaboration. Finally, the fact that Ia antigens are oligosaccharide in nature must be borne in mind when attempts are made to develop a relationship between Ia antigens and *Ir* genes and their role as recognition units. In this light, it should be recalled that carbohydrate determinants have been shown to be involved in a variety of recognition processes, such

as sponge cell reaggregation (28), plant pathogen-host recognition (29), embryonic development (30), hemagglutination by plant lectins (30, 31), platelet-collagen adhesion (30, 32), and the removal from the circulation of glycoproteins lacking their terminal sialic acid groups (30, 33). The carbohydrate nature of the Ia specificities and their possible role in T-B cell collaboration may provide another example of the important role of carbohydrates as informational molecules. Superficially, it might appear that Ia antigens possess certain genetic characteristics which are difficult to reconcile with the *Ia* gene product being a glycosyl transferase. First, is it possible for one *I* subregion to code for multiple Ia antigenic specificities (1-3) if glycosyl transferases are involved? The simplest explanation is that each *I* subregion consists of a cluster of genes coding for glycosyl transferases which act very specifically in sequence to construct an oligosaccharide sidechain. Certainly our observation that two of the *I-A* subregion specificities (i.e., specificities one and three) in the *H-2^k* haplotype are defined by different sugars is consistent with this subregion containing a number of glycosyl transferase genes. Second, if the *Ia* gene product is a glycosyl transferase would it be possible for the Ia antigenic specificities from each *I* subregion to be expressed on separate protein molecules on the cell surface (14, 34)? Before answering this question it must be remembered that glycosyl transferases have strict specificity requirements and thus the attachment of a sugar by one transferase rigorously determines the sequence of transferases which subsequently extend the carbohydrate chain (17, 21, 30). Thus, if it is assumed that the Ia-bearing protein molecule in the lymphocyte membrane is glycosylated at only one site in its polypeptic chain, then the first transferase that attaches a sugar predetermines the complete oligosaccharide sidechain which is constructed. The net result is that only one *I*-subregion oligosaccharide is attached to each protein. The observation that the lymphocytes of F_1 mice can express the subregion specificities of each parent on separate molecules (14, 24) can also be explained in this way.

Summary

We have previously reported that the Ia specificities, coded for by the *I* region within the *H-2* complex, appear to consist predominantly of carbohydrate. This conclusion was reached by examining low molecular weight Ia-bearing oligosaccharides isolated from mouse serum. We now report hapten-inhibition studies which indicate that the binding of both allogeneic and xenogeneic anti-Ia antibodies to the Ia glycoproteins found predominantly on B lymphocytes can be specifically inhibited by certain free sugars. Both inhibition assays revealed that the specificity for the following Ia antigens resides predominantly in the following sugars: (a) Ia.1: N-acetyl-D-mannosamine or related sugars; (b) Ia.3: α-D-galactose and related sugars; (c) Ia.7: L-fucose; and (d) Ia.15: N-acetyl-D-glucosamine. It seems likely that these sugars are found at the terminal nonreducing ends of the carbohydrate portion of the Ia-bearing glycoproteins present in the lymphocyte membrane. In contrast, several public and private *H-2* antigenic specificities did not appear to be sugar defined.

These studies imply that at least some of the *Ia* genes from both the *I-A* and *I-C* subregions of the *I* region code for glycosyl transferases which modify oligosac-

charide structure and impart specificity to the Ia antigens by alteration of their terminal sugar residues.

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