Resistance to Denaturation by Periodate of a Dialyzable Form of BALB/c Histocompatibility Antigen[1] (36739)

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Studies of the chemical composition of soluble preparations of histocompatibility antigens (HCA) of the mouse have suggested that this was glycoprotein (1, 2), lipoprotein (3) or polypeptide (4) in nature. Recently several investigators have indicated the presence of carbohydrate in human (5, 6) and murine (7, 8) HCA. In the case of human HCA the presence of carbohydrate in the immunochemical determinant area has been suggested on the basis of substantial inactivation of this antigen by periodate (6), a reagent which causes a considerably higher rate of denaturation of carbohydrate than of protein (9).

Solubilization of BALB/c HCA by the detergent Triton X-100 and *n*-butanol from the lymph nodes, livers and spleens of rabbits (10, 11) and inbred mice (12) has been reported from this laboratory. More recently, it was reported that proteolytic digestion of Sephadex fractions of both these detergent-solubilized antigens yielded several smaller molecular species which retained specific inhibiting activity. Of these, the most striking was a product of papain digestion of a Sephadex G-200 excluded fraction of Triton solubilized antigen. This fraction was excluded by Sephadex G-10 but not by G-15 (hereafter referred to as G 15-10), and appeared to have a molecular weight slightly less than vitamin B_{12} (13).

In the present study the denaturing effect of periodate on mouse HCA (2) has been confirmed for detergent-solubilized macromolecular BALB/c antigen. Further, the availability of a small molecule derived from these preparations, and retaining BALB/c specificity, has made possible a test of the significance of the carbohydrate moiety for the determinant area of this HCA.

Materials and Methods. BALB/c and CBA mice were obtained from The Jackson Laboratory, Bar Harbor, ME. CBA anti-BALB/c globulins were obtained from ascitic fluids of CBA mice 11–13 days after a second injection of BALB/c spleen cells, and a second injection of a peritoneal irritant containing Bayol (Humble Oil & Refining Co., Linden, NJ), and Arlacel (Hill Top Research, Inc., Miamiville, OH) 3:1, with oleic acid at a final concentration of 0.5% (12). The BALB/c soluble histocompatibility antigen was prepared from cell membrane fragments of BALB/c spleens by extraction with Triton X-100 and precipitation with acetone (12). This preparation has been shown to cause accelerated rejection of BALB/c skin grafts in normal CBA mice after 3 injections suitably spaced. BALB/c antigenic activity was detected and estimated by the inhibition of CBA anti-BALB/c antibody in 3 *in vitro* tests: (a) agglutination of BALB/c RBC by CBA anti-BALB/c antibody, in plastic plates, as described recently (13); (b) cytotoxicity of such globulins for BALB/c spleen cells, as described elsewhere (14); (c) suppression by such alloantibodies of the ability of spleen cells to produce hemolytic antibody plaques to sheep RBC (15). In each case, the inhibition test was done against approximately 3 units of antibody.

Results. Denaturation of the BALB/c anti-

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TABLE I. Effect of $NaIO_4$ on Inhibiting Activity of BALB/c Histocompatibility Antigen (Triton X-100 Extract from Spleen), and Its G 15-10 Derivative.[a]

$NaIO_4$								
Active	Inactive	Time exposed	Inhibiting titer of original BALB/c spleen Triton antigen (log_2 of 1 mg/ml)			Inhibiting titer of BALB/c spleen Triton antigen, G 15-10 derivative (log_2 of $OD_{280} = 1$)		
(*M*)	(*M*)	(min)	Hemagglutination	Suppression	Cytotoxicity	Hemagglutination	Suppression	Cytotoxicity
—	—		4.0	2.6	2.8	2.5	1.4	1.6
0.005		30	4.0	2.6	2.3	2.5	1.4	1.3
	0.005	30	4.0	2.5	2.3	3.0	1.5	1.7
0.05		30	2.0	0.4	0.4	2.5	1.3	1.6
		60	2.0	0.3	0.6	3.0	1.4	1.6
		90	2.0	0.4	0.4	2.5	1.4	1.6
	0.05	30	4.0	2.5	2.4	2.5	1.1	1.4
		60	4.0	2.5	2.5	2.0	1.1	1.2
		90	3.5	2.5	2.2	2.5	1.3	1.4

[a] No inhibition of anti-BALB/c alloantibody in any of these 3 tests by antigen solubilized from CBA spleen cells by Triton X-100 at concentrations up to 4 mg/ml, or by a G 15-10 derivative of this CBA antigen up to OD = 4, respectively, *i.e.*, 20× to 10× the concentrations at the end points shown above for the BALB/c antigen preparations. Also, the preparations shown did not inhibit these effects of BALB-anti CBA alloantibody on CBA spleen cells in any of these tests at the same level of concentrations, 4 mg/ml or OD = 4.

gens by periodate. Samples of the BALB/c spleen Triton antigen at 1 mg/ml, or the G 15-10 derivative at $OD_{280} = 1$, in phosphate buffered saline (pH 7.0) were treated at 37° with $NaIO_4$ at either 0.005 or 0.05 *M*, for periods of 30, 60 and 90 min. The $NaIO_4$ was used either in an untreated, active form, with the excess $NaIO_4$ inactivated after the reaction time by addition of 2.5% glucose, or, for control preparations, with $NaIO_4$ inactivated by incubating with 2.5% glucose prior to treatment of the BALB/c antigens. After exposure to $NaIO_4$, the preparations were tested for antigenic activity by their ability to inhibit allogeneic antibodies from causing agglutination of BALB/c RBC (13), cytotoxicity, and suppression of the ability of BALB/c spleen cells to produce hemolytic antibody plaques to sheep RBC (12).

Table I (left) shows that in all 3 reactions the original spleen Triton antigen showed no reduction in the titer of inhibition from the control level on exposure to 0.005 *M* $NaIO_4$. However, on increasing the concentration of $NaIO_4$ to 0.05 *M*, exposure for 30 min or longer caused a reduction of approximately $\log_2 2$, a loss of about 75%, in the inhibiting activity. Further, no loss of inhibiting activity was found if the $NaIO_4$, at the higher level, had been inactivated by the prior addition of glucose. In contrast, the G 15-10 product, as shown in Table I (right) showed no reduction of inhibition titer from its original level as a result of any of these treatments.

The concentrations of the small antigen are given in Table I as OD_{280} since we had not as yet obtained sufficient quantities at an adequately confirmed level of purity to express concentrations in terms of dry weight. Meaningful comparison of specific activity of the original macromolecular HCA and the G 15-10 form can not be made, both for this reason and because the OD_{280} of a small molecule can vary widely relative to its dry weight, depending on the percentage of tyrosine and tryptophan. The periodate effect on each form of the antigenic material must therefore be considered in relation to its own starting material. It may be pointed out in this connection that no indication of the actual or expected relation of specific activities of the two forms of the antigen can be made now, both because the concentration of one is expressed in relation to dry weight and the other as OD_{280}, and because we do not know the number of repeating units of the BALB/c antigenic determinant on the macromolecular soluble antigen as obtained by Triton X-100.

Gas chromatographic analysis for carbohydrate. Because of the indication of denaturation by periodate in the case of the macromolecular antigen, but not of the G 15-10 fraction, the two preparations were subjected to carbohydrate analysis. Briefly, 2.5-ml samples of the original BALB/c spleen Triton antigen at 2 mg/ml, or of the G 15-10 derivative at $OD_{280} = 2$, were hydrolyzed by treatment with 1.5 *N* HCl in methanol. Fatty acids were extracted with hexane, the methanol layer taken to dryness and the trimethylsilyl derivative made (16). Analysis was performed on a Hewlett-Packard gas chromatograph, using an OV-1 column. Compounds were identified by comparison of retention time with those of standards. Carbohydrate analysis indicated that the original antigen contained 2.82% glucose, 6.34% mannose, 2.0% glucuronic acid and 2.0% *n*-acetyl galactosamine. In contrast, the G 15-10 material had no detectable carbohydrate (Fig. 1). Since the amounts of the 4 sugars detected in the 5-mg sample of the macromolecular antigen were, respectively, 141, 317, 100 and 98 μg, and since none of these was detected at all in the G 15-10 preparation (with a threshold of detection of <1 μg) the results clearly signified an absence of these sugars in the G 15-10 molecule. These results were confirmed in two repetitions of these analyses.

Discussion. The data reported here show that BALB/c antigen, solubilized by Triton X-100, is largely inactivated on short exposure to periodate, at low concentration, indicating glycoprotein structure. [Some levels of denaturation of a number of proteins by periodate have been described, but these have been of minor degree, and have required exposure to a higher range of concentration, and for considerably greater periods of time

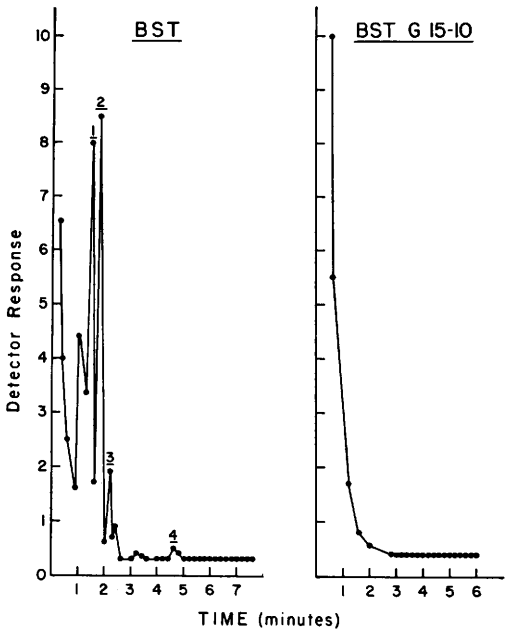

FIG. 1. Gas chromatogram of whole BALB/c Triton-solubilized HCA and of its papain digestion product (G 15-10). The following assignments were made by comparison with chromatograms of the individual sugar derivatives: (1) glucuronic acid, (2) mannose, (3) glucose, (4) *N*-acetyl galactosamine.

(17–19).] On the other hand, a considerably smaller molecular unit, produced by papain digestion of the detergent-solubilized HCA and retaining BALB/c specificity, was not inactivated by this treatment. Further, gas chromatographic analysis, which indicated the presence of 4 sugars in the macromolecular BALB/c antigen, gave no evidence of these in the small molecule. It would appear, on the basis of these preliminary data, that an area of BALB/c HCA which includes the antigenic determinant does not contain the carbohydrate moieties of the antigen as originally solubilized. Evidence was recently presented by Shimada and Nathenson (20) that of the three known kinds of carbohydrate components in macromolecular mouse HCA, one of them, sialic acid, could be removed without loss of inhibition of immune cytolysis. That observation is confirmed and extended by the present findings, which indicate that, at least in the case of BALB/c specificity, none of the carbohydrate moieties of the macromolecular HCA are included in the determinant area.

The observations reported here would suggest that the substantial degree of inactivation of macromolecular mouse HCA by periodate may be due to deformation of the molecule in carbohydrate-containing areas outside the antigenic determinant, with loss of structural support on which the configurational integrity of the antigenic determinant may depend.

Summary. BALB/c histocompatibility antigen solubilized by Triton X-100, and a small molecular form derived from it by digestion with papain, were examined for inactivation by periodate and for carbohydrate content. Treatment with a low concentration of periodate reduced by about 75% the antigenic activity of the original macromolecular form of the antigen, as judged by inhibition of anti-BALB/c alloantibodies in several *in vitro* tests, but did not have this effect on the small molecular unit. Also, carbohydrate analysis of the macromolecular antigen showed it to contain mannose, glucose, glucuronic acid and *n*-acetyl galactosamine, whereas no sugars were detectable in the small molecule.

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