PREPARATION OF SINGLE, SOLUBLE ANTIGENS OF THE MOUSE HISTOCOMPATIBILITY-2 COMPLEX

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Chemical analysis of the principal transplantation antigens of the mouse, the Histocompatibility-2 antigens,[1] has been hampered by the complexity of immunologically active cell fractions. Though soluble H-2 antigens have been prepared from both normal adult[2-4] and embryo[5] tissues and from tumors,[6,7] these extracts are chemically complex and antigenically diverse. It appears that the antigenic diversity of these preparations is not likely to be resolved by physicochemical techniques, and indeed that the array of antigenic determinants present in a single H-2 complex may be linked to a common backbone.[8]

Further analysis of the H-2 antigens would be facilitated by the isolation of single determinants from the complex. An approach to this is offered by the availability of antisera of limited specificity, often putatively monospecific,[1] which may be used as specific isolating agents. This paper describes a procedure for the extraction from adult mouse tissues of soluble H-2 antigen complexes that are active as inhibitors of cytotoxic alloantisera. The soluble complexes have been resolved into single antigens using antisera.

Materials and Methods.—Antigenic starting materials: A stable, insoluble starting material for solubilization (stroma) was made by extracting pooled lymph nodes, spleen, liver, and thymus of 50 to 200 BALB/cJ mice (H-2.3,4,6,8,10,13,14, 27,28,29,31) five times with 0.02 per cent EDTA–0.14 M phosphate-buffered NaCl, pH 7.2 (EDTA-PBS), and then four times with 1.14 M phosphate-buffered NaCl, pH 7.2, following Kandutsch and Reinert-Wenck.[9] Extracted stromata were washed in distilled water and lyophilized for storage prior to further treatment. The lyophilized materials themselves were specific inhibitors of cytotoxic antisera; 100 μg of stroma absorbed 80 per cent of a standard antiserum dose ($R = 0.20$).

Assay of antigens: The microcytotoxic test of Boyse, Old, and Thomas[10] was used for all determinations of antiserum activity. Ten microliters of a suspension of 1×10^6 mouse mesenteric lymph node cells, suspended in 1 per cent bovine serum albumin-tris-buffered Hanks solution (pH 7.2 at 37°C), was added to 10 μl of antiserum on a microscope slide. After incubation for 15 minutes at 37°C in a moist box, 10 μl of guinea pig serum at $^1/_2$ to $^1/_4$ dilution was added to the drops, and incubation continued for 45 minutes. At the end of this period one drop of 0.6 per cent nigrosin in heated mouse or calf serum was added, and the cells were examined at 200–250× magnification. One hundred to two hundred cells per drop were counted and classified as damaged (stained) or undamaged (unstained). Five replicate tests were placed on a single slide.

Antigen activity was assayed by mixing a standard volume of extract with a standard amount of antiserum. The serum standard was arbitrarily set as 0.10 ml of a $^1/_{10}$ dilution of a serum with end point $^1/_{200}$. A serum with endpoint $^1/_{400}$ would be used at 0.10 ml of $^1/_{20}$ dilution, and so on. The effect of antigenic extracts

of standard antiserum is expressed in terms of R, the relative potency of the extract-treated serum. This value is computed from the comparison of a serum's cytotoxicity at a given dilution, after treatment with antigen, with that of a standard, untreated serum; this method has been discussed fully elsewhere.[11] Cytotoxic inhibition is particularly suitable to the assay of large numbers of chromatographic fractions of antigen, and is quite accurate when used to assess the location of peaks of activity. However, relatively large random variations in R occur. Hence, the comparison of the inhibition of two different sera in equivalent amounts is at best an approximation. Only if a fraction shows no activity when tested with a particular serum are absolute indications of activity obtained.

Antisera: Six antisera were used for the assays described here; the hosts and donors used for their production, together with their putative specificities, are listed in Table 1. AS-245E, thought at the time it was prepared to be monospecific, may well be polyspecific, and this same caution may be applicable, though with less force, to the other putatively monospecific reagents.[12]

Preparation of soluble H-2 antigens from stroma: The solubilization of antigen method was based on observations that EDTA released soluble H-2 antigens from young embryos but not from embryos older than 11 days of gestation and that the activity of this material was not destroyed by trypsin though its apparent molecular weight was reduced.[5] Therefore, trypsin and EDTA were applied in sequence to stroma as described below. If either step was omitted or if excess calcium ion was present together with EDTA, no H-2 activity went into solution.

Soluble antigen was prepared from cell stroma by mixing in a Waring blendor 300–350 μg of dry stroma in 25–30 ml of 5 M urea–0.05 M $CaCl_2$ to which was added 25–30 ml of 0.5 per cent crude trypsin (Difco 1:250). After adjustment of the pH to 7.2 with Tris, the mixture was incubated at 37°C for two to three hours with continuous stirring on a magnetic stirrer. The stromata were spun down (8,000 g for 30 minutes), washed once with 0.14 M phosphate-buffered saline (PBS), and resuspended in the blendor in 60 ml of EDTA-PBS. This suspension was stirred overnight in the cold and was recentrifuged 16 hours later. At this step the stromata were discarded and the opalescent 8,000 g supernatant was deproteinized by ×3 extraction with 88 per cent phenol. Traces of phenol were removed with ether, traces of ether with N_2, and the supernatant was lyophilized to dryness.

After reconstituting in a small volume, usually 3 ml, of distilled water, the deproteinized extracts were loaded on a 45 × 1 cm BioGel P-2 column (Calbiochem) and eluted with PBS. The main peak of antigen activity, as measured by inhibition of A-anti-BALB/c serum, was late-eluting; some activity, not necessarily specific and not further examined, occasionally eluted at the void volume. The

TABLE 1

Antiserum	Host	Donor	Principal H-2 specificities	Other possible H-2 specificities
AS-239F	(C57B1/10 × A/Sn)F_1	B10.D2-New	31	—
AS-245E	(C3H.Sw × A.CA)F_1	A.Sw	3	19
AS-248D	(AKR.M × C57B1/10)F_1	B10.A	4	10
A-anti-BALB/c	A/HeJ	BALB/cJ	31	—
WAS-97	C57B1/10J	A/J	1, 3, 4, 8, 10, 11*	
BALB/c-anti-A	BALB/cJ	A/HeJ	1, 5, 11, 25*	

* According to H-2 tables. Titers of individual specificities have not been determined for these sera.

late-eluting antigen was readily dialyzable and soluble in 5 per cent TCA. Its *Rf* on P-2 and its behavior on Sephadex G-25 and G-10 suggest that it is adsorbed to some extent on both types of sieve. Hence, the separations described here using BioGel or Sephadex are not strictly separations by molecular size.

Figure 1*a* shows the activity of P-2 fractions tested against three different sera, two of which, WAS-97 and A-anti-BALB/c, are expected to react with BALB/c extracts, and the third of which is a specificity control. The preparation illustrated shows some activity in the void volume fractions, but in contrast to the main peak of reactivity, the void volume fractions are not specific. The main peak was pooled, dried, and desalted in BioGel Ag 11A8 ion retardation resin (Calbiochem). On the basis of the inhibition of a standard dose of serum by 0.1 ml of each of the pooled fractions, sufficient antiserum, either A-anti-BALB/c or WAS-97, was added to complex all of the activity present. (The sera used here had been eluted from Sephadex G-50 in order to remove low-molecular-weight, nonantibody compounds.) The mixture of antigen and antibody was rerun on P-2, and void volume fractions, presumably containing antigen-antibody complexes, were pooled, dried, dissolved in distilled water, and taken to pH 2.0 with 1 *N* HCl. The acidified mixture was run once again on P-2, this time eluting with 0.01 *N* HCl. The antibody eluted in the void volume. After the solution was neutralized and brought to isotonicity, antigen activity was detected in a late-eluting and somewhat tailing peak. This peak inhibited both WAS-97 and A-anti-BALB/c, regardless of the serum used for isolation; it was never seen to inhibit the specificity control, BALB/c-anti-A (Fig. 1*b*). Thus, reaction of an antigenically diverse mixture with antisera to one or several components of that mixture seems to result in complexing of many if not all antigens present. This suggests that the determinants share a common backbone; such a suggestion is borne out by the observation that the late-eluting fractions from the P-2 column on which antigen-antibody complexes were first isolated contained no detectable activity specific for any $H\text{-}2^d$ antigens.

Isolation of single antigenic determinants: An attempt to isolate single antigenic determinants from the complex was made by digesting active fractions with Cal-

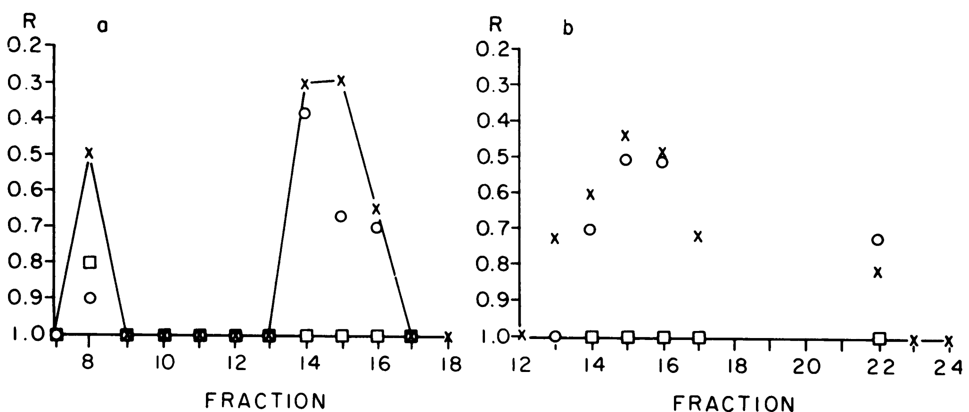

FIG. 1—(*a*) Fractionation of crude soluble antigen; fractions eluted with PBS. ×, Tested with A-anti-BALB/c; ○, tested with WAS-97; □, tested with BALB/c-anti-A. (*b*) Antigen-A-anti-BALB/c antibody complex dissolved in HCl and chromatographed on P-2, eluting with 0.01 *N* HCl. Symbols as for (*a*). *Note:* fractions 18–21 were too acid to be determined.

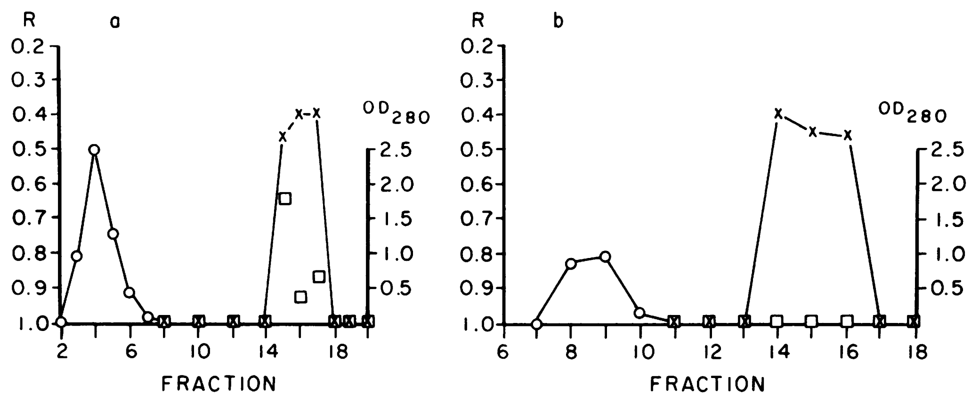

FIG. 2.—(*a*) Antigen-AS-239F antibody complex of Pronase-digested antigen split in HCl as described. ×, tested with AS239F; □, tested with WAS-97; ○, OD 2800 Å. (*b*) Pronase digest as eluted from P-2, after removal of H-2.31. ×, Tested with WAS-97; □, tested with A-anti-BALB/c; ○, OD 2800 Å.

biochem "Pronase" (10 mg/ml for 3 hr, at pH 7.2) and rechromatographing the digest on P-2. After digestion the peak of activity was slightly retarded, compared to that of undigested antigen, and nonspecific inhibitors were once again evident at low levels. The latter are apparently due to autodigestion of Pronase, since Pronase alone yields a nonspecific inhibitor after P-2 chromatography. This nonspecific activity was smeared along the length of the column and was not sufficient to account for all the activity of the antigen-containing fractions.

The activity of Pronase-digested fractions seems to be as diverse as that before digestion. However, when fractionation with antisera was repeated, antigenic specificities separated from each other to a considerable extent. Thus, after mixing antigen with an excess of AS-239F (anti-31), isolating the complex on P-2, and dissociating it with acid, fractions were collected that were good inhibitors of AS-239F and A-anti-BALB/c, but reacted poorly with WAS-97 (Fig. 2*a*). The scavenged antigen fractions, i.e., those eluting from P-2 behind the antigen antibody complex, reacted with WAS-97, but not at all with AS-239F (Fig. 2*b*). This differential change in reactivity contrasts with that seen after antibody treatment of undigested fractions.

Further chromatography of the isolated H-2.31 activity on Sephadex G-10 yielded fractions that reacted well with AS-239F but still gave a feeble reaction with WAS-97. Similar isolation of H-2.4 led to activity in two peaks on G-10, one of which also reacted with a reagent for all BALB/c antigens other than 4 and 31 (WAS-97 used against AKR.M cells, which lack H-2.4 and H-2.31). The second peak reacted only with anti-H-2.4 and did not inhibit the test for all BALB/c antigens other than 4 and 31 (Fig. 3). Here separation of antigenic determinants seems to have been complete.

The materials isolated as outlined above have been examined by a variety of chemical techniques. Fractions containing activity show no absorbance in the range 2500–2800 Å, and give inconsistent reactions with aniline hydrogen phthalate and ninhydrin after hydrolysis in 2 *N* HCl for two to three hours. The amounts of

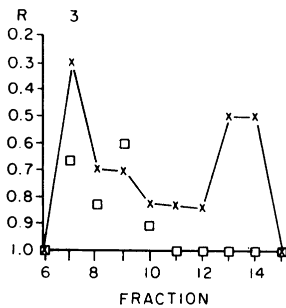

FIG. 3.—G-10 fractions of antibody-isolated H-2.4. ×, Tested with AS248D; □, tested with WAS-97 against AKR.M cells, for BALB/c antigens other than 4 and 31.

antigen detected as serum inhibitors appear to be below those needed for microchemical study.

Discussion.—The techniques presented here yield soluble, though complex, H-2 antigen preparations. These seem to consist of many specific determinants linked on a single molecule, confirming previous observations.[8] The determinants' linkage may be peptide in nature, since it is broken to some extent by Pronase, though the difficulty of separation of H-2.31 and other H-2 antigens after Pronase digestion (Fig. 2*a*) suggests that the effect could be due to traces of other enzymes in the Pronase used. However, a separation was effected of H-2.4 from other antigens, and it may be that in fact peptide bonds of limited accessibility are involved.

A second linkage is also involved in the procedure described, that of the entire antigen complex to the cell surface. The linkage appears to be rather complex, since neither proteolytic digestion alone, nor a chelating agent alone, but the combination of the two is required to effect solution. This contrasts with other reports of autolytic or proteolytic solubilization of H-2,[4, 7, 8] though the methods used involved extensive washes in calcium magnesium-free saline and in distilled water, and may have removed sufficient calcium ion to allow solubilization. Experience with embryo H-2 antigens suggests that the initial bond holding the H-2 antigen complex to the cell surface is EDTA-sensitive, and indicates that this is supplanted by another, EDTA-resistant link in later embryonic development.[5] The picture that emerges from all these studies is that of an antigenic complex carrying many if not all the antigens determined by a given H-2 locus, linked by several different types of bonds to the cell surface. Perhaps more will be learned about the identity of the complex and its mode of linkage when sufficient quantities of a soluble, single antigenic determinant become available for chemical study.

Summary.—Tryptic digestion of insoluble tissue stroma, followed by treatment with a chelating agent, releases specific inhibitors of anti-Histocompatibility-2 cytoxic alloantisera, presumably complexes of H-2 antigenic determinants. These inhibitors may be purified by isolation from antigen-antibody complexes, and may be resolved into single antigens by further proteolysis followed by a second isolation using monospecific antisera.

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