Chemical and Serological Characterization of Purified H-2 Antigens

by

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Chemical and Serological Characterization of Purified H-2 Antigens[1]

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The connection between studies on the purification of histocompatibility antigens and the field of immunological tolerance is based mainly on the hope that with future understanding the purified antigens can be used to render an animal tolerant to homografts. As suggested by the observation of Dresser (1) and Mitchison (2) and Billingham and Silvers (3), it may be necessary only to find some means of introducing large amounts of antigen in the proper state to achieve tolerance in the adult.

It would be best if we could focus efforts on purification of the H-2 antigens, as these are the strongest histocompatibility antigens of the mouse. An H-2 difference alone, between donor and recipient of a graft, is sufficient to bring about as rapid and complete a rejection as differences at many other H-loci (4). As a number of workers have shown that in the absence of H-2 incompatibility it is possible to achieve successful homotransplantation (5, 6), should it be feasible to specifically neutralize the H-2 antigen, or desensitize the graft recipient to it, grafts between genetically non-identical individuals might become generally possible.

Perhaps the major obstacle, though, so far, to the purification of histocompatibility antigens has been the lack of methods of assay which are specific for individual antigens. There are in excess of fifteen genetic loci in the mouse (7), each of which may determine a distinct histocompatibility antigen difficult to distinguish with respect to the homograft reaction. For this reason, using accelerated homograft rejection as an assay for antigen in an extract, under conditions where the donor and the recipient animals come from ordinary inbred mouse strains and therefore differ from each other at many histocompatibility loci, makes interpretation of purification and inactivation data very difficult. A given extract could contain any or all of the reasonably strong histocompatibility antigens and therefore separation of transplant sensitizing activity into two active fractions could mean either that two antigens have been separated or that a single antigen has been partitioned between the two fractions.

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2. Summary of this paper presented by Dr. G. J. V. Nossal.

This objection can be overcome by the use, as donor and recipient, of coisogenic mouse strains such as the "IR" lines developed by Snell (8), in which a difference at only a single histocompatibility locus separates members of a given pair of lines. Still, using skin grafting as an assay for antigen during purification is not entirely satisfactory, as grafting is a laborious and time consuming procedure which requires large numbers of animals and gives only semiquantitative estimates of antigenic activity.

Of course, it is by no means unreasonable to use grafting as an assay for purification of antigens for which no serological methods are available, but in the case of the H-2 antigen, where the hemagglutination system is so well studied, preliminary screening of fractions by serological methods is more efficient (9—11). Not only are these serological methods simpler and more rapid than grafting, but they are specific for these antigens. It is possible to assay for H-2 antigen using either inhibition of hemagglutination or absorption of hemagglutinating activity from a serum with negligible interference from other histocompatibility antigens.

Using hemagglutinin absorption to follow the purification of H-2 antigen from mouse liver, we found the bulk of the H-2 activity associated with the membranes of the cell (11). The membranes were isolated by successive differential centrifugation of the tissue homogenate in 0.25 M sucrose in 0.013 M phosphate buffer, pH 7, then in buffer alone, and finally floated on a solution of potassium bromide of a density of 1.21 to 1.22.

A similar procedure applied to spleen, ascites tumor cells (L-1210), cell free ascites fluid induced with this tumor and in vitro cultured cells (ML 38) also yields H-2 activity in the flotsam.

Membranes prepared in this fashion from a number of mouse strains including A/J, C57Bl/6J, C57Bl/10Sn, B10.D2/Sn, C3H/Sn, C3H.SW/Sn, DBA/2 and F_1 hybrids of some of these all contain the expected H-2 antigens. For some unknown reason, we find C57Bl/6J and C57Bl/10J mice yield considerably less on both a weight and activity basis, although C3H.SW (which like C57B is H-2^b) and B6AF$_1$ (C57Bl/6J $\times$ A/J) give apparently normal yields.

The preparation procedure indicates, to a certain extent, the composition of the membrane fraction. With the initial, low speed centrifugation, we bring down whole nuclei, large cell membrane fragments, some whole cells and erythrocytes. We discard, with the supernatants, the mitochondria, microsomes, and the soluble components of the cell including free fats and lipoproteins. Resuspension in dilute phosphate buffer lyses the nuclei, the erythrocytes and the few whole cells which may remain, and low speed centrifugation then sediments the membranes of the cell along with chromatin granules and uncharacterized debris. If the membranes are broken, (e.g. by more vigorous homogenization) into smaller pieces, more intensive centrifugation is necessary to prevent large losses of antigenic material thro

the above procedure. At homogenization of the fraction in potassium bromide solution of density 1.22 (~ 2.7 M), the nucleoprotein dissolves, then, during centrifugation, any material whose density exceeds 1.22 sediments, while the lighter membrane fraction floats to the top of the tube.

On attempting further purification and characterization of the H-2 antigen from the membrane fraction, using hemagglutinin absorption to follow the antigen, we were severely limited by certain factors. The physical state of the material greatly influences the detection of the antigenic activity. Heavily clumped materials do not absorb well. Finally divided and soluble fractions cannot be separated from the antiserum.

Discovery that after disruption of the membranes by sonication, antigen can be measured by a simple, one-step inhibition test, enabled us to apply the hemagglutination inhibition assay to this system. For the assay, serial dilutions of a test fraction are mixed with antiserum at a constant dilution and then erythrocytes are added. The lowest dilution of a fraction which prevents agglutination defines its antigenic activity. To control for non-specific inhibitors, each fraction is checked for inhibition of hemagglutination in an H-2 system which does not cross react with the antigen.

Table 1 shows that the antigen weight and units of antibody in the final inhibited tube are roughly constant. (A unit is that amount of antiserum which is present in the last positive tube of a serum titration.) At present, with our most active fractions, 0.1—1 μg. of protein inhibits 10—20 hemagglutinin units.

Because of the difficulties inherent in the mouse isohemagglutination system we were surprised to find so little non-specific inhibition by the various types of cell extracts. Provided erythrocytes are prepared the same day and the test tubes are well washed and rinsed, the only fractions which consistently give even two or three tubes of non-specific inhibition are the ones containing large amounts of free lipid.

If the membranes are not adequately disrupted, and relatively large pieces remain, instead of inhibition of hemagglutination, the early tubes in the antigen

Table 1. Inhibition of hemagglutination by sonicated membranes.
Serum: C3H.SW anti C3H/Sn (H-2^b anti H-2^k). Erythrocytes: DBA/2J (H-2^d). Membranes: DBA/2J (H-2^d), from liver.

Serum dilution	μg. of membranes per tube								
	64	32	16	8	4	2	1	0.5	None
1/50	+	+	+	+	+	+	+	+	+
1/100	±	±	±	+	+	+	+	+	+
1/200	±	—	—	—	+	+	+	+	+
1/400	±	—	—	—	—	±	+	+	+
1/800	±	—	—	—	—	—	+	+	+
1/1600	±	—	—	—	—	±	+	+	+

Table 2. Immunogenicity of membrane fraction.
C3H/Sn recipients were injected i.p. with membranes prepared from $B6AF_1/J$ livers

	Membranes injected (mg.)		Reciprocal of hemagglutinin titer
	Day 0	Day 21	Day 35
Group I	2	3	< 4, < 4, 4, 256, 512, 1024, 102 > 2048, > 2048
Group II	0.2	0.2	< 4, < 4, < 4, < 4, < 4

dilution series show clear mixed-agglutination of red cells and membra fragments.

A large variety of treatments with detergents and organic solvents, sor under conditions where protein denaturation is minimal, completely destr activity. For example, extraction of lyophilized antigen with dry methan ethanol, or n-hexane at —72° C or mixture of fresh antigen in saline with equal volume of n-butanol at —10° C and immediate lyophilization remov all trace of hemagglutination inhibiting activity. This suggests that the lip is intimately concerned with the maintenance of antigenic activity, or is fact, a part of the antigenic determinant.

These H-2 antigen preparations have been shown, where tested, to eli the formation of H-2 isohemagglutinins upon injection into appropriate mou strains. In some cases two injections and in others one injection is sufficient demonstrate this immunogenic activity.

In a typical experiment (12), two injections, spaced three weeks apa of two to three mg. of membranes from $B6AF_1/J$ ($H\text{-}2^a/H\text{-}2^b$) elicited average titer in six out of nine C3H/Sn($H\text{-}2^k$) animals of greater than 1/1(against $H\text{-}2^a$ erythrocytes (see table 2). The positive sera, when tested agai $H\text{-}2^b$ erythrocytes, were uniformly negative, indicating that probably response was directed against the strong antigenic components of $H\text{-}2^a$.

Detailed speculations on the chemical nature of the H-2 antigenic det minants would be premature. However, the association of these antigens w the cell membranes of a variety of cell types and the importance of the li components for detectable activity may yet yield information on their chem make-up.

References

1. Dresser, D. W., Immunology, *4* : 13, 1961.
2. Mitchison, N. A., personal communication, 1961.
3. Billingham, R. E. and Silvers, W. K., J. Immunol., *85* : 14, 1960.

4. Berrian, J. H. and McKhann, C. F., Ann. N.Y. Acad. Sci., *87* : 106, 1960.
5. Russel, E. S., *in* "Methodology in Mammalian Genetics", in press.
6. Linder, O., J. Nat. Cancer Inst., *27* : 351, 1961.
7. Barnes, A. D. and Krohn, P. L., Proc. Roy. Soc., B, *146* : 505, 1957.
8. Snell, G. D., J. Nat. Cancer Inst., *21* : 843, 1958.
9. Brent, L., Medawar, P. B. and Ruszkiewicz, M., Brit. J. Exp. Pathol., *42* : 464, 1961.
10. Davies, D. A. L. and Ann M. Hutchison, in press.
11. Herzenberg, L. A. and Herzenberg, L. A., Proc. Nat. Acad. Sci., *47* : 762, 1961.
12. Ozer, H. L. and Herzenberg, L. A., submitted for publication.