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A Genetic and Immunologic Approach to the Purification of an Histocompatibility Antigen*

LEONARD A. HERZENBERG
Department of Genetics
Stanford University School of Medicine
Palo Alto, California

The H-2 antigen is the only one of the mouse histocompatibility antigens that can readily be detected on red blood cells by hemagglutination with isoantisera. As Gorer has shown, injection (or grafting) of tissues between strains differing at the H-2 locus results in the production, by the recipient, of hemagglutinins for the donor strain erythrocytes (1). With the aid of a developing agent, such as human serum and dextran (which Gorer used) or polyvinylpyrrolidone (Stimpfling method) (2), reproducible titers in the order of 1/5000 or more are routinely observed with hyperimmune serum.

The presence of an H-2 antigen on cells other than erythrocytes, or in purified tissue fractions, can be demonstrated by incubating these materials with appropriate isoantiserum and observing the decrease in hemagglutinin titer when the absorbed serum is then tested with the corresponding red cells (3, 4, 5).

The application of the absorption method is shown in Table I. The decrease in hemagglutinin titer after ab-

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TABLE I

Absorption of Hemagglutinins[a] by Membrane Fraction

M_1 (Membrane Fraction) Mg Protein	Antiserum Dilution (After Absorption)							
	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
4.0	1	1	±	-	-	-	-	-
3.0	1	1	1	-	-	-	-	-
2.0	2	3	2	±	-	-	-	-
1.0	2	4	2	2	1	-	-	-
0.5	3	3	2	2	1	-	-	-
0.25	2	4	4	4	3	2	1	-
Unabs. Antiserum	4	3	3	2	3	3	2	1
Normal Serum	-	-	-	-	-			

[a]Antiserum used: H-2^b anti H-2^d; 0.2 ml of serum was absorbed at 1/20 dilution for 30 minutes at 8° C.

sorption of an antiserum with an extract of mouse liver cells is roughly proportional to the amount of absorbing material. Absorption is carried on for 30 minutes at 8° C, and the titering of the absorbed sera requires another 30 minutes incubation, this time at room temperature. However, it is essential that a control of the specificity of absorption be used along with this test.

Fortunately, the extensive knowledge of the cross reactions of the various H-2 antigens makes this control easy to devise. Table II shows a recent summary by Snell and Stimpfling of the immunogenetics of the H-2 locus, garnered from their own work (2, 6) as well as that of others, including Amos (7), Gorer (8) and Dr. Hoecker (9) who is here today. On the left is a list of the known alleles, that is, alternate forms of the gene, H-2. In the body of the table are listed the serologically distinguishable antigenic components for each allele. (These components are really the conceptual units which indicate that sera have been found

TABLE II

Antigen Components of the H-2 Locus (after Snell and Stimpfling) (29)

H2 Allele	Antigenic Components	Typed Strains
H-2^a	A C D E F - H - J K M N - - - - Y Z	A AKR.K
H-2^b	- - D^b E F - - - - K^b - N - - - V - Z	A.BY C57BL/10 B10.LP B10.BY C57BL/6 C57L C3H.SW LP D1.LP 129
H-2^c	C D E - - - -	D1.C
H-2^d	- C D E^d F - H - J - M N - - - - - Z	BALB/c C57BL/6Ks B10.D2 DBA/2 ST.T6 WH
H-2^d	C D E^d F - M	YBL Rr YBR/Wi
H-2^e	C - E F - - - Y Z	STOLI
H-2^f	- - E?F?G H I - - - - - Z	A.CA B10.M
H-2^g	- - D^b E^d F - - - - N - V	From F_1 (H-2^d/H-2^b)
H-2^h	A C D^b E F - H - K - N? - -	From F_1 (H-2^a/H-2^b)
H-2^i	C D E F - K^b M N? - V	From F_1 (H-2^a/H-2^b)
H-2^j	- - - - F - - - - - - - V	JK/St
H-2^k or H-2^k	A C D^k E - - H - - K - - - - - Y Z	C3H C3H.K AKR AKR.ALB C57BR/a C57BR/cd C58 CH1 CBA D1.ST MA MA/My RF ST CE
H-2^l	- - - - F - J - - - V	I/St
H-2^m	C - E - - H K M -	AKR.M
H-2^{no}	A - - E F H J - - N -	F/ST
H-2^p	A C - E - - - - P - -	BDP? P C3H.NB
H-2^q	- C - E F - - - - - M - - Q - -	DBA/1 C/St BUB B10.Y
H-2^r	C?- E - - H - K - - Y -	RIII/Wy LP.RIII
H-2^s	C - E F G - - - - - - S - Z	A.SW

TABLE III

Relevant H-2 Components and Expectation of Antibody Specificities (6).

Serum	Strain	H-2 allele	Relevant Antigenic Components	Erythrocytes Agglutinated
	C57B1/6	H-2^b	- D^b E F- - - N Z	
	DBA/2	H-2^d	C D E^d F H J M N Z	
H-2^b anti H-2^d			*anti* C D E^d - H J M - -	H-2^d, H-2^k
	C3H	H-2^k	C - - - H - - - -	
H-2^b anti H-2^d absorbed with H-2^k			*anti* - D E^d - - J M - -	H-2^d

which can detect as many differences as there are components assigned.) The inbred strains which carry each allele are presented on the right. I would like to emphasize that this chart represents a detailed analysis of just the H-2 locus, and that other histocompatible loci, of which there are probably upwards of 14 in the mouse (10), may be equally complex.

In Table III, I have abstracted a portion of the last table to indicate one specificity test which we use. Serum prepared in a strain carrying the H-2^b allele against tissues of an H-2^d strain agglutinates H-2^d erythrocytes and also H-2^k erythrocytes, due to the presence of components C and H in the H-2^k allele. Absorption of this serum with materials containing H-2^k antigenic activity removes agglutinating activity (that is for components C and H) against red cells of the absorbing type, H-2^k, but leaves hemagglutinins for H-2^d red cells. Thus, in a single tube the test and control absorptions are performed.

Figure 1 shows our procedure for obtaining a 50-100-fold purification of H-2 antigen from mouse liver, using hemagglutinin absorption to evaluate the alternative procedures tried (5).

In fractionation, we find the bulk of the H-2 antigen of mouse liver is associated with the cell membrane fraction,

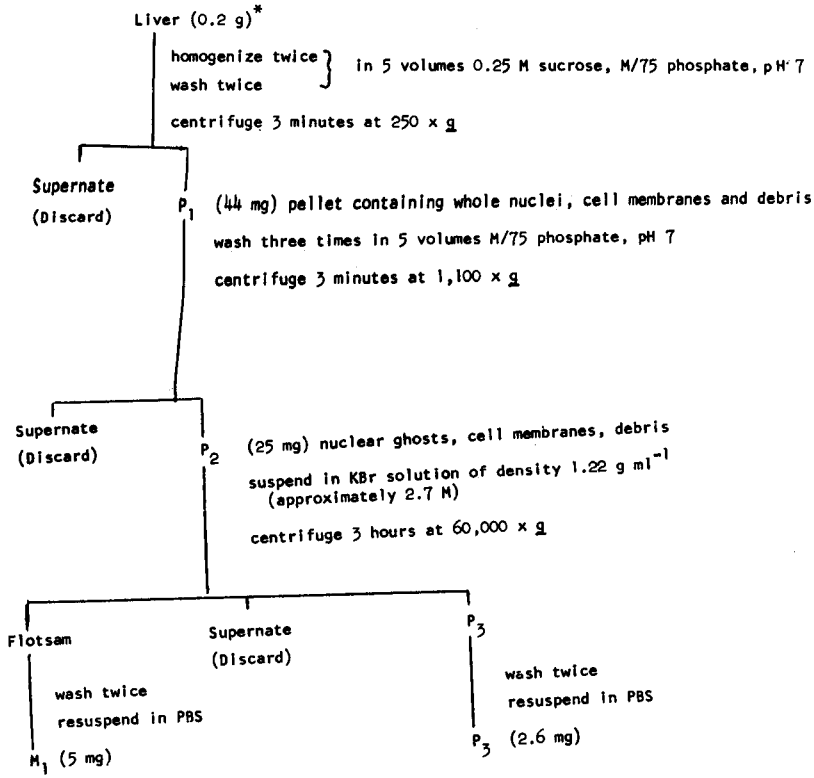

Fig. 1. Isolation flow sheet. The figures in parentheses give protein content expressed per g fresh weight of liver.

which appears to be mainly lipoprotein. Analysis of the liver membrane fraction shows it to contain about 60% protein, 40% lipid, 0.7% total carbohydrates, and less than 0.2% hexosamine. We have obtained a similar fraction from mouse spleen, ascites tumor (L-1210) and *in vitro* cultured cells (ML-388) and in all cases find H-2 activity concentrated there.

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 by absorption. Heavily clumped materials don't absorb well, whereas finely divided and soluble substances 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 develop a hemagglutination inhibition assay for 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 nonspecific inhibitors each fraction is checked for inhibition of hemagglutination in an H-2 system which does not cross-react with the antigen. Table IV 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, one μg of protein inhibits 10-20 hemagglutinin units.

TABLE IV

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	±	−	−	−	−	±	+	+	+

The purification and characterization of homograft antigens have been proceeding in several laboratories since the definitive dispelling by Billingham, Brent and Medawar (11) in 1958 of the then current, neovitalist idea that these antigens exist only in living cells. These workers employed the straightforward procedure of injecting tissue homogenates, or separated fractions therefrom, from one mouse strain into a second strain and subsequently grafting skin of the donor strain to the pretreated animals. The presence of a transplant antigen in the injected material was evidenced by an accelerated graft rejection compared to similar grafts placed on control individuals.

The directness of this assay for histocompatibility antigens is unfortunately compensated for by its lack of specificity and difficulty in its quantification. The chief difficulty with the transplantation test is that one antigen cannot be distinguished from another with the usual laboratory strains. This becomes evident from the fact that there are at least 15 histocompatibility loci in the mouse, many of which will be detected in a transplantation test. For purification purposes, it is clear that a test specific for a single genetically, and therefore chemically, distinct transplant antigen must be used. A direct test for transplant antigens by induction of a second set (accelerated) homograft rejection is specific when mice are used from pairs of isogenic resistant lines such as Snell's (6) which differ from each other only by the allele carried at one histocompatibility locus.

Skin grafting does remain, however, a laborious and time-consuming procedure which, even with large numbers of test animals, is at best a semi-quantitative assay for antigen. For this reason, other direct or grafting assays have been devised which are more quantitative and appear to be extremely sensitive (12).

Another method of measuring the immunogenic potency of H-2 preparations which we have tried to use depends on the elimination of labeled erythrocytes from the circulation of an animal immunized against the H-2 type of the erythrocytes. However, as Figure 2 shows, even in a model situation, using passive immunization with a hyperimmune serum, immune elimination of Cr^{51}-labeled red cells is only partial. Furthermore, there is considerable variation in the extent and kinetics of elimination in different animals. In all our experience, whether with passively or actively im-

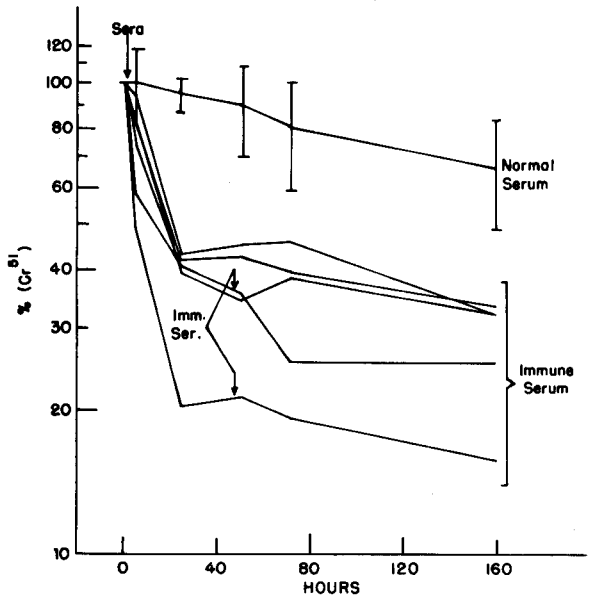

Fig. 2. Immunological Clearance of Labeled Erythrocytes. Cr^{51}-labeled B10·D2 H-2^d erythrocytes were injected (i.p.) into B10 H-2^b mice 24 hours before the start of the experiment. At time 0, 0.2 ml of normal mouse serum or H-2^b anti H-2^d hyperimmune erum diluted with saline to 1 ml was injected into each of five animals in each group. The per cent of radioactivity remaining was obtained from 10 μl samples of blood obtained from the tip of the tail. At 48 hours, 0.2 ml of the same hyperimmune serum was again given to two animals as indicated by the arrows.

munized animals, with newly matured erythrocytes or with different labeling techniques, we have always observed a fraction of from 20%-50% of the red cells resistant to immune elimination (13).

There appears to be a direct parallel here with the observation of others on immune cytotoxicity *in vitro* with lymphomas or normal lymphoid cells (14). I do not know of any cytotoxicity system where isoimmune sera and complement kill 100% of the cells. As a good example, consider some recent work carried on by Dr. Howard Cann in our laboratory. We were interested in using the H-2 antigens as cellular genetic markers on cells cultured *in vitro* for carrying out studies on somatic cell genetics. We showed by hemagglutinin absorption that a lymphoma, ML 388 (15), cultured *in vitro* since 1956, retains the H-2^d antigens of the DBA/2 strain from which it was derived. In order to determine whether a small number of non-H-2^d cells existed in the population tested, we exposed the culture to an anti-H-2^d isoantiserum and guinea pig complement. When specific killing was completed, we plated the survivors in growth medium. Contrary to what would be expected if the survivors were genetically non-H-2^d, their progeny were neither more nor less sensitive to anti-H-2^d serum than the population from which the survivors were selected. As a matter of fact, 6 or 7 such cyclings did not change the sensitivity of the population at all.*

If partial resistance to isoimmune destruction seems to be a general phenomenon in the mouse cell, one can't help but be curious as to why. Is the antigen masked, not expressed phenotypically, not reacting with antibody in such a way as to bring about cell death? With the data available, there is little to choose between one hypothesis and another.

*After 14 cyclings, resistance to the cytotoxicity of this antiserum did develop.

I would like to return now to studies of the immunogenicity of purified H-2 preparations. Enhancement of the growth of tumors in mice has been used as a very sensitive test for a strain specific antigen which may well be the H-2 (16, 17). We have used, as evidence of H-2 antigen in a preparation, the development of specific H-2 hemagglutinins upon homologous injection. The data in Table V (18) show that a membrane fraction containing H-2^a antigen elicits the formation of antibodies which agglutinate H-2^a (A/J) erythrocytes.

TABLE V

Immunogenicity of Memrane Fraction. C3H/Sn Recipients Were Injected IP with Membranes Prepared from $B6AF_1/J$ Livers.

	Membranes Injected (mg)		Reciprocal of Hemagglutinin Titer with A/J Erythrocytes
	Day 0	Day 21	Day 35
Group I	2	3	<4, <4, 4, 256, 512 1024, 1024, >2048, >2048
Group II	0.2	0.2	<4, <4, <4, <4, <4

As yet, we have not tested the transplant-antigen activity of the concentrated H-2 materials by the accelerated homograft reaction. We hope to do so soon using an H-2 isogenic-resistant pair.

One of the major hopes for medicine in general, and in the treatment of radiation injury in particular, is the development of means to insure the long-term survival of grafts of normal tissues between genetically non-identical individuals. In recent years, as we all know, Owen, Medawar, Burnet and others have shown that, in spite of the basic law of transplantation that a graft will be rejected if it contains one or more antigens not represented in the tissues of the recipient, under certain restricted conditions

grafts between genetically non-identical individuals will be accepted.

Owen first recognized that natural grafts of hematopoetic tissues occur between cattle twins *in utero* (19). Two types of red blood cells were found to be circulating in adult cattle twins, one type determined by the genotype of one twin, and the other by the genotype of the second twin. The presence of both erythrocyte types in these adults with no apparent immune response against either was correctly interpreted by Owen as meaning that a genetically different graft is accepted by a fetus although it would be rejected by the matured individual.

The elaboration of the self-marker theory by Burnet and Fenner (20) gave a working hypothesis to explain permanent acceptance of foreign tissue grafted to an immature individual. They proposed that the developing immunological system of an animal acquires, as it matures, the essential information as to which antigens are "self" and against which it should not react later in life. Any antigen present at the critical time in development when this acquisition of information occurs is then considered to be "self". The hematopoetic grafts studied by Owen thus were accepted as "self" by the recipient individuals and therefore persisted without encountering a homograft reaction.

Medawar brilliantly predicted and performed the experiment to prove that Owen's chimeric cattle twins would accept skin grafts from their fetal partners (21, 22). He then went on to show in other species that immunological tolerance could be experimentally produced by the injection of lymphoid tissue into fetuses *in utero* or into newborns (23). These animals at maturity accept skin grafts which have the same genetic constitution as the donor of the previously injected lymphoid cells.

It was soon found that immunological tolerance could be produced in adults (24). Animals which have been subjected to a lethal dose of irradiation and saved by hematopoetic grafts subsequently accept skin transplants of the same genetic constitution as the hematopoetic graft.

Immune tolerance, or as it is now more appropriately called, immunological unresponsiveness, can be produced for non-living antigens as well. Perhaps the most exciting example of this is the recent finding of Mitchison that soluble bovine serum albumin injected into adult mice does not elicit an immune response, but rather makes these mice immunologically unresponsive to a subsequent injection of the same antigen in a form which does immunize previously untreated mice (25).

The truly significant finding in this work is that unresponsiveness is produced by merely introducing the antigen in a non-immunizing form. In Mitchison's experiment, as well as in all the other examples of immunological tolerance, the antigen is always present in the unresponsive animal.Once that antigen disappears from the body, the state of immunological responsiveness is simultaneously lost (26).

The homograft problem, then, can be stated in a manner which suggests its own solution: how to introduce, prior to grafting, the histocompatibility antigens in such fashion as not to immunize the recipient, but rather to render him unresponsive to those antigens and, hence, tolerant to the graft.

We have made some progress in purification and concentration of H-2 antigen, and with this material it may be possible to induce graft tolerance in H-2 IR strains. The general solution of the homograft problem may depend on purification, or at least the concentration, of several other antigens as well.

Fortunately, it may not be necessary to achieve a prior state of unresponsiveness to *all* histocompatibility antigens which differ between graft and host. Recent work of Linder (27) in Klein's laboratory at the Karolinska Institute and of Elizabeth Russell (28) at Bar Harbor, shows that in mice it is possible to successfully homograft ovaries and hematopoetic tissues when some weak histocompatibility gene differences exist, providing there are no strong ones, like H-2. Future work will have to determine which other, if any, histocompatibility antigens will have to be purified to allow interstrain grafting.

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