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LEUKOCYTE AGGLUTINATION IN MICE

DETECTION OF H-2 AND NON-H-2 ISOANTIGENS[1]

ROBERT I. MISHELL,[2] LEONARD A. HERZENBERG AND LEONORE A. HERZENBERG

From the Departments of Medicine and Genetics, Stanford University School of Medicine, Palo Alto, California

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Isoantibodies which cause specific agglutination of leukocytes have been found, following immunization, in both humans and mice (1). The leukocyte agglutination test can detect a variety of human isoantigens, distinct from those responsible for red cell agglutination. Isoantibodies which agglutinate human leukocytes appear after sensitization by pregnancy (2) or transfusion (3).

The recent report that human leukocyte isoantibodies, demonstrable by the antiglobulin consumption test, occur after the rejection of a skin homograft suggests that antigens responsible for the homograft reaction can be detected with the leukocyte agglutination reaction (4).

Much of the work on the homograft reaction has been done using the controlled genetic systems available in mice. It is estimated that there are at least 15 separate genetic loci determining histocompatibility antigens in mice (5, 6), the best characterized of which is the H-2 locus. This locus determines a strong histocompatibility antigenic difference between mouse strains, and its expression is detectable by a hemagglutination reaction. Mice differing at the H-2 locus develop hemagglutinins when immunized with living or killed cells, cell fractions (7), skin or tumor homografts and when sensitized by pregnancy isoimmunization (8).

Amos (1) in 1953, described the detection of isoantibodies in mice by leukocyte agglutination as well as by hemagglutination. It is not certain from this work whether the leukocyte agglutination observed was due to H-2 antigens, non-H-2 antigens or both. Since then, isogenic resistant lines of mice, pairs of which are genetically identical except for the chromosome segment carrying the H-2 locus, have been developed and made generally available (9). This paper describes work using the isogenic-resistant lines which shows that both H-2 and non-H-2 isoantigens are separately detectable with the mouse leukocyte agglutination reaction.

MATERIALS AND METHODS

Mouse Strains. The inbred mouse strains listed in Table I were used.

Sera. Hyperimmune sera were prepared by repeated injections of spleen and thymus cell suspensions as previously described (7).

Pregnancy serum pools were made by combining serum of C57BL/6J females breeding with DBA/2J males. The sera all had strong hemagglutinin titers as defined by Herzenberg and Gonzales (8).

Sera from mice receiving skin homografts were collected 8 days following the operation.

Solutions. Gelatin 4%, (Knox brand) in isotonic saline was employed. Polyvinylpyrrolidine in phosphate buffered saline (PVP-PBS) as previously described (7) was used, diluted to final concentrations of 1 and 0.5% polyvinylpyrrolidone (type NP-K60, Antara Chemicals, General Aniline Company).

Hemagglutination. Hemagglutination was done using PVP by the method of Stimpfling (10) except that the incubation time was 60 min and the pH 6.5.

In vivo absorption. Immune serum, 0.3 ml, was injected intraperitoneally (i.p.) in the absorbing mouse. Four to 18 hr later the mouse was exsanguinated and serum collected.

Leukocytes. Leukocytes were obtained by injecting mice i.p. with 1 ml of 4% gelatin. Eighteen

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[2] Postdoctoral Fellow, Arthritis and Rheumatism Foundation, 1961 to 1962, Department of Medicine, Stanford University School of Medicine, Palo Alto, California.

TABLE I

Mouse strains used in experiments

Strain	Source	H-2 Allele
C3H/SnHz	Colony[a]	k \ coisogenic
C3H.SW/SnHz	Colony	b / pair
C57BL/10SnHz	Colony	b \ coisogenic
B10.D2/SnHz	Colony	d / pair
C57BL/Ka	Kallman[b]	b
C57BL/6J	Simonsen[c]	b
DBA/2J	Simonsen	d
A/J	Jackson Mem. Lab	a
129/CrglGa	Gates[d]	b
CBA/J	Jackson Mem. Lab	k
BALB/c CrglGa	Gates	d

[a] Bred at Stanford from animals supplied four or fewer generations ago by G. D. Snell of Jackson Memorial Laboratories.

[b] Kindly supplied by R. Kallman, Department of Radiology, Stanford Medical School.

[c] Simonsen Farms, Gilroy, California.

[d] Kindly supplied by A. Gates, Department of Obstetrics and Gynecology, Stanford Medical School.

hours later, the cells were harvested by sacrificing the animals and washing the peritoneal cavity with 0.5% PVP-PBS. The leukocyte suspension was diluted to a final concentration of 700 to 1000 cu mm. Only preparations free of erythrocytes and made immediately before testing were used.

Skin homograft. Full thickness skin grafts were exchanged for the production of certain antisera using essentially the method of Billingham and Medawar (11).

Leukocyte agglutination. Test sera were serially diluted with doubling dilutions in 0.05 ml of 1% PVP-PBS in 10- by 75-mm Pyrex tubes. Diluted leukocyte suspension, 0.05 ml, was added to each tube and the mixture incubated for 90 min at room temperature. The tubes were flicked with the fingers to dislodge adherent cells and part of each suspension was transferred to an alcohol-cleaned glass slide with a Pasteur pipette. The suspension was allowed to settle for 2 min and then read microscopically at low power (10 × 8). In later experiments, cell suspensions were transferred, after flicking, by inverting the tube and pouring the contents on the slides.

Because individual cell preparations were sometimes nonspecifically clumped at higher serum concentrations, in all readings the appropriate normal serum control was read on the same slide as the test.

Results were scored on a 0 to 4 scale, based partly on the size and tightness of aggregation and partly on the number of loose cells not aggregated.

Interpretation of leukocyte agglutination. The leukocyte agglutination reaction is subject to a number of variables, some of which are not easily controlled. High concentrations of normal serum cause nonspecific clumping and because of this a normal control must be directly compared with each reading. Nonspecific clumping may occur about debris present either in the serum or in the peritoneal wash; serum can be filtered to remove debris, although usually it does not present a problem at the dilutions used; peritoneal washes that contain large amounts of debris are discarded and fresh preparations made. Detergent present in minute amounts inhibits the reaction. Various cell preparations differ in apparent stickiness; thus comparisons of titer using different cell preparations are difficult and of questionable significance. In addition to differences within a strain, the various strains appear to differ in stickiness, again making titer comparisons difficult. After strong reagent sera were developed, positive controls as well as negative controls were always done in order to make comparisons as meaningful as possible.

RESULTS AND DISCUSSION

Leukocyte agglutination with a hyperimmune isoantiserum. The data in Table II show that B10.D2 anti-C3H hyperimmune serum agglutinates C3H leukocytes to a serum dilution of 1:1000; this serum does not agglutinate B10.D2 leukocytes. Since there is a wide genetic disparity between B10.D2 and C3H, including a strong difference at the H-2 locus, the agglutination observed in this system can be due to H-2 isoantibodies, non-H-2 isoantibodies, or both.

Leukocyte agglutination by H-2 antibodies. To determine whether a serum containing antibodies directed only at H-2 antigens can cause agglutination, we made use of the isogenic resistant (IR) pairs of strains developed by Snell (8). The IR pairs used for this work differ with respect to the H-2 antigen which each carries; otherwise their genetic background is assumed

TABLE II

Leukocyte agglutination with a hyperimmune isoantiserum

Serum	Leukocyte Donor	Serum Dilution										
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1000	1:2000	1:4000
B10.D2 anti-C3H/Sn	C3H/Sn (H-2^k)	1[a]	3	3	2	3	3	2	2	1	±	0
B10.D2 anti-C3H/Sn	B10.D2 (H-2^d)	1	±	0	0	0	0	0	0	[b]	[b]	[b]
Normal B10.D2	C3H/Sn	±	±	0	0	0	0	0	0	0	0	0
Normal B10.D2	B10.D2	±	0	0	0	0	0	0	0	[b]	[b]	[b]

[a] Strength of agglutination from 0 (negative) to 4; see Methods.
[b] Not read.

TABLE III

Leukocyte and erythrocyte agglutination by H-2 antibody

Serum	Cell Donor	Reciprocal of Agglutination Titer	
		Erythrocytes	Leukocytes
C3H anti-C3H.SW	C3H (H-2^k)	Neg.[c]	Neg.
	C3H.SW (H-2^b)	5000	4000
	C57BL/10 (H-2^b)	5000	2000
Normal C3H	C3H	Neg.	Neg.
	C3H.SW	Neg.	Neg.
	C57BL/10	Neg.	Neg.

[a] Negative is less than 10.

TABLE IV

Leukocyte agglutination by non-H-2 antibodies[a]

Recipient Strain	Immunizing Strain	Reciprocal of Leukocyte Agglutination Titer	
		Recipient leukocytes	Donor leukocytes
B10.D2 (H-2^d)	DBA/2 (H-2^d)	Neg.[b]	>2,500
C3H.SW (H-2^b)	C57BL/10Sn (H-2^b)	Neg.	1,280
D57Bl/10Sn	C3H.SW	Neg.	640
BALB/c (H-2^d)	DBA(H-2^d)	Neg.	>320
BALB/c (H-2^d)	B10.D2 (H-2^d)	Neg.	320
B10.D2 (H-2^d)	BALB/c (H-2^d)	Neg.	320
C3H (H-2^k)	CBA (H-2^k)	Neg.	20–40?

[a] See text footnote 3.
[b] Negative is less than 10.

to be close to identical. By immunizing one member of an IR pair with tissues from the other, antisera presumably containing only H-2 antibodies are prepared.

Using the pair C3H (H-2^k) and C3H.SW (H-2^b) we prepared a hyperimmune serum containing H-2^k anti-H-2b antibodies. As seen in Table III, leukocytes from C3H.SW and C57BL/10 (also H-2^b) are agglutinated by this serum while control C3H leukocytes are not, demonstrating that anti-H-2 antibodies can cause leukoagglutination.

Leukocyte agglutination by non-H-2 isoantibodies. Where the H-2 allele of two strains is the same, immunization of one with the other can produce immunity only to antigen(s) other than the H-2. Presumably then, agglutination of leukocytes of the donor strain by serum from the immunized strain would be due to isoantibodies directed at non-H-2 antigens. Thus agglutination of C57BL/10 (H-2^b) leukocytes by a C3H.SW (H-2^b)-anti-C57BL/10 antiserum would be due to non-H-2 antigen(s) present in the C57BL/10 line and absent from the C3H.SW line.[3]

We prepared seven such hyperimmune antisera, and tested them as shown in Table IV. With six, strong leukocyte agglutinations were observed. (The variations in titer indicated probably do not reflect the nature of the antigenic difference(s). See Methods.) With the

[3] The validity of this argument rests on the assumption that the H-2b antigens present in C57BL/10 and C3H.SW are identical. This assumption is probably correct but definitive genetic proof is lacking. This problem is presented and discussed by Snell (8).

TABLE V

Cross-reaction of non-H-2 leukocyte agglutinins[a]

Recipient Strain	Immunizing Strain	DBA/2	C3H H-2^k	C3H.SW H-2^b	CBA	C57BL/10 H-2^b H-2^d	BALB/c	129	A
B10.D2	DBA/2	+[b]	+	+	+	−[c]	+	+	+
C3H.SW	C57BL/10Sn	+	−	−	−	+	+	+	+
C57BL/10	C3H.SW	+	+	+	+	−	+	[d]	+
BALB/c	B10.D2	+	+	[d]	[d]	+	−	+	[d]
BALB/c	DBA/2	+	+	[d]	[d]	+	−	[d]	[d]

[a] Because of variations with different preparations of leukocytes, comparison of titer is not justified (see Methods).

[b] Indicates titer of 1:160 or more.

[c] <1:10 (see Methods).

[d] Not tested.

seventh, C3H-anti-CBA, we were unable, even after repeated trials, to demonstrate that the serum was definitely weakly positive, or, conversely, that it was clearly negative.

Cross-reacting non-H-2 isoantibodies. Each of the antisera described in the previous paragraph was tested with leukocytes of several mouse strains as shown in Table V. Since these antisera presumably contain no H-2 isoantibodies, the agglutinations observed should be due to non-H-2 isoantigens which the immunizing strain and leukocyte donor strain share, but which the recipient strain lacks.

All of our clearly positive sera cross-react to cause agglutination of leukocytes from many or all of the strains in our panel. There was no agglutination of the control leukocytes from the serum donor, nor, as expected, was there agglutination of leukocytes from a strain forming an IR pair with the serum donor (antibody-producing) strain; such strains differ only at the H-2 locus. Thus, C3H.SW-anti-C57BL/10 serum, in addition to agglutinating C57BL/10 leukocytes, agglutinates cells from DBA/2, BALB/c and 129, but does not agglutinate leukocytes from C3H. One serum, C3H.SW-anti-C57BL, failed to agglutinate CBA cells, suggesting again that CBA and C3H are similar with respect to the leukocyte antigens, within the sensitivity of the assay.[4]

Selective in vivo absorption. We used the technique of *in vivo* absorption to see if the non-H-2 leukocyte antibodies could be selectively absorbed. A C3H-anti-C57BL/10 antiserum was absorbed separately in C3H, C57BL/10, DBA/2, BALB/c and 129 mice. The absorbed sera were then tested with leukocytes of each of the strains as shown in Table VI. Absorption in C3H mice results only in a drop in titer, due to dilution. Absorption in C57BL/10 mice removes all agglutinins. Absorption in BALB/c mice removes anti-BALB/c agglutinins, but the absorbed serum still agglutinates C57BL/10, 129 and DBA/2 leukocytes. Absorption in 129 mice removes 129 and DBA/2 agglutinins; the serum still agglutinates BALB/c and C57BL/10 leukocytes. Absorption in DBA/2 mice removes all anti-DBA/2 leukocyte agglutinins, and

TABLE VI

Selective in vivo absorption of C3H.SW-anti-C57 BL/10 antiserum in several inbred strains

Absorption Strain	Reciprocal of Leukocyte Agglutination Titer with Leukocytes from Indicated Strain[a]				
	C3H	C57/BL	BALB/c	DBA/2	129
Not absorbed	−	>320	>320	>320	>320
C3H	−	40, 80	40, 80	40, 160	80, 80
C57BL/10	−	Neg.[b]	Neg.	Neg.	Neg.
BALB/c	−	40, 80	Neg.	40, 80	80, 160
DBA/2	−	40, 80	80, 80	Neg.	±40[c]
129	−	20, 40	40, 80	±20[c]	Neg.

[a] Several determinations done for each example; range of titer presented.

[b] Negative is less than 10.

[c] Signifies a weak (+1) reaction in which there is doubt if the difference from negative controls is significant (see Methods).

[4] We have examined the homograft reaction between the C3H and CBA lines and have found a rapid (11 to 14 days) primary rejection time (12).

TABLE VII

Leukocyte agglutinins in pregnancy-sensitized mice

Serum	H-2 Antibody Activity	Non-H-2 Antibody Activity	Donor Cells	H-2 Antigen	Non-H-2 Antigen(s)	Reciprocal of Agglutination Titer	
						Erythrocytes	Leukocytes
Pool A[a]	H-2[b]-anti-H-2*d*	C57 Black-anti-DBA/2	B10.D2 DBA/2	H-2*d* H-2*d*	C57 Black DBA/2	160 160	128 128
Pool A absorbed in B10.D2[b]	None if absorption complete	C57 Black anti-DBA/2	B10.D2 DBA/2		 DBA/2	Neg.[c] [d]	Neg. 128
Pool A absorbed in DBA/2	None if absorption complete	None if absorption complete	B10.D2 DBA/2			Neg. [d]	Neg. Neg.

[a] Pool A is pooled serum of C57BL/6 outcrossed to DBA/2 which had relatively high hemagglutination titers.

[b] *In vivo* absorption.

[c] Negative is less than 10.

[d] Not tested, insufficient serum, presumably negative.

TABLE VIII

Non-H-2 isoantibodies induced by skin homograft sensitization: B10.D2 (H-2[d]) mice grafted with DBA/2 (H-2[d]) skin

No. of Skin Grafts	Donor Leukocytes	Reciprocal of Agglutination Titer					
		10	20	40	80	160	320
0	DBA/2	±[a]	0	0	0	0	0
2	DBA/2	2	2	±	1	±	±
3	DBA/2	3	2	2	2	1	2
3	B10.D2	±	0	0	0	0	0

[a] Readings on a 0 to 4 scale, see Methods.

most or all of the 129 agglutinins but leaves intact the antibodies which react with BALB/c and C57BL/10 leukocytes.

These results suggest that C57BL/10 strain shares some antigen(s) with strains 129 and DBA/2 which are not present in the BALB/c strain and shares other(s) with the BALB/c strain that are not present in 129 or DBA/2 mice.

Mode of sensitization. Most sera which we have tested have been prepared by repeated injections of spleen and thymus cell suspensions, however, we have, in addition, prepared active sera from animals sensitized by pregnancy or skin grafting.

Pregnancy: A serum pool was made of sera from C57BL/6 female breeders that had been outcrossed with DBA/2 males for F_1 hybrid production. The sera had previously been tested for hemagglutinins and were strongly positive (7). The serum pool was separately absorbed by the *in vivo* technique in B10.D2 (to remove only anti-H-2d antibodies) and in DBA/2 (to remove all antibodies). The unabsorbed and both of the absorbed sera were then tested with DBA/2 leukocytes and B10.D2 leukocytes and erythrocytes as shown in Table VII. The unabsorbed sera agglutinated DBA/2 and B10.D2 leukocytes and B10.D2 erythrocytes, all of which can be ascribed to anti-H-2d antibodies. After absorption in B10.D2, the serum agglutinated only the DBA/2 leukocytes, indicating absorption of the anti-H-2d antibodies and the continued presence of non-H-2 antibodies. Absorption in DBA/2 removed all agglutinins.

Skin grafting: Gorer (13) reported finding leukocyte agglutinins following sensitization with a skin homograft between mice differing at the H-2 locus. We have examined sera produced in B10.D2 mice grafted with DBA/2 skin twice and three times. The grafts were made 3 or more weeks apart and the serum collected 8 days after grafting. As shown in Table VIII, a marginal reaction was observed

after rejection of the second graft and a modreately strong reaction was observed after three grafts. These experiments were done with only two mice in each group and the results are considered as preliminary findings.

DISCUSSION

Amos (1) showed that the leukocyte agglutination reaction was capable of detecting some isoantigens which were not demonstrable by the hemagglutination technique. The data presented above amplifies this finding and shows that some of the antigens are controlled by genes at loci other than the H-2. Whether the non-H-2 antigens so demonstrated are histocompatibility antigens remains to be determined. It is apparent from the data of the C3H-anti-CBA experiment that all major histocompatibility loci are not detected by the method as we have used it.

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SUMMARY

Development of a method for the detection of H-2 and non-H-2 isoantigens with the leukocyte agglutination reaction is described. Non-H-2 leukocyte agglutinins were induced by sensitization with injections of living spleen and thymus cells, by pregnancy and by skin homografts. Cross reaction of non-H-2 antisera with leukocytes of many inbred mouse strains was observed, indicating that these strains share some of the non-H-2 isoantigens. Selective *in vivo* absorption was done with one non-H-2 antiserum and a minimum of two separate agglutinating factors was found.

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