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A New Erythrocytic Antigen of C57BL/10 (B10) Mice[1] (42023)

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Abstract. A new antigen, detectable on murine erythrocytes by hemagglutination assay with a (BALB/cCrl × SWR/J)F_1 anti-B10.D2n/Sn alloantiserum, is described. Among the inbred and congenic mouse strains tested for reactivity with the antiserum, only the immunizing strain, B10.D2, and its congenic resistant partner, C57BL/10 (B10), reacted. Three other C57 strains, C57BL/6J, C57BL/6By, and C57L, were negative for the antigen. F_1 hybrids between B10 and BALB/c, an antigen-negative strain, were positive for the antigen indicating that its expression is dominant. Typing of 39 (BALB/c × (BALB/c × B10)F_1) and 62 ((BALB/c × B10)F_1 × BALB/c) backcross mice revealed that a single gene controls expression of the antigen. The gene is autosomal and not linked to H-2, Ly-4, or the c (albino) or b coat color genes. © 1985 Society for Experimental Biology and Medicine.

Nine loci controlling erythrocyte alloantigens of mice and designated Ea-1, Ea-2, and so forth have hitherto been identified by classical serological techniques (1–5). Among inbred laboratory mice each alloantigen has a unique inbred strain distribution pattern (SDP) with the exception of Ea-1 whose products have thus far been detected only in wild mice (1, 4).

In studies of the genetic control of resistance to the BALB/c plasmacytoma MPC-11 by hybrids between BALB/c and the seven Bailey Recombinant Inbred Strains (6), we found that the SDP for resistance to the tumor corresponded to that of three unlinked genes: Ea-4, Ly-4, and H-2 (7). In the course of producing alloantiserum against the antigens controlled by these genes for linkage studies, we prepared a (BALB/cCrl × SWR/J)F_1 anti-B10.D2n/SnJ antiserum by a modification of the procedure of Snell *et al.* (8). This antiserum was expected to detect Ly-4.2 on lymphocytes and Ea-4.2 on erythrocytes. As we here report, however, the antiserum reacts with Ly-4.2 but completely lacks anti-Ea-4.2 activity. Instead, it detects another, previously undescribed erythrocytic antigen, in addition to Ly-4.2.

Materials and Methods. *Mice.* A/J, AKR/J, C57BL/6By (B6/By), C57BL/6J (B6/J), C57BL/10SnJ (B10), C3H/HeJ, CXBD/By, CXBE/By, CXBG/By, CXBH/By, CXBI/By, CXBJ/By, CXBK/By, C57L/J, DBA/1J, DBA/2J, 129/J, SJL/J, and SWR/J mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. B10.D2n/SnJ were the gift of Dr. Dan Meruelo, New York University Medical Center, New York, New York. BALB/cCrl mice were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts. All F_1 and hybrid mice were bred in our own colony. The maternal strain is listed first in hybrid crosses and backcrosses.

Antisera. An antiserum which was expected to detect both the lymphocyte alloantigen Ly-4.2 and the non-cross-reactive erythrocytic alloantigen Ea-4.2 was prepared by immunizing (BALB/cCrl × SWR/J)F_1 hybrid mice with B10.D2n/SnJ lymphoid cells according to the method of Snell *et al.* (8) with the following modifications: (a) BALB/cCrl mice were used in place of BALB/cJ or BALB/cByJ mice, (b) B10.D2 spleen and peripheral lymph node cells were injected along with thymus cells in the primary immunization as well as in the booster immunizations, the rationale for this change being that only 5% of thymocytes express Ly-4.2 (9) and none express Ea-4.2 (5). Many of the immunized mice proved to be weakly or nonresponsive to the antigens. Therefore, in order to increase the yield of antiserum against the antigens,

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naive (BALB/cCrl × SWR)F_1 mice were irradiated (750 rad) using a cesium source (Radiation Machinery Corp., Parsippany, N.Y.), reconstituted with 1.5×10^7 spleen cells from selected immunized syngeneic mice which were producing both anti-B10.D2 lymphocyte and anti-B10.D2 erythrocyte antibodies, and challenged with pooled B10.D2 spleen, thymus, and peripheral lymph node cells suspended in RPMI-1640 medium at a 1:5 donor to recipient ratio. These mice were test bled at 7 and 10 days and bled out at 12 days. Individual sera from both the conventionally immunized and the irradiated, reconstituted, immunized mice were examined for antibody activity against B10.D2 lymphocytes by cytotoxicity test and against B10.D2 erythrocytes by hemagglutination. In general, an antiserum which reacted with lymphocytes also reacted with erythrocytes. Therefore, as the two antibody populations were not expected to cross-react (and in fact did not), all the reactive antisera were pooled.

The D Ly-4.2/Cy antiserum, prepared by Dr. M. Cherry on contract from the National Institutes of Health was obtained from the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, as a reference antiserum for comparative studies with our antiserum. D Ly-4.2/Cy is a (BALB/c × SWR)F_1 anti-B10.D2 antiserum specific for Ly-4.2. Dr. Cherry (personal communication) predicted that it would also express anti-Ea-4.2 activity as well. Double blind hemagglutination studies using an appropriate mouse strain panel (see Table I) indicated that the antiserum reacts with all the strains which express Ea-4.2 and that there are no extraneous reactions (4).

Anti-H-2^b antiserum (B10.D2 anti-B10) was the gift of Dr. R. Basch, New York University Medical Center, New York, New York.

Serological tests. Typing for the erythrocyte antigens and H-2 was done by hemagglutination assay. The dextran-facilitated hemagglutination assay of Gorer and Mikulska (10) as modified by Lilly (11) was used. The erythrocytes were suspended to 2% in 60% fetal calf serum in saline and the antisera were diluted in a solution of 3% dextran (110,000 mol wt, kindly provided by Dr. Z. Ovary, New York University Medical Center, New York, New York). The anti-Ea and anti-H-2 antisera were used at a 1/40 dilution and the D Ly-4.2/Cy antiserum at a 1/30 dilution. One-tenth milliliter of diluted antiserum was reacted with 0.05 ml of 2% red blood cells at 37°C for 2 hr. Agglutination was scored after streaking on glass slides. BALB/c and (BALB/c × B10)F_1 red cells as well as normal mouse serum controls were included for each group of backcross mice typed.

TABLE I. COMPARISON OF THE REACTIVITY BY HEMAGGLUTINATION OF OUR (BALB/cCrl × SWR/J)F_1 ANTI-B10.D2n/SnJ ANTISERUM AND THE D Ly-4.2/Cy ANTISERUM WITH ERYTHROCYTES OF 23 DIFFERENT MOUSE STRAINS

Mouse strain	(BALB/cCrl × SWR/J)F_1 anti-B10.D2n/SnJ antiserum	D Ly-4.2/Cy antiserum
A/J	–	–
AKR	–	–
BALB/c	–	–
B6/By	–	+
(BALB/c × B6/By)F_1	–	+
B6/J	–	+
(BALB/c × B6/J)F_1	–	+
B10/Sn	+	+
(BALB/c × B10)F_1	+	+
B10.D2	+	+
C3H/He	–	–
CXBD	–	–
CXBE	–	+
CXBG	–	+
CXBH	–	–
CXBI	–	+
CXBJ	–	+
CXBK	–	+
C57L	–	–
DBA/1	–	–
DBA/2	–	–
129	–	–
SJL	–	–

The endpoint hemagglutination titer (1–2 + reactivity) of our antiserum pool was found to be 1/160 when assayed against either B10.D2 or B10 peripheral blood erythrocytes from which the buffy coat cells had been removed; however, the titer on (BALB/c × B10)F_1 erythrocytes was consistently lower (1/80), suggesting that less antigen might be expressed on the erythrocytes of heterozygotes. The specificity of the antiserum pool for Ea-4.2 was tested by hemagglutination assay in a double-blind study using a panel

of mouse strains positive and negative for the antigen. However, as will be described in this paper, instead of detecting Ea-4.2, our antiserum detects another, previously undescribed erythrocyte antigen in addition to Ly-4.2 (see Table I).

Typing for Ly-4.2 was done by the trypan blue vital dye exclusion cytotoxicity test of Takahashi *et al.* (12) on peripheral blood lymphocytes. Ten drops of blood were collected from the tail into 0.5 ml heparinized saline and the erythrocytes were removed by centrifugation on Ficoll–Hypaque (density: 1.085) followed by distilled water lysis of residual red blood cells. The lymphocytes were washed twice in 1 ml of RPMI-1640 medium and then suspended to a final volume of 0.25 ml in medium 199. Cytotoxicity tests were done in duplicate or triplicate with 0.05 ml aliquots of the lymphocyte suspension. Our antiserum pool was used at a 1/40 dilution. The source of complement was rabbit serum obtained 5 days after whole body X irradiation of the rabbit (900 rad) and used at a 1/30 dilution. BALB/c and (BALB/c × B10)F_1 hybrid controls were included with each group of backcross mice typed. Results were expressed as a cytotoxicity index (CI) calculated as follows: CI = (% dead after incubation with anti-Ly-4.2 antiserum plus complement minus % dead after incubation with complement alone)/(100 − % dead after incubation with complement) × 100. The control CI observed on different days were variable and averaged 23.6 for the Ly-4.2 negative BALB/c controls and 52.6 for the Ly-4.2 positive (BALB/c × B10)F_1 controls. Backcross mice were considered positive for Ly-4.2 when their CI were clearly distinguishable from those of BALB/c and undistinguishable from those of the F_1 hybrid controls. Mice showing intermediate values were retyped on different days until their Ly-4.2 negativity or positivity was unequivocally established.

The endpoint cytotoxicity titer of our antiserum on B10.D2 lymphocytes, defined as the highest antiserum dilution which consistently yielded a positive CI as compared to a negative strain, usually BALB/c, was 1/80. The same titer was obtained for B10.D2 whether the lymphocytes were from spleen, peripheral lymph nodes, or peripheral blood. The specificity of the antiserum for Ly-4.2 was determined by testing its activity at a 1/40 dilution in double-blind fashion against a number of strains known to be positive or negative for the antigen. In agreement with the published SDP for Ly-4.2 (8) the pooled antiserum reacted with lymphocytes from B6, B10, (B10 × BALB/c)F_1, B10.D2, and C57L mice but not those of BALB/c, DBA/1, or AKR mice. In addition, when the antiserum was quantitatively absorbed and then tested for cytotoxic activity on B10.D2 peripheral lymph node cells by the procedure of McKenzie and Snell (9), it retained cytotoxic activity after absorption with B10.D2 thymocytes but not after absorption with B10.D2 splenocytes in agreement with the findings of McKenzie and Snell (9) for Ly-4.2.

Statistical analysis. The number of genes controlling expression of the erythrocytic antigen segregating in the backcross mice and the possible linkage of the erythrocytic antigen gene to various other genes were assessed using the χ^2 test.

Results and Discussion. Twenty-three different kinds of inbred, congenic, and hybrid mice were coded and tested for reactivity with the (BALB/cCrl × SWR/J)F_1 anti-B10.D2n/Sn antiserum and the D Ly-4.2/Cy antiserum in a hemagglutination assay. The distribution of reactivity of the two sera with the mouse strain panel is shown in Table I. The SDP of the antigen is different from that thus far reported for any mouse erythrocytic antigen (2–5) and for any lymphocyte (Ly) alloantigen (4, 5) including Ly-4 (8): only the erythrocytes of B10, B10.D2, and (BALB/c × B10)F_1 mice were agglutinated by our (BALB/c × SWR)F_1 anti-B10.D2 antiserum. It is especially interesting that none of the three other C57 strains compared with B10 and B10.D2, namely C57BL/6J, C57BL/6ByJ, and C57L/J express the antigen. C57BL/10 and C57BL/6 strains are closely related and few alloantigenic differences have been observed between them (4, 5), but of the few alloantigens known to be expressed in only one of these two strains, one is an erythrocytic antigen with a SDP opposite to that detected by our antiserum; i.e., it is expressed in all inbred mouse strains tested with the exception of B10 and congenic

TABLE II. A SINGLE GENE CONTROLS THE EXPRESSION OF THE ERYTHROCYTIC ANTIGEN (Ea Ag) IN (BALB/c × (BALB/c × B10)F_1) AND ((BALB/c × B10)F_1 × BALB/c) BACKCROSS MICE

Mice	No. positive for Ea Ag/ No. tested (%)	Expected No. positive if single gene controls Ea Ag expression (%)	χ^2 for fit to one gene model[a]
BALB/c × (BALB/c × B10)F_1	17/39 (44%)	19.5 (50%)	0.41
(BALB/c × B10)F_1 × BALB/c	32/62 (52%)	31 (50%)	0.20
Combined backcross populations	49/101 (49%)	50.5 (50%)	0.04

[a] None of the χ^2 values achieved significance ($P < 0.5$), consistent with a single gene controlling the expression of the antigen.

strains on the B10 background (2). It is not inconceivable, therefore, that the two antigens could be allelic.

As previously noted, both anti-Ly-4.2 antibodies and anti-erythrocyte antibodies are present in the same antiserum pool. Since the SDP of reactivity of our (BALB/c × SWR)F_1 anti-B10.D2 antiserum with the erythrocytes and lymphocytes of various mouse strains is quite different, it is apparent that the two antibody populations are distinct. Ly-4.2 is not expressed on erythrocytes (8, 9) and there is no difference in the reactivity of our antiserum pool with lymphocytes from strains expressing or not expressing the erythrocyte antigen. Thus, the two antibody populations do not cross-react and the erythrocytic antigen is probably not expressed on lymphocytes. The SDP of the erythrocyte antigen detected by the D Ly-4.2/Cy antiserum, also a (BALB/c × SWR)F_1 anti-B10.D2 antiserum, is identical to that of Ea-4 (1). Thus the two antisera; i.e., ours and the D Ly-4.2/Cy antiserum, detect different erythrocyte antigens. While differences in the origin of the BALB/c parent of the immunized hybrids might explain this discrepancy, it seems more likely that our method of expanding the immune lymphoid cell pool during immunization has fortuitously selected for cells producing antibody to this previously undetected erythrocyte antigen.

As BALB/c mice are negative for the erythrocyte antigen detected by our antiserum while (BALB/c × B10)F_1 hybrids are positive, antigen expression is a dominantly inherited characteristic. To determine the number of genes controlling the expression of the antigen, 39 (BALB/c × (BALB/c × B10)F_1) and 62 ((BALB/c × B10)F_1 × BALB/c) backcross mice were typed for the erythrocytic antigen (Table II). The proportion of mice positive and negative for the antigen in each individual backcross and in the combined backcrosses was not statistically significantly different by χ^2 analysis from the 50:50 distribution expected if antigen expression were controlled by a single gene. Since the antigen was equally

TABLE III. INDEPENDENT SEGREGATION OF THE GENES CONTROLLING EXPRESSION OF THE Ea ANTIGEN, Ly-4, H-2, AND THE b AND c COAT COLORS IN 101 PROGENY OF THE BACKCROSS OF (BALB/c × B10)F_1 HYBRIDS TO BALB/c

	Number expressing traits (Proportion expected to express traits)					
Second trait	Ea Ag^+/2nd $Trait^-$	Ea Ag^+/2nd $Trait^+$	Ea Ag^-/2nd $Trait^-$	Ea Ag^-/2nd $Trait^+$	Total	χ^2
Ly-4[b]	18 (1/4)	31 (1/4)	26 (1/4)	26 (1/4)	101	1.67[b]
H-2[b]	25 (1/4)	24 (1/4)	31 (1/4)	21 (1/4)	101	0.80
Albinism (c)	21 (1/4)	28 (1/4)	28 (1/4)	24 (1/4)	101	1.20
Black coat (B)[a]	12 (1/4)	9 (1/4)	13 (1/4)	13 (1/4)	49	0.18

[a] There were 49 nonalbino colored mice, 22 expressed the black (B) coat color and 27 the brown (b) coat color. All the backcross mice carried the agouti gene.

[b] All χ^2 values were nonsignificant; i.e., P value greater than 0.05.

distributed in males (27/55, 49%) and females (22/46, 48%), this single gene is autosomal.

The backcross populations were also typed for H-2^b and Ly-4^b and the white, black agouti, or brown agouti coat color of the mice was noted (Table III). The Ea Ag was not linked to Ly-4 (chromosome 2), H-2 (chromosome 17), or the b (chromosome 4) or c (chromosome 7) coat color genes.

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