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Induction of Peritoneal Ascites Fluid by Freund's Adjuvant: Genetic and Hormonal Influences[1] (38761)

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Intraperitoneal injections of Freund's complete adjuvant (FCA) and antigen in the laboratory mouse may induce large volumes of antibody-containing ascites fluid (1, 2). During the course of our using this procedure for obtaining alloantibodies we noted large strain and sex differences in the volumes of fluid produced by different inbred strains of mice. This study reports the results of experiments designed to elucidate possible hormonal and genetic influences underlying the differences.

Materials and Methods. Inbred AKR/J and A/J mice were obtained from the Jackson Laboratory and C57e/HaJa yellow mutant, ICR/Swiss/HaJa (inbred 17 generations), and BALB/c mice were bred in the American Medical Center colony. Animals were 2–5 mo old at the time of the initial FCA injection but groups were matched for age within each experiment.

All mice, except those of the A strain, received two intraperitoneal (ip) injections, 0.25 ml each, 7 days apart of an emulsion containing three parts FCA (DIFCO, Detroit, Michigan) and two parts crystalline bovine serum albumen (10 mg/ml of 0.15 *M* NaCl). The A strain mice received three ip injections at 3-day intervals, 0.2 ml per injection, of FCA containing 50 mg/ml dry weight of a B10.D2 sarcoma.

For studies of hormonal influences, the mice were castrated 2 wk before the first FCA injection. In addition, groups of AKR castrates received either ovary grafts (two ovaries subcutaneously in the left flank) or testosterone propionate (TP) injections subcutaneously, 15 μg in 0.1 ml sesame oil per injection. The first TP and FCA injections were given on the same day; subsequent TP injections were given thrice weekly during the first month and twice weekly the second month.

In order to avoid unnecessary irritation, ascites fluid was collected only when abdominal distention became pronounced, usually beginning 2.5–3 wks after the first FCA injection. Fluid was withdrawn with a syringe fitted with an 18-gauge perforated needle and collections were made at periodic intervals. Since ascites production ceased in most of the mice by 2 mo after the initial FCA injection, the recorded total volumes include only the collections for a 2-mo period.

Results. Differences between strains. During the first 2 mo following 2 weekly inoculations with the adjuvant-antigen mixture, strain AKR and strain A mice produce large volumes of ascites, C57 produced very little, and BALB/c and Swiss produced essentially none (Table I). An analysis of variance shows significant differences between the AKR and C57 strains ($P < 0.01$) (Expts. 1 *vs.* 3; 2 *vs* 3; 1 plus 2 *vs.* 3). A third inoculation of the FCA-BSA mixture extended ascites production in the C57 strain and induced a small amount of ascites in 17 of 20 Swiss mice.

Effect of hormonal status. Female AKR and C57 mice developed significantly more ascites fluid than did males of the corresponding strain (Table I, Expts. 1–3). To determine the basis for the sex difference, ascites production was assayed in intact and castrate animals and in castrate mice bearing ovary grafts (Table II). There was considerable variation in total volumes between replicate experiments, but levels were significantly higher in castrate than in intact males in both the AKR and C57 strains (Table II,

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TABLE I. STRAIN AND SEX DIFFERENCES AMONG INBRED MICE IN ASCITES PRODUCTION INDUCED BY ADJUVANT.

Expt. No.	Strain	No. of mice		Mean ± SE ml ascites fluid		P[a]
		Female	Male	Female	Male	
1	AKR	5	5	25.8 ± 7.98	11.5 ± 4.71	<0.01
2	AKR	9	9	24.1 ± 2.68	13.6 ± 4.20	<0.05[b]
3	C57[c]	5	5	1.5 ± 0.72	0.08 ± 0.08	<0.05[b]
10	A	18	—	11.5 ± 1.58	—	—
11	BALB/c	10	10	None[d]	None	—
12	Swiss[e]	5	6	None[d]	None	—

[a] By Student's *t* test for difference between sexes.
[b] One-sided.
[c] Collections over 3-month period; third inoculation after 2 mo resulted in further ascites production in some of the females but in none of the males.
[d] One mouse produced 1 ml.
[e] A third inoculation after 2 mo resulted in ascites production for 10 days; no significant difference between groups.

TABLE II. EFFECT OF CASTRATION, ADMINISTRATION OF TESTOSTERONE, AND OVARY TRANSPLANTS ON ASCITES PRODUCTION.

Exp. No.	Strain and sex	Treatment	No. of mice	Mean ± SE ml ascites fluid	P[a]
1	AKR ♀	Intact	5	25.8 ± 7.98	
		Castrated	5	38.6 ± 9.63	NS
		Castrated and ovary graft	5	31.2 ± 8.80	NS
	AKR ♂	Intact	5	11.5 ± 4.71	
		Castrated	4	27.9 ± 2.80	<0.025
		Castrated and ovary graft	5	29.3 ± 6.72	<0.05
2	AKR ♀	Intact	9	24.1 ± 2.68	
		Castrated	10	17.6 ± 2.36	NS
	AKR ♂	Intact	9	13.6 ± 4.20	
		Castrated	10	19.7 ± 2.13	NS
3	C57BL/6 ♀	Intact	5	1.54 ± 0.72	
		Castrated	6	5.82 ± 2.71	NS
	C57BL/6 ♂	Intact	5	0.08 ± 0.08	
		Castrated	6	3.48 ± 1.65	<0.05[b]
4	AKR ♂	Intact	8	4.48 ± 1.07	
		Castrated	8	12.56 ± 2.07	<0.005
		Castrated and testosterone	9	1.81 ± 0.94	<0.0005[c]

[a] By Student's *t* test for difference between intact and treated mice; NS, not significant at the 5% level.
[b] One-sided.
[c] Difference between castrated and castrated plus testosterone.

Expts. 1, 3, and 4). Castration did not affect ascites yields in females, nor did the presence of ovary grafts in castrate AKR males (Table II, Expts. 1 and 2). In contrast, castrate AKR males given TP (male hormone) produced significantly lower volumes of ascites than did the male castrates not receiving TP (Exp. 4, Table II). Apparently the presence of male hormone in the intact male, rather than an absence of female hormones, influences the levels of ascites generated. Sex also is a factor in response levels in F_1 and backcross mice (Table III) (considered further below).

Genetic basis for response differences between inbred strains. Assays of ascites fluid

TABLE III. ASCITES PRODUCTION IN F_1 HYBRIDS AND BACKCROSS (BC) MICE.

Exp. No.	Cross[a]	No. of mice		Mean ± SE ml ascites fluid		P[b]
		Female	Male	Female	Male	
5	(AKR × C57)F_1	7[c]	10	1.2 ± 0.87	0.67 ± 0.50	NS
7	(C57 × AKR)F_1	12	12	32.4 ± 3.64	7.8 ± 2.01	<0.001
8	(AKR × C57)F_1	15	11	9.7 ± 3.23	0.35 ± 0.20	<0.05
8	(C57 × AKR)F_1	9	13	0.44 ± 0.27	0.02 ± 0.01	<0.05[d]
9	(AKR × C57)F_1	8	9	32.8 ± 7.31	0.80 ± 0.33	<0.001
9	(C57 × AKR)F_1	8	8	17.0 ± 4.27	4.4 ± 1.54	<0.02
6	BC C57 × (C57 × AKR)F_1	11[e]	12	1.7 ± 0.83	0.75 ± 0.40	NS
6	BC AKR × (C57 × AKR)F_1	12	12	17.8 ± 5.14	6.0 ± 2.02	<0.05

[a] Female strain listed first in all crosses.
[b] By Student's *t* test for the difference between sexes; NS, not significant at the 5% level.
[c] Excludes one mouse that produced 23.0 ml of fluid.
[d] One-sided.
[e] Excludes one mouse that produced 31.5 ml of fluid.

levels were made in reciprocal F_1 hybrids of AKR by C57 matings and in backcrosses (BC) of the F_1 to each parental strain. The difference between strains (Tables I and II) and variations within strain (Table II, Expts. 1 and 2) and among hybrid groups (Table III, Expts. 5 and 7–9) indicate that both genetic and epigenetic factors influence the ascites response.

To emphasize the difference in individual reactions, each mouse was designated as either a "high responder" (producing a total of at least 5 ml of fluid) or a "low responder." Combining intact and castrate animals of both sexes, 80 AKR mice were high responders and seven were low responders (Table IV). For the smaller number of C57 mice, five were high responders and 18 low responders ($P < 0.005$ by chi-square analysis for the difference between the two strains).

Of the (AKR ♀ × C57 ♂)F_1 hybrids, 18 were high responders and 41 low responders; offspring of the reciprocal C57 ♀ × AKR ♂ mating gave 28 high and 43 low responders. The difference between the reciprocal hybrids is not statistically significant, but differences between the hybrids and the AKR parental strain are highly significant ($P < 0.01$). The data of Expt. 8 and 9 (see Table III) which were run simultaneously suggest the presence of a maternal influence on response levels. In both experiments offspring of AKR mothers produced more fluid than did those of C57 mothers.

For further genetic analysis backcrosses were made of (AKR ♀ × C57 ♂)F_1 males with females of each of the parental strains. Of the offspring of C57 mothers, two were high responders and 22 low responders; offspring of the AKR mothers gave 15 high and nine low responders. The significantly larger number of high responders in the BC to AKR than the BC to C57 females ($P < 0.005$) supports the conclusion that genetic factors are a significant determinant in the ascites response. A maternal influence may also have contributed to the BC results.

Discussion. The induction of antibody-containing ascites fluid in mice by ip injection of antigen and adjuvant was first reported by Munoz (1) and later used by others for yielding a variety of antibodies in large quantities (2). Munoz, using Swiss Webster female mice, noted that only half of the animals produced ascites following the same inoculation regimens that we employed. Lieberman *et al.* (2) obtained a more consistent production of ascites in "general purpose" Swiss mice, but they used a more frequent and prolonged immunization schedule. They reported good ascites yields in both male and female Swiss, C57BL/6, and A/LN mice, but much smaller volumes in A/He mice of either sex. It is possible that a sex difference was obscured by the more intense treatment these mice received.

Our Swiss mice did not produce ascites after two inocula but did after a third inoculation. (Dr. H. S. Shin induced ascites in

TABLE IV. SUMMARY OF RESPONSE TO ADJUVANT-ANTIGEN IN INDIVIDUAL AKR, C57, F_1 HYBRID, AND BACKCROSS MICE.

Strain	Sex and hormonal status	Number of responders	
		High[a]	Low[b]
AKR	Intact ♀	14	0
	Intact ♂	9	5
	Castrated ♀	15	0
	Castrated ♂	14	0
	Castrated ♀ + ovary graft	14	1
	Castrated ♂ + ovary graft	14	1
	Total	80	7
C57	Intact ♀	0	5
	Intact ♂	0	5
	Castrated ♀	3	3
	Castrated ♂	2	4
	Total	5	17
(AKR × C57)F_1	Intact ♀	17	14
	Intact ♂	1	29
(C57 × AKR)F_1	Intact ♀	18	12
	Intact ♂	10	23
	Total	46	78
(AKR × C57)BC to AKR ♀	Intact ♀	9	3
	Intact ♂	6	6
	Total	15	9
(AKR × C57)BC to C57 ♀	Intact ♀	2	10
	Intact ♂	0	12
	Total	2	22

[a] High responder: producing a total of at least 5 ml of ascites fluid.
[b] Low responder: producing less than 5 ml of fluid.

C57BL/6 (*H-2*^{b}) mice by repeated inoculations of FCA but was unable to do this in C57BL/Ks (*H-2*^{d}) mice (personal communication).

In retrospect, our BALB/c mice might have been more desirable in crosses for genetic analysis since they were even less responsive to adjuvant-antigen stimulation than were the C57 mice. Nevertheless, our data do substantiate the existence of a genetic influence upon levels of ascites produced. Since the F_1 mice responded to a degree intermediate between the two parental strains and the backcross to AKR yielded only 15 responders out of 24 animals, we conclude that genetic control of the expression of the "response" trait is very likely multifactorial.

Variability in response can not be attributed to genetic inhomogeneity since our strains are highly inbred. It could be due in part to physiological differences which are characteristic even of highly inbred animals (3). Certainly fluctuations in endogenous hormone levels might contribute to variability, and in addition other undefined endogenous and exogenous influences may be operative. A maternal influence also may have affected the F_1 response (4). In summary, several factors, genetic and epigenetic, interplay in the expression of the response to FCA, and the relative degree to which each component influences the reaction differs among the inbred strains of mice.

Demonstration of the role of male hormone in delimiting the response level is clear cut (Table II). Similarly, male hormone has been shown to suppress serum antibody production (5), and hormonal status may be implicated in the more intense humoral and cellular immune responses characteristic of females of several species of animals (5–7). When investigating immune responses, these

factors should be taken into consideration in selecting the most appropriate experimental animals.

Summary. Strain differences among inbred mice is demonstrated for the relative amounts of peritoneal ascites fluid induced by the injection of Freund's adjuvant and antigen. Females tend to produce more fluid than males; the lower level in males is attributable to the action of male hormone. Both genetic and epigenetic factors are determinants in the response levels; genetic factors differentiate the inbred strains of mice, epigenetic factors underlie the variations within a strain and the difference between sexes.

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