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ANTIBODY AFFINITY IN MICE WITH THE CBA/N DEFECT

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The CBA/N mouse carries an X-linked immune defect that results in a profound inability to respond to certain thymus-independent antigens. It has been suggested that this B cell defect might also lead to an inability to produce high affinity antibody in response to immunization with thymus-dependent antigens. To investigate this issue, the anti-hapten antibody response to hapten-protein conjugates was studied at various times after immunization. Defective (CBA/N × DBA/2N)F_1 male and control (DBA/2N × CBA/N)F_1 male mice were immunized with dinitrophenyl (DNP) hemocyanin and bled at 2, 4, and 6 weeks after immunization. They were boosted at 6 weeks or 6 months after primary immunization. The affinity of anti-DNP antibodies was measured in an ammonium sulfate precipitation assay with ^{3}H-DNP-lysine. No significant differences in the affinity of anti-DNP antibodies in defective and control mice were found at any of the times tested. Similarly, defective F_1 male and control F_1 female mice immunized with (4-hydroxy-5-bromo-3-nitrophenyl) acetyl (NBrP) chicken γ-globulin (CGG) or with azobenzenearsonate-5-hydroxyphenylacetyl (ABA-HOP) CGG developed anti-hapten antibodies of similar relative affinity as measured by a haptenated-phage inactivation assay. It was concluded that mice with the CBA/N immune defect are capable of producing high affinity antibodies to haptens on protein carriers and that within the period studied, the appearance of high affinity antibody occurs with the same time course in defective and control animals.

The CBA/N mouse strain carries an X-linked gene (*xid*) (1) that results in a profound inability to mount an immune response to thymus-independent (TI)[3] antigens, such as dextran, levan, and haptenated Ficoll, which have been classified as type 2 TI (TI-2) antigens (2). This deficiency is associated with an absence of B lymphocytes that express the cell surface antigens Lyb3 (3), Lyb5 (4), and Lyb7 (5). These lymphocytes are thought to represent a mature or late developing subset of B cells that are lacking in neonatal mice (2, 6). Based on the finding that CBA/N mice lack a mature subset of B lymphocytes, investigators have been led to question whether, in addition to the failure to respond to TI-2 antigens, abnormalities of the response to thymus-dependent (TD) antigens might also be found. Indeed, Scher *et al.* (7) have recently found that the early IgG anti-sheep erythrocyte (SRBC) response is profoundly depressed in mice with the CBA/N defect. Furthermore, Gershon and Kondo (8) have reported that CBA/N mice that had been hyperimmunized with SRBC failed to produce a cross-reacting antibody to horse erythrocytes (HRBC), which was found in high titer in the hyperimmune sera of control mice, and which was of high avidity (9). It was proposed that the absence of this cross-reacting antibody might be due to a general inability of CBA/N mice to produce high affinity antibodies to TD antigens. In addition, it has been reported that mice with the CBA/N immune defect have a lower percentage of high avidity anti-trinitrophenyl (TNP) plaque-forming cells (PFC) than control animals after either *in vitro* (10) or *in vivo* (11) immunization with TNP-lipopolysaccharide (TNP-LPS).

We have approached the question of whether the CBA/N defect results in a general inability to produce high affinity antibody by immunizing mice with conventional hapten-protein conjugates and directly measuring the affinities of the anti-hapten antibodies produced. We find that mice with the CBA/N defect immunized under both optimal and limiting conditions produce anti-hapten antibodies of high affinity; indeed no significant differences from those produced by control animals were observed.

MATERIALS AND METHODS

Mice. All mice were obtained from the Small Animal Section, Division of Research Services, National Institutes of Health, and were immunized at 8 to 12 weeks of age.

Antigens and immunizations. DNP-keyhole limpet hemocyanin (KLH), containing an average of 10 DNP groups per 100,000 daltons, and DNP-bovine serum albumin (BSA), containing 12 DNP groups per molecule of BSA were prepared as described by Benacerraf and Levine (12). Anti-DNP antibodies were raised by immunization with 100 or 1 μg of DNP_{10}-KLH in complete Freund's adjuvant (CFA) in the hind footpads (0.05 ml/footpad) and subcutaneously behind the neck (0.1 ml) and were boosted with 50 or 1 μg, respectively, in incomplete Freund's adjuvant (IFA), given i.p. Other mice were immunized with 100 μg of DNP_{12}-BSA in CFA and boosted with 10 μg in IFA as described above. Immunization schedules for animals immunized with DNP conjugates are described in the tables. Anti-NBrP antibodies were raised by immunization with (4-

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[3] Abbreviations used in this paper: DNP, dinitrophenyl; NBrP, (4-hydroxy-5-bromo-3-nitrophenyl) acetyl; CGG, chicken γ-globulin; ABA-HOP, azobenzenearsonate-5-hydroxyphenylacetyl; TI, thymus-independent; TD, thymus-dependent; HRBC, horse erythrocyte; ABC, antigen-binding capacity.

hydroxy-5-bromo-3-nitrophenyl)acetyl-chicken γ-globulin (NBrP-CGG) containing an average of 12 NBrP groups per molecule of CGG as previously described (13). Anti-ABA-HOP antibodies were produced by immunization with ABA-HOP-CGG containing an average of eight ABA-HOP groups per molecule of CGG as previously described (14). Mice immunized with $NBrP_{12}$-CGG and $ABA\text{-}HOP_8$-CGG were boosted 8 weeks after the primary immunization and were bled 8 weeks after the secondary challenge.

Antibody determinations. Antibody to DNP was measured by an ammonium sulfate precipitation method using ^{3}H-DNP-lysine (10^{-8} M) as the hapten, as described (15). Results are reported as ABC-33, which is 1/dilution of serum required to bind 33% of added ligand multiplied by the bound concentration of ligand (0.33×10^{-8} M). Equilibrium constants were also determined by an ammonium sulfate precipitation method (16). Scatchard plots of the equilibrium binding of ^{3}H-DNP-lysine to anti-DNP antibodies from individual defective and normal male mice are shown in Figure 1. Association constants were calculated from the relation

$$B/F = K_0 (Ab_0 - B)$$

where B = bound ligand concentration; F = free ligand concentration; K_0 = average intrinsic association constant; Ab_0 = total concentration of antigen-binding sites. K_0 is determined when half of the combining sites are occupied, i.e., when $B = \frac{1}{2} AB_0$. Relative affinities were calculated according to the expressions derived by Paul and Elfenbein (15) as follows:

$$K_a = \frac{R(B/F)_x B_i - (B/F)_i B_x}{(1\text{-}R) B_i B_x}$$

where R is the ratio of the antigen-binding capacity (ABC; defined as 1/serum dilution multiplied by the concentration of ligand that is bound at that serum dilution) at a given experimental condition to the ABC determined at an index condition; B/F is the (% ligand bound/100-% ligand bound); B is (% ligand bound × total ligand concentration)/100 under experimental, x, and index, i, conditions. Antibody titers and relative affinities of anti-NBrP and anti-ABA-HOP antibodies were determined as previously described (13, 14).

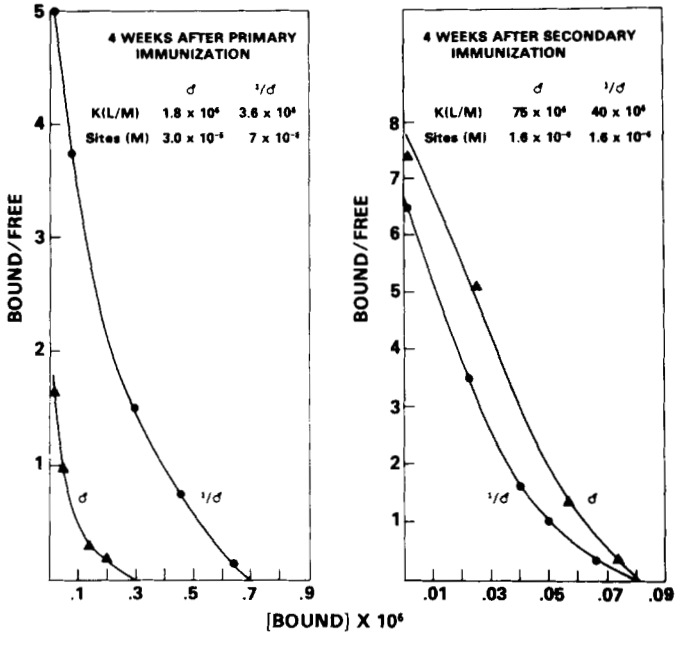

Figure 1. Equilibrium binding of ^{3}H-DNP-lysine by anti-DNP antibodies from defective and normal mice. Sera were obtained from (CBA/N × DBA/2)F_1 defective male (♂) and (DBA/2 × CBA/N)F_1 normal male (1/♂) mice at 4 weeks after a primary immunization (100 μg DNP_{10}-KLH in CFA, *left-hand panel*) or a secondary challenge (50 μg DNP_{10}-KLH in IFA, *right-hand panel*) and assayed by the ammonium sulfate precipitation method.

RESULTS

Equilibrium binding of ^{3}H-DNP-lysine to anti-DNP antibodies from (CBA/N × DBA/2)F_1 defective male and (DBA/2 × CBA/N)F_1 normal male mice is illustrated in the Scatchard plots in Figure 1. ABC-33, the number of hapten-binding sites, and K_0 obtained from the analysis of individual sera from a large number of mice bled at various times during primary and secondary immune responses are compiled at Table I. Data from representative individual mice bled and assayed at a series of times throughout the course of 1° and 2° immunization are shown in Table II. The ABC-33 data and the number of hapten-binding sites show that during the primary response and at certain times in the course of the secondary response, defective mice produce somewhat less antibody than control animals. The data clearly show, however, no significant differences in the K_0 of anti-DNP antibodies from defective and control mice at any time during a primary or secondary response. These data also demonstrate, both in groups of mice (Table I) and in individual mice (Table II), a time-dependent increase in antibody affinity that is characteristic of the normal maturation of the immune response (17).

The results presented in Tables I and II were obtained with sera from mice that had been immunized under optimal conditions (100 μg DNP_{10}-KLH). In order to rule out the possibility that this immunization might have been sufficient to overcome any differences that existed between defective and normal mice, two other immunization protocols were utilized. One involved a low dose of DNP_{10}-KLH (1 μg) and the second involved immunization with DNP coupled to a "weaker" carrier than KLH (DNP_{12}-BSA). The data obtained from these two groups of mice are shown in Tables III and IV. In these experiments defective mice produced substantially less antibody than controls. The relative difference between normal and defective mice is greater in these groups than in the group immunized with 100 μg of DNP_{10}-KLH. Nevertheless, no significant differences in antibody affinities are seen between defective and control groups immunized either with a low dose of DNP_{10}-KLH or with DNP_{12}-BSA.

Because the calculation of the average intrinsic association constant (K_0) depends on an accurate measurement of the total concentration of serum antigen-binding sites and because such measurements are difficult when the serum antibody concentration and affinity are low, we considered the possibility that we had failed to detect differences in K_0 of antibodies from defective and normal mice because of an inaccuracy in our measurement of the antibody concentration in the sera from the defective animals. We, therefore, measured the relative affinities of anti-DNP antibodies using the dilutional assay of Paul and Elfenbein (15), which is designed specifically for the measurement of affinities of heterogeneous populations of relatively low affinity antibodies present at low concentration. Under such conditions, K_0 determined by this technique may be substantially higher than those obtained by Scatchard analysis (15). Nevertheless, no differences in the affinity of anti-DNP antibodies from defective and control mice are observed by using the dilutional assay (Table V), thus confirming the results obtained by Scatchard analysis.

In another set of experiments we used a different method to

TABLE I

Amount and affinity of anti-DNP antibody

	K × 10^{-6} (L/M)		Sites[a] × 10^{6} (M)		ABC-33 × 10^{8} (M)	
	♂[b]	1/♂[c]	♂	1/♂	♂	1/♂
1° Response						
2 weeks	4.7 ± 1[d] (5)[e]	4.0 ± 0.8 (7)	1.6 ± 0.6 (5)	2.6 ± 1 (7)	2.4 ± 1 (5)	6.4 ± 2 (5)
4 weeks	4.0 ± 1 (6)	7.2 ± 2 (6)	2.5 ± 0.6 (6)	4.2 ± 2 (6)	26 ± 2 (3)	49 ± 25 (4)
6 weeks	6.8 ± 1 (6)	6.0 ± 1 (6)	2.0 ± 0.5 (6)	4.4 ± 1 (6)	24 ± 4 (2)	62 ± 7 (3)
2° Response (boosted at 6 weeks)						
8 days	12 ± 4 (5)	9.3 ± 1 (5)	2.5 ± 0.5 (5)	4.3 ± 0.6 (5)	31 ± 1 (2)	53 ± 16 (2)
2 weeks	11 ± 1 (5)	17 ± 6 (5)	3.2 ± 0.7 (5)	4.8 ± 1 (5)	76 ± 24 (4)	86 ± 17 (3)
4 weeks	55 ± 11 (6)	29 ± 8 (4)	2.5 ± 0.5 (6)	3.8 ± 1 (4)	92 ± 18 (3)	90 ± 20 (3)
8 weeks	145 ± 42 (5)	79 ± 22 (5)	1.2 ± 0.2 (5)	1.4 ± 0.3 (5)	122 ± 5 (3)	117 ± 28 (2)
2° Response (boosted at 6 months)						
	♂[f]	♀[g]	♂	♀	♂	♀
8 days	54 ± 16 (5)	61 ± 36 (4)	1.2 ± 0.6 (5)	2.0 ± 1.1 (4)	61 ± 2 (2)	167 ± 19 (2)

[a] Concentration of hapten-binding sites.
[b] (CBA/N × DBA/2)F_1 male (defective).
[c] (DBA/2 × CBA/N)F_1 male (normal).
[d] Arithmetic mean ± S.E.
[e] Number of animals.
[f] (CBA/N × BALB/c)F_1 male (defective).
[g] (CBA/N × BALB/c)F_1 female (normal).

TABLE II

Amount and affinity of anti-DNP antibody produced by individual mice

	K × 10^{-6} (L/M)				Sites[a] × 10^{6} (M)				ABC-33 × 10^{8} (M)			
	♂[b]		1/♂[c]		♂		1/♂		♂		1/♂	
Animal No.	1	2	1	2	1	2	1	2	1	2	1	2
1° Response												
2 weeks	3.3	2.0	2.8	2.1	1.2	3.1	1.2	2.6	3	4	3	17
4 weeks	3.7	1.8	3.5	2.4	2.2	3.0	3.0	8.2	25	28	25	74
6 weeks	9.5	4.2	6.5	7.6	1.0	2.4	3.5	3.5	28	20	33	70
2° Response (boosted at 6 weeks)												
8 days	11	4.8	13	11	3.6	3.1	1.2	6.0	32	30	43	85
2 weeks	12	8.4	19	18	3.4	3.8	3.5	3.8	40	43	39	117
4 weeks	43	75	40	30	2.9	1.6	1.6	2.2	40	120	69	110
8 weeks	179	245	73	135	0.8	1.1	1.5	0.8	96	116	89	145

[a] Concentration of hapten-binding sites.
[b] (CBA/N × DBA/2)F_1 male (defective).
[c] (DBA/2 × CBA/N)F_1 male (normal).

TABLE III

Amount and affinity of anti-DNP antibody produced under limiting conditions

	K × 10^{-6} (L/M)		Sites[a] × 10^{6} (M)		ABC-33 × 10^{8} (M)	
	♂[b]	1/♂[c]	♂	1/♂	♂	1/♂
1 μg DNP_{10}-KLH						
1° response						
4 weeks	17 ± 8[d] (5)[e]	20 ± 4 (6)	0.9 ± 0.4 (5)	1.4 ± 0.3 (6)	9.6 ± 4 (4)	44 ± 12 (3)
2° response (boosted at 8 weeks)						
12 days	241 ± 133 (5)	102 ± 23 (4)	0.8 ± 0.3 (5)	2.1 ± 0.3 (4)	24 ± 6 (4)	116 ± 28 (3)
100 μg DNP_{12}-BSA						
1° response						
4 weeks	19 ± 5 (5)	17 ± 9 (4)	0.4 ± 0.1 (5)	1.3 ± 0.3 (4)	8.8 ± 3 (4)	45 ± 18 (3)
2° Response (boosted at 8 weeks)						
12 days	49 ± 31 (5)	64 ± 20 (5)	0.8 ± 0.4 (5)	2.3 ± 1 (5)	28 ± 9 (3)	50 ± 9 (4)

[a] Concentration of hapten-binding sites.
[b] (CBA/N × DBA/2)F_1 male (defective).
[c] (DBA/2 × CBA/N)F_1 male (normal).
[d] Arithmetic mean ± S.E.
[e] Number of animals.

TABLE IV

Amount and affinity of anti-DNP antibody produced by individual mice under limiting conditions

	K × 10^{-6} (L/M)				Sites × 10^{6} (M)[a]				ABC-33 × 10^{8} (M)			
	♂[b]		1/♂[c]		♂		1/♂		♂		1/♂	
	597[d]	601	610	612	597	601	610	612	597	601	610	612
1 μg DNP_{10}-KLH												
1° response												
4 weeks	33	5.4	18	18	0.2	0.2	1.0	1.5	2.1	16	50	66
2° response (boosted at 8 weeks)												
12 days	436	505	163	58	0.3	0.4	2.5	2.1	18	11	158	127
	605	606	617	620	605	606	617	620	605	606	617	620
100 μg DNP_{12}-BSA												
1° response												
4 weeks	8.6	5.4	35	10	0.1	0.4	1.0	2.3	2.5	17	34	49
2° response (boosted at 8 weeks)												
12 days	43	170	150	74	0.3	0.4	0.6	1.8	5.3	28	30	61

[a] Concentration of hapten-binding sites.
[b] (CBA/N × DBA/2)F_1 male (defective).
[c] (DBA/2 × CBA/N)F_1 male (normal).
[d] Animal no.

TABLE V

K calculated from dilutional assay

Immunogen	Time of Bleeding	Animal No. and K × 10^{-6} (L/M)			
		♂[a]		1/♂[b]	
		No.	K	No.	K
DNP-KLH (1 μg)	4 week 1°	601	125	612	79
				616	39
DNP-BSA (100 μg)	4 week 1°	602	104	617	309
		605	25	618	33
		606	107	620	123
		607	42	622	191

[a] (CBA/N × DBA/2)F_1 male (defective).
[b] (DBA/2 × CBA/N)F_1 male (normal).

TABLE VI

Amount and affinity of anti-NBrP antibody

	Animal No.	♂[a]	Animal No.	♀[b]
Antibody Concentration				
50% inactivation titer	1	1166	11	596
[(1/dilution) × 10^{-3}]	2	375	12	712
	3	598	13	1190
	4	307	14	713
			15	514
Geometric mean ×÷ S.E.		532 ×÷ 1.3		712 ×÷ 1.2
Antibody Affinity				
[NBrP caproate] for	1	7.8	11	7.7
50% inhibition of	2	7.7	12	6.8
haptenated phage	3	7.2	13	13
inactivation	4	5.1	14	5.1
(nmoles/ml)				
Geometric mean ×÷ S.E.		6.8 ×÷ 1.1		7.7 ×÷ 1.2

[a] (CBA/N × BALB/c)F_1 male (defective).
[b] (CBA/N × BALB/c)F_1 female (normal).

measure the relative affinities of antibodies to the haptens NBrP and ABA-HOP. This was done by measuring the concentration of hapten required to cause 50% inhibition of the inactivation of haptenated phage by antibody. In this assay, the relative affinity is inversely related to the concentration of hapten required for 50% inhibition. Antibody concentrations were determined by calculating the reciprocal of the serum dilution which caused 50% inactivation of haptenated phage. In this group of experiments, defective (CBA/N × BALB/c)F_1 male and control (CBA/N × BALB/c)F_1 female mice were immunized with 100 μg of CGG conjugates of NBrP or ABA-HOP. The data are shown in Tables VI (anti-NBrP) and VII (anti-ABA-HOP). There is no significant difference in the amount of anti-NBrP or ABA-HOP antibodies between defective males and normal females and again, as in the other experiments, there is no difference in the relative affinity of anti-hapten antibodies in the two groups.

DISCUSSION

It is well known that in the course of immune responses to a variety of antigens, the affinity of the antibody produced increases with the duration of time after immunization (17). The cellular dynamics of this process are still poorly understood but recent work has suggested that the memory cells that are the precursors of high affinity antibody-producing cells have a distinctive complement of immunoglobulins on their surface when compared with precursors of low affinity antibody-secreting cells. The difference in the two populations is that the "low affinity precursors" possess membrane IgD whereas the "high affinity precursors" appear to lack IgD (18). The possibility that high affinity antibody production is a function of a specialized subpopulation of B lymphocytes has also been raised by the findings of Gershon and Kondo (8) that mice with the CBA/N defect fail to produce the high avidity anti-SRBC antibody, cross-reactive with HRBC, which is a feature of the normal response. In addition, Quintans (11) has shown that CBA/N mice have a normal number of low avidity TNP PFC in response to TNP-LPS immunization but a reduced number of high avidity anti-TNP PFC. These results also suggested

TABLE VII

Amount and affinity of anti-ABA-HOP antibody

	Animal No.	♂[a]	Animal No.	♀[b]
Antibody Concentration				
50% inactivation titer	6	72	16	90
[(1/dilution) × 10^{-2}]	7	22	17	92
	8	40	18	97
	9	460	19	81
	10	85	20	25
Geometric mean $\overset{\times}{\div}$ S.E.		75.6 $\overset{\times}{\div}$ 1.7		69.4 $\overset{\times}{\div}$ 1.3
Antibody Affinity				
[ABA-HOP] for 50% inhibition of haptenated phage inactivation (nmoles/ml)	6	48	16	82
	7	16	17	275
	8	50	18	5.7
	9	15	19	284
	10	24		
Geometric mean $\overset{\times}{\div}$ S.E.		26.6 $\overset{\times}{\div}$ 1.3		77.5 $\overset{\times}{\div}$ 2.5

[a] (CBA/N × BALB/c)F_1 male (defective).

[b] (CBA/N × BALB/c)F_1 female (normal).

that the precursors of high affinity antibody-producing cells are confined to a subset of lymphocytes that is deficient in CBA/N mice.

We have immunized mice with the CBA/N defect with a series of defined haptens that were coupled to several different protein carriers and measured the affinities of the anti-hapten antibodies in a variety of assays. Our results show that mice with the CBA/N-immune defect make anti-DNP, anti-NBrP, and anti-ABA-HOP antibodies that are indistinguishable in affinity from those produced by appropriate normal controls at various times in the course of primary and secondary immune responses. These studies indicate that this is true even under limiting conditions of immunization.

These results are in apparent conflict with those of others (9, 11). Gershon and Kondo (9), however, did not measure the affinities of the anti-SRBC antibodies produced in CBA/N mice and it is possible that small amounts of high affinity antibodies against SRBC were produced by the defective mice but not detected in their assay. An alternative explanation is that the cross-reactive antibodies to HRBC produced after immunization with SRBC are directed against a carbohydrate determinant to which CBA/N mice are unresponsive. The data of Quintans (11) show that by hapten inhibition of PFC, CBA/N mice showed fewer inhibitable PFC at very low concentrations of hapten, suggesting the CBA/N mice have fewer high avidity PFC than controls. Nevertheless, if one uses his data to obtain the concentration of hapten that results in 50% inhibition of PFC, there is not a significant difference between CBA/N (2.6 nM) and CBA/CaJ (1.6 nM) mice.

Our results do not rule out the possibility that affinity maturation is a function of a specialized B lymphocyte population; however, if this function is uniquely expressed in a B cell subpopulation, it must be one possessed by mice with the CBA/N defect.

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