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Immunology

Inheritance of acquired immunological tolerance to foreign histocompatibility antigens in mice

(cytotoxic T cells/genetic transmission)

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ABSTRACT **CBA mice were rendered tolerant of major histocompatibility antigens of A/J mice by neonatal injection of 100×10^6 lymphoid cells of (CBA × A/J)F_1 followed by repeated injections of F_1 cells at 2-week intervals throughout the study. When adult (8 weeks old), 10 tolerant or normal CBA males were mated to normal CBA females. Spleen cells of the progeny were tested for their ability to mount a cytotoxic T lymphocyte response *in vitro* against A/J antigens or against C57BL/6J and B10.A (2R) antigens in a cell-mediated lympholysis (CML) assay. A significant proportion (50–60%) of first-generation offspring of tolerant fathers failed to produce detectable anti-A/J cytotoxic responses but responded in the normal range to stimulation by C57BL/6J or B10.A (2R). Second-generation offspring derived from mating animals born of tolerant male parents—either brother × sister matings (incross) or matings to normal CBA mice (outcross)—also showed a high proportion (20–40%) with diminished anti-A/J CML responses when similarly tested *in vitro*. Thus, a specific acquired somatic characteristic in the immune system (tolerance to major histocompatibility antigens) induced in male mice shows significant transmission to first- and second-generation offspring.**

Data published by several independent laboratories (1–4) on the apparent inheritance of somatically generated idiotypes of antibodies raised in rabbits by hyperimmunization with bacterial vaccines lend support to the idea that acquired states of the immune system may be inherited (5), a concept that challenges the classical evolutionary notion of the isolation of the soma from the germ line (Weismann's doctrine). To investigate the possibility that a soma → germ plasm mode of inheritance can occur, we investigated whether acquired neonatally induced allograft tolerance (6) could become inherited. Tolerance to strain A/J histocompatibility antigens (K^k D^d) was induced in male CBA mice (K^k D^k) by neonatal injection of (CBA × A/J)F_1 lymphoid cells (K^k $D^{k/d}$). These A/J-tolerant CBA males were then mated to normal nontolerant CBA females, and spleen cells of the offspring were used to produce anti-A/J specific cytotoxic T lymphocytes (CTLs) in a 5-day *in vitro* cell-mediated lympholysis (CML) assay. The CML test is an *in vitro* correlate of *in vivo* allograft reactivity (7–9). The data below show that tolerance to A/J antigens appears without prior antigenic exposure and at high frequency (40–60%) in the first- and second-generation offspring.

MATERIALS AND METHODS

Mice. CBA/Cum mice were obtained at 5–6 weeks of age from Cumberland View Farms (Clinton, TN). CBA/OCI mice were bred and raised in our animal room from CBA/Cum foundation stock. C57BL/6J and A/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). (CBA/Cum × A/J)F_1 (hereafter designated simply "F_1") mice and B10.A (2R) mice (a gift from Marc Feldmann) were bred and raised in our animal room. All mice were allowed food and water ad lib.

Acquired Neonatal Tolerance to (CBA × A/J)F_1 Cells.

Table 1. Cytotoxic responses of spleen cells from mice shown in Fig. 1*B*

Spleen cell donor	% specific cytotoxicity to C57BL/6 At 3:1	% specific cytotoxicity to A/J At 3:1	% specific cytotoxicity to A/J At 12:1
Normal CBA/OCI	3.4	8.3	16.9
	17.6	7.3	15.1
	26.8	5.6	12.6
	24.1	4.7	10.1
	21.9	6.9	14.2
	20.6	6.6	13.9
Normal F_1	8.1	1.8	2.1
	12.6	1.7	1.9
	18.4	1.8	2.9
	16.9	1.2	2.3
	17.4	1.4	0.1
Outcross:			
Normal CBA × ♂ F_1	28.4	0.8	1.0
	21.3	0.4	0.7
	18.6	0.1	0.6
	11.9	2.4	4.1
	20.6	1.6	2.4
	17.6	1.4	2.1
Incross:			
$♀_q ♀_r$ × $♂_s$	16.7	7.1	12.9
	19.3	9.9	20.6
	29.3	8.6	16.1
	12.3	2.3	4.2
	16.2	0.9	2.1
	16.9	7.3	12.9
	16.5	4.9	8.4

Spleen cells (1×10^6) of the type shown (details in Fig. 1*B*) were used in standard CML assays. After 5 days of culture, the cells were tested at effector/target cell ratios of 3:1 and 12:1 with 1×10^4 ^{51}Cr-labeled target cells. All values shown are arithmetic means of triplicate determinations.

Abbreviations: CML, cell-mediated lympholysis; CTL, cytotoxic T lymphocyte(s); CBA/Cum, CBA mice obtained from Cumberland View Farms; CBA/OCI, CBA mice bred at the Ontario Cancer Institute from CBA/Cum foundation stock; MHC, major histocompatibility; F_1, (CBA/Cum × A/J)F_1; Tol-progeny, offspring of tolerant males and normal female CBA/Cum mice; *H-2*, mouse genes coding for MHC antigens located on chromosome 17. The serologically defined K-end and D-end H-2 specificities for the mouse strains used are: CBA, K^k D^k; A/J, K^k D^d; (CBA × A/J)F_1, K^k $D^{k/d}$; C57BL/6J, K^b D^b; and B10.A (2R), K^k D^b.

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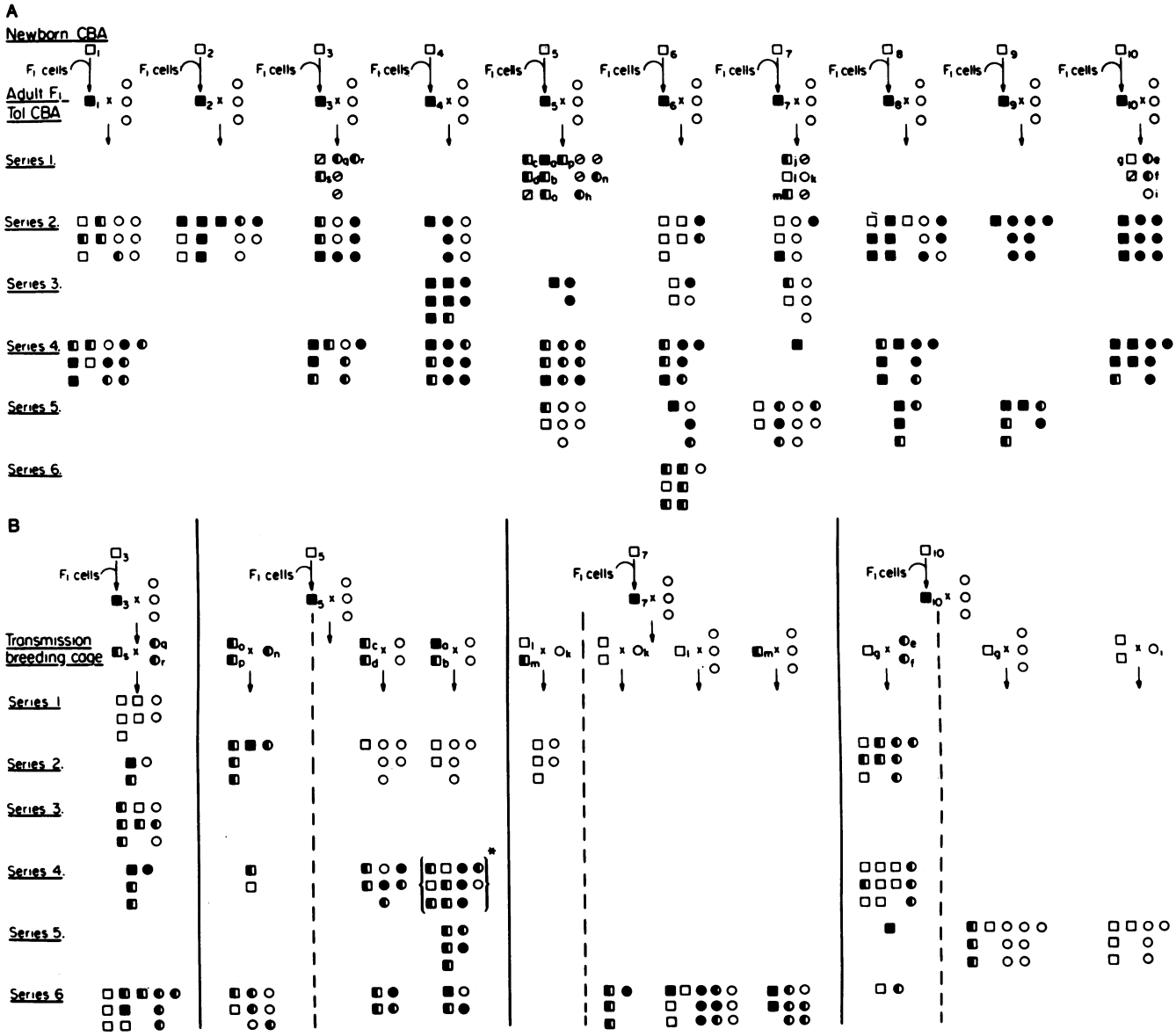

FIG. 1. Genetic transmission of acquired tolerance to A/J histocompatibility antigens. Males are represented by squares and females, by circles. (*A*) Series of progeny of the first generation; (*B*) series of progeny of the second generation. □ ○, Normal responders to A/J; ◧ ◐, hyporesponders; ■ ●, tolerant animals; ⊘, spleen not tested. The criteria used to make these classifications are described in *Materials and Methods*. The mean ± SD for the anti-A/J cytotoxic response of spleen cells from normal (CBA, F_1) mice tested alongside each progeny series were: (*A*) series 1, normal CBA 16.3 ± 5.4 (n = 4), normal F_1 1.6 ± 1.0 (n = 4); series 2 and 3, 6.9 ± 3.1 (32), 0.61 ± 0.91 (35); series 4, 13 ± 2.8 (17), 1.3 ± 1.3 (10); series 5, 11.6 ± 3.3 (13), 1 ± 0.7 (7); series 6, 12.1 ± 3.7 (28), 1 ± 1 (12); (*B*) series 1, 11.6 ± 3.3 (13), 1 ± 0.7 (7); series 2, 12.1 ± 3.7 (28), 1 ± 1 (12); series 3, 17 ± 4.5 (9), 0.2 ± 0.8 (4); series 4, 11.4 ± 2.3 (8), 2.2 ± 0.9 (6); series 5, 22.4 ± 5.4 (5), 1.8 ± 1.7 (4); series 6, 7.5 ± 1.5 (10), 1.4 ± 0.6 (5). The mice in series 1 in *A* were tested retrospectively at 31 weeks of age after producing the series 6 second-generation progeny (in *B*); all other mice shown were tested at 6–8 weeks of age. In *B*, outcross transmission cages are to the right of the dashed line and incross transmission cages are to the left. The normal animals used to initiate breeding in *A* and *B* were CBA/Cum stock. *, Some of the series 4 outcross progeny of δc, δd were mixed at weaning with the series 4 outcross progeny of δa, δb.

CBA/OCI mice were injected intraperitoneally, within 24 hr of birth, with 50×10^6 spleen and 50×10^6 bone marrow cells of F_1 mice as described (7–9). All recipients received 50×10^6 F_1 lymphoid cells at 2-week intervals to maintain a state of chimerism.

Breeding Program. At 8 weeks of age, 10 tolerant males were selected at random and each was mated to three normal female CBA/Cum mice. During a 6-month breeding period, several series of age-matched offspring (designated "Tol-progeny" and never deliberately exposed to F_1 cells) were produced (see Fig. 1*A*). Spleen cells of these animals were routinely tested at 6–8 weeks of age in CML assays.

Early in the breeding program, some of the Tol-progeny from A/J-tolerant fathers 3, 5, 7, and 10 were allowed to inbreed by brother × sister matings (incross transmission breeding cage) or by mating to normal control CBA/Cum mice (outcross transmission breeding cage). CML responsiveness to A/J antigens in these parental mice was determined retrospectively by assaying their spleen cells at 31 weeks of age (after they produced the series 6 second-generation progeny; see Fig. 1*B* for details).

CML Assay Test for CTL. Responder spleen cells (1×10^6) were stimulated for 5 days in tissue culture with 5×10^5 irradiated [1500 rads (15 grays)] A/J, C57BL/6J, or B10.A (2R)

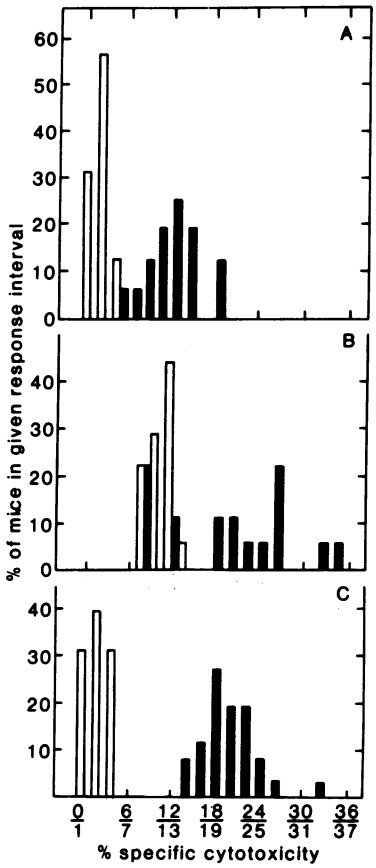

FIG. 2. Distribution of cytotoxic responses produced by normal F_1 or CBA/Cum mice and neonatally tolerized CBA/OCI mice given repeated injections of F_1 cells. These data show responses to A/J antigens (open columns) and C57BL/6 antigens (solid columns) in 16 normal F_1 mice (*A*) and 18 normal CBA/Cum mice (*B*) (6–8 weeks of age) and in 26 CBA mice given F_1 lymphoid cells neonatally and at 2 week intervals thereafter (*C*). The mice in this last group include 5 of the original F_1-tolerant males (numbers 1, 5, 6, 7, and 8), which were 10 months of age at the time their spleens were tested, and 7 other males and 14 females (8 months old at the time of testing). Comparisons between any two response profiles were made by using a Mann–Whitney test (11). The |Z| statistic and corresponding *P* values for the various comparisons are as follows. Response to A/J antigens: CBA/Cum vs. F_1 tolerant-chimeric CBA mice, $|Z| = 5.59$, $P < 0.001$; CBA/Cum vs. F_1, $|Z| = 4.97$, $P < 0.001$. F_1 vs. F_1 tolerant-chimeric CBA mice, $|Z| = 2.07$, $P \approx 0.05$. Response to C57BL/6J antigens: CBA/Cum vs. F_1 tolerant-chimeric CBA mice, |Z| 0.05, $P > 0.1$; CBA/Cum vs. F_1, $|Z| = 2.71$, $P < 0.01$; F_1 vs. F_1 tolerant-chimeric CBA mice, |Z| 5.05, $P < 0.001$.

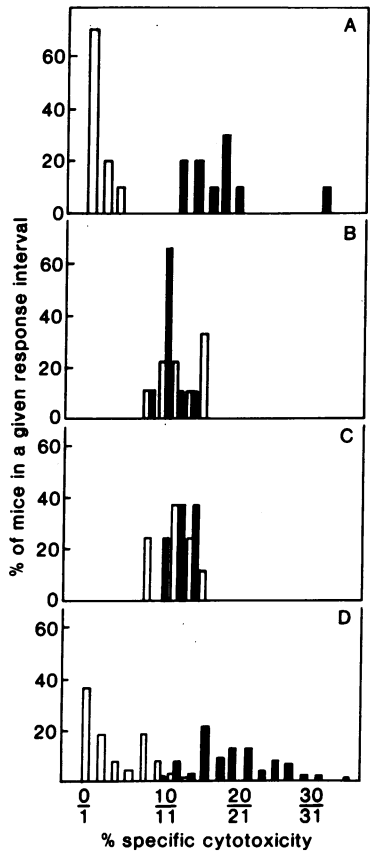

FIG. 3. Cytotoxic responses of spleen cells from 10 normal F_1 (*A*), 9 CBA/Cum (*B*), or 8 CBA/OCI (*C*) mice and 63 first-generation Tol-progeny (*D*). These data were obtained from the Tol-progeny of series 4, first generation (Fig. 1*A*). All mice were tested at 6–8 weeks of age. Open columns, response to A/J antigens (left of interval); solid columns, response to C57BL/6J antigens (right of interval). Response to A/J antigens: CBA/Cum vs. Tol-progeny, $|Z| = 5.06$, $P < 0.01$; CBA/Cum vs. CBA/OCI, $|Z| = 0.48$, $P > 0.1$; CBA/Cum vs. F_1, $|Z| = 3.67$, $P < 0.01$; F_1 vs. Tol-progeny, $|Z| = 2.3$, $P < 0.02$. Response to C57BL/6J antigens: CBA/Cum vs. Tol-progeny, $|Z| = 4.62$, $P < 0.01$; CBA/Cum vs. CBA/OCI, $|Z| = 2.07$, $P \approx 0.05$; CBA/Cum vs. F_1, $|Z| = 3.27$, $P < 0.01$; F_1 vs. Tol-progeny, $|Z| = 1.9$, $0.05 < P < 0.1$.

spleen cells (7, 8, 10). The recovered cells were tested at varying dilutions in a 4-hr ^{51}Cr release assay with 1×10^4 ^{51}Cr-labeled concanavalin A-induced blasts of stimulator cell genotype [A/J, C57BL/6J, or B10.A (2R)]. Except where stated, the specific cytotoxicity shown for any target represents an effector/target cell ratio of 3:1.

Statistics. During this study it became clear that both our normal and test mice displayed individual variability with respect to the magnitude of their cytotoxic responses. The data presented below represent response profiles—i.e., histogram plots of the frequency of mice in either a group of normal or Tol-progeny displaying a CML response (% specific cytotoxicity) of a given magnitude. Mann–Whitney ranking tests were performed on control and test data and the |Z| statistic (11) and probability (*P*) were calculated.

We have operationally defined a hyporesponsive state to A/J as a level of specific cytotoxicity <1 SD away from the mean control CBA level and a complete tolerant state to A/J antigens as a level of specific cytotoxicity within 1 SD of the control F_1 anti-A/J response.

RESULTS

At the outset we decided to follow various guidelines thought to be important in this type of experiment (see ref. 5, pp. 40–41). First, to avoid the possibility of maternal transmission of specific suppressor cells or factors via the placenta or milk, only the transmission of tolerance from male mice mated with normal female mice was studied. Second, to ensure that the tolerant breeding fathers were exposed continuously, rather than merely neonatally, to F_1 antigens, we gave regular injection of F_1 cells at 2-week intervals. Both for historical purposes and because these strain combinations consistently produce operational allograft tolerance, we used Medawar's classical combination of $(CBA \times A/J)F_1 \rightarrow CBA$ (6).

The pedigree diagram summarizing this study (Fig. 1) indicates that tolerance to A/J antigens, initially induced in male CBA mice, appeared at high frequency in both the first- and

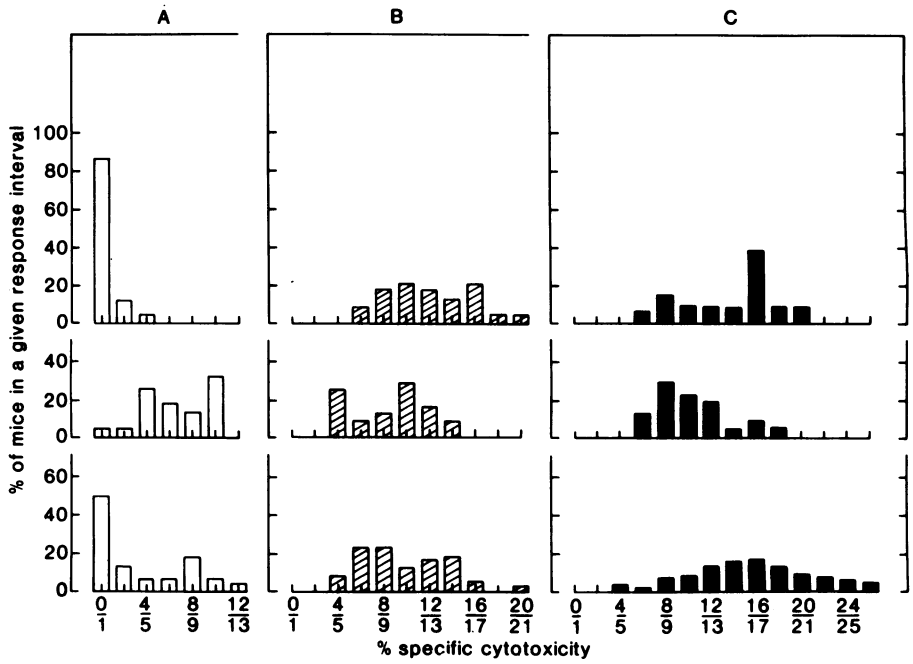

FIG. 4. Cytotoxic responses produced by 35 normal F_1 (*Top*) or 32 CBA/Cum mice (*Middle*) and 83 first-generation of Tol-progeny (*Bottom*). These data were obtained from the Tol-progeny of series 2 first generation (Fig. 1*A*). See legend to Figs. 2 and 3 for further details. CTL responses were measured to A/J (*A*), B10.A (2R) (*B*), and C57BL/6J (*C*) antigens. Response to A/J antigens: CBA/Cum vs. Tol-progeny, $|Z| = 4.27$, $P < 0.001$; CBA/Cum vs. F_1, $|Z| = 6.74$, $P < 0.001$; F_1 vs. Tol-progeny, $|Z| = 3.37$, $P < 0.001$. Response to B10.A (2R) antigens: CBA/Cum vs. Tol-progeny, $|Z| = 1.07$, $P > 0.1$; CBA/Cum vs. F_1, $|Z| = 3.24$, $P < 0.002$; F_1 vs. Tol-progeny, $|Z| = 2.94$, $P < 0.01$. Response to C57BL/6J antigens: CBA/Cum vs. Tol-progeny, $|Z| = 4.89$, $P < 0.001$; CBA/Cum vs. F_1, $|Z| = 3.20$, $P < 0.002$; F_1 vs. Tol-progeny, $|Z| = 1.47$, $P > 0.1$.

second-generation offspring originating from matings of these animals with normal females.

CML Response Phenotype of Parental and First-Generation Mice. The data of Figs. 2 and 3 show the CML response phenotype (with A/J or C57BL/6 as antigen) of normal F_1 animals, of normal and tolerant CBA animals used in the breeding program, and of 63 first-generation Tol-progeny [these animals represent series 4 (first generation) of Fig. 1*A*].

The anti-A/J CML response profile (open columns, Fig. 3) of the Tol-progeny resembled the response profiles of F_1 mice and of CBA mice made tolerant neonatally (Fig. 2); 60% of the Tol-progeny gave no detectable anti-A/J cytotoxic response. Tolerance in both parents (Fig. 2) and offspring (Fig. 3) was specific in that the anti-C57BL/6J cytotoxic response (solid columns) was not suppressed. The data of Fig. 3 also show that the anti-A/J and anti-C57BL/6J response profiles of CBA/OCI mice (bred in cages alongside the Tol-progeny) were not significantly different from those of CBA/Cum mice (see also Fig. 5).

The cytotoxic responses of normal CBA/Cum mice and Tol-progeny mice for two H-2 D-end disparate stimulator cell populations [A/J or B.10A (2R)] was investigated (Fig. 4). Although cytotoxicity was lower than for the H-2 (k + d) disparate stimulator C57BL/6J, the Tol-progeny nevertheless responded normally to B.10A (2R) antigens and not to A/J antigens. Thus, the tolerance phenotype of the individual progeny mice is exquisitively specific for H-2D^d and is independent of the quantitative level of cytotoxicity measured (Table 1).

Transmission of A/J-Specific Tolerance to the Second-Generation. We investigated whether the A/J-specific tolerance of first-generation Tol-progeny could be transmitted to the second generation (Figs. 1*B* and 5) in both incrosses (Tol-progeny × Tol-progeny) and outcrosses (Tol-progeny × normal CBA/Cum). For outcross progeny, 20% were tolerant and 34% were hyporesponders after stimulation with A/J antigens; for incross progeny the corresponding values were 7 and 40%. Thus, about 50% of these second-generation mice were hyporesponders or tolerant to A/J antigens (specificity being assessed by using stimulation by C57BL/6J antigens) (Fig. 5). Typical data used in the construction of the pedigree diagram (Table 1) indicate that the phenotype (tolerant, hyporesponder, normal) assigned to the mice is independent of the level of cytotoxicity assayed.

DISCUSSION

The approach to genetics investigated in this paper is unorthodox—modifications acquired somatically during the lifetime of an animal are not expected to undergo hereditary transmission (Weismann's doctrine, 5). We have shown, however, that a neonatally acquired and actively maintained state of antigen-specific tolerance to foreign H-2 antigens in male CBA mice is transmitted to a high proportion (50–60%) of first-generation offspring. All 10 of the A/J tolerant males produced such progeny [although there was some variation among the fathers in the efficiency of transmission (Fig. 1*A*)]. Further breeding (incrossing and outcrossing) from first-generation mice showed that a high proportion (≈50%) of second-generation animals were also specifically tolerant or hyporesponders to the original antigen. This specific tolerant state was transmitted in the absence of further exposure to F_1 cells and presumably represents the relatively stable inheritance of a trait derived from the original parental CBA male. More recent studies have demonstrated a similar transmission of tolerance (to B10.D_2 or B10.BR antigens) in B10 male animals exposed neonatally to foreign lymphocytes (unpublished data).

If a genetic mechanism is responsible for the phenomenon observed, it requires a process whereby somatic genes (normal or mutated) enter the germ line. A hypothetical scheme based on two current biological theories has been discussed elsewhere (5). (*i*) The clonal selection and somatic mutation theory of antibody (idiotype) diversity [see Burnet (12) and others (13–19)]. (*ii*) Temin's protovirus and provirus hypotheses on the origin and biological significance of vertebrate type C RNA tumor viruses (20–22). In this scheme, clonal expression and somatic selection of a given gene copy (e.g., RNA sequences) enhances the probability of its capture by endogenous RNA virus particles, leading ultimately to integration of somatic RNA gene copies (via reverse transcriptase) into germ-line DNA.

Two additional points need consideration. First, what is the nature of the "message" transmitted by tolerant male animals?

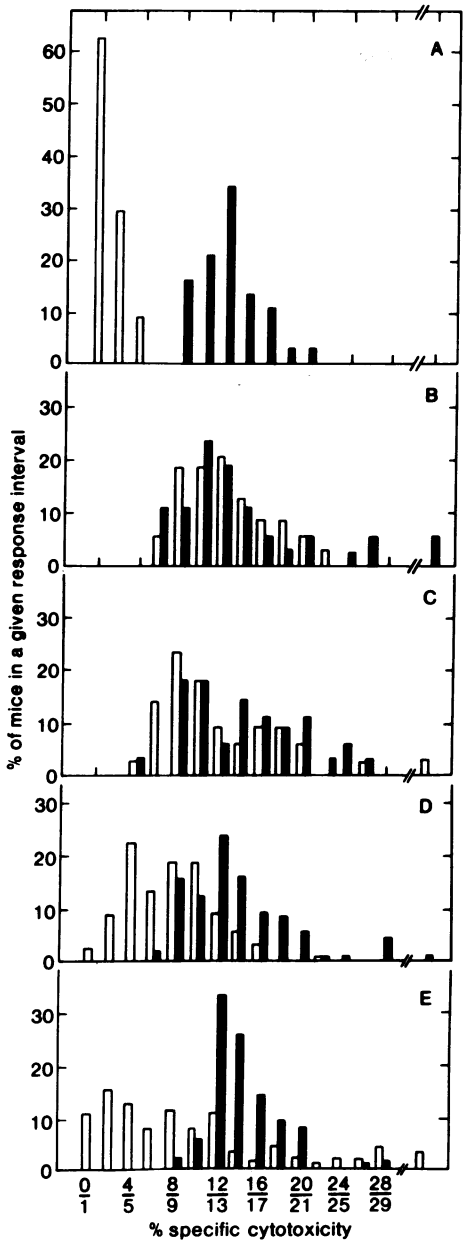

FIG. 5. Cytotoxic responses produced by 38 normal F_1 (*A*), 38 CBA/Cum (*B*), or 35 CBA/OCI (*C*) and 89 incross (*D*) or 87 outcross (*E*) second-generation Tol-progeny. These data were obtained from the Tol-progeny of the second generation in Fig. 1*B*. More details are given in the legends to Figs. 2 and 3. Included in the 89 incross progeny are 76 mice from the incross breeding cages of Fig. 1*B* and 13 additional mice from a cage containing δ_j, φ_h, and φ_i, (see series 1 in Fig. 1*A*). Response to A/J antigens: CBA/Cum vs. outcross progeny, $|Z| = 2.93$, $P < 0.01$; CBA/Cum vs. incross progeny, $|Z| = 5.18$, $P < 0.01$; CBA/Cum vs. CBA/OCI, $|Z| = 0.8$, $P > 0.1$; CBA/Cum vs. F_1, $|Z| = 7.5$, $P < 0.001$; CBA/OCI vs. outcross progeny, $|Z| = 2.24$, $P < 0.05$; CBA/OCI vs. incross progeny, $|Z| = 3.89$, $P < 0.001$. CBA/OCI vs. F_1, $|Z| = 7.34$, $P < 0.001$; incross progeny vs. outcross progeny, $|Z| = 1.63$, $P > 0.1$; F_1 vs. outcross progeny, $|Z| = 6.97$, $P < 0.001$; F_1 vs. incross progeny, $|Z| = 8.51$, $P < 0.001$. Response to C57BL/6J antigens: CBA/Cum vs. outcross progeny, $|Z| = 2.42$, $P < 0.02$; CBA/Cum vs. incross progeny, $|Z| = 0.94$, $P > 0.1$; CBA/Cum vs. CBA/OCI, $|Z| = 0.98$, $P > 0.1$; CBA/Cum vs. F_1, $|Z| = 0.09$, $P > 0.1$; CBA/OCI vs. outcross progeny, $|Z| = 0.13$, $P > 0.1$. CBA/OCI vs. incross progeny, $|Z| = 0.69$, $P > 0.1$. CBA/OCI vs. F_1, $|Z| = 1.65$, $P \approx 0.1$; incross progeny vs. outcross progeny, $|Z| = 1.86$, $0.1 < P > 0.05$; F_1 vs. outcross progeny, $|Z| = 3.35$, $P < 0.001$; F_1 vs. incross progeny, $|Z| = 1.41$, $P > 0.1$.

This could be genetic information involved in the active regulation of tolerance in these males (e.g., see refs. 7–9) or genes coding for expression of the MHC antigens (H-2D^d) of the F_1 cells coexisting within these animals [perhaps as seen earlier by Kanazawa and Imai (23)]. Second, of 219 first-generation mice tested, 44% were tolerant and 29% were hyporesponders to A/J antigens; in the second generation (176 mice), the corresponding values were 13 and 40%. Perhaps the "germ-line" stability of transmitted information depends upon continued exposure to the inductive stimulus.

Irrespective of the answer to these questions, we believe that the data document that a specifically acquired somatic characteristic (tolerance to MHC antigens) can be transmitted, via male mice, to subsequent generations at high frequency.

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