Is acquired immunological tolerance genetically transmissible?
(tetraparental/parabiont/Lamarch/histocompatibility)

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ABSTRACT We have attempted to verify that acquired characteristics can be transmitted through the male germ line by using as a model system the vertical transmission of specific immunological tolerance to major histocompatibility antigens. Tolerant males were a tetraparental mouse and separated parabionts, in each case showing stable lymphoid chimerism. Tolerance in the progeny was assessed by two in vivo assays, rejection of cardiac allografts and clearance of 125I-labeled tumor cells. We were unable to find evidence for heritability of the tolerant state in tetraparental or parabiont males, with either assay system.

The recent work of Gorczynski and Steele (1, 2) has indicated that acquired tolerance to major histocompatibility antigens can be vertically transmitted through the male germ line for at least two generations, a finding which appears to violate Weisman's doctrine of the separation of soma from germ line. As a possible mechanism for this phenomenon, Gorczynski and Steele proposed a variant of Temin's hypothesis (3, 4) concerning the possible role of type C RNA viruses in the transduction of genetic material.

In view of the great importance of such a finding, if verified, we have attempted to reproduce this phenomenon with a somewhat different system. In our protocol, the tolerant male was a C3H/HeJ × BALB/cCr tetraparental mouse and was mated to normal C3H/HeJ females. The homozygous C3H/HeJ progeny were subsequently tested for tolerance of H-2d major histocompatibility antigens by their ability to reject BALB/cCr fetal heart grafts and by the kinetics of clearance of 125I-labeled L1210 leukemia cells (5).

In this paper we report that the first-generation progeny were indistinguishable from normal age-matched homozygotes in their first-set rejection of cardiac allografts and in their ability to be primed for immune clearance of tumor bearing the relevant antigen in vivo. Similar results were obtained in limited studies on heart graft rejection by the progeny derived from mating separated parabiont males with normal females. Thus, these results place strict limits on the general applicability of Gorczynski and Steele's theory.

MATERIALS AND METHODS

Mice. C3H/HeJ, DBA/2J, and BALB/cCr mice were obtained from the Small Animal Breeding Program (The University of Alberta, Edmonton, Canada). Plugged female mice of strains C3H/HeJ and BALB/cCr and pseudopregnant females of strain ICR were obtained from the same source.

Tetraparental Chimeras. Tetraparental mouse chimeras were made according to standard procedures (6). Briefly, eight-cell-stage embryos of strains C3H/HeJ and BALB/cCr were obtained by flushing the oviducts and proximal uterine segments of female mice on the second day of pregnancy (day of plugging = day 0). The zona pellucida of the recovered embryos were removed by brief exposure to acidic Tyrode's solution, pH 2.5, and pairs of embryos were aggregated in drops of Whitten's WK-14 medium under a layer of mineral oil. After in vitro culture for 24–36 hr, mosaic blastocysts were transferred surgically to ICR foster mothers on the second day of pseudopregnancy. On day 19 of gestation, the mothers were killed, and the young were delivered by Caesarian section. Cannibalism was sharply reduced by using lactating ICR females as foster mothers for the litters.

Parabiont Mice. Adult male mice of strains DBA/2J and (C3H/HeJ × DBA/2J)/F1, were surgically parabionted by suturing the wound margins of opposed longitudinal incisions in the skin of the lateral thoracic and abdominal walls (7). After approximately 3 months of parabiosis, the animals were surgically separated under penthrane anesthesia, and the skin wounds were closed by wound clips.

Fetal Heart Grafting. Seventeen-day BALB/cCr or C3H/HeJ fetal hearts were implanted subcutaneously in pockets at the base of the external ears of the recipient mice (8). Graft acceptance was assessed at various times in anesthetized animals by electrocardiography, using difference electrodes inserted through the pinnae. This technique, developed by K. G. Pearson (Department of Physiology, The University of Alberta), allows recording of the electrical activity of the graft without interference from the host heart. Spontaneous electrical activity could be detected in grafts by 7–10 days; in case of rejection, no impulses could be recorded after 14–17 days.

RESULTS

Experimental Design. During the course of other studies, we identified a male C3H/HeJ ↔ BALB/cCr tetraparental mouse which, despite an overall chimerism of (BALB/C3H, 80:20), showed a pure C3H germ line as assessed by mating with BALB/cCr females. All of the 58 progeny of these test matings were agouti. This animal, which is most likely an XX/XY ch-
The results of parabiont matings gave comparable results, with similar kinetics of rejection for C3H, H2K allografts (Fig. 2).

**Tumor Clearance.** BALB/cCr animals showed slow clearance of the L1210 leukemia, consistent with failure of rejection. Indeed, all animals eventually died from the tumor. By contrast, C3H and C3H TOL animals showed a rapid loss of label, consistent with second-set (accelerated) rejection. The slopes of the clearance lines for normal and putatively tolerant animals did not differ from each other (Fig. 3), arguing against even partial tolerance to H-2d antigens or a deficit in priming.

Thus, the ability to mount both first- and second-set allograft responses was not impaired in the progeny of the mating of tetraparental tolerant male with a number of normal females, compared to normal syngeneic animals. Similar findings with respect to first-set responses were obtained in matings with the parabiont males.

**DISCUSSION**

The work of Gorczynski and Steele (1, 3) has a substantial precedent in the studies by Gutmann et al. (11) and by Kanazawa and Imai (12), the former group having studied the heritability of induced immunological tolerance and the latter, that of foreign H-2 antigens. Further evidence in support of genetic fixation of environmental influences comes from the classic studies by Evans and his collaborators who described nuclear changes associated with induced phenotypic changes in the flax *Stormont cirrus* (13).

All of these results are consistent with a Lamarckian interpretation, yet the immunological studies leave unanswered cer-
tain key points—chiefly, technical aspects of tolerance induction and assay and the mechanisms by which induced changes become fixed in the germ line.

Our own studies reported here give a contrary view—namely, that the H-2 tolerant state in stable tetraparental mouse chimeras is not heritable, despite the fact that chimerism extends throughout most tissues of the body. None of the progeny of the tetraparental matings were tolerant of BALB/cCr MHC antigens, as measured by prolongation of heart allograft survival. Although one of the 18 putatively tolerant progeny had a weakly functioning graft at 14 days (but not by 17 days), we do not consider this evidence of significant hyporesponsiveness ($P > 0.1$ by the 4-fold table test). Similarly, the kinetics of rejection of the L1210 leukemia were identical for tolerant and control animals and were characteristic of second-set (accelerated) rejection.

Our parabiont matings studies also clearly show that heritable tolerance cannot be fixed in the germ line of male animals made tolerant by parabiosis to semiallogeneic partners. No evidence was found among these animals for variation in the ability of different males to transmit tolerance, as reported initially by Gorczynski and Steele (1) and claimed by Steele (14) in the data of Brent et al. (15).

It is possible that differences in experimental conditions may account for the discrepancy between our results and those of Gorczynski and Steele. The time relationship for tolerance induction, and perhaps its mechanism, is strikingly different in tetraparental as opposed to neonatally tolerant animals, and it may be postulated that, unlike the active tolerance of the Billingham and Brent model (16), the passively acquired tolerance of the tetraparental mouse is incapable of being fixed in the germ line. If, as suggested by Steele (17), environmental pressure is of particular importance in forcing genetic transduction, it may be supposed further that the repeated injections of semiallogeneic cells used by these authors to maintain the chimeric state (1, 2) would tend to favor this process. Nonetheless, the work of Guttman et al. (11) suggests that a transmissible state of tolerance can be induced by a single injection of tolerizing cells immediately after birth, although this tolerance tended to wane with time. The means by which tolerance is assessed is also of importance. The Guttman et al. studies used direct in vivo assays of skin graft acceptance and tumor take (10); Gorczynski and Steele's work relied on a more indirect criterion, the ability to generate cytoxic T lymphocytes against the putatively tolerated H-2 type in vitro (1, 2), an assay which has been claimed to correlate with in vivo graft rejection (18). In the present study, we used in vivo assays similar to Guttman and co-workers' although more accurately quantifiable, which allow us to discriminate between first-set rejection and the ability to be primed for accelerated rejection of a second graft. Our results with both assays argue strongly against the possibility of soma → germ-line transmission of tolerance, at least in our experimental system.

Since the preparation of this manuscript, Brent et al. (17) and McLaren et al. (19) have reported results similar to ours, with neonatally tolerant and tetraparental male mice, respectively.

The Brent et al. paper (15) is significant in that it includes a direct comparison of the in vitro cytotoxic T lymphocyte and in vivo skin graft rejection assays, obtaining concordant and negative results in both systems. It therefore seems unlikely that our failure to reproduce the Gorczynski and Steele (1, 2) results is a reflection of our assay systems. Similarly, McLaren et al. (19) found no evidence for vertical transmission of immunological hyporesponsiveness in breeding studies with tetraparental males, as assayed by the in vitro generation of cytoxic lymphocytes.

Thus, the work suggesting that acquired tolerance can be transmitted through the male germ line does not appear to represent a general phenomenon. Our results, together with those in other recent reports, place apparently strict limits on the conditions under which it can be observed, particularly with physiologically meaningful assay systems.

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