Brief Definitive Report

CELLULAR BASIS OF NEONATAL INDUCTION OF IN VITRO TOLERANCE*

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The differential function of LD (L determinant) and SD (S determinant) antigens of the major histocompatibility complex (MHC) in stimulation of proliferation in mixed leukocyte culture (MLC) and in acting as targets in cell-mediated lympholysis (CML), respectively, has been recently reviewed (1). Whereas the basis of this differential function is not understood, it is clear that collaboration between two subpopulations of T lymphocytes (proliferating helper cells and cytotoxic T lymphocytes) responding to LD and SD antigens, respectively, functions in the optimal generation of cytotoxicity in CML (1–4).

Whereas it has been clearly established that the SD antigens, or the phenotypic product of genes very closely linked to those determining the SD antigens, are the prime targets for cytotoxic T lymphocytes (1), several groups (1, 5–9) have recently demonstrated that significant cytotoxicity can also be associated with LD differences. Although it is not clear that the LD antigens themselves are the target for the CML reaction (1), for the sake of simplicity we shall assume this to be the case. Since it has already been demonstrated that MLC tolerance (presumably of the helper cell population) can be induced neonatally (10–13), and since development of CML against the SD antigens is intimately linked to the helper cell response to LD antigens, the present study was designed to test whether neonatally induced CML tolerance (12, 13) is due to tolerance of helper cells or tolerance of cytotoxic T lymphocytes. In addition, we investigated the relative ease of inducing CML tolerance against SD- and LD-target antigens.

Materials and Methods

The following mouse strains were used (the *H-2* genotype, listing the *K, I-A, I-B, I-C, S,* and *D* regions, is given in each case): B10.T(6R) *(qqqqqd),* AQR *(qkkddd),* B10.A *(kkkddd),* B10.S *(ssssss),* and C57BL/10 *(bbbbbb).* Strains AQR and B10.A are referred to as SD different and as identical for the strong LD antigens coded in the *I* region although they may differ for weak LD-like antigens coded in the *K* region (1). AQR and B10.T(6R) are LD different for the strong *LD* locus in the *I* region and are SD identical. The mice studied were maintained in our own colony.

Induction of Tolerance. Newborn B10.A mice were injected with 1×10^7 AQR spleen cells i.v. into the sinus orbitalis to provide an SD stimulus; B10.T(6R) newborns were immunized with the same dose of AQR cells to give an LD stimulus. The treated mice were sacrificed 10–16 wk later,

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and their spleen cells were stimulated in vitro as previously described (14). Both MLC proliferation and cytotoxicity on various targets were measured 5 days after initiation of culture. The activity of cells from the neonatally immunized animals was compared with that of normal spleen cells from animals of the same strain. We shall use the term "tolerance" when a marked reduction in immunological reactivity in vitro is seen in adult life after neonatal administration of cells; our interest in this study is related to understanding tolerance at the cellular level rather than in vivo where tolerance is usually tested.

Results

Role of SD Antigens in Tolerance Induction. Spleen cells from B10.A mice which had been injected neonatally with AQR cells (an SD stimulus) were stimulated in vitro with the same SD stimulus alone (AQR X-irradiated cells), as used in tolerance induction, or with the same SD antigen plus an LD antigen to which the mice had never been previosly exposed (B10.T(6R) X-irradiated cells) (see Table I). The neonatal treatment with cells differing by SD antigens leads to tolerance (unresponsiveness) upon restimulation in adulthood. That is, there is a marked decrease in the ability of the animals to generate a CML response to the tolerizing SD antigens whether these antigens are presented alone (stimulation with AQR) or in association with a strong allogeneic LD stimulus (stimulation with B10.T(6R)). Tolerance induction was specific in that the tolerant cells responded well to third party cells (B10.S or C57BL/10). It is also clear from Table I that the proliferative response toward B10.T(6R) stimulators remains intact. On the other hand, the MLC response toward AQR cells (used as the tolerogen) was abrogated. These data, in a tolerance induction system, support the dichotomy of subpopulations responding to SD and LD antigens. Neonatal treatment with SD antigens above leads to CML tolerance (elimination of CTL precursors) against those antigens, but retains proliferative helper cell (PHC) activity intact against LD antigens not included on the neonatally administered cells as in the case of stimulation in adult life with B10.T(6R). Stimulation of "tolerant" cells from B10.A animals with AQR cells demonstrates tolerance in both MLC and CML. The MLC reactivity in this

TABLE I
Tolerance Induced by SD

	CML			MLC	
	Targets			cpm	SI
	B10.T(6R)	AQR	C57BL/10		
Normal					
B10.A + B10.A$_x$				5,126 ± 506	
B10.A + B10.T(6R)$_x$	39 ± 2	36 ± 6		16,665 ± 737	3.25
B10.A + AQR$_x$	24 ± 6	24 ± 5		10,292 ± 1,116	2
B10.A + C57BL/10$_x$			60 ± 5	41,357 ± 3,019	8
Tolerant					
B10.A + B10.A$_x$				3,637 ± 94	
B10.A + B10.T(6R)$_x$	−1 ± 2	−2 ± 5		17,659 ± 1,467	4.8
B10.A + AQR$_x$	−9 ± 2	4 ± 4		3,603 ± 945	0
B10.A + C57BL/10$_x$			58 ± 4	59,661 ± 9,437	16

B10.A *(kkkddd)*; B10.T(6R) *(qqqqqd)*; AQR *(qkkddd)*; C57BL/10 *(bbbbbb)*.
MLC and CML responses of cells taken from normal B10.A mice and from "tolerant" B10.A mice that were injected neonatally with cells from strain AQR. SI refers to the stimulation index. x refers to X-irradiated.

strain combination is presumably attributed to weak LD differences associated with the *K* region.

Failure of LD Antigens to Effectively Induce Tolerance. Adult spleen cells from B10.T(6R) mice immunized neonatally with AQR cells and stimulated in vitro with either AQR or B10.A X-irradiated cells were tested and compared to the response of spleen cells from normal animals (see Table II). Although it has been demonstrated that significant CML is associated with differences for the central regions of *H-2* (i.e., LD differences) (5–9), the level of CML generated in normal adult spleen cells given no SD differences is sometimes rather low. Presented in Table II are the results of an experiment in which relatively very high level CML associated with LD differences was induced in the normal adult spleen cells. Obviously, neonatal administration of the AQR cells led to no tolerance in terms of reduced reactivity of the adult cells. In four other experiments the percent CML on AQR target cells when normal adult B10.T(6R) cells were stimulated in vitro with AQR cells varied from 6 to 23%. Neonatal administration of AQR cells did not lead to a significantly decreased CML against the AQR target cells in any of these cases either. The results thus indicate that no significant decrease in CML reactivity "against the LD antigens" has taken place. In addition, no significant reduction of the cytotoxic activity was detected when B10.T(6R) "AQR-tolerant" cells were stimulated with B10.A X-irradiated cells (providing the same LD differences as the tolerogen plus an SD stimulus to which the mice had never been previously exposed). This failure to induce tolerance on the cytotoxic level by a neonatal LD stimulus in 12 different experiments (in 7 of which only B10.A cells were used as adult stimulating cells and in 5 AQR and B10.A cells were tested after neonatal administration of AQR cells) was in contrast to the marked reduction in the MLC proliferative activity toward B10.A and AQR cells in the 8 experiments of this series in which the MLC response was examined (data not shown).

Discussion

The findings in this paper suggest that SD antigens are highly effective at inducing tolerance in the cytotoxic T lymphocytes which can recognize those SD antigens as CML targets. In contrast, the LD antigens that appear to function as tragets in "LD-induced" CML are relatively ineffective at inducing CML tolerance. In confirmation of the findings of others (10–13), the results in all of the strain combinations indicate that neonatal administration of cells differing by

TABLE II
Failure to Induce Tolerance by LD

	B10.A	AQR
Normal		
B10.T(6R) + B10.A$_x$	67 ± 10	28 ± 6
B10.T(6R) + AQR$_x$	17 ± 1	33 ± 4
Tolerant		
B10.T(6R) + B10.A$_x$	66 ± 6	21 ± 4
B10.T(6R) + AQR$_x$	19 ± 2	33 ± 4

Genotypes: B10.T(6R) *(qqqqqd)*; B10.A *(kkkddd)*; AQR *(qkkddd)*.
CML reactivity of cells from normal B10.T(6R) animals and cells from B10.T(6R) animals injected neonatally with AQR cells, referred to as "tolerant" animals. x refers to X-irradiated.

LD antigens results in decreased reactivity in adult life to these LD antigens in MLC.

Since the optimal development of CML is in some measure dependent on the helper cell response to foreign LD antigens in the stimulating MLC (1), it might be argued that the decreased CML seen after neonatal tolerance induction by cells differing by the entire MHC, as in the work of others (12, 13) or in the SD-antigen-different combinations reported here, is due to "tolerance" that is induced against the LD antigens, thereby decreasing the helper effect in the final test mixture, i.e., that the cytotoxic T lymphocyte itself is not rendered tolerant. That this is not the sole explanation of the findings is indicated by the data in Table I. Cells from B10.A animals which had been injected neonatally with AQR cells and were stimulated in adult life with 6R cells (thereby providing a very strong helper signal against which tolerance was never induced), are still tolerant in their CML directed at the SD antigens. The most likely explanation of these findings is that the cytotoxic T lymphocytes themselves are rendered tolerant by the SD antigens. This does not eliminate the possibility that the decreased proliferative (helper) response to LD may not in some cases affect CML, as in the case of in vitro stimulation of B10.A cells rendered tolerant to AQR and stimulated in vitro with AQR cells.

The finding in these preliminary studies that neonatal administration of cells differing by a strong LD antigen leads to a markedly decreased MLC reactivity in adult life but to either no decrease in the ability to generate a cytotoxic response against these LD antigens points once again to the dichotomy of expression of these two types of MHC determinants. It may well be, of course, that greatly increased numbers of LD-different cells might be able to induce tolerance.

The fact that CML remains normal against AQR (with B10.T(6R) cells from animals injected neonatally with AQR) after stimulation with AQR or against B10.A after stimulation with those cells while MLC is reduced could be due to the existence of a relatively low threshold requirement of helper cell function. That is, even a reduced MLC reaction results in the proliferation of enough helper cells to allow the full CML response to occur. In fact, in this combination neonatal administration of AQR cells resulted in a marked reduction of MLC reactivity in adult life but not the elimination of such activity. Our finding of "incomplete" MLC tolerance is in contrast to the findings of others (12, 13) and could be explained in at least two ways. First, there may be a need for LD-SD collaboration in optimizing MLC-tolerance induction. Others have tested only strains differing by LD and SD antigens. Second, the difference could perhaps be due to the more sensitive MLC culture technique (14) employed in our laboratory. We have discussed the problem of equating proliferation with helper effect previously (15).

It would appear that the proliferating helper cell (the monolayer nonadherent, Ly-1^+ cell) which is responsive to LD antigens is susceptible to tolerance induction no matter whether the LD stimulus provided at birth is given with or without an SD antigen. The cytotoxic T lymphocyte (monolayer adherent, Ly-$2,3^+$ cell) is responsible for the cytotoxicity directed in CML against the SD antigens and presumably also for the weaker cytotoxicity associated with LD

differences. The SD antigens thus function as much stronger inducing stimulus for CML than LD antigens; similarly, in terms of neonatal tolerance induction the SD antigens are highly effective at inducing tolerance, whereas the LD antigens are relatively ineffective, suggesting an innate differential ability of CTLs to respond to LD and SD antigens. Interestingly, unpublished studies by Noel Rose in which he attempted to induce tolerance in B cells toward a number of different antigens also showed that those antigens that were the best immunogens in adult life for antibody production were also the best tolerogens in neonatal life.

Summary

The LD and SD antigens of the major histocompatibility complex subserve differential roles in the induction of the proliferative phase in mixed lymphocyte culture and in the cytotoxic reaction seen in cell-mediated lympholysis. The present study suggests that they also behave differently in the neonatal induction of tolerance. SD antigens appear to induce tolerance in the cytotoxic T lymphocytes very effectively, whereas LD antigens (or the cytotoxic targets coded by genes in the *I* and/or *S* regions) are relatively ineffective in this regard. LD antigens presented neonatally are effective at inducing tolerance in the proliferating helper cells.

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References

1. Bach, F. H., M. L. Bach, and P. M. Sondel. 1976. Major histocompatibility complex antigens: differential function on T lymphocyte activation. *Nature (Lond.)*. 259:273.
2. Bach, F. H., M. Segall, K. S. Zier, P. M. Sondel, B. J. Alter, and M. L. Bach. 1973. Cell mediated immunity: separation of cells involved in recognitive and destructive phases. *Science (Wash. D. C.)*. 180:403.
3. Cantor, H., and E. A. Boyse. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of Ly^+ cells in the generation of killer activity. *J. Exp. Med.* 141:1390.
4. Wagner, H., M. Röllinghoff, and G. J. V. Nossal. 1973. T-cell mediated immune responses induced in vitro: a probe for allograft and tumor immunity. *Transplant. Rev.* 17:3.
5. Peck, A. B., and F. H. Bach. 1975. Mouse cell-mediated lympholysis assay in serum-free and mouse serum-supplemented media: culture conditions and genetic factors. *Scand. J. Immunol.* 4:53.
6. Schendel, D. J., and F. H. Bach. 1975. H-2 and non-H-2 determinants in the genetic control of cell-mediated lympholysis. *Eur. J. Immunol.* 5:880.
7. Bach, M. L., C. Grillot-Courvalin, K. S. Zier, and B. J. Alter. 1975. Genetic control of CML restimulation. *In* Histocompatibility Testing 1975. F. Kissmeyer-Nielsen, editor. Munksgaard, Copenhagen. 871.
8. Wagner, H., D. Götze, L. Ptschelinzew, and M. Röllinghoff. 1975. Induction of cytotoxic T lymphocytes against *I*-region-coded determinants: in vitro evidence for a third histocompatibility locus in the mouse. *J. Exp. Med.* 142:1477.
9. Nabholz, M., H. Young, A. Rynbeek, R. Boccardo, C. S. David, T. Meo, V. Miggiano, and D. C. Shreffler. 1975. I-region-associated determinants: expression on mitogen-stimulated lymphocytes and detection by cytotoxic T cells. *Eur. J. Immunol.* 5:594.

10. Wilson, D. B., W. K. Silvers, and P. C. Nowell. 1967. Quantitative studies on the mixed lymphocyte interaction in rats. II. Relationships of the proliferative response to the immunological status of the donors. *J. Exp. Med.* 126:655.
11. Schwartz, H. R. 1968. The mixed lymphocyte reaction: an in vitro test for tolerance. *J. Exp. Med.* 127:879.
12. Beverley, P. C. L., L. Brent, C. Brooks, P. B. Medawar, and E. Simpson. 1973. In vitro reactivity of lymphoid cells from tolerant mice. *Transplant. Proc.* 5:679.
13. Brooks, C. G. 1975. Neonatally induced transplantation tolerance: in vitro evidence supporting a clonal inactivation mechanism. *Eur. J. Immunol.* 5:741.
14. Peck, A. B., and F. H. Bach. 1973. A miniaturized mouse mixed leukocyte culture in serum-free and mouse serum supplemented media. *J. Immunol. Methods.* 3:147.
15. Schendel, D. J., and F. H. Bach. 1974. Genetic control of cell-mediated lympholysis in mouse. *J. Exp. Med.* 140:1534.