Brief Definitive Report

ALTERNATIVE PATHWAYS OF T LYMPHOCYTE ACTIVATION

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Separate subpopulations of T lymphocytes are responsive to I region encoded lymphocyte defined (LD) and K/D region encoded cytotoxicity defined (CD) determinants (1) as based on studies with sera against the Ly1 and Ly2 antigens (2, 3) as well as monolayer adsorption protocols (4). Development of a maximal allogeneic cytotoxic response in mouse is dependent on an $Ly1^+2^-$ T_h cell (Ly1) and an $Ly1^-2^+$ T_c cell (Ly2); the interactive events between these cells lead to the strong cytotoxic response (2). The concept that an Ly1 T_h cell collaborates with an Ly2 T_c cell in the generation of a cytotoxic response to H-2 encoded CD determinants has become the accepted cellular model for T lymphocyte differentiation in response to H-2 encoded alloantigens.

In view of our earlier observation that K/D differences alone between the stimulating and responding cell populations, without I region differences, can result in a cytotoxic response in vitro, we investigated whether the cellular pathway of T lymphocyte differentiation under these circumstances was identical to that described by Cantor and Boyse. We have previously published data (5) which we interpreted as consistent with the concept that the development of a proliferative response of K/D differences alone may be dependent on an Ly1,2 cell which is apparently not needed, or plays a minimal role, in the development of a proliferative response against an entire H-2 difference. The recent report by Wettstein et al. (6) studying the cellular basis of the mixed leukocyte culture (MLC) proliferative response to H-2 mutants is likewise consistent with involvement of an Ly1,2 cell. We now present data substantiating this hypothesis not only for the proliferative response which develops to K/D differences but also for the development of the cytotoxic response.

Materials and Methods

Mice. Inbred strains used in this study and maintained in this laboratory are B10.A (kkdd), B10.T(6R) (qqqd), AQR (qkdd), B10.G (qqqq), and B10.M (ffff). Lower case letters refer to the K, I, S, and D regions of the major histocompatibility complex (MHC).

MLC. Mouse spleen or lymph node cell populations are sensitized in MLC as has been described elsewhere (7).

Cell-Mediated Lympholysis. The culture conditions for the generation of cytotoxic T lymphocytes are as has been described elsewhere (8) or with the following modifications. Given numbers of responding spleen or lymph node cells are incubated in 16-mm wells (Costar 3524, Costar, Broadway, Mass.) with indicated numbers of irradiated splenic stimulating cells in 2.5-ml vol of EHAA media (Eagle-Hanks'-amino acids) containing 0.6% mouse serum and 5×10^{-5} M 2-

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TABLE I

MLC Proliferative Response of Ly Subpopulations against Selected MHC Region Differences on Stimulating Cells

AQR Responding cells treated with§	MHC Region difference of stimulator* net cpm‡		
	K	I + S	K + I + S + D
C (5) ‖	2,565	14,885	14,207
αLy1.2 + C (5)	−4	4,695	1,118
αLy2.2 + C (5)	−178	20,773	15,903
αLy1.2 + C (2.6) + αLy2.2 + C (2.6)	317	13,183	12,153
αLy1.2 + C (4.0) + αLy2.2 + C (3.6)	312	10,423	11,733

* K Region difference = B10.A; I + S = B10.T(6R); K + I + S + D = B10.M.

‡ Net cpm refers to cpm remaining after background cpm of responding cells incubated with syngeneic irradiated cells has been subtracted. Background cpm are as follows: C(5) = 2,725; αLy1.2 (5) = 1,155; αLy2.2 (5) = 1,868; αLy1.2 (2.6) + αLy2.2 (2.6) = 1,660; αLy1.2 (4.0) + αLy2.2 (3.6) = 2,608

§ Before culture AQR spleen cell aliquots are treated separately with each antiserum plus complement (C) or C alone after which they are stimulated with 5×10^5 irradiated spleen cells for 4 days.

‖ Responding cells $\times 10^5$ per well given in parentheses.

mercaptoethanol for 5 days in 5% CO_2 and air. Effector cells are combined with ^{51}Cr-labeled target cells for cell-mediated lympholysis (CML) as previously described (9). Radioactivity released into the supernate is collected by using a Skatron Supernatant Collection System (Flow Laboratories, Inc., Rockville, Md.).

Treatment of Cells with Antisera. All responding cell populations were either untreated or treated before culture with anti-Ly1.2 or anti-Ly2.2 sera plus complement or complement alone. Lymph node or spleen cells were suspended in Hank's balanced salt solution (HBSS) containing 5 mM Hepes at a concentration ranging between 20 and 50 $\times$ 10^6 cells/ml, in predetermined dilutions of antisera, incubated at room temperature for 35 min, washed once in HBSS, and resuspended in an equal volume of diluted rabbit complement and incubated at 37°C for an additional 30–40 min after which the cells were washed three times in HBSS and resuspended in media for viable cell counts and for culture. Preparation and testing of antisera and rabbit complement used have been previously described (10) except that a 1:6 dilution of complement was used in some experiments. We thank Doctors Shen and Boyse for providing these reagents.

Results

Results shown in Table I are representative of the effect of anti-Ly1 or anti-Ly2 treatment of precursor T lymphocytes involved in the proliferative response in MLC where stimulating and responding cells differ for either K or D region alone (a K region difference is shown in Table I), for the I and S regions with K/D identity or for the entire H-2 region. As demonstrated by Cantor and Boyse (2, 3), the great majority of the proliferating cells in an MLC differing for the entire H-2 complex are Ly1; these cells presumably represent the T_h cells although heterogeneity in the Ly1 population may well exist. Similar results are observed when stimulator and responder cells differ for the H-2 I region.

In marked contrast to these findings are those when stimulator and responder differ by only a K or D region difference. Under these conditions treatment with either anti-Ly1 or anti-Ly2 sera removes the great majority, and in some cases all, of the proliferating cells. These results are interpretable, as previously discussed (5), either on the basis that an Ly1,2 cell plays an important role in the generation of the proliferative response or on the basis that Ly1 T_h cells and Ly2 T_c cells are responding. Under the latter interpretation, however, one would have to argue that the Ly2 T_c cells make up the vast majority (in fact, virtually all) of the proliferating cells, however, this proliferation is totally dependent on the activation of a very small

TABLE II

$Ly1^+2^+$ Cells are not Required for CTL Development in Cultures between Completely H-2 Disparate Cells

B10.T(6R) Responding cells‡	E:T§	%CML ± SD Targets*	
		B10.T(6R)	B10.M
C Treated (2.5) ‖	40:1	−3.2 ± 4.1	49.2 ± 3.3
	20:1	NT¶	42.9 ± 3.9
	5:1	NT	31.2 ± 3.0
C Treated (1.25)	40:1	−5.5 ± 3.3	33.4 ± 2.1
	20:1	NT	31.9 ± 4.4
	5:1	NT	20.8 ± 3.2
αLy2.2 + C (2.0)	40:1	−6.1 ± 3.6	13.1 ± 3.0
	20:1	NT	13.0 ± 2.3
	5:1	NT	7.3 ± 4.2
αLy2.2 + C (1.25)	40:1	−6.0 ± 3.8	9.2 ± 2.5
	20:1	NT	10.8 ± 2.4
	5:1	NT	9.0 ± 3.1
αLy1.2 + C (1.25)	6:1	NT	2.6 ± 1.4
αLy1.2 + C (1.25) + αLy2.2 + C (1.25)	40:1	−3.4 ± 3.5	44.9 ± 4.5
	20:1	NT	38.7 ± 2.6
	5:1	NT	24.8 ± 3.6

* B10.T(6R) Target: SR = 291 and MR = 840 cpm.
B10.M target. SR = 443 and MR = 1,287 cpm.

‡ B10.T(6R) Lymph node cell aliquots are treated separately with each antiserum plus complement or complement alone after which they are stimulated with 2.5×10^6 $B10.M_x$ spleen cells per 16 mm well.

§ Effector:target cell ratio.

‖ Responding cells $\times 10^6$ in each 16 mm culture well.

¶ Not tested.

number of Ly1 T_h cells. Since mixing of the two treated populations and then adding the stimulating cells (Table I) does not restore the proliferative response, one must conclude that the T lymphocyte differentiative pathway involves an Ly1,2 cell.

Given in Table II are results of cytotoxicity studies in strain combinations that differ by the entire H-2 complex. The data presented in Table II are confirmatory of those published by Cantor and Boyse (2, 3). It should be noted that pretreatment of the responding cells with either anti-Ly1 or anti-Ly2 sera causes a very marked inhibition of the development of a cytotoxic response, however when the two populations of pretreated cells are admixed before stimulation with the entire H-2 different X-irradiated cells, a synergistic effect is noted which is presumably due to help provided by Ly1 cells (remaining in the population pretreated with anti-Ly2 sera) to the Ly-2 cells (remaining in the population pretreated with anti-Ly1 sera). Pretreatment with anti-Ly1 sera which removed Ly1 and Ly1,2 cells prevents development of a proliferative response. If such cells, i.e. Ly2 cells, are cultured alone, very low recovery is obtained at the end of 5 days. Thus in the experiment shown in Table II, the Ly2 cells cultured alone could only be tested at a 6:1 effector to target cell ratio. Clearly, even the results obtained at this one ratio indicate that a very weak cytotoxic response remains at the end of 5 days, a finding consistently observed in all experiments; in other experiments enough Ly2 cells were recovered at 5 days of culture to allow testing at several effector to target cell ratios.

Once again in contrast with these findings, are the results shown in Table III. Under these conditions responder cells are stimulated with cells differing by only the D region. Pretreatment of the responding cells with either the anti-Ly1 or the anti-Ly2 sera essentially eliminates the development of a cytotoxic response in the MLC,

TABLE III

$Ly1^+2^+$ Cells are Required for CTL Development of Cultures between H-2 D Region Disparate Cells

B10.T(6R) Responding cells‡	E:T§	%CML ± SD Targets*	
		B10.T (6R)	B10.G
Untreated (30) ‖	50:1	−3.2 ± 5.0	38.4 ± 4.8
	5:1	7.6 ± 3.1	29.1 ± 6.6
αLy1.2 + C (30)	50:1	NT¶	2.6 ± 5.2
αLy2.2 + C (30)	50:1	0.7 ± 5.8	12.8 ± 6.3
αLy1.2 + C (15) + αLy2.2 + C (15)	50:1	−2.3 ± 4.1	8.1 ± 4.3

* B10.T(6R) target: SR = 231 and MR = 1018 cpm.
B10.G target: SR = 139 and MR = 721 cpm.

‡ B10.T(6R) Spleen cell aliquots are treated separately with each antiserum plus complement or left untreated after which they are stimulated with 30 × 10^6 B10.G_s spleen cells per flask.

§ Effector:target cell ratio.

‖ Responding cells × 10^6 in each flask given in parentheses.

¶ Not tested.

however admixture of the two pretreated populations does not result in a synergistic effect.

Discussion

The results we have presented in this paper are most easily interpreted by assuming that an $Ly1^+2^+$ cell plays a pivotal role as a precursor T cell in the development of a cytotoxic response against K/D region differences in the absence of I region encoded differences on the stimulator cells. At least four different models are consistent with these results. First, it is possible that an Ly1,2 cell is the only responding cell and no cell interactions between Ly1 or Ly2 T-cell subsets are involved; alternatively, that two Ly1,2 populations interact. Second, that an Ly1,2 cell is not a precursor of the differentiated T_c but rather plays a role in controlling the response of Ly1 and Ly2 cells. Third, that an Ly1 precursor T_h cell collaborates with an Ly1,2 presursor T_c. Fourth, that Ly1,2 precursor T_h cells collaborate with Ly2 precursor T_c cells. (We have not considered the possibility of an Ly1 precursor T_c cell.)

Preliminary data show that pretreatment of the cytotoxic cells with either anti-Ly1 or anti-Ly2 sera on day 5 immediately before their use as effectors in the 3.5-h chromium release assay very markedly reduces cytotoxicity. This finding, consistent with an Ly1,2 phenotype of the effector, would argue that the T_c precursor is Ly1,2 as well. Also, to the extent that proliferation in MLC can be equated with help, the findings reported in Table I mitigate against participation by an Ly1 T_h cell in the response. We are thus inclined to favor model one of the four listed above.

Involvement of Ly1,2 cells in cytotoxic responses has been previously noted, although not in cytotoxicity directed at alloantigens. Cantor and Boyse (11) noted that an Ly1,2 cell was needed for the development of a cytotoxic response to trinitrophenyl modified syngeneic target cells, although the actual effector was Ly2. Studies of Shiku et al. (12) implicated an Ly1,2 cell in cytotoxicity against a syngeneic sarcoma; Stutman and collaborators (13) showed that effector cells against a syngeneic mammary adenocarcinoma were Ly2 although an Ly1,2 cell was probably also involved. Cantor and Boyse (11) explained the role of the Ly1,2 cell by postulating

that allorecognition is special and different from recognition of altered-self; Bach and Alter have suggested (5) that the same rules of recognition and response may well apply in the two systems. The present data demonstrating a role for an Ly1,2 cell in the development of a cytotoxic response to alloantigens (K/D region different allogeneic stimulating cells) is consistent with the notion that factors governing the cellular pathway chosen is a reflection neither of a fundamental difference between allo- and altered-self response (11) nor of the different lengths of cytotoxic assays used in different systems (13).

The evaluation of Ly phenotype of cells responsive in vitro to MHC encoded allogeneic K/D difference thus suggests that there is an alternative pathway of T lymphocyte activation in the generation of a cytotoxic response in addition to that described when stimulator and responder cells differ by an entire H-2 complex. The only factor which we understand as playing a role in the development of a cytotoxic response which is different in these two situations (stimulating with K/D differences plus an I region difference versus with K/D differences alone) is the presence or absence of I region encoded LD determinants on the stimulating cells. It is thus tempting to hypothesize that a control element which helps determine which pathway of T lymphocyte differentiation will proceed in any particular situation is the level of help which is provided by I region responsive Ly1 T_h cells. Clearly other factors, such as the strength of the CD antigenic stimulus which is presented to the responding cells, may also play a role in determining which pathway is favored. It seems most probable that under any given situation there is a balance between the quantitative role assumed by each of the alternative pathways of T lymphocyte activation.

Summary

Data presented in this paper suggest that there may be two alternative pathways which T lymphocytes can use in generating a cytotoxic response to alloantigens in vitro. First, there is the pathway taken when stimulator and responder cells differ by an entire H-2 complex where $Ly1^+2^-$ helper T lymphocytes respond to I region encoded lymphocyte defined differences and provide help to the $Ly1^-2^+$ cytotoxic T lymphocytes responsive primarily to K/D region encoded cytotoxicity defined determinants. Second, there is the pathway taken when stimulator and responder cells differ by only K or D region differences without an I region encoded difference; under these conditions, an $Ly1^+2^+$ cell, which does not appear to play a significant role in the development of a cytotoxic response to an entire H-2 difference, appears to play a pivotal role.

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References

1. Bach, F. H., M. L. Bach, and P. M. Sondel. 1976. Differential function of major histocompatibility complex antigens in T-lymphocyte activation. *Nature (Lond.).* **259:**273.
2. Cantor, H., and E. A. Boyse. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. *J. Exp. Med.* **141:**1376.
3. Cantor, H., and E. A. Boyse. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperative between subclasses of Ly^+ cells in the generation of killer activity. *J. Exp. Med.* **141:**1390.

4. 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.
5. Bach, F. H., and B. J. Alter. 1977. T lymphocyte activation by major histocompatibility antigens: the allograft reaction as a model for altered-self. *In* Immune System: Genetics and Regulation. E. E. Sercarz, L. A. Herzenberg, and C. F. Fox, editors. Academic Press, Inc., New York. p. 631.
6. Wettstein, P. J., D. W. Bailey, L. E. Mobraaten, J. Klein, and J. A. Frelinger. 1978. T-lymphocyte response to H-2 mutants. I. Proliferation is dependent on $Ly1^+2^+$ cells. *J. Exp. Med.* **147:**1395.
7. 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.
8. 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.
9. Alter, B. J., D. J. Schendel, M. L. Bach, F. H. Bach, J. Klein, and J. H. Stimpfling. 1973. Cell-mediated lympholysis: importance of serologically defined H-2 regions. *J. Exp. Med.* **137:**1303.
10. Shen, F. W., E. A. Boyse, and H. Cantor. 1975. Preparation and use of Ly antisera. *Immunogenetics* **2:**591.
11. Cantor, H., and E. A. Boyse. 1977. Regulation of cellular and humoral immune responses by T-cell subclasses. *Cold Spring Harbor Symp. Quant. Biol.* **41:**23.
12. Shiku, H., T. Takahashi, M. A. Bean, L. J. Old, and H. F. Oettgen. 1976. Ly phenotype of cytotoxic T cells for syngeneic tumor. *J. Exp. Med.* **144:**1116.
13. Stutman, O., F.-W. Shen, and E. A. Boyse. 1977. Ly phenotype of T cells cytotoxic for syngeneic mouse mammary tumors: evidence for T cell interactions. *Proc. Natl. Acad. Sci. U.S.A.* **74:**5667.