Brief Definitive Report

IN VITRO GENERATION OF CYTOTOXIC CELLS SPECIFIC FOR HUMAN MINOR HISTOCOMPATIBILITY ANTIGENS BY LYMPHOCYTES FROM A NORMAL DONOR POTENTIALLY PRIMED DURING PREGNANCY

By WILHELM A. TEKOLF and STEPHEN SHAW

From the Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Minor histocompatibility antigens are defined as antigens that elicit tissue-graft rejection in the absence of differences at the major histocompatibility complex (MHC) (1). Analysis of T cell responses to minor antigens is potentially informative for two reasons: (*a*) as one model system in which to analyze the basic mechanisms of T cell responses, and (*b*) as an in vitro model of immune responses that occur in clinical transplant recipients of tissue from an MHC-matched allogeneic donor. T cell recognition of minor histocompatibility antigens normally requires identity of a major histocompatibility antigen between responder and stimulator and an in vivo priming of the responding individual (2, 3). The best characterized human minor antigen is designated H-Y, the male-associated antigens. Goulmy and colleagues (4) first described human cell-mediated cytoxicity specific for H-Y in the blood of a female patient with aplastic anemia who had rejected a bone marrow transplant from a male HLA-identical sibling. Subsequent studies have shown that multiple transfusions are sufficient to prime for cellular responses to the H-Y antigen. Several individuals have been found to have HLA-A2-restricted H-Y-specific cytotoxic effectors, and at least one donor was also found to have a subset of HLA-B7-restricted H-Y-specific cytotoxic effectors (5). Minor histocompatibility antigens different from H-Y were first described by Parkman et al. (6); specific cytotoxic T lymphocytes (CTL) were derived from patients with aplastic anemia, who had rejected bone marrow transplants from HLA-identical siblings. Elkins et al. (7) described a patient whose lymphocytes were primed to recognize an HLA-B7-restricted minor antigen. Similar to the circumstances in H-Y priming, this patient had received repeated transfusions from HLA-identical siblings (some of whose cells expressed this minor antigen).

The present study reports and analyzes HLA-restricted cell-mediated lympholysis (CML) against a human minor antigen(s) that can be readily generated in vitro from the blood of a normal female. Since she is a multiparous female whose children are virtually HLA identical to her but express the minor antigen, it is plausible that her response reflects in vivo priming during pregnancy.

Materials and Methods

Human peripheral blood mononuclear cells were obtained by batch leukapheresis, separated by flotation on Ficoll-Hypaque, and cryopreserved as previously described (8). Plasma from six or more male donors was pooled, frozen in aliquots at −20°C, and used as the normal human

TABLE I

Cytotoxicity Generated between Cells from HLA-A, -B, -C-Identical Donors

Effectors				Targets (Percent cytotoxicity)						
Responder	HM1*	Stimulator	HM1	B17	F2	H7	H9	K4	M14	W7
S11	−	H9	−				7.2			
F1	−	B17	−	15.1						
W7	+	H9	−				3.7			
F2	+	B17	−	13.6						
W7	+	M14	−						6.1	
PM1	+	M14	−						6.7	
H7	+	K4	−					2.4		
F2	+	K4	−					12.8		
W7	+	K4	−					8.8		
K4	−	H7	+			5.7				
K4	−	W7	+							6.8
H9	−	W7	+							48.0
B17	−	F2	+		12.2					
K4	−	F2	+		2.4					
F2	+	W7	+							12.3
FC4		FR4		59.4	50.6	57.3	46.0	47.4	41.7	52.4

FC4: HLA-A2,2, -B7,7, -C-, -. FR4: HLA-A1,1, -B8,8, -C-, -. All other donors type as HLA-A1,2, -B7,8, -C7, -.

* Determined by whether target cells from these donors were lysed by H9/W7.

plasma pool. Serotyping for HLA-A, -B, -C, and -DR was kindly performed by Dr. Rene Duquesnoy, Blood Center of Southeastern Wisconsin, Milwaukee, WI.

CTL were generated as previously described (9). Briefly, 9×10^6 responding cells were cultured for 10 d with 3×10^6 2,000-rad-irradiated stimulator cells in a 25-cm^2 tissue culture flask. To generate secondary effectors primary cultures were harvested after 10 d and either cryopreserved or restimulated immediately. 3×10^6 primed cells (fresh or thawed after cryopreservation) were restimulated with 9×10^6 2,000-rad-irradiated stimulator cells in 8 ml culture medium supplemented with 1.5 ml heat-inactivated human plasma, and harvested after 4 d.

Cytotoxicity was determined in a standard 4-h ^{51}Cr-release assay (8) using 1×10^4 phytohemagglutinin (PHA)-stimulated target cells and effector to target cell ratios of 40:1, 15:1, and 4:1. CML assays were performed in triplicate and percent specific lysis was calculated as [(mean counts per minute, experimental − mean counts per minute, media)/(mean counts per minute, detergent − mean counts per minute, media)] × 100.

Results and Discussion

Detection of a CML Target Antigen by a Unique Responder/Stimulator Combination. As part of a systematic analysis of histocompatibility antigens that differ between 12 donors completely matched for the serologically defined HLA-A, -B, and -C antigens, mixed lymphocyte cultures (MLC) were generated between many combinations of these donors, restimulated in secondary MLC, and the lymphocytes tested for CTL activity with target cells from the donor used as stimulator. CTL FC4/FR4, directed against HLA-A1 and -B8, was used as control to show comparable lysability of the different target cells (Table I). Strong cytotoxicity (>20% lysis) on PHA-stimulated lymphoblasts was seen in only one combination: donor H9 anti-donor W7. Detection of this antigen by CML seemed to require secondary restimulation, because little cytotoxicity was seen after primary stimulation (data not shown). To maximize reproducibility in subsequent studies of the specificity of this cytotoxic activity, large batches of primed cells were frozen after primary stimulation, and were used as

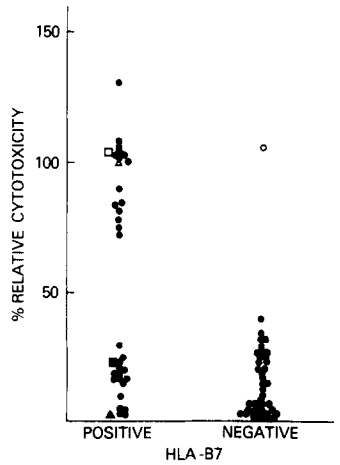

FIG. 1. Lysis of HLA-B7-positive and HLA-B7-negative donors by H9/W7, expressed as relative percent cytotoxicity compared with lysis of W7 (100%). Lysis of W7 cells in the four experiments ranged between 45 and 65% specific lysis. (▲) Donor H9 (HLA-A1,2, -B7,8, -C7,-, -DR2,3, SB1,2) (□) Donor FH3 (HLA-A1,2, -B7,8, -C7,-, -DR2,3, SB1,4) (■) Donor FH4 (HLA-1,2, -B7,8, -C7,-, -DR2,3, SB1,2) (○) Donor W1 (HLA-A23,24, -B44,-, -C4,-, -DR7,-) (△) Donor W7 (HLA-1,2, B7,8, -C7,-, -DR2,3, SB1,4) (●) all other unrelated normal donors.

cytotoxic effectors after thawing and secondary restimulation with W7 cells.

Fig. 1 summarizes the results of four experiments testing H9/W7 effectors on panels of target cells from unrelated donors. The pattern of lysis was clearly bimodal, which indicates that these effectors were recognizing predominantly the presence or absence of a single antigen or group of genetically associated antigens in the population of donors. Target cells from 17 of 35 HLA-B7-positive donors were lysed, while only 1 of 40 B7-negative donors' cells was lysed. Thus, with a single exception, the antigen was detected only on B7-positive cells. The single exception is donor W1, with the serologically defined phenotype of HLA-A23, -24, -Bw44, -. Family studies of the only available family member failed to distinguish whether this reflects homozygosity for Bw44 or the presence of a serologically undefined B locus antigen.

Family Studies Indicate Control by Gene(s) Independent of HLA. The pattern of lysis observed in the population was consistent with at least three possible specificities of the CTL: (*a*) for a molecular variation in the B7 molecule that is not yet serologically defined; (*b*) for the product of an allele of another HLA-linked gene in strong positive linkage disequilibrium with the HLA-B7 allele; or (*c*) for a human 'minor' histocompatibility antigen recognized in conjunction with an HLA antigen highly associated with B7. Studies of three generations of family B (Table II) prove that the antigen recognized by H9/W7 is controlled by at least one gene distinct from B7. The donors in the family whose cells express the cytotoxic target antigen are those that have inherited the 'a' haplotype, which encodes HLA-B7. However, one of the six members (FB12) who has inherited this haplotype does not express the antigen recognized by the CTL H9/W7; since the typing indicates clearly that FB12 inherited the B7 haplotype from his mother FB4, failure of his cells to express the CTL antigen must indicate that the B7 gene per se is not sufficient for expression of the CTL antigen. Furthermore, these data make hypothesis *b* unlikely; there has been no recombination on the B7 haplotype between HLA-Aw24 and DR4, so the postulated HLA-B7-

TABLE II

Family Studies of the Antigen Detected by CTL H9/W7

Family B

		FB2 a/b 81.9*				FB1 c/d 9.2		
				I				
FB5 b/d 10.1	FB7 a/d 49.2	FB6 b/c 6.7	FB11 ba/c 2.0	FB3 a/c 63.1	FB8 a/c 58.3	FB4 a/c 61.1‡		FB21 c/f ND§
							I	
							FB12 a/e 10.8	

a: Aw24,Cw-,B7,DR4,SB1.
b: A25,Cw-,B15,DR4,SB4.
c: Aw31,Cw3,Bw40,DR4,SB-.
d: Aw33,Cw3,B17,DR5,SB4.
b/a: A25,Cw-,B15,DR4/SB1.
e: A2, Cw5,Bw44,DRw6,SB?.
f: not determined.

Family W

	FW1 a/b 56.7	FW2 c/d 4.1
	I	
FW3(=W7) a/d 50.1	FW4 a/c 51.8	FW5 b/c 46.0

a: A2,Cw7,B7,DR2
b: Aw30,Cw5,B18,DR3
c: A3,Cw7,B7,DR2
d: A1,Cw7,B8,DR3

* Percent cytotoxicity.
‡ Percent specific lysis by an HLA-B7-specific CTL combination was 37.4% for FB4 and 36.2% for FB12.
§ Not determined.

associated allele would have to be of a gene outside this region, and therefore unlikely to show such extraordinarily strong linkage disquilibrium with B7.

The results on the second family (W), although less conclusive, are most consistent with minor antigen recognition (Table II). In this family, both parents are positive for B7, but only the father expresses the antigen recognized by H9/W7. If the target antigen were related only to expression of a particular structurally distinct subset of the B7 molecule, then only the children who inherited the B7 from the father ought to express the antigen recognized by H9/W7. Instead, all children, including one who inherited B7 from the mother and not from the father (FW5), express the antigen. A plausible interpretation of this data is that although the mother expressed an appropriate B7, she lacked the relevant allele of the minor antigen; FW5 expresses the full antigenic complex since he inherited the B7 gene from his mother and the minor antigen from his father. There is an alternate explanation: that the CTL antigen that is recognized on the father's cells is encoded by genes on both his HLA-B7 and -B18 haplotypes; however, it is statistically improbable ($P \sim 1/40$) that his

A30-B18 haplotype should encode the antigen when most B7-negative haplotypes in the population do not. Data from these two families suggest that there is another gene, provisionally designated HM1 for human minor 1, that controls the expression of the complete antigen. This antigen is not identical to the H-Y antigen because it is expressed on cells of female donors as well. The phenotype frequency for HM1, calculated from the phenotype frequency among HLA-B7-positive donors (17/35) (Fig. 1), is ~50%, which implies a gene frequency of ~30%, if the gene is expressed codominantly.

Evidence for Possible In Vivo Priming During Pregnancy. The combination H9/W7 is unusual in its ability to recognize HM1. As can be seen from Table I, four other combinations of HM1-negative cells against HM1-positive cells, e.g., B17/F2 or K2/W7, do not detect HM1. Furthermore, other potential minor antigen differences are not recognized strongly in any of the 14 other combinations of HLA-matched donors. The lower level of lysis (<20%), seen in many combinations, is consistent with Ia-specific kill on the PHA-stimulated targets (10). Is the detection of a minor histocompatibility antigen by the CTL response H9 anti-W7 really due to an unusual responsiveness of H9 or perhaps due to a uniqueness of cells from donor W7? Two additional data argue for the former. First, W7 did not stimulate cells from other donors against minor histocompatibility antigens (Table I). Second, although H9/W7 preferentially recognized only one antigen, the relative differences within the group of HLA-B7-negative donors suggest further complexity (Fig. 1). Indeed, another donor was identified, whose cells stimulated cells from H9 against at least two other minor histocompatibility antigens (manuscript in preparation). Thus, H9 is unique in the ability of her cells to recognize multiple minor histocompatibility antigens.

Studies of minor histocompatibility antigens in mice indicate that detection of these antigens normally requires both an initial in vivo priming and H-2 identity between responder and stimulator during priming. We therefore investigated whether an in vivo priming of donor H9 could account for the donor's unusual reaction against minor histocompatibility antigens. As H9 never received any blood transfusions or organ transplants, pregnancy would have been the only known allogeneic exposure. It has already been demonstrated, by detection of Y chromosome-containing lymphocytes in the blood of mothers with male fetuses, that an exchange of small amounts of white cells can occur (11). Theoretically it is likely that these cells are removed from the maternal circulation quite rapidly by humoral and cellular responses against antigens controlled by the paternal HLA haplotype of the fetal cells. Studies of available members of H9's family revealed the postulated conditions for an in vivo priming against minor histocompatibility antigens (Table I). Both children are HLA-A, -B, -C, and -DR identical with their mother. One child (FH3) is SB different and expresses the HM1 target antigen. The other child is SB identical with her mother and does not express HM1. These data not only prove that one of the children carries the relevant antigen necessary for a priming during pregnancy but also show a virtual HLA identity between mother and children, which may have provided optimal conditions for priming against minor histocompatibility antigens during pregnancy. No other unusual features of H9's pregnancies could be identified.

Although minor histocompatibility antigens are thought to play an important role in transplantation biology, our knowledge about these antigens in humans is very limited. Cells from donors like the one described in this study, i.e., in whom a priming

against minor histocompatibility antigens has occurred, will facilitate identification of "immunodominant" minor histocompatibility antigens and investigation of the biological role of these antigens.

Summary

A normal female donor (H9) is described, whose cells generate strong cytotoxicity against a human minor histocompatibility antigen in vitro. These cytotoxic T lymphocytes are generated after secondary restimulation with cells from an HLA-A, -B, -C, and -DR-matched donor and are HLA restricted (HLA-B7). No other donor could be identified whose cells responded to this antigen. The two children of donor H9 are virtually HLA identical to her and one of the children expresses the relevant minor histocompatibility antigen. These data suggest that priming in vivo during pregnancy has facilitated cytotoxic T cell response to human minor histocompatibility antigens in vitro.

Dedicated to Professor W. Doelle on the occasion of his 60th birthday.

Received for publication 28 February 1983 and in revised form 5 April 1983.

References

1. Graff, R. J., and D. W. Bailey. 1973. The non-H-2 histocompatibility loci and their antigens. *Transplant. Rev.* **15**:26.
2. Bevan, M. J. 1975. Interaction antigens detected by cytotoxic T cells with the major histocompatibility complex as modifier. *Nature (Lond.).* **256**:419.
3. Bevan, M. J. 1975. The major histocompatibility complex determines susceptibility to cytotoxic T cells directed against minor histocompatibility antigens. *J. Exp. Med.* **142**:1108.
4. Goulmy, E., A. Termijtelen, B. A. Bradley, and J. J. van Rood. 1977. Y-antigen killing by T cells of women is restricted by HLA. *Nature (Lond.).* **266**:544.
5. Goulmy, E., J. D. Hamilton, and B. A. Bradley. 1979. Anti-self HLA may be clonally expressed. *J. Exp. Med.* **149**:545.
6. Parkman, R., F. S. Rosen, F. S. Rappeport, B. Camitta, R. L. Levey, and D. G. Nathan. 1976. Detection of genetically determined histocompatibility antigen differences between HL-A identical and MLC nonreactive siblings. *Transplantation.* **21**:110.
7. Elkins, W. L., G. Pierson, W. Negendank, and K. S. Zier. 1982. Recognition of human minor histocompatibility antigen(s) by cytotoxic lymphocytes in vitro. *Immunogenetics.* **15**:485.
8. Shaw, S., D. L. Nelson, and G. M. Shearer. 1978. Human cytotoxic responses in vitro to trinitrophenyl-modified autologous cells. I. T Cell recognition of TNP in association with widely shared antigens. *J. Immunol.* **121**:281.
9. Tekolf, W. A., W. E. Biddison, R. D. Aster, and S. Shaw. 1982. Two subgroups of HLA-Bw44 defined by cell-mediated lympholysis that differ in Bw44 expression on platelets and in patterns of genetic linkage disequilibrium. *J. Immunol.* **129**:1474.
10. Shaw, S., A. H. Johnson, and G. M. Shearer. 1980. Evidence for a new segregant series of B cell antigens that are encoded in the HLA-D region and that stimulate secondary allogeneic proliferative and cytotoxic responses. *J. Exp. Med.* **152**:565.
11. Schroeder, J. 1975. Review article: transplacental passage of blood cells. *J. Med. Genet.* **12**:230.