Brief Definitive Report

ANTI-SELF HLA MAY BE CLONALLY EXPRESSED*

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In man, gene products of the major histocompatibility complex (MHC) are involved in the T-cell-mediated cytotoxicity directed to minor histocompatibility antigens. In a previous publication, we demonstrated that self HLA antigens were required to obtain cell-mediated cytotoxicity directed to the male-associated antigen, H-Y. (1, 2). Analogous restriction phenomena in man have also been shown for virus-infected target cells and for DNCB-modified target cells (3, 4).

To explain the restriction phenomenon, two hypotheses have been put forward (5). According to one hypothesis (altered self) the antigens recognized can be regarded as neoantigens created by molecular interactions between self compatible HLA-A, -B, or -C gene product and the foreign antigen. This altered self concept is a logical consequence of classical theories of immunology such as the clonal selection theory (6, 7), and this interpretation is favored by some investigators (8, 9). According to another hypothesis (dual recognition) the antigens are seen by cells which have two receptors; one recognizes self compatible HLA-A, -B, or -C, and the other recognizes the foreign antigen. This concept is supported by other authors (10–13) but at the present moment a firm choice cannot be made between these two hypotheses. Whichever is true, two aspects of the specificity of T-cell recognition in cell-mediated lympholysis (CML) are important to evaluate. First, is the anti-self or altered-self reaction clonal implying the existence of multiple populations of specific immune cells (14, 15)? Second, is the anti-self or altered-self reaction attributable to lymphocytes with polyvalent specificities as suggested by others (16)? The discovery of a case of HLA-restricted CML reactivity directed to the H-Y antigen in which the killing was restricted by a requirement either for self HLA-A2 or B7, and the development of a monolayer absorption technique by one of us (15), gave us the opportunity to answer these questions.

Materials and Methods

Lymphocytes of two female patients (patients A and B) were used. The HLA-restricted H-Y killing phenomenon as described in previous studies was demonstrated in both (2, 17). In reference 17, patient 1 is equivalent to patient A and patient B is the individual referred to as the third patient. Patients A and B were both suffering from aplastic anemia and had received multiple transfusions, a situation which severely limited our access to materials. Lymphocytes from A and B were cultured for 6 d with lymphocytes from HLA-A, -B, and -C identical, but

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HLA-D different male individuals. After this in vitro sensitization, specific anti-HLA-A2-H-Y cytotoxic cells were generated in the case of patient A (17). Patient B formed cytotoxic cells which specifically recognized HLA-A2 male target cells and HLA-B7 male target cells (Table II). We demonstrated, that the effector cells of the patients studied retained their specificities for HLA-A2 males for a long period of time and that by recalling immunological memory (using lymphocytes from HLA-D different unrelated male donors) it was possible to reinduce high levels of specific killing (17). After culturing, the sensitized effector cells were overlaid on monolayers, according to the techniques described elsewhere (15), which were based on the mouse system introduced by Brondz et al. (16). The nonadherent cells were removed and tested on a panel of target cells, which had been incubated with phytohemagglutinin for 3 d and then labeled with sodium ^{51}chromate. Cytotoxicity as exemplified in Tables I and III, was measured and calculated as a percentage according to the methods described elsewhere (17).

Preparation of the Monolayer. Monolayers were made of fresh peripheral blood lymphocytes adhered to Petri dishes treated with poly-L-lysine; the nonadherent cells were removed after 1 h at room temperature. The effector cells were overlaid and incubated for 1 h at 37°C and nonadherent cells were removed and tested (reference 15). The calculation of the depletion of cytotoxicity, referred to in the text, was calculated as a percentage of the unabsorbed population.

Results

After 6 d of in vitro sensitization, the responder cells of patient A reacted specifically against HLA-A2 males, as illustrated in Table I and in reference 1. These were absorbed on four different monolayers, which were numbered one to four and were, respectively, HLA-A2 male, non-HLA-A2 male, HLA-A2 female, non-HLA-A2 female. The nonadherent cells were removed and tested on a panel of unrelated target cells, in which the same specificities were represented.

Effector cells absorbed on monolayer 1 (HLA-A2 male) gave strongly reduced cytotoxic activity directed to all HLA-A2 male-positive target cells. The depletion varied from 47 to 64%. Absorption on monolayer 2 (non-HLA-A2 male), 3 (HLA-A2 female), and 4 (non-HLA-A2 female) showed no significant reduction in cytolysis (Table I). These results illustrated that only the appropriate monolayer, i.e. an HLA-A2 male monolayer, could absorb the specific killer cells.

Patient B lymphocytes showed cytotoxic activity against two independent phenotypes namely, HLA-A2 and HLA-B7 male target cells (Table II). Using these cells, we investigated whether it was possible to specifically absorb one clone of cytotoxic cells and leave the other intact, thus demonstrating the clonal expression of these specific killer cells. Table III showed that when effector cells recovered from monolayer 1 (HLA-A2 male) were tested on the panel of target cells, almost complete removal of the cytotoxic activity could be demonstrated against male HLA-A2 target cells (target 5–8). The converse effect was found after absorption with monolayer 3 (HLA-B7 male) and, in this case, the reaction directed to HLA-B7 males was specifically depleted. Double absorption further depleted the killer cell clones, but because of the limited amount of material available, it was not possible to perform this in all cases (E. Goulmy, unpublished results). The most effective reduction (up to 82%) in cytotoxicity was obtained with monolayer 4 (HLA-A2 and HLA-B7 male). Killing activity directed to the target cells which carried both restricting HLA antigens (HLA-A2 and HLA-B7), was only partially reduced after absorption with an HLA-A2 male monolayer (monolayer 1), or HLA-B7 male monolayer (monolayer 3). These results provide strong evidence that the anti-B7 clone could be absorbed independently of the anti-A2 clone.

TABLE I

Absorption of the Anti-H-Y; HLA-A2 Cytotoxicity

Target cell	HLA-A2	Sex	0*	Monolayers			
				1 A2 ♂	2 NonA2 ♂	3 A2 ♀	4 Non A2 ♀
			Ψ				
1	+	♂	61 ± 1.4	26 ± 3.1	61 ± 1.6	54 ± 3.7	57 ± 3.9
2	+	♂	82 ± 1.5	29 ± 1.1	80 ± 0.9	67 ± 2.6	77 ± 1.2
3	+	♂	70 ± 1.3	26 ± 1.2	71 ± 1.8	65 ± 1.7	69 ± 0.4
4	+	♂	66 ± 0.7	35 ± 3.4	73 ± 1.2	67 ± 3.9	72 ± 3.5
5	−	♂	−9 ± 4.9	−7 ± 9.3	−5 ± 2.6	−6 ± 9.2	−4 ± 3.5
6	−	♂	−10 ± 0.7	−7 ± 3.1	−5 ± 1.1	−7 ± 2.8	−4 ± 2.2
7	−	♂	−10 ± 6.6	−8 ± 4.5	−6 ± 3.8	−8 ± 5.5	−5 ± 1.6
8	+	♀	−8 ± 4.6	−10 ± 3.5	−4 ± 3.8	−9 ± 3.1	−2 ± 4.3
9	+	♀	−6 ± 5.3	−9 ± 0.7	−3 ± 3.0	−9 ± 4.9	−2 ± 5.3
10	+	♀	−1 ± 3.7	−4 ± 5.1	0 ± 8.4	−2 ± 4.4	+1 ± 3.3
11	+	♀	−6 ± 2.0	−2 ± 7.7	−6 ± 3.8	−8 ± 1.9	−4 ± 4.0
12	−	♀	−4 ± 9.1	−3 ± 3.7	0 ± 4.5	−2 ± 2.2	−1 ± 5.5
13	−	♀	−6 ± 3.2	−8 ± 3.6	−6 ± 2.4	−6 ± 1.8	−4 ± 6.1
14	−	♀	−7 ± 3.2	−7 ± 4.4	−3 ± 3.5	−6 ± 3.5	−3 ± 3.0

Sensitized cells of patient A before and after absorption on different monolayers, tested on a panel of target cells (1–14). Phenotype of patient A: ♀, HLA-A2, −; B12, w40; Cw3, −. Effector: target ratio = 50:1. Different ratios were tested (Not shown). The experiments were repeated at least once. Ψ, percent lysis ± variation coefficient.

* Without absorption. Monolayers: 1. HLA-A2 male. 2. Non HLA-A2 male. 3. HLA-A2 female. 4. Non HLA-A2 female.

TABLE II

HLA-A2 and HLA-B7 Restricted H-Y Killing Shown by Patient B, Phenotype: HLA-A2, 28; B7, 15; Cw3, −.*

Serological type of target

HLA-A2

CML	+	−
+	15	0
−	8	11

HLA-B7

CML	+	−
+	11	0
−	3	11

Sex of the target

HLA-A2

CML	♂	♀
+	15	0
−	0	8

HLA-B7

CML	♂	♀
+	11	0
−	0	3

* Patient B was sensitized in vitro with lymphocytes of an HLA-A, -B, and -C identical, HLA-D different, unrelated male donor.

TABLE III

Independent Absorption of the Anti-HLA-A2; H-Y and Anti-HLA-B7; H-Y Cytotoxicity

Target cell	HLA-A2/-HLA-B7	Sex	O*	Monolayers			
				1 A2 ♂	2 Non A2 & B7 ♂	3 B7 ♂	4 A2, B7 ♂
			Ψ				
Patient	+/+	♀	0 ± 3.1	−1 ± 4.9	+1 ± 3.0	0 ± 4.2	−3 ± 1.1
1	+/+	♂	89 ± 3.8	63 ± 3.2	85 ± 5.6	54 ± 4.6	38 ± 3.0
2	+/+	♂	84 ± 2.8	51 ± 2.8	78 ± 3.5	42 ± 3.3	24 ± 2.5
3	+/+	♂	87 ± 2.0	61 ± 3.5	81 ± 1.4	48 ± 3.7	28 ± 2.2
4	+/+	♂	60 ± 4.3	43 ± 4.7	52 ± 3.1	27 ± 1.1	15 ± 3.6
5	+/−	♂	47 ± 1.1	9 ± 2.9	43 ± 3.5	33 ± 3.5	16 ± 3.0
6	+/−	♂	47 ± 2.2	8 ± 4.8	41 ± 1.3	31 ± 3.7	12 ± 1.1
7	+/−	♂	47 ± 4.0	14 ± 4.1	43 ± 3.1	32 ± 2.1	14 ± 2.4
8	+/−	♂	57 ± 2.4	13 ± 3.9	44 ± 2.0	34 ± 1.6	16 ± 2.7
9	−/+	♂	65 ± 3.9	47 ± 2.4	61 ± 1.3	25 ± 3.4	14 ± 2.4
10	−/+	♂	82 ± 3.5	64 ± 4.4	78 ± 2.6	32 ± 3.2	27 ± 0.7
11	−/+	♂	73 ± 0.6	52 ± 3.4	62 ± 2.7	24 ± 3.0	13 ± 0.8
12	−/−	♂	7 ± 1.5	0 ± 5.3	2 ± 2.1	3 ± 5.4	1 ± 5.2
13	−/−	♂	9 ± 3.5	4 ± 5.1	8 ± 5.0	0 ± 0.8	2 ± 2.1
14	−/−	♂	2 ± 6.1	0 ± 4.0	1 ± 2.6	−1 ± 6.8	−2 ± 2.2

Sensitized cells of patient B before and after absorption on different monolayers, tested on a panel of target cells (1–14). Phenotype of patient B: ♀, HLA-A2, 28; B7, 15; Cw3, −. Effector: target ratio 50:1. Different ratios were tested (not shown). The experiments were repeated at least once. Ψ, percent lysis ± variation coefficient.

* Without absorption. Monolayers: 1. HLA-A2 male. 2. Non HLA-A2, Non HLA-B7 male. 3. HLA-B7 male. 4. HLA-A2, HLA-B7 male.

Discussion

The reduction obtained in the level of cytotoxicity in these experiments was thought to be attributable to removal of specific subpopulations of effector cells by the monolayer and not by admixed soluble antigen or cells detached from the monolayer. Experiments in mouse using a similar system demonstrated that cells detached from the monolayer were incapable of cold target inhibition (18). These observations were confirmed in the human monolayer absorption techniques used here (15).

We cannot draw conclusions from these data which favor altered self or dual recognition. In the former case, the clonal expression of killing would fulfill the prediction of the clonal selection theory and altered self would simply be regarded as an alloantigen. In the latter case, the clonal expression of anti-self would imply that allelic exclusion existed with regard to anti-self HLA receptors, i.e. T-cell clones specialize in different HLA-A, -B, and -C gene products of the self type and are thus clonally expressed. Furthermore, the avidity of the two receptors (self and foreign) would be interdependent.

Our findings are in agreement with those of others who observed that cytotoxic effector cells directed to virus-infected syngeneic cells could be blocked in cold target inhibition experiments only by the virus-infected syngeneic cells and not by virus-infected allogeneic cells. Thus, self and foreign determinants are required on the same cell to obtain blocking (19). This general rule is confirmed here and extended by our experiments because they suggest that in man this anti-self or altered-self activity is clonally expressed. The advantage of the absorption technique as used here is that

clones can be physically removed from cell suspensions, thus giving evidence for separate populations. In contrast, cold target inhibition studies result only in blocking of receptors and theoretically cannot distinguish between polyvalent and monovalent effector cells. Of great interest was the observation that a small but significant reduction in lysis of B7 positive cells occurred after absorption on A2 monolayers and similarly, a reduction occurred in the killing of A2-positive cells after absorption on B7 monolayers. There was no clear explanation of this phenomenon but the possibility existed that it was attributable to clones of killer cells which recognized antigens that were shared by both A2 and B7 bearing molecules (20, 21). No reduction at all has been found using nonrelevant male or female monolayers.

The possible relevance of these findings to the mechanisms involved in the initiation of autoimmune disease in man has been discussed elsewhere (22).

Summary

A monolayer absorption technique was used to test the hypothesis that killer cells directed to self HLA-associated minor histocompatibility antigens (H-Y) were divisible into subsets. The results showed that sensitized killer cells, which recognized two combined antigens HLA-A2; H-Y and HLA-B7; H-Y could indeed be divided into two populations. One was directed to HLA-A2; H-Y and the other to HLA-B7; H-Y. These results can be interpreted in the context of the altered self hypothesis. However, when interpreted in the context of the dual recognition hypothesis, they strongly suggest that independant clones of killer T cells exist which are committed to the recognition of self HLA.

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References

1. Goulmy, E., A. Termijtelen, B. A. Bradley, and J. J. van Rood. 1976. Alloimmunity to human H-Y. *Lancet.* **II:**1206.
2. 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.
3. McMichael, A. J., and A. Ting. 1977. HLA restriction of cell mediated lysis of influenza virus-infected human cells. *Nature (Lond.).* **270:**524.
4. Dickmeiss, E., B. Soeberg, and A. Svejgaard. 1977. Human cell mediated cytotoxicity against modified target cells is restricted by HLA. *Nature (Lond.).* **270:**526.
5. Zinkernagel, R. M., and P. C. Doherty. 1974. Immunological surveillance against altered self components by sensitized T lymphocytes in lymphocytic choriomeningitis. *Nature (Lond.).* **251:**547.
6. Burnet, F. M. 1959. The clonal selection theory of acquired immunity. Vanderbilt University Press and Cambridge University Press (Nashville, Tenn. and London).
7. Jerne, N. K. 1971. The somatic generation of immune recognition. *Eur. J. Immunol.* **1:**1.
8. Bevan, M. J. 1975. The major histocompatibility complex determines susceptibility to cytotoxic T cells directed against minor histocompatibility antigens. *J. Eur. Med.* **142:**1349.
9. Schrader, J. W., B. A. Cunningham, and G. M. Edelman. 1975. Functional interactions of

viral and histocompatibility antigens at tumor cell surfaces. *Proc. Natl. Acad. Sci. U.S.A.* **72:** 5066.

10. Janeway, C. A., H. Wigzell, and H. Binz. 1976. Two different V_h gene products make up the T-cell receptors. *Scand. J. Immunol.* **5:**993.
11. Zinkernagel, R. M., G. N. Callaham, J. Klein, and G. Dennert. 1978. Cytotoxic T cells learn specificity for self H-2 during differentiation in the thymus. *Nature (Lond.).* **271:**251.
12. Howard, J. C. 1978. H-2 restriction, the thymus and the immune response. *Nature (Lond.).* **272:**11.
13. Hurme, M., C. M. Hetherington, P. R. Chandler, and E. Simpson. 1978. Cytotoxic T cell responses to H-Y: mapping of the Ir genes. *J. Exp. Med.* **147:** 758.
14. Golstein, P., E. A. J. Svedmyr, and H. Wigzell. 1971. Cells mediating specific in vitro cytotoxicity. 1. Detection of receptor-bearing lymphocytes. *J. Exp. Med.* **134:**1385.
15. Hamilton, J. D., B. A. Bradley, and J. J. van Rood. 1979. Clonality of human anti HLA killer T cells. *Tissue Antigens.* In press.
16. Brondz, B. D., and N. E. Golberg. 1970. Further in vitro evidence for polyvalent specificity of immune lymphocytes. *Folia Biol. (Prague).* **16:**20.
17. Goulmy, E., B. A. Bradley, Q. Lansbergen, and J. J. Van Rood. 1978. The importance of H-Y incompatibility in human organ transplantation. *Transplantation (Baltimore).* **6:**315.
18. Neefe, J. R., and D. H. Sachs. 1977. Specific elimination of cytotoxic cells. II. Reduction of cytotoxic activity by absorption on monolayers results from removal of cytotoxic cells and not from inhibition of their activity. *Cell. Immunol.* **29:**129.
19. Zinkernagel, R. M., and P. C. Doherty. 1975. H-2 incompatibility requirement for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. *J. Exp. Med.* **141:**1427.
20. Belvedere, M., P. Mattiuz, and E. S. Curtoni. 1975. An antibody cross-reacting with LA and four antigens of the HLA system. *Immunogenetics.* **1:**538.
21. Scalamogna, M., F. Mercuriali, C. Pizzi, and G. Sirchia. 1976. Cross reactivity between the first and second segregant series of the HLA system. *Tissue Antigens.* **7:**125.
22. Bradley, B. A., E. Goulmy, A. van Leeuwen, A. Munro, A. Termijtelen, and J. J. van Rood. 1977. The role of HLA gene products in the recognition of altered self and their hypothetical role in autoimmune disease. *In* The Menarini Series on Immunopathology. First Symposium on organ Specific Autoimmunity. Cremona, Italy. Miescher et al, editors. Schwabe & Co., Basel **1:**247.