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THE IMPORTANCE OF H-Y INCOMPATIBILITY IN HUMAN ORGAN TRANSPLANTATION[1]

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SUMMARY

As an extension of our first observation in which the peripheral blood lymphocytes of an aplastic amaemia patient with a transplant were able to show HLA-restricted H-Y killing in a cell-mediated lympholysis assay, we report here a second case showing exactly the same phenomenon.

A multitransfused woman suffering from aplastic anaemia was shown to have in vitro killing after priming her lymphocytes with her HLA-identical brother. This killing was directed to all male target cells carrying the HLA-A2 antigen. Marginally, killing was also directed to some HLA-A2 female target cells, but this was at a considerably lower level than that directed to male cells. The level of HLA-restricted H-Y killing declined with time. However, it was possible to reactivate the H-Y specific killing by in vitro stimulation with lymphocytes from an HLA-A, -B, and -C-identical, but HLA-D-different male donor. That these findings could be relevant for renal transplantation was supported by renal allograft survival data obtained at 2 years after transplantation. Male allografts from HLA-A2-positive donors in A2-positive females survived for a significantly shorter time than non-A2 male kidneys in non-A2 female recipients. This was only apparent in recipients who produced antileukocyte antibodies.

Observations in the mouse have shown that gene products coded for on the Y chromosome can play a role in graft rejection (*4*). In various strains, skin grafts from males are rejected by isologous females and it is possible to generate cytotoxic T cells which specifically kill male cells (*5, 15*). We have recently described the case of a woman who had been hyperimmunized by pregnancies, blood transfusion, and a bone marrow graft from an HLA-identical sibling donor (*7*). She had developed effector cells against all male phytohaemagglutinin blasts that were positive for HLA-A2, an antigen that she shared with her brother who donated the bone marrow.

In this publication we show that, although with time the level of killing declined almost to background, it could be recalled by restimulation with HLA-A and -B-identical, but HLA-D non-identical lymphocytes from an unrelated male donor. A second patient showing the same phenomenon is also described. Furthermore, the possible relevance of this finding for renal transplantation is discussed.

MATERIALS AND METHODS

The cell-mediated lympholysis (CML) assay used has been previously described in detail (*6*). Briefly, cytotoxicity was measured using an isotope release assay with target cells that had been incubated with phytohaemagglutinin for 3 days and then labelled with 100 μc of ^{51}Cr(^{51}Cr Na_2 Cr O_4, 5 mc/5 ml; specific activity 100 to 350 mc/mg, Amersham CJS 1P) for 1 hr at 37 C. The effector cells were collected from the tissue culture flasks after 6 days in culture using 8×10^6 responder cells and 16×10^6 stimulator cells. Effector cells (70, 50, and 25×10^4) and target cells (1×10^4) in RPMI-1640 medium plus 20% heat-inactivated human AB serum were incubated for 4 hr in round-bottomed microtiter plates. After incubation the plates were centrifuged for 5 min at 200 *g*, and the supernatant was removed and counted in a gamma counter. All combinations were tested in triplicate. The variation coefficient within the triplicates was less than 10%.

The degree of chromium release of a target was found to vary between target cells. Some cells gave very high spontaneous release while others gave low spontaneous release. Because of this, the degree of specific release could not simply be expressed as an index of spontaneous release. It was further noted that a linear relationship existed between the level of spontaneous release and the level of maximum release on freeze-thawing (Fig. 1). Thus the data were expressed on a scale on which 0% was made equivalent to the spontaneous release value and 100% was made equivalent to the freeze-thaw value. This was calculated for each target cell using the following formula:

$$\frac{\text{experimental mean} - \text{mean of spontaneous release}}{\text{freeze-thaw mean (100\% release)} - \text{mean of spontaneous release}} \times 100\% = \%\ \text{kill}$$

Percentages lower than 10% were considered negative. All experiments were repeated at least once.

Effector cells from two female patients are described in this paper.

Patient 1. Mrs. R. had been suffering from a severe aplastic anaemia. She had had four pregnancies, received more than 100 units of blood and blood products, and, after antithymocyte globulin pretreatment, bone marrow from her HLA-identical brother.

Patient 2. Mrs. K. suffered from aplastic anaemia. She had had three pregnancies and received more than 20 units of blood or blood products. She did not receive a transplant.

All individuals were typed for *HLA-A, -B,* and *-C* and in some cases also for *HLA-D* locus products. The results of cadaveric renal allograft survival were collected under the auspices of the Eurotransplant Organization. No distinction was made between first and second grafts and all were included in the analysis.

RESULTS

Until now we have studied a group of 14 aplastic anaemia patients including males and females and HLA-A2-positive and -negative individuals. In five patients positive CML reactions

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were detected against HLA-identical target cells, and in three of these killing was also detected against cells of HLA-identical siblings. These latter three were female patients carrying HLA-A2 and they were able to kill the cells of their HLA-identical male siblings. Extensive segregation studies have now been completed in two of these individuals, using families and panels of unrelated individuals. They indicated that the target antigen was coded for by the Y chromosome and was a probable human equivalent of the H-Y histocompatibility antigen in rodents.

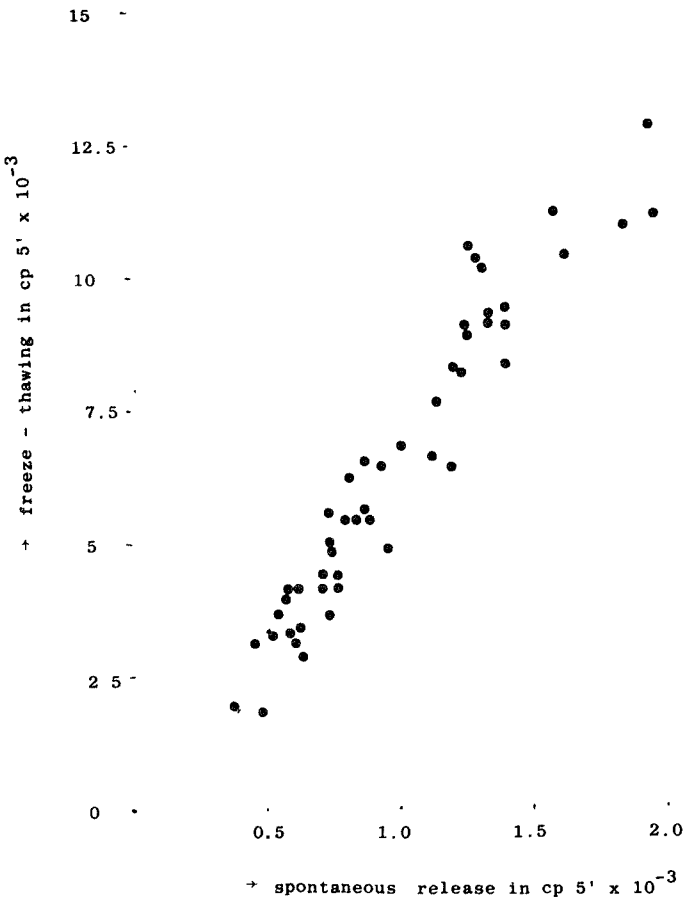

FIGURE 1. Relationship between spontaneous release and freeze-thaw values.

Preliminary data also suggested that a similar phenomenon occurred in a recently studied third patient.

H-Y-specific cytotoxicity. The pattern of killing obtained with effector cells of patient 2 (Mrs. K.) after restimulating in vitro against members of her family showed that the target antigen was not a product of the HLA region per se (Table 1). The patient had two HLA-identical siblings (sib), a sister (sib 1) and a brother (sib 2). When cells from sib 1 were used for restimulating, they failed to generate cytotoxic cells but when sib 2 cells were used cytotoxic cells were generated. They were capable of killing target cells from both sib 2 and the father. Furthermore, the cytotoxic cells generated when the father's lymphocytes were used for restimulation were able to kill cells from sib 2 but not from sib 1.

Despite this nonsegregation with the HLA haplotype when panel studies were performed using a series of target cells derived from unrelated individuals, an association with HLA was found. Effector cells were made using patient 2 restimulated to sib 2 and these were tested on the panel (Table 2). All male HLA-A2-positive target cells gave a high percentage of killing (32 to 53%), whereas non-A2 male cells gave zero killing (−1 to −2%). A variable degree of killing at a low level was shown by HLA-A2-positive female target cells (−2 to 17%), but non-A2 female target cells gave zero killing (−3 to +1%).

Memory and recall of the phenomenon. Patient 1 was studied on seven occasions between the 25th and the 74th week after transplantation. A consistent decline was found in the killing of the HLA-identical brother's cells (Table 3). After 1½ years the level of kill was only slightly above background. Nevertheless, it was possible to recall the H-Y-specific effector cells by an in vitro stimulation using lymphocytes from an HLA-A, -B, and -C-identical but HLA-D-different donor (Table 4). Such effector cells retained their specificity for HLA-A2 males as seen in the repeat panel study shown in Table 5.

Possible relevance in renal transplantation. Cadaveric renal allograft survival data, collected under the auspices of Eurotransplant, were examined 2 years after transplantation for the influence of sex. It was found that a significant difference existed between HLA-A2 females who received HLA-A2 male kidneys and non-HLA-A2 females who received non-HLA-A2 male kidneys (Table 6). This occurred only in those patients who produced antibodies to leukocytes but not in the antibody-negative group. The data are compatible with the assumption that H-Y incompatibility in donor-recipient combinations that

TABLE 1. Killing pattern of cells from patient 2 (Mrs. K. against her family)

Responder × stimulator (sensitizing haplotype)	Stimulation index in mixed leukocyte culture	Target cells[a] (haplotypes-sex)				
		Father (*ab*)	Mother (*cd*)	Patient (*ac*-female)	Sib 1 (*ac*-female)	Sib 2 (*ac*-male)
Patient × father (b)	4,8	72[b]	5	−2	1	62
Patient × mother (d)	3,4	2	1	−2	−1	−1
Patient × sib 1 (−)	1,8	NT[c]	NT	−2	−2	NT
Patient × sib 2 (−)	3,1	45	NT	−2	−2	45
Sib 2 × patient (−)	1,1	NT	NT	NT	NT	NT

[a] HLA haplotypes:

a:	2	W15.2	W6	CW3
b:	9		W6	
c:	3	W40	W6	CW3
d:	28	7	W6	

[b] Figures represented percentage of Cr release; effector to target ratio = 70:1.

[c] NT, not tested.

TABLE 2. Killing of target cells from unrelated individuals by cells from patient 2 (Mrs. K.), stimulated in vitro with her HLA-identical male sib

Targets	HLA phenotypes					Sex	% kill[a]
	HLA-A		*HLA-B*		*HLA-C*		
Patient 2	2[b]	3	W15.2	W40	CW3	Female	0
Brother	2	3	W15.2	W40	CW3	Male	44
1	2	3	W15.2	W40	CW3	Male	32
2	1	2	8	W40	CW3	Male	53
3	2	3	7	12	—	Male	43
4	2		12	W40	CW3	Male	48
5	1	2	8	27	CW2	Male	51
6	3	W25	7	W39	—	Male	−2
7	—	3	18	W17	—	Male	−2
8	—	3	18	27	CW2 CW3	Male	−2
9	1	9	8	27	CW1	Male	−1
10	2	W32	8	W15.2	CW3	Female	7
11	2	3	7	12	—	Female	17
12	2	3	7	W40	CW3	Female	10
13	2	3	7	12	—	Female	13
14	2	3	W17	W35	CW4	Female	10
15	2	3	7	W35	CW4	Female	8
16	2	11		W35	CW4	Female	6
17	2	3	W22	W35	CW4	Female	−2
18	2	9		5	—	Female	11
19	2	—	8	12	—	Female	8
20	1	28	5	8	—	Female	−3
21	11	W31	W15.2	W17	CW3	Female	−1
22	1	3	8	27	CW2	Female	0
23	1	9	8	W40	CW3	Female	1

[a] Effector to target ratio = 70:1.
[b] The HLA antigens shared with patient 2 are underlined.

TABLE 3. Killing pattern of patient 1 (Mrs. R.) against her HLA-identical sibling donor

Time post-transplant (weeks)	Mixed leukocyte culture stimulation index	CML[a]	
		Direct	Indirect (specific stimulation)
31	2.8		67% kill[b]
42	1.1	18% kill	60% kill
52	1.6		48% kill
72	1.1		13% kill
74	1.0		3% kill

[a] In the indirect CML effector cells of patient 1 (Mrs. R) were made using cells from her HLA-identical brother as stimulator cells. In the direct CML Mrs. R.'s cells were tested without in vitro stimulation against her HLA-identical brother.
[b] To facilitate the comparison between the percentage of Cr release, the results are given only of the effector to target ratio, 70:1.

share HLA-A2 have a poorer graft survival than those who do not share HLA-A2.

DISCUSSION

We have concluded from these data that lymphocytes from two, possibly three patients suffering from aplastic anaemia, recognized HLA-A2-positive male target cells. The low level of killing obtained against some HLA-A2 female target cells suggested that at least one other clone was present that recognized other HLA-A2-associated gene products. This was not surprising, since all such patients received many blood transfusions; 20 in the case of patient 2 and more than a 100 in the case of patient 1. One would have expected that this would have led to many more reactions directed to non-HLA antigens. The detection of a predominant specificity was thus a surprising observation. It was even more surprising to find that 3 of 14 patients who demonstrated non-HLA killing recognized the same predominant specificities, namely, HLA-A2 and maleness. Patient 2 suffered from a severe aplastic anaemia and was immunized by multiple blood transfusions and pregnancies. Patient 1, however, had received in addition a bone marrow graft from her HLA-identical brother, which was subsequently rejected. Thus, it follows that blood transfusions and possibly pregnancies are sufficient in themselves to induce HLA-restricted anti-H-Y cytotoxicity and that bone marrow transplantation was not a necessary prerequisite.

Positive cytotoxic tests directed to HLA-identical siblings have been described before in multi-transfused patients, but these have not been examined for the HLA restriction phenomenon (*8, 11, 16*). However, it was remarkable that in all three studies killing was only obtained with patients who carried HLA-A2. Some indication for preferential killing of a male sibling's cells by a female patient's cells was indicated in one case (case 5, Reference (*11*)). Of the 14 patients studied in our series (7 male and 4 female patients carrying the HLA-A2 antigen), 3 cases were found showing preferential killing of male cells by female cells. Several hypotheses are suggested to explain these findings. They include: (1) The presence of a Ir gene

TABLE 4 Reinduction of A2-restricted anti-H-Y effector cells[a]

Responder	Stimulator	Target	% Kill
Bone marrow recipient Mrs R (patient 1)	HLA-identical male sibling donor	HLA-identical male sibling donor	13
Bone marrow recipient Mrs R (patient 1)	Unrelated SD-identical male donor	HLA identical male sibling donor	68
Bone marrow recipient Mrs R (patient 1)	Unrelated SD-identical male donor	Unrelated SD-identical male donor	53

[a] HLA phenotypes

	HLA A	*HLA B*	*HLA C*
Patient 1	2	12 w40	Cw3
Identical sibling donor	2	12 w40	Cw3
Unrelated male donor	2	12 w40	Cw3

TABLE 5 Killing pattern of cells of patient 1 (Mrs R) stimulated in vitro on HLA-A, B, and C-identical but HLA D-different unrelated individuals[a]

Targets	HLA Phenotypes				Sex	% kill
	HLA A		*HLA B*	*HLA C*		
Patient 1	—	2[b]	12 W40	CW3	Female	−4
sib[c]	—	2	12 W40	CW3	Male	68
1	—	2	— W40	CW3	Male	39
2	—	2	12 W40	CW3	Male	53
3	1	2	8 27	CW2	Male	46
4	2	11	5 12	—	Male	42
5	2	3	7 12	—	Male	43
6	2	—	5 12	CW5	Male	70
7	1	3	7 8	—	Male	−2
8	28	29	7 W15 2	CW3 CW4	Male	−8
9	11	29	8 W15 2	CW3	Male	−2
10	2	3	W17 W35	CW4	Female	−5
11	2	9	5 W40	CW3	Female	7
12	2	W31	12 W35	CW4	Female	2
13	2	W31	W35 W40	CW3 CW4	Female	4
14	—	2	12 W40	CW3	Female	10
15	2	3	7 W35	CW4	Female	6
16	2	11	W35	CW4	Female	6
17	2	3	W22 W35	CW4	Female	0
18		2	8 12	—	Female	6
19	2	3	7 W40	CW3	Female	7
20	2	3	7 12	—	Female	7
21	1	2	8 12	—	Female	4
22	3	W26	W35	CW4	Female	1
23	11	W31	W15 2W17	CW3	Female	−1

[a] HLA phenotype of the stimulator cell HLA-A2, B12, Bw40, Cw3
[b] The HLA antigens shared with patient 1 are underlined
[c] HLA-identical bone marrow donor

TABLE 6 Two-year actuarial cadaveric renal graft survival in Eurotransplant patients sex and HLA-A2 data for male donors and female recipients

Donor	A2 positive	A2 negative	T	p
Recipient	A2 positive	A2 negative		
Leukocyte antibody positive group				
	38%	58%	1 96	0 05
	$n = 48$[a]	$n = 50$		
Leukocyte antibody negative group				
	57 9%	61 0%	0 24	0 08
	$n = 53$	$n = 53$		

[a] At risk after 2 years

for H-Y linked to HLA-A2 (2) A peculiar immunogenecity of the A-2-H-Y complex (3) The high frequency of HLA-A2 and H-Y antigens in blood transfusions

Without further knowledge of the nature of the effector and target cells, it is at the moment difficult to choose between these three suggestions The major histocompatibility complex restriction phenomenon first described in the mouse for virally coded antigens (*17*) and later for minor histocompatibility antigens (*2*), including H-Y (*5*), also occurs in man as demonstrated here by our data and those of others These other examples include the killing of influenza-infected target cells (*10*) and dinitrochlorobenzene-treated target cells (*3*) In both cases there seemed to be an indication that there was a pref-

erence for sharing of a specific HLA antigen between killer and target cells. The low level of killing of some female target cells in our study indicated that the restriction was not exclusive to H-Y, but nevertheless HLA-A2 seemed to be the restricting antigen.

With regard to the immunological memory of this phenomenon, it was considered of interest to ask how long such reactions persisted after immunization. If they became undetectable, was it possible to reinduce the specific killing? In one case (unpublished data) we were able to detect HLA-restricted H-Y killing 3 years after the last blood transfusion, suggesting that memory was very long-lived.

In the above study patient 1 was monitored for over 74 weeks after the bone marrow transplantation. Although a few blood transfusions were administered during this period, there was a progressive decline in the level of killing obtained with her cells. Despite this it was possible to reinduce high levels of specific killing by priming in vitro with cells from an HLA-A, -B, and -C-identical and HLA-D-different, unrelated male individual. This anamnestic response to the HLA-identical male sibling was not only found in patient 1 but was also demonstrated in the other cases. These findings indicated that after primary sensitization, the development of cytotoxic effector cells is dependent on the effect of T helper cells. These would respond to the foreign HLA-D determinant on the unrelated A2 male cells used for in vitro priming. It is possible that our observations find clarification in the hypothesis of associative recognition proposed by Lake and Mitchison (*9*), in which they suggested that immunity against non-major histocompatibility complex determinants may more readily be evoked in the presence of major histocompatibility complex alloantigens.

There is some indication that the observations contained in this study may be of relevance in the context of clinical bone marrow and kidney transplantation. In this context it is perhaps relevant to note that bone marrow grafts between HLA-identical siblings have a significantly poorer graft survival if transplanted across a sex difference (*1, 12, 14*). Furthermore, a retrospective study of cadaveric renal allograft survival at 2 years showed a significant influence of sex difference between donor and recipient who shared HLA antigens. This only occurred in the antibody-producing group. In previous studies it has been shown that matching for the HLA-A and -B antigens influences graft survival, especially in patients who had formed leukocyte antibodies. In this group matching for HLA-A and -B antigens seemed to be more important than in the antibody-nonproducing group, where other as yet unidentified HLA gene products seemed to play a role. The fact that the effect of male incompatibility operated only in the antibody-producing HLA-A2 group suggested that this group alone could exhibit the HLA-restricted killing. Nonantibody producers on the other hand had a better graft survival than antibody producers (*13*). Thus, it could be suggested either that this latter group was incapable of exhibiting the restricted killing phenomenon or simply that higher levels of suppressor cells were generated in these cases.

One phenomenon remained for which we have no explanation and that was the failure of patient 2 to generate a positive CML when sensitized in vitro to her mother's cells (Table 1). A similar failure to develop positive CML in instances where one might expect this to occur across HLA-incompatible situations was a frequent finding in our studies of patients who had received blood transfusions.

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