MAJOR HISTOCOMPATIBILITY COMPLEX-RESTRICTED ANTIBODY REACTIVITY MAINLY, BUT NOT EXCLUSIVELY, DIRECTED AGAINST CELLS FROM MALE DONORS*

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Extensive studies in the mouse have shown that cell-mediated lympholysis can occur against virus-infected (1) or chemically modified (2) target cells if the cytolytic effector cell shares an H-2 determinant with the target cell. The same holds true for the minor histocompatibility antigens such as H-Y (3, 4). More recent studies have shown that the same phenomenon can be observed in man. Our group has described a patient (Mrs. R.) suffering from aplastic anemia whose lymphocytes were able to kill the lymphocytes of her HLA-identical brother and all other HLA-A2-positive males (5). Cells from HLA-A2-negative males and all females (with two possible exceptions, *vide infra*) were not killed.

When the serum of Mrs. R. (serum R.)[1] was tested for the presence of lympholytic antibodies it was found that complement-dependent cytolytic antibodies were present that reacted with part of the cells stained with anti-Ig fluorescein isothiocyanate (FITC). It appeared possible that this antibody recognized a hitherto undescribed, possibly non-HLA specificity.

In this article we will describe the results obtained so far with the serum R. This serum demonstrates the presence of an antibody with an almost perfect correlation with HLA-A2-restricted H-Y killing in the cell-mediated lympholysis (CML) test described by Goulmy et al. (6) This serum is unique because it is the first example of an HLA-A2-restricted antibody directed against a non-HLA antigen We will show furthermore that this serum reacts only with part of the mononuclear cells from peripheral blood and that the antibody in it can be found in the IgM fraction

Materials and Methods

Serum The serum R used for the experiments was obtained from a female patient suffering from aplastic anemia She was 46 yr old and married From five pregnancies, two boys and three girls were delivered From March 25th, 1975 until April 25th, 1975 she received 19 buffy coat-free blood transfusions and from March 14th, 1975 until May 1st, 1975, she received 40 platelet transfusions

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[1] *Abbreviations used in this paper* ALG, anti-lymphocyte globulin, BSA, bovine serum albumin, CML, cell-mediated lympholysis, CTL, cytotoxic T lymphocytes, FITC, fluorescein isothiocyanate, NIH, National Institutes of Health, PBS, phosphate-buffered saline, serum R , serum from a female patient, Mrs R , suffering from aplastic anemia, TCF, two-color fluorescence

From these platelet transfusions, 36 were donated by male donors Her HLA type is HLA-A2, B40, Bw6, Cw3, DRw6/A2, B12, Bw4, DRw4 On May 9th, 1975 she received a bone marrow graft from her HLA-identical brother after anti-lymphocyte globulin (ALG) pretreatment There was a temporary take, but the graft was rejected after 20 d The serum we used for the experiments to be described, however, was obtained before the bone marrow transplantation

Cells Mononuclear cells were obtained from HLA-A, -B, -C, and -DR typed healthy panel donors and from healthy family members From each donor, 3–5 ml heparin blood was drawn, diluted with an equal amount of phosphate buffered saline (PBS) and after layering over a Ficoll-Isopaque (Pharmacia Fine Chemicals, Div of Pharmacia, Inc , Piscataway, N J) (7) gradient (density, 1 077), the tubes were centrifuged for 15 min at 1,000 *g* The cells in the interface were, after washing with PBS-1% bovine serum albumin (BSA) used in the two-color fluorescence (TCF) method as described by van Rood et al (8) with a few modifications As a screening method, we used a microtest in HAMAX plates (Moss, Norway) Because only part of the cells reacted, the results could only be evaluated by counting the percentage of the cells that had been lysed For that reason a macro TCF method was used

Macro TCF Test The test was performed as follows two drops (± 0 05 ml) of antiserum were incubated with 1 or 2 million mononuclear cells These cells were labeled (in a volume of 0 5 ml) for 5 min at 37°C with FITC-labeled anti-Ig (Behring Werke AG, Marburg/Lahn, West Germany) diluted 1 3 with PBS in a siliconized test tube (8 × 0 8 cm) for 60 min at 20°C After this incubation time, 10 drops of rabbit complement were added, the suspension carefully mixed with a Pasteur capillary pipette, and incubated again for 2 h at 20°C

Finally, two drops of ethidium bromide, 1 70 dilution in 5% EDTA in PBS of a concentrated solution (100 μg/ml) were added, gently mixed with a Pasteur pipette, incubated for 15 min at room temperature and the tube was centrifuged for 5 min at 600 *g* The supernate was removed and the sediment was transferred to a slide and covered with a coverglass With a normal microscope (Carl Zeiss, Oberkochen, West Germany) with a two-wavelength illuminator after Ploem (9), and 500 × magnification 200 to 300 cells were evaluated

As a control, AB serum from a nontransfused, healthy male donor was used In a TCF preparation, one can identify T cells (not labeled by the anti-Ig-FITC, these cells can thus only be seen with phase contrast) and monocytes (small green spots on the membrane, these cells do not cap under the conditions described above), and B cells (green caps, and occasional interrupted green lines) When a cell is dead the ethidium bromide stains the nucleus with a red fluorescence The green and red fluorescence can be seen simultaneously by appropriate use of filters (8)

National Institutes of Health (NIH) Cytotoxicity Method We used the normal standard NIH method as described in the manual of tissue-typing techniques (10) The method used was, briefly serum and cells were incubated for 30 min at room temperature in a micro-culture tissue plate (catalog No 1/5682, C A Greiner und Sohn, West Germany) under paraffin oil Then normal rabbit complement was added and the mixture was incubated for another 60 min at room temperature The reading was done with an inverted phase contrast microscope after the addition of eosin and after formalin fixation

Serum Absorptions Frozen mononuclear cells obtained from several (five–six) donors as described above and pooled, were used for absorption of serum R 40 million cells were used to absorb 0 1 ml serum All absorptions were done at room temperature The lymphocytes were divided into aliquots and spun for 10 min at 20,000 *g* The serum was added to the dry sediment in the first tube, mixed well, incubated for 30 min, and centrifuged The supernatant serum was aspirated and added to the second tube with lymphocytes, mixed again, incubated for 30 min, and centrifuged The absorbed serum was stored at −20°C until further use

Indirect Immunofluorescence One drop (± 0 025 ml) serum and one drop mononuclear cells containing 0 5–1 million cells were incubated in a siliconized glass tube (8 × 0 8 cm) for 30 min at 20°C The sediment was washed three times with PBS-1% BSA, and then incubated with one drop of the $F(ab')_2$ preparation of a tetramethyl rhodamine isothiocyanate labeled goat anti-human IgG (Fc), or one drop of the $F(ab')_2$ preparation of a FITC-labeled goat anti human anti IgM (Fc), or one drop of FITC-labeled goat anti-human anti IgA (Fc) (Nordic, Tilburg, The Netherlands)

Marking of Monocytes with Latex 5 ml heparin blood was shaken for 30 min in a 37°C waterbath with 0 05 ml latex suspension (1 4 × 10^{10} particles per ml, DIFCO-BACTOLATEX

0 81 Difco Laboratories, Detroit, Mich) Then the blood was diluted with an equal volume of PBS and layered on Ficoll Isopaque Subsequently the tubes were spun for 15 min at 1,200 *g* The cells from the interface were washed twice with PBS 1% BSA and then incubated for 5 min at 37°C with FITC labeled anti Ig (Behring Werke AG), diluted 1 3 with PBS

Serum Fractionation 5 ml of serum R was fractionated on a Sephadex G 200 column (Pharmacia Fine Chemicals, Div of Pharmacia, Inc , 2 5 × 90 cm) and eluted with PBS Fractions of 99 drops were collected and the IgM , IgG , and albumin rich fractions were pooled These pools were desalted on a Sephadex G 25 coarse column (2 5 × 45 cm) in 0 1 M NH_4HCO_3 The protein rich void volume fractions were pooled and lyophilized For testing in the serological assays the lyophilized pools were dissolved in 1 ml PBS Particulate matter was removed by centrifugation at 5,000 *g* for 20 min The three fractions were also subjected to immuno electrophoresis to detect contaminations in the fractions with other Ig

Results

First, we established whether the cells which were killed by serum R were T cells, B cells, and/or monocytes From Table I it appears that only a part of the mononuclear cells labeled with anti Ig was reacting with the antibody The monocytes were differentiated from the B cells by treatment of the blood with latex Because serum R did not react with latex phagocytosing cells, it was concluded that it reacted with the B cells only

Next, the mononuclear cells of unrelated individuals was reacted with serum R 14 cells showed a kill which was 19% or higher of the Ig labeled mononuclear cells and 16 cells (including the patients' own cells) showed a kill of 6% or lower Of two donors, one of which was Mrs R 's mother, 13% of the Ig-labeled cells were killed It was decided that if 10% or less of the cells were killed, the reaction would be scored as negative and that if 15% or more were killed, the reaction would be scored as positive A percentage kill of 10–15% would be considered as doubtful or unclassifiable

To determine the optimal temperature for the reaction in the TCF method, serum R was titrated in normal AB serum and tested at 10°C, 20°C, and 37°C At 10°C, only weak antibody activity was demonstrated Incubation at 37°C resulted in a lower titer than incubation at 20°C Moreover at 37°C a high background of dead T cells and monocytes was found On the basis of these findings all tests were done at 20°C Serum R did not contain an autologous antibody as was demonstrated by testing the serum with Mrs R 's own cells

From serum R IgM-, IgG-, and albumin rich fractions were prepared Table II shows the results of the reactions with HLA-A2-positive male and HLA A2 positive female cells with the IgM, IgG, and albumin serum fractions It is apparent that the activity was found in the IgM fraction This fraction was not completely free of IgG, but because the IgG fraction did not react with HLA-A2 positive male cells, we must conclude that the antibody activity is located in the IgM fraction of the serum Moreover, we could prove with the indirect immunofluorescence method using $F(ab')_2$ fragments of anti-IgM, anti-IgG, and anti-IgA that the HLA-A2 positive male cells only react with anti-IgM and not with anti-IgG or anti IgA

Several sera samples were obtained from patient R over a period of >1 yr Antibody activity was found in the anti IgM fraction only (Fig 1), and declined after ≅1 yr The antibody was stable at −20°C for several years

When the serum of Mrs R was tested in the macro TCF test against a panel of unrelated individuals consisting of 14 HLA A2 positive male donors, 7 HLA A2 negative male donors, 7 HLA A2 positive female donors, and 4 HLA A2 negative

TABLE I

Distribution of the Cells Killed by Serum R. over the Classes of Mononuclear Cells

Donor	Sex	HLA-A, -B	Percentage of cytotoxicity		
			Anti-Ig labeled cells	Monocytes (latex-containing cells)	T cells
				%	
H.	♂	3, 31, 35, 40	0	1	2
P.	♂	2, 3, 7, 12	22	1	3
Z.	♂	2, 5, 7	43	7	1
G.	♀	9, 12, 40	4	1	0

The cells have been incubated with latex

female donors, all the results that typed for HLA-A, -B, -C, and -DR were obtained as shown in Table III. The serological data are given in the right-hand column. This column indicates the percentage of anti-Ig FITC-labeled cells that were killed.

An almost perfect correlation between the lysis induced by the lymphocytes of Mrs. R. in the CML test and the complement-dependent lysis with the serum of the same patient was observed.

All HLA-A2-positive male donors were positive, and all HLA-A2-negative male donors were negative. From the HLA-A2-positive female donors, two gave a borderline positive reaction (A. J. and M. S., both 13%). The last one is the mother of the patient. The HLA-A2-negative female donors all gave negative reactions.

Comparison of the last two columns from this table show that there exists excellent agreement between the results obtained with the CML method and those obtained with the TCF method.

Table IV shows the results of the absorption with pools of different cell combinations. Only HLA-A2-positive males were able to absorb the antibody from the serum. The only exception was found when serum R. was absorbed with the lymphocytes of the mother. After absorption, no antibody activity was demonstrated anymore in the serum R. against an HLA-A2 male donor.

Discussion

The complement lymphocytotoxic activity found in serum R. is, in all probability, a result of an antibody. This conclusion is based on the findings that it reacted with part of the mononuclear cells, recognized a polymorphic determinant, was stable at −20°C for several years, and could be absorbed by cells carrying the corresponding specificities (A2-positive males), but not by other cells from other donors. Lastly, the antibody activity was shown to be in the IgM fraction, both on the basis of serum fractionation and immunofluorescence studies. To the best of our knowledge, this is the first description of an MHC-restricted antibody.

That the reaction observed is antibody dependent appears thus to be well established. Far less clear is the character of the antigen(s) with which the antibody reacts. That the HLA-A2 antigens are involved seems to be certain. The problem lies in the definition of the characterization of the other antigen. In the beginning it was thought

TABLE II

Reactivity of Fractions of Serum R with Anti-Ig-labeled HLA-A2 Cells in the TCF Assay

Cell donor	Serum fractions	Percentage of cytotoxicity of anti-Ig-labeled cells, serum dilution				
		1 1	1 2	1 4	1 8	1 16
HLA-A2-positive male	IgM	20	14	9	6	0
	IgG	0	0	0	0	0
	Albumin	0	0	0	0	0
HLA-A2-positive female	IgM	0	0	0	0	0
	IgG	0	0	0	0	0
	Albumin	0	0	0	0	0

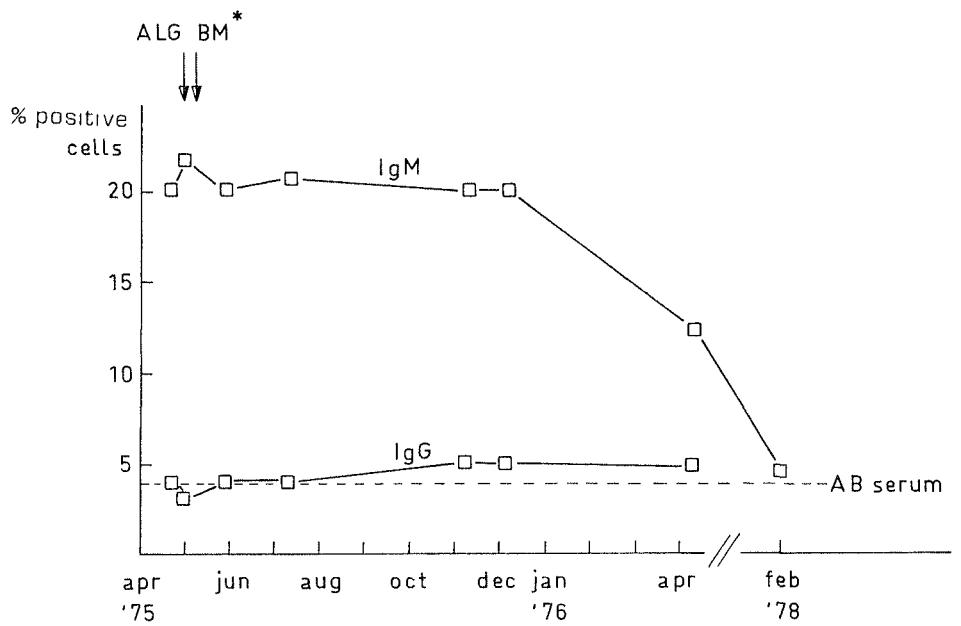

FIG 1 The percentage of positive cells obtained when serum samples from patient R were tested in the indirect immunofluorescence method against a male HLA-A2-unrelated donor (M G) (□), subsequent serum samples of patient R IgM is IgM fraction, IgG is IgG fraction *BM, bone marrow transplantation

that this would be the H-Y antigen, which has been shown to be present on human male leukocytes (11, 12). The weak reactions with the two female cells were assumed to be due to an extra antibody with another specificity. This assumption was analogous to the one made by Goulmy et al (5, 6) when they described the HLA-A2-restricted, cell-mediated, anti-H-Y killing of the lymphocytes of Mrs. R Then also, a few HLA-A2-positive female cells were found which were lysed by Mrs R.'s lymphocytes, although less strongly than the HLA-A2-positive male cells. The assumption of

TABLE III

Correlation between Lysis in the CML Test and Complement-dependent Lysis in Serum R, Tested with Several Male and Female Donors all Typed for HLA-A, -B, C, and DR

Donor	Sex	HLA					Percentage of specific ^{51}Cr in the CML test	Percentage of cytotoxicity of the anti-Ig labeled cells in the TCF method
		-A	B	Bw4/Bw6	C	DR		
							%	%
H J P	♂	2, 3	7, 12	4, 6	—	2, 6	+47	22
J P	♂	2, 3	7, 40	6, 6	3	2	+41	32
D B	♂	1, 2	8, 40	6, 6	3	1, 3	+44	37
K M	♂	2, 2	12, 4	4, 6	3	4, 6	+63	33
T S	♂	1, 2	8, 27	4, 6	2	2, 3	+46	38
O E	♂	2, 11	15, 40	6, 6	4	4 (5 × 8)	+33	25
T D	♂	1, 2	8, 40	6, 6	—	7	+28	19
Z W	♂	2	5, 7	4, 6	—	2, 4	86	43
M G	♂	2	8, 15	6, 6	3	3, 4	ND	29
P K	♂	1, 2	8, 21	6, 6	6	3, 7	ND	25
F C	♂	2, 32	15, 40	6, 6	3	4, 8	ND	30
N O	♂	2, 31	5, 40	4, 6	3	2, (4 × 7)	ND	36
S T	♂	2, 30	7, 39	6, 6	—	1 5	ND	19
S A	♂	2, 2	7, 35	6, 6	4	1, 8	ND	23
E L	♂	11, 31	15, 17	4, 6	3	4, 7	−1	0
V D H	♂	3, 31	35, 40	6, 6	3, 4	1, 4	+9	0
H B	♂	11, 29	8, 15	4, 6	3	3, 4	−2	1
D O	♂	3	18, 27	4, 6	2, 3	4	−3	2
A M	♂	1, 3	7, 8	6, 6	—	3, 6	+6	0
J P	♂	1, 10	8, 12	4, 6	—	1 3	ND	0
V R	♂	1, 33	17, 18	4, 6	—	5, 7	ND	1
A J	♀	2, 9	5, 40	4, 6	3	4	+15	13
M S*	♀	2, 31	12, 35	4, 6	4	1, 4	4	13
K P	♀	1, 2	8, 12	4, 6	5	2, 6	+3	5
W I	♀	2, 3	7, 40	6, 6	3	1, 4	−1	2
K A	♀	2, 32	8, 15	6, 6	3	6, 8	−3	0
R‡	♀	2, 2	12, 40	4, 6	3	4 6	−1	1
A V L	♀	2, 32	15	4, 6	3	4, (5 × 8)	ND	6
B G	♀	9	12, 40	4, 6	3	6, (5 × 8)	+6	4
A M	♀	1, 9	8, 40	6, 6	3	1, 3	0	4
D I	♀	3, 26	35	6, 6	4	1, (4 × 7)	+1	4
H O	♀	10, 28	35, 53	4, 6	4	3, (5 × 8)	0	5

ND, not done
* Mother of patient
‡ Patient (Mrs R)

the existence of a weak extra antibody becomes, however, rather unlikely because at least one of these female cells was able to absorb all antibody reactivity against the male A2-positive cells from the serum We could assume the presence of two potentiating or complementing antibodies, one an anti-HLA-A2 and the other an anti-H-Y One would then have to postulate that, with a few exceptions, only when a cell presents both the HLA-A2 and H-Y antigen will lysis occur Occasionally, an HLA-A2-positive female cell will be lysed by the anti-HLA-A2 alone This explanation is,

TABLE IV

*Reactivity of Serum R with HLA A2 Male Lymphocytes after Absorption with Various Lymphocyte Pools**

Lymphocyte pool	Percentage of cytotoxicity of anti Ig labeled cells‡
	%
HLA A2 positive males	2
HLA A2 negative males	27
HLA A2 positive females	27
HLA A2 positive females	26

* Each pool consists of cells from five to six individuals

‡ The percentage of cytotoxicity of anti Ig labeled cells before absorption was 29%

in all probability, incorrect because we then expect that HLA-A2-negative males and HLA-A2-positive females would be able to absorb the antibody, and this appears not to be the case (Table IV)

Furthermore, Mrs R is HLA-A2 homozygous positive, which almost excludes the possibility that she can make an anti-HLA-A2 antibody A possibility which cannot be ruled out is that the non-HLA antigen is not coded for by the H Y chromosome, but by an autosomal chromosome that is involved in either the development of maleness or the expression of H-Y This explanation is not very satisfying either because it would imply that the two females, A J and M S , would be, in this regard, different from the other four HLA A2 positive females and we have no evidence for this

It is clear that serum R should not only be studied by cytotoxicity, but by other techniques as well We have started such a study with immunofluorescence (Fig 1)

More-sensitive techniques, including cell-surface-labeling techniques, will be necessary to study not only the distribution of the antigen(s) involved, but the character of the antigen as well If we assume on the basis of the absorption experiments that only one antibody is responsible, then logically the next question is whether this antibody reacts with an antigen that arises through an interaction of HLA-A2 with the non HLA antigen (whether this is H Y or something else is, in this context, irrelevant) or whether the simultaneous presence of HLA-A2 and the non-HLA antigen in the same cell induces a neo antigen

The striking similarity of the results in the CML test and the complement dependent cytotoxicity test shown in Table III raises the question, which cannot yet be answered What is the relation of the antigen recognized by the antibody vis-a vis the determinant interacting with the cytolytic cell?

The apparent contradiction of the good correlation between the serological detection of a polymorphic antigen present on part of the B cells and the killing in the CML test of phytohemagglutinin blasts which are thought to arise predominantly from T cells becomes understandable in the light of our finding that the antigen can be shown to be present on T cells by immunofluorescence (A van Leeuwen and J J van Rood Manuscript in preparation)

It should be of interest to look for H-Y antibodies in the sera of other patients whose lymphocytes show an HLA-restricted, anti H-Y CML killing and for that

matter in the sera of all patients whose lymphocytes show a direct positive CML test One could even speculate that (some) CML reactions are a result of similar antibodies and are thus in fact not cytotoxic T lymphocytes but antibody-dependent cell-mediated cytotoxicity reactions This seems, however, unlikely because the CML reactions are done with three-times-washed lymphocytes in the absence of serum from patients Furthermore, absorptions on monolayers demonstrated the clonality of cytotoxic T lymphocytes with MHC-restricted, anti-H-Y specificity (13) It is, in this context, appropriate to emphasize that we would have missed the antibody in the serum of Mrs R if we had not used the TCF test that enabled us to detect that a small part of the mononuclear cells from peripheral blood were killed

Preliminary results with cells from patients with abnormal chromosome patterns have provided already interesting information on sexual differentiation It might well be that this serum and other similar ones might open new avenues in immunogenetics and might be essential in the unraveling of the altered-self versus dual-recognition dilemma by immunological methods

Summary

An IgM antibody, present in the serum of a female patient with aplastic anemia, is described that reacted in a modified complement-dependent cytotoxicity test with a subset of the B cells from HLA-A2-positive, but not HLA-A2-negative males With the exception of two HLA-A2-positive females, the antibody did not react with other cells from either HLA-A2-positive or HLA-2-negative females The cells of one of these and from HLA-A2-positive males were able to absorb the antibody from the serum Cells from other donors were unable to absorb the antibodies

The mononuclear cells of the same patient were cytolytic in cell-mediated lympholysis (CML) for phytohemagglutinin blasts from all HLA-A2-positive males and one of the females reacting with antibody, but not with blasts from HLA-A2-negative males and all other females Thus, the results obtained with the antibody in the complement-dependent cytotoxicity test showed an almost perfect correlation with cytolysis in CML tests

These results suggest that the IgM antibody may be the first example with major histocompatibility complex restriction Because the antibody reacted with the cells from two female donors, the restricting determinant is not, in all probability, the H-Y determinant

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References

1 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

2 Shearer, G M 1974 Cell mediated cytotoxicity to trinitrophenyl modified syngenic lymphocytes *Eur J Immunol* **4**:257

3 Gordon, R D , L Simpson, and L L Samelson 1975 In vitro cell mediated immune

responses to the male specific (H-Y) antigen in mice *J Exp Med* **142:**1108
4 von Boehmer, H , C G Fathmann, and W Haas 1977 H-2 gene complementation in cytotoxic T cell responses of female against male cells *Eur J Immunol* **7:**443
5 Goulmy, E , A Termijtelen, B A Bradley, and J J van Rood 1976 Alloimmunity to human H-Y *Lancet* **II:**1206
6 Goulmy, E , A Termijtelen, B A Bradley, and J J van Rood 1977 Y antigen killing by women is restricted by HLA *Nature (Lond)* **266:**544
7 Boyum, A 1960 Separation of leukocytes from blood and bone marrow *Scand J Clin Lab Invest Suppl* **21:**97
8 van Rood, J J , A van Leeuwen, and J S Ploem 1976 Simultaneous detection of two cell populations by two colour fluorescence and application to the recognition of B cell determinants *Nature (Lond)* **262:**795
9 Ploem J S 1975 General Introduction *Ann N Y Acad Sci* **254:**4
10 van Rood, J J 1974 Microlymphocytotoxicity test *In* Manual of Tissue Typing Techniques J G Ray, D B Hare, P D Pederson, and D E Kayhoe, editors National Institutes of Health, Bethesda, Maryland 75
11 Goldberg, E H , T Arrington, and S Tokuda 1978 Detection of H-Y antigen on human male leukocytes *J Immunol Methods* **23:**203
12 Fellous, M , E Gunther, R Kemler, J Wiels, R Berger, J L Guenet, H Jakob, and F Jakob 1978 Association of the H-Y male antigen with $\beta 2$ microglobulin on human lymphoid and differentiated mouse teratocarcinoma cell lines *J Exp Med* **148:**58
13 Goulmy, E , J D Hamilton, and B A Bradley 1979 Anti self HLA may be clonally expressed *J Exp Med* **149:**545