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Recognition of a human T-lymphocyte differentiation antigen by an IgM monoclonal antibody

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SUMMARY

A monoclonal antibody directed at a determinant on human T cells was produced and characterized. This IgM antibody, MBG6, bound to human peripheral blood T lymphocytes and to medullary thymocytes. It was unreactive with normal B cells, B-cell lines and granulocytes. Apart from T lymphocytes, bone marrow cells (including cells positive for the terminal transferase marker, myeloid colony-forming cells, myeloblasts, and differentiating myeloid and erythroid cells) were negative. Peripheral blood cells that were treated with MBG6 and rabbit complement were no longer capable of proliferating in response to phytohaemagglutinin or concanavalin A; MBG6 did not have any direct mitogenic action on T lymphocytes. Double immunofluorescence studies using IgM MBG6 and OKT3, and IgG2a monoclonal antibody that recognizes all peripheral T cells, showed that these two antibodies identified exactly the same cell populations. Competitive binding studies, however, indicated that MBG6 and OKT3 recognized different epitopes. The antibody may have clinical applications in bone marrow transplantation.

INTRODUCTION

It has been known for many years that some membrane glycoproteins are expressed selectively on T lymphocytes or their subpopulations. Thy 1 was found on murine T cells but not B cells (Reif & Allen, 1964) and T-cell subpopulations have been defined by the Ly 1, 2, 3 antigens (Boyse *et al.*, 1968; Cantor & Boyse, 1975). Similar characterization of human T-cell surfaces has been made using spontaneously occurring autoantibodies (Strelkauskas *et al.*, 1978; Reinherz *et al.*, 1979) and heteroantisera (Schlossman *et al.*, 1976; Chess & Schlossman, 1977). These antisera have been of limited value, however, because of difficulty in standardization. A far more useful technique has been the fortuitous finding that human T lymphocytes spontaneously form rosettes with sheep erythrocytes.

The application of the hybridoma technique (Kohler & Milstein, 1975) to the study of human cell surface antigens has led to the generation of a series of monoclonal antibodies, specific for human T lymphocytes (McMichael *et al.*, 1979; Kung *et al.*, 1979). The best characterized set of T-cell-specific antibodies are the Ortho (OKT) reagents (Reinherz *et al.*, 1980) which can be used as standards. The principal T-lymphocyte differentiation antigens are thus defined by OKT3 which recognizes all mature T lymphocytes, OKT4 which binds to inducer T cells and OKT8 which binds to suppressor and cytotoxic T lymphocytes. Immature T lymphocytes in the thymus cortex also express a unique antigen, HTA-1, defined by Na1/34 (McMichael *et al.*, 1979) and OKT6 (Reinherz

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et al., 1980). These reagents are already very useful research tools; their value in immunodiagnosis and therapy is currently being assessed (Cosimi, Coloin & Goldstein, 1981).

In this paper we describe a monoclonal antibody, MBG6, which is specific for mature T lymphocytes. Because it is of the IgM class it might have special clinical applications.

MATERIALS AND METHODS

Growth and selection of hybridoma clones

Spleen cells of a BALB/c mouse immunized with human thymocytes were fused to cells of the 8-azaguanine-resistant murine myeloma cell line PS-NS1/Ag4.1 according to the technique of Kohler & Milstein (1975). The fused cells were plated out into 48 tissue culture wells and hybrid cells grown in selective HAT medium. In one culture well the antibody in the supernatant bound to glutaraldehyde-fixed human thymocytes and to peripheral blood lymphocytes but not to a B-lymphocyte cell line (Bay 5). Cells from this culture were twice cloned by limiting dilution to give the cell line MBG6. This line has remained stable for over 10 months and has been grown in BALB/c mice to yield ascitic fluid with an antibody titre of 10^{-4}.

Culture supernatants and ascitic fluids were assayed according to the method of Morris & Williams (1975), first binding antibody to the cells and then adding ^{125}I-labelled rabbit F(ab′)$_2$ anti-mouse F(ab′)$_2$ (1·25 μCi/ml, 0·125 μg/ml) (gift of Dr J. Fabre).

Other antibodies

In addition to MBG6, the following monoclonal antibodies were used. PA2.6 recognizes a common determinant on HLA-A, B, C antigens (Brodsky *et al.*, 1979). NA1/34 recognizes an antigen, HTA-1, expressed on 85% of human thymocytes (McMichael *et al.*, 1979). J15 is an IgM anti-platelet antibody (Vainchenker *et al.*, 1981). OKT3, 4 and 8, which recognize T-cell differentiation antigens on all T, inducer T and suppressor/cytotoxic T lymphocytes respectively, were a gift from Dr G. Goldstein (Ortho Pharmaceuticals, Raritan, New Jersey, USA).

An antiserum to HLA-DR antigens (also referred to as 'Ia-like' or p28,33 antigens) was made in chicken and absorbed with thymocytes (Janossy *et al.*, 1979). A rabbit antiserum specific for terminal transferase was a gift from Dr F. J. Bollum. Anti-μ antiserum was a gift from Dr H. N. A. Willcox.

Complement-mediated cytotoxicity

In cytotoxicity assays, 250 μl monoclonal antibody in doubling dilutions were incubated with 250μl freshly prepared cells (either lymphocytes or thymocytes) at 20°C. After 30 min the cells were washed once and 500 μl rabbit serum complement were added to each tube. After 45 min at 37°C the number of viable cells was calculated by trypan blue exclusion.

Radiobinding assays

Monoclonal antibody was tested using the indirect binding assay (Morris & Williams, 1975) on glutaraldehyde-fixed single-cell suspensions of lymphoid, blood or bone marrow cells. B lymphoblastoid (Bay 5, 8866) and T leukaemic (HSB2) cell lines were also used as target cells.

Binding of monoclonal antibody to human liver and brain homogenates was measured by absorption according to the method of Morris & Williams (1975).

Immunofluorescence (IF)

Single IF. Tissues obtained at autopsy (within 24 h of death) or biopsies were frozen onto cryostat chucks in liquid N_2. Seven-micron cryostat sections were dried at 37°C for 1 hr and fixed in acetone at −20°C for 15 min and air-dried at 22°C. The supernatant from a hybridoma culture was placed on the section for 1 hr in a humidified atmosphere and the slide washed with phosphate-buffered saline (PBS); fluorescein-labelled rabbit anti-mouse immunoglobulin (diluted 1:15) was applied to the section for 30 min. After further washing in PBS, the section was mounted

in 70% glycerol/PBS and examined by fluorescence microscopy. Controls were sections incubated with a monoclonal antibody to HLA-A, B, C, PA2.6 or second layer alone.

Double IF. In these assays the reactivity pattern of MBG6 was studied in relation to other conventional monoclonal antibodies. Cells (2×10^6 in 50 μl PBS containing 0·2% azide) were incubated for 10 min at 20°C with MBG6 and the other antibody, washed three times and resuspended. The suspension was then stained with second-layer goat antibody conjugated with fluorescein (FITC) or rhodamine (TRITC).

When monoclonal antibodies of different Ig classes were applied, the second layers were goat anti-mouse IgM-TRITC or -FITC (μ-specific), goat anti-mouse IgG2-FITC (γ_2-chain-specific) and goat anti-mouse IgG1-TRITC (γ_1-specific). These reagents were from Meloy Laboratories and were conjugated at the Royal Free Hospital (0·9–1 fluorochrome/protein ratios). The specificity and titre were checked by showing that they stained relevant myeloma cells at a dilution greater than 1:100 and did not stain myeloma cells of irrelevant class at a 1:10 dilution (Tidman *et al.*, 1981).

In other combinations of monoclonal antibody and conventional antisera the labelled layers were goat anti-mouse Ig-FITC and goat anti-chicken or human Ig-TRITC (1:20–1:40 final dilutions).

Finally, some samples were labelled with MBG6 (using goat-anti-M-Ig-TRITC second layers) and then smeared. The smears were fixed in cold methanol (30 min) and incubated in a moisture chamber with an affinity-purified rabbit anti-terminal deoxynucleotidyl transferase antibody (anti-TdT; Bollum, 1975), washed for 30 min in PBS and stained with goat anti-rabbit-Ig-FITC (Janossy *et al.*, 1979).

Immunoperoxidase

Tissue sections ($1–4 \times 10^6$) were incubated with 100 μl monoclonal antibody for 1 hr at 4°C, washed, and fixed in buffered formol acetone for 30 sec (Mason, Farrell & Taylor, 1975). After rinsing in distilled water and washing in 0·15 M saline (buffered with 0·05 M Tris–HCl, pH 7·6) the sections were incubated for 1 hr at room temperature with rabbit anti-mouse IgG conjugated with horseradish peroxidase (Nakane & Kawaoi, 1974). Slides were then washed in Tris buffer and the peroxidase developed for 8 min in a diaminobenzidine tetrahydrochloride solution (0·6 mg/ml) and H_2O_2 (0·01%). Slides were washed, counterstained with haematoxylin and mounted in DPX mounting medium (Raymond Lamb, London).

Competitive binding studies

OKT3 immunoglobulin, purified by DEAE Sepharose chromatography, was iodinated by the chloramine T method as described by Jensenius & Williams (1974). Fresh peripheral blood lymphocytes, 1×10^6 in 25 μl PBS/0·5% BSA, were incubated with 25 μl unlabelled antibody—OKT3, MBG6 or J15, each at a super-saturating concentration of 1:10 diluted ascitic fluid. After incubating for 1 hr at 4°C, various dilutions (two-fold from 0·5 μg/ml) of radioiodinated OKT3 were added. After a further incubation of 1 hr at 4°C the cells were washed twice in PBS/0·1% BSA and gamma-counted.

Functional assays

Granulocytic–monocytic colony-forming cells (CFUc). Normal bone marrow samples were cultured using the double-layer agar technique with human peripheral blood leucocyte feeder layers (Pike & Robinson, 1970).

Mitogenic responses. These were measured using PHA (Wellcome) at 9 μg/ml, Con A (Pharmacia) at 2 μg/ml and OKT3 to stimulate triplicate aliquots of 10^5 lymphocytes in round-bottomed microtitre trays. Incorporation of tritiated thymidine was measured at day 3.

RESULTS

Characterization of monoclonal antibody MBG6

Fourteen days after cell fusion the supernatant in one well was found to bind to human thymocytes

and peripheral blood lymphocytes but not to B-cell lines. These cells were cloned by limiting dilution. The clone MBG6 was selected for further characterization and grown, first in culture and then as an ascitic tumour in the pristane-treated peritoneal cavities of BALB/c mice. The immunoglobulin class of MBG6 was determined to be IgM by the formation of a precipitin line when ascitic fluid was tested with anti-μ antiserum in an Ouchterlony plate. This was later confirmed by the specific staining achieved in immunofluorescence studies using an anti-μ second layer.

Purification of MBG6 was readily achieved by passing ascitic fluid through a Sephacryl 200 column. The antibody that was recovered in the void volume (molecular weight > 250,000 daltons) was highly purified (Fig. 1). There was some binding activity in the later fractions, which was possibly monomeric IgM. The total yield of the pure antibody was relatively low (0·2 mg protein/ml ascites) when compared to yields that we have obtained with IgG monoclonal antibodies.

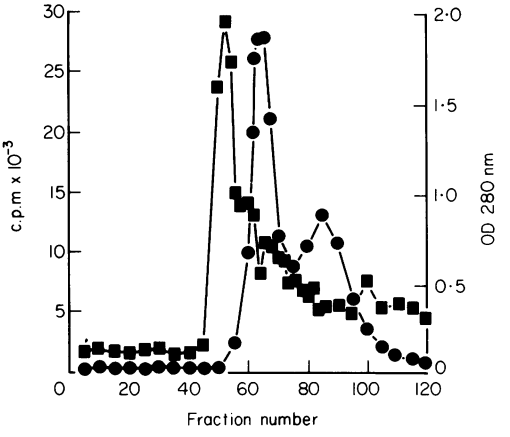

Fig. 1. Purification of MBG6. (●——●) Optical density (E_{280}) of fraction samples from S-200 column, (■——■) radiobinding assay activity of fraction samples on human thymocytes.

Tissue distribution of the antigen identified by MBG6 (Table 1)

When tested for its binding to a wide range of human cells, analysed in suspension as well as in tissue sections, it was found that significant amounts of antigen identified by MBG6 were detectable only on thymocytes, T lymphocytes, chronic lymphatic leukaemia cells of T type (T-CLL and Sézary syndrome) and the T-lymphoid line HSB2. All non-lymphoid organs tested were negative, as were erythrocytes, granulocytes and platelets.

In lymphoid organs, such as tonsil, MBG6 stained only the T-cell areas. In infant thymus, MBG6 reacted with 24% of cells. These were mostly medullary thymocytes, which were negative with NA1/34 and OKT6, although a minority population of cortical thymocytes which express HTA-1 antigen did bind MBG6. A 16-week-old fetal thymus section was totally negative, indicating that MBG6 sees a T-cell antigen that arises relatively late in fetal development.

Distribution of the antigens recognized by MBG6, OKT3, anti-terminal transferase and anti-HLA-DR

The tissue distribution of the antigen defined by MBG6 was reminiscent of that found with OKT3 (Kung *et al.*, 1979). A comparison was therefore made between these two antibodies, making use of goat anti-mouse IgM-TRITC (red) to stain for MBG6 and goat anti-mouse IgG-FITC (green) to identify OKT3. The results indicate that the two antibodies stain the same T-cell population (Table 2). This was true in blood, tonsil and infant thymus. Only 24% of thymocytes were positive with both antibodies, corresponding to the mature cell population in the thymus (see above). In the bone marrow, MBG6 stained neither B lymphocytes nor the cells which were positive for terminal transferase (TdT) and HLA-DR. Two per cent of cells in bone marrow were stained with both

Table 1. Distribution of MB6 reactivity*

	Membrane fluorescence	Radiobinding assay	Tissue section immunoperoxidase	Absorption
(a) Normal lymphoid tissue				
Fetal thymus (16 week)	−			
Infant thymus	15–20†	+		
Blood T cells	>95	+		
Blood B cells	−	−		
T-cell area in tonsil	+		+	
B-cell area in tonsil	−		−	
(b) Leukaemias/cell lines				
HSB2 (T line)		+		
T-ALL (T line)	−			
T-CLL		+		
Sézary cells		+		
B-CLL	−			
8866 (B line)		−		
Bay 5		−		
(c) Haemopoietic cells				
Granulocytes	−	−		
Erythrocytes	−	−		
Platelets	−	−		
Immature myeloid cells in marrow	−			
(d) Other tissues				
Brain	−			−
Pituitary	−			
Liver	−			−
Lung	−			
Kidney	−			
Placenta	—			

* See Materials and Methods for further details. (+) Positive binding of MBG6, (−) negative binding. No result indicates not tested.

† Percentage of lymphoid cells.

MBG6 and anti-HLA-DR antibodies but as these were TdT-negative they were probably activated T cells and not precursor cells.

It was concluded from these studies that MBG6 bound only to mature T lymphocytes.

Effect of MBG6 on T-cell functions

MBG6 was found to be cytotoxic to T lymphocytes in the presence of rabbit complement. The optimal conditions for cytotoxicity were incubation with antibody alone for 30 min followed (after one wash in saline/FCS) by addition of a selected batch of lyophilized rabbit serum (diluted 1:2 in complement fixation diluent) for 45 min at 37°C. No lysis was observed when human serum was used; suboptimal lysis was observed when rabbit serum was further diluted or rabbit–human serum mixtures were used. The ascitic fluid titred out to a dilution of 1:100. MBG6 and OKT3, in the presence of complement, destroyed the same peripheral T-cell population (Table 3). Having established these conditions, the effect of removing MBG6-positive cells on various functional assays could be determined.

Mitogenic responses. Human peripheral blood lymphocytes were treated with MBG6 and complement and the response of the remaining cells to PHA and Con A was measured. As shown in Table 4 these proliferative responses were completely abolished by treatment with MBG6 or OKT3

Table 2. Double staining using MBG6 and other reagents in cell suspension

	Combination		Percentage of cells with different staining patterns*			
	TRITC	FITC	+/+	+/−	−/+	−/−
Blood	MBG6	OKT3	76†	0	0	24
mononuclear cells	MBG6	OKT4+ OKT8	75†	0	0	25
	Ia-like	MBG6	4	10	72	14
Tonsil cells	MBG6	OKT3	56†	0	0	44
	Ia-like	MBG6	0	39	58	3
Thymocytes	MBG6	OKT3	24†	0	0	76
	MBG6	OKT6	14	10	74	2
	MBG6	NA1/34	13	11	74	2
Bone marrow	MBG6	TdT	0	32	4	64
	MBG6	SmIg	0	30	9	61
	Ia-like	MBG6	2	14	16	68

* +/+ and −/− refer to cells which are reactive or unreactive with both antibodies; +/− and −/+ refer to cells which react only with the first (TRITC) or the second reagent (FITC) respectively.

† The proportions of $OKT3^+$ T cells in these tissues are: blood 76%, tonsil 56%, thymocytes 24%, bone marrow 18–32%.

Table 3. Complement-mediated lysis of human T cells using MBG6*

Cells	Antibody dilution	Complement used	Per cent lysis
Human peripheral blood lymphocytes	MBG6 1/20	Human C′ neat	8
	MBG6 1/20	Rabbit C′ neat	71†‡
	MBG6 1/20	Rabbit C′ 1/5	52
	MBG6 1/20	Rabbit C′ 1/10	24
		Hu:rabbit 3:1 neat	24
	MBG6 1/20	Rabbit C′ neat	72†
	OKT3 1/20	Rabbit C′ 1/5	45
Human bone marrow cells	MBG6 1/20	Rabbit C′ neat	12†
Human thymocytes	MBG6 1/100	Rabbit C′ neat	20

* The proportions of T lymphocytes detected as $OKT3^+$ by IF in the samples studied are: 70% in blood, 10–13% in bone marrow and 20% in thymus.

† After C′ treatment < 1% $OKT3^+$ lymphocytes could be detected in these samples.

‡ Similar observations were seen in six other experiments using a selected batch of rabbit C′. This batch was similarly effective when diluted 1:2 with complement-fixing diluent.

Table 4. The effect of MBG6 and C′ on the PHA and Con A response of blood lymphocytes

Treatment of cells	Con A (c.p.m.)	PHA (c.p.m.)	Medium (c.p.m.)
MBG6 alone	76,463 ± 5,567	90,919 ± 8,046	3,302 ± 186
MBG6 + C′	1,000 ± 572	2,898 ± 123	349 ± 17
OKT3 alone	72,136 ± 4,922	110,507 ± 1,242	18,398 ± 826*
OKT3 + C′	823 ± 190	809 ± 111	386 ± 55
J15 alone	71,701 ± 3,348	81,806 ± 3,178	3,724 ± 483
J15 + C′	103,430 ± 2,789	107,240 ± 9,712	2,005 ± 392
C′ alone	87,161 ± 3,487	102,609 ± 15,087	751 ± 138

Figures represent mean ^{3}H-thymidine incorporation ± s.e.m. measured 72 hr after initiating the culture.

* Shows clearly mitogenic effect of OKT3; see also Fig. 2.

but not by control antibody J15 (IgM anti-platelet). OKT3 has been reported to be directly mitogenic to T lymphocytes (van Wauwe, de Mey & Goossens, 1980). MBG6 and OKT3 were therefore tested in the absence of complement on cultured peripheral blood lymphocytes (Fig. 2). While OKT3 was a potent mitogenic agent even at low doses, MBG6 had no such property. Furthermore, MBG6 did not inhibit the mitogenic properties of OKT3 (data not shown).

Bone marrow myeloid colony-forming cells. Preliminary experiments showed that treatment of bone marrow (BM) with MBG6 and rabbit complement did not diminish the proportion of recognizable early myeloid cells (myeloblasts) and terminal-transferase-positive precursor cells. Further experiments were therefore carried out to test the recovery of CFUc (granulocytic–monocytic colony-forming cells) after treatment with MBG6 and complement (Table 5). A slight increase in CFUc was seen in treated samples.

Competitive binding studies between OKT3 and MBG6

Attempts were made to radiolabel purified MBG6 with iodine-125 but although the protein was readily labelled, all binding activity was lost. Twenty-five micrograms of purified OKT3 IgG were therefore iodinated with ^{125}I and found to react with T lymphocytes. In the presence of a large (greater than 40-fold) excess of MBG6, no inhibition of binding of ^{125}I-OKT3 to peripheral blood lymphocytes was observed; binding of ^{125}I-OKT3 was completely inhibited by an excess of unlabelled OKT3 (Fig. 3). The results clearly indicate a lack of competitive binding by the two antibodies.

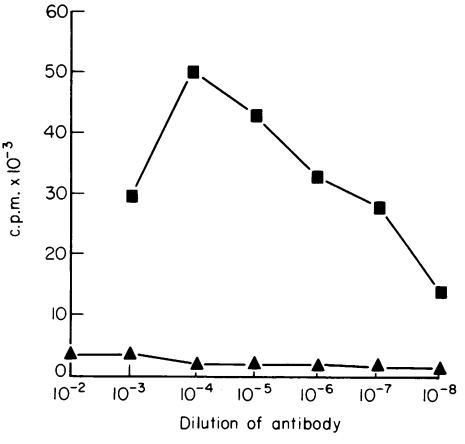

Fig. 2. Proliferation (^{3}H-thymidine incorporation) of human peripheral blood lymphocytes incubated for 3 days in the presence of MBG6 (▲——▲) and OKT3 (■——■) at dilutions of ascitic fluid from 1:100 to 1:10^8.

Table 5. The effect of MBG6 and C′ on myeloid colony-forming cells in BM

	CFUc in BM*
Untreated cells	100
No antibody + C′	87
MBG6 + C′	130
MBG6 without C′	86

* These are percentage recovery; 100% is 117 colonies/10^5 cells plated.

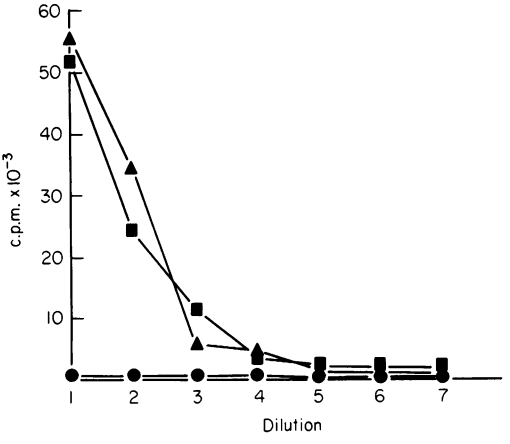

Fig. 3. Competitive binding studies using human peripheral blood lymphocytes. Cells were preincubated with OKT3 (●——●), J15 (anti-platelet) (■——■) and MBG6 (▲——▲), all at concentrations of 1 : 10 ascites. The plot shows the binding of ^{125}I-OKT3 to the pretreated cells at two-fold dilutions from 0·5 μg/ml.

DISCUSSION

This paper reports the generation and characterization of a stable cloned hybridoma line that produces an IgM monoclonal antibody, MBG6, that is specific for mature T lymphocytes. This conclusion is based on extensive binding studies on a wide range of normal human tissues and cell lines using radiobinding techniques, immunofluorescence and immunoperoxidase. Particular attention has been paid to the reactivity of this antibody to bone marrow cells, with a view to its possible use, in the presence of complement, to destroy T cells prior to transplantation. MBG6 did not bind to putative lymphoid cell precursors, which carry the terminal transferase markers, nor did it lyse, in the presence of complement, the granuloid–monocytic colony-forming cells (Table 5) (Janossy *et al.*, 1981). Likewise, immature T cells in the thymic cortex of fetal thymus were also negative. The antigen thus appears late in T-cell development.

A comparison was made between MBG6 and the Ortho anti-T-cell antibodies OKT3, OKT4 and OKT8 (Reinherz *et al.*, 1980) which may be used as reference reagents. The binding characteristics of MBG6 appeared to be closely similar to those of OKT3 and when advantage was taken of their different immunoglobulin classes to perform double fluorescence-labelling experiments, there was complete concordance in their reactivity (Table 2).

It is of interest to know, therefore, whether MBG6 and OKT3 recognize the same antigen or whether there could be sets of differentiation antigens defining various cell types. We were unable to precipitate the structure defined by MBG6 from radiolabelled cells, possibly because of low affinity binding in the presence of detergents. We therefore had to rely on indirect evidence. Binding of

radiolabelled OKT3 to T cells was not inhibited by a 40–80-fold excess of unlabelled MBG6. OKT3 is potently mitogenic for T cells (van Wauwe *et al.*, 1980; Chang *et al.*, 1981) and MBG6 was not; nor did it inhibit the mitogenic properties of OKT3. These results imply, therefore, that the MBG6 antigen is different from that seen by OKT3. It is possible, however, that the two antibodies might see different epitopes on the same molecule.

Callard *et al.* (1981) have described a third antibody, UCHT1, with a similar specificity and this was mitogenic (P. Beverley, personal communication). There are therefore three antibodies, of different immunoglobulin subclass, that exclusively recognize mature T lymphocytes: OKT3 (IgG2a), UCHT1 (IgG1) and MBG6 (IgM). The IgM class of MBG6 has some advantages. This antibody can be used in double-fluorescence experiments with any of the other, IgG, anti-T-cell reagents for a detailed phenotypic analysis of cell suspensions or tissue sections, as described in Table 2. It is particularly effective in complement-mediated cell lysis and it can be readily purified (Fig. 1).

The last two properties offer the possibility of using it to destroy T lymphocytes in bone marrow prior to transplantation with the aim of preventing graft-versus-host disease. The results shown in Table 3 indicate that rabbit, rather than human, complement would be needed for such treatment *in vitro*. It might therefore be advantageous to use the MBG6 as a carrier of covalently linked toxins, using the large molecule to advantage, to target these agents to T lymphocytes. Preliminary studies of this type are now in progress.

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