Immunology 1981 **43** 493

A monoclonal antibody to the HLA-DR product recognizes a polymorphic Ia determinant in mice

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Accepted for publication 22 January 1981

Summary. A rat monoclonal antibody which recognizes a product of the HLA-DR locus is described. The determinant recognized by this antibody is monomorphic in man but polymorphic in mice. Mapping studies with inbred and recombinant strains of mice show that this antigen is associated with H-2 haplotypes b, d and q and is coded for by genes within the I-A subregion.

INTRODUCTION

The HLA-D/DR locus in man codes for cell-surface glycoproteins which are involved in the control of immune responses at a variety of levels. The products of this locus can be recognized antigenically either by T lymphocytes, as in the mixed lymphocyte reaction (Yunis & Amos, 1971) or with allo- and xenogenic antisera.

The use of such antisera against human Ia antigens has demonstrated their presence on the surface of B lymphocytes, subsets of monocytes and 'null cells' (Winchester, Wang, Halper & Hoffman, 1976), haematopoietic precursor cells (Winchester, Ross, Jarowski, Wang, Halper & Broxmeyer, 1977), epidermal Langerhans cells (Rowden, Lewis & Sullivan, 1977) and T lymphocytes (Mann & Sharrow, 1979; Greaves, Verbi, Festenstein, Papasterianis, Jaraquemada & Hayward, 1979). Since conventional antisera are not monospecific their use to detect Ia antigens, especially on T lymphocytes, has been controversial. Monoclonal antibodies raised against human Ia antigens by the cell fusion technique of Köhler & Milstein (1976) are proving very useful in clarifying the cellular distribution of these antigens and in studying the functions of Ia-positive and Ia-negative cell populations (Reinherz, Kung, Pesando, Ritz, Goldstein & Schlossman, 1979; Engleman, Charron, Benike & Stewart, 1980). Furthermore, monoclonal antibodies are of considerable value in obtaining purified samples of these molecules for further characterization of their structure (Charron & McDevitt, 1980).

We describe here a potent monoclonal antibody, raised in the rat, which recognizes the human Ia antigen and precipitates in addition to the expected 34,000 and 28,000 Dalton polypeptides, a third component of mol. wt 25,000 Daltons. It has the additional interesting property of recognizing a polymorphic determinant coded for by the mouse H-2 I-A locus, as well as detecting a polymorphic determinant on sheep lymphocytes.

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0019-2805/81/0700–0493$02.00

MATERIALS AND METHODS

Rats and mice

AO and (AO × LOU) F_1 rats were bred and main-

tained in the animal unit of the MRC Laboratory of Molecular Biology, Cambridge.

AJ, B10A, B10D2, B10M, C3H/HE, C57B1, CSW, CWB and SJL mice were kindly provided by Dr A. Munro, Department of Pathology, Cambridge. AQR, B10A (4R), B10A (5R) and B10G mice were a gift from Dr E. Simpson, Clinical Research Centre, Harrow and DBA/1 mice were obtained from OLAC 1976 Ltd, Bicester. All other strains were bred and maintained by Central Animal Services, MRC Laboratory of Molecular Biology, Cambridge.

Cell lines and media

Y3-Ag 1.2.3 is a cell line derived from the LOU rat myeloma line R210. The cells die in medium containing hypoxanthine (10^{-4} M), aminopterin (4×10^{-4} M) and thymidine ($1{\cdot}6 \times 10^{-5}$ M) (HAT medium; Szybalska & Szibalski 1962). They secrete κ light chains (Galfrè, Milstein & Wright, 1979).

Medium for culture of Y3-Ag 1.2.3 and established hybrid cell lines was Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum (FCS).

All other cell lines used in this study (with the exception of K45 T and 117 which were gifts from Dr A. Karpas, MRC Laboratory of Molecular Biology, Cambridge) are grown routinely in our laboratory in RPMI 1640 medium with 10% newborn calf serum (NBCS), both from Gibco Biocult, Ltd, Paisley, Scotland. Ramos is a B-cell lymphoma line established from an Epstein-Barr Virus (EBV) negative Burkitt's lymphoma (BL) biopsy (Klein, Giovanella, Westman, Stehlin & Mumford, 1975). EHR-B-Ramos is one of a series of lines derived by *in vitro* transformation of Ramos with EBV (Klein *et al.*, 1975). Raji, EB4 and Daudi are B-cell lymphoma lines established from EBV positive BL biopsies (Epstein, Achong, Barr, Zajac, Henle & Henle, 1966; Epstein, Barr & Achong, 1966; Klein, Klein, Nadkarmi, Nadkarmi, Wigzell & Clifford, 1968). Daudi lacks membrane HLA-A, B, C antigens but expresses membrane HLA-DR antigens (Goodfellow, Barnstable, Jones, Bodmer, Crumpton & Snary, 1976). 117 is a lymphoid line with B-cell characteristics but lacking surface membrane immunoglobulin (Karpas, Hayhoe, Greenberger & Neumann, 1978). Molt 4 and K45T are T-lymphoma cell lines established from patients with acute lymphocytic leukaemia (Minowada, Ohnuma & Moore, 1972; Karpas, Hayhoe, Greenberger, Barker, Cawley, Lowenthal & Moloney, 1977). Before use, cells were harvested, washed three times in Hanks's balanced salt solution (HBSS) and were resuspended at the desired concentration in HBSS with 10% NBCS and 0·01 M NaN_3.

Preparation of lymphoid cells and cells from peripheral blood

Mice were killed by cervical dislocation and single cell suspensions prepared from spleen, thymus and peripheral and mesenteric lymph nodes and made to the desired concentration in HBSS + 10% NBCS + 0·01 M NaN_3.

Human and sheep peripheral blood leucocytes (PBL) were prepared from freshly taken defibrinated blood by Ficoll-Hypaque (Pharmacia) centrifugation and washed three times in HBSS. Human peripheral blood monocytes were prepared (by Mr B. Shiels) by incubating this suspension for 1 hr at +37° in 10% CO_2 air in Lab-Tek tissue culture chamber/slides (Miles Laboratories, Ltd, Bridgend, Glamorgan). The adherent cells were predominantly monocytes. Platelets were recovered from platelet rich plasma prepared from fresh human blood by centrifugation in HBSS 0·01 M EDTA + 10% NBCS at pH 6·5 in siliconed glass tubes and red blood cells were obtained from outdated O and AB human blood.

Blood from leukaemic patients was provided by Dr J. Rees, Addenbrooke's Hospital, Cambridge. Blood from a patient with Bruton's agammaglobulinaemia was provided by Dr D. L. Brown, Department of Clinical Immunology, Addenbrooke's Hospital, Cambridge.

Sheep peripheral blood lymphocytes were prepared as above from sheep blood given by Dr B. Tucker, ARC Institute of Animal Physiology, Babraham, Cambridge.

Immunization of spleen cell donors

AO rats were immunized on six occasions at intervals of 2–5 weeks with 1×10^7–5×10^8 EHR-B Ramos cells. Half the cells were injected subcutaneously (sc) at two sites and half intraperitoneally (i.p.). A final intravenous boost of 5×10^7 cells was given and four days later the rat was killed by cervical dislocation, its spleen removed and a suspension of spleen cells prepared for fusion.

Fusion

The fusion was carried out essentially as described by Galfrè, Howe, Milstein, Butcher & Howard (1977). Y3-Ag 1.2.3 cells ($4{\cdot}5 \times 10^7$) were pelleted with $1{\cdot}8 \times 10^8$ spleen cells, gently resuspended in 1 ml of polyethylene glycol 1500 at a concentration of 1 g/ml

and, after fusion, distributed into 49, 2 ml cultures in DMEM + 20% FCS in Linbro plates (Flow Laboratories, Irvine, Scotland). After 24 hr, 1 ml of medium in each cup was replaced with 1 ml of DMEM + 20% FCS + HAT containing 5×10^5 spleen cells from a non-immune AO rat, as feeders. HAT medium was used to maintain cultures for the following 6 weeks, after which time cultures were adapted to growth in normal medium by transferring first to HT medium (HAT medium without aminopterin) and then, after about 5 days, to normal medium.

Hybrid cell lines derived from this fusion are prefixed YE2.

Cloning and freezing of hybrids

Cloning of hybrid cultures was performed in soft agar essentially as described by Cotton, Secher & Milstein (1973).

For storage at −70° cells were suspended in DMEM + 10% FCS + 10% dimethylsulphoxide and frozen in aliquots of 10^6 cells.

Antibody production in rats injected with hybrid clones

Male (AO × LOU) F_1 rats were injected i.p. with 5×10^7 cloned hybrid cells, 2 weeks after an i.p. injection of 2 ml pristane (2,6,10,14-tetra methyl pentadecane, Aldrich Chemical Co., Milwaukee, Wis.). Hybrid cells grew as ascites tumours, and ascitic fluid was harvested 5–7 days later.

Indirect radioactive binding assay

Culture supernatants (50 μl) were incubated at 4° for 1 hr in U-bottom plastic microtitre trays with fresh target cells in 50 μl of HBSS + 10% NBCS + 0·01 M NaN_3 (5×10^5 cells for cell lines; 1×10^7 cells for normal lymphoid cells). After two washes the cells were resuspended in 25 μl of ^{125}I-labelled sheep anti-rat $F(ab)_2$ (a gift from Dr D. Secher) at 50,000 c.p. 100s and incubated for a further 45 min at 4°. The cells were washed three times and individual cells were transferred to tubes for γ-counting. Y3-Ag 1.2.3 supernatant was used as a negative control.

Indirect immunofluorescence

Fresh target cells ($2–4 \times 10^6$) were pelleted in glass Rh tubes (5 mm × 50 mm), resuspended in 50 μl of culture supernatant and incubated for 1 hr at 4°. After two washes in HBSS + 10% NBCS + 0·01 M NaN_3 the cells were resuspended in 25 μl of fluorescein isothiocyanate (FITC)—conjugated rabbit anti-rat Ig and incubated for a further 45 min at 4°. Cells were then washed twice, resuspended in medium and analysed by fluorescence microscopy.

Analysis of chain composition of Ig secreted by hybrids

[^{14}C] lysine was incorporated into secreted Ig essentially as described by Cotton *et al.* (1973).

Labelled clonal products were reduced by boiling with dithiothreitol and analysed by SDS-polyacrylamide electrophoresis (SDS-PAGE) according to the method of Laemmli (1970).

Radiolabelling and immunoprecipitation of cell surface proteins

Aliquots of 10^8 cells were surface-labelled with 2 M Ci Na ^{125}I (Radiochemical Centre, Amersham, Bucks.) by the lactoperoxidase method (Haustein, Marchalonis & Harris, 1975). After washing in HBSS, cells were lysed by incubation for 30 min at +4° in 1 ml 0·5% Nonidet P40 (NP40)/PBS containing 10 mM phenylmethanesulphonylfluoride (PMSF), 100 mM EDTA, pH 7·2 and 10 mM iodoacetamide. Cell debris was removed by centrifugation at 10,000 *g* for 15 min.

Ten microlitres of normal rat serum (NRS) was added to 200 μl of lysate and incubated for 6 hr at +4°. An optimal amount of rabbit anti-rat immunoglobulin was added and the mixture left at +4° overnight. Fifty micrograms Sepharose protein A beads (SpA, Pharmacia, Sweden) were incubated for 1 hr at +4° with the mixture which was then centrifuged at 10,000 *g* for 15 min.

The 'cleaned' supernatant was then transferred to a fresh tube and treated in an identical manner, using 10 μl ascites fluid from hybridoma-bearing rats, or 10 μl NRS as a negative control. Immune complexes bound to SpA were washed once in each of 0·5% NP40/1 mM EDTA/0·1 M Tris-HCL pH 8·0/10 mM NaN_3 (NET), NET/150 mM NaCl and NET/0·5 M LiCl, and after a final wash in NET were eluted and reduced by incubation for 30 min at 37° in 8 M urea/5% SDS/50 mM dithiothreitol/0·1 M Tris-HCl pH 8·0/0·01% bromophenol blue.

SDS-PAGE analysis of these samples was then performed on a 10% gel according to Laemmli (1970). Gels were stained with 0·25% Coomassie blue in methanol:acetic:water (20:5:75) to visualize molecular weight marker proteins runs in adjacent slots and, after destaining and drying, were exposed to presensitized Fuji X-ray film at −70° for 7 days (Laskey & Mills, 1975).

RESULTS

Derivation of hybrid clone YE2/36 HLK

Of the forty-five cultures set up after fusion, forty showed growth of hybrid cells. When hybrids had grown to high density, tissue culture supernatants were tested for their ability to bind to EHR-B-Ramos cells in the indirect radioactive binding assay. Out of thirty-seven cultures tested, eighteen had binding activity above background (× 3–30) but six of these lost their activity after further growth. Supernatants from culture number 28 and 36 consistently bound to EHR-B-Ramos cells at 10–20 times background even after prolonged culture.

YE2/28 was cloned and is described in Table 1. YE2/36 was cloned in soft agar. All of the clones reacted similarly to YE2/36. Clone YE2/36.7 was re-cloned in soft agar and YE2/36.7.8 selected for further study.

YE2/36.7.8 has now been cultured for 18 months without loss of activity, active cells being readily recoverable from frozen stocks. It has also been grown as an ascites tumour in pristane-primed (AO × LOU) F_1 rats. The ascitic fluid bound maximally to EHR-B-

Table 1. Reactivity of YE2/36 HLK with human cells by indirect immunofluorescence

Cells	% Immunofluorescence positive cells YE2/36 HLK	YE2 28.8*	Y3-Ag 1.2.3
Normal PBL	16	100	0
Normal peripheral blood monocytes (adherent cells)	40	100	0
T-cell acute lymphoblastic leukaemia PBL	0	100	0
Bruton's agammaglobulinaemia PBL	0	100	0
Normal platelets	0	100	0
Normal red blood cells (O and AB)	0	0	0
IgM-coated sheep red blood cells	0	0	0
IgG-coated sheep red blood cells	0	0	0

* YE2 28.8 is a hybrid recognizing a monomorphic determinant common to the HLA-A, B and C molecules (our unpublished observations).

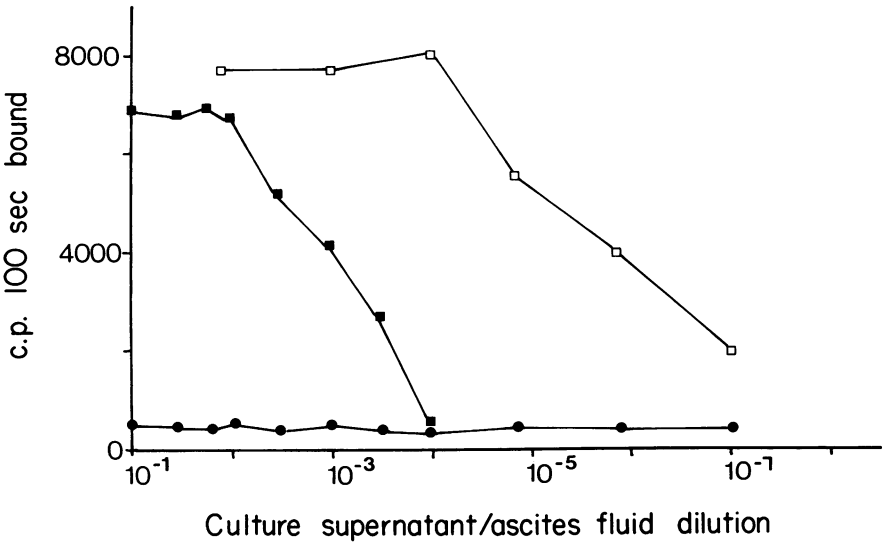

Figure 1. Titration of YE2/36 HLK binding activity on EHR-B-Ramos cells in indirect radioactive binding assay. (■) YE2/36.HLK culture supernatant; (□) ascites fluid from (AO × LOU) F_1 rats bearing YE2/36.HLK hybridomas; (●) Y3-Ag 1.2.3 culture supernatant.

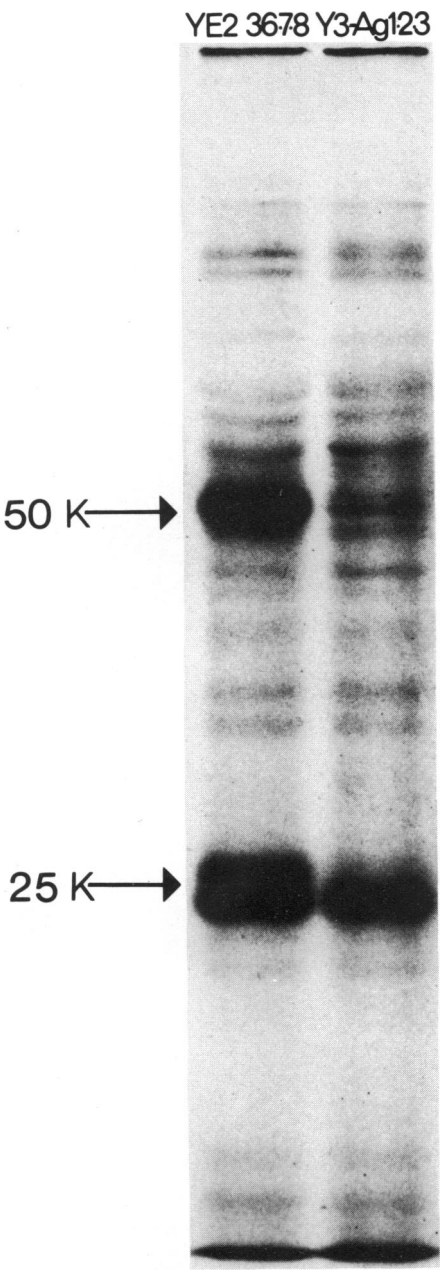

Figure 2. Analysis by SDS-PAGE of the reduced products of (a) YE2/36 HLK and (b) Y3-Ag 1.2.3.

Ramos cells at about thirty times background in the indirect radioactive binding assay and gave 50% maximal binding at a dilution of 1 in 10^6 (Fig. 1).

SDS-PAGE analysis of [^{14}C] lysine labelled YE2/36.7.8 culture supernatant showed that YE2/36.7.8 secretes a γ-like heavy chain and two distinct light chains, one of which is identical to Y3-Ag 1.2.3 κ chain. The clone is therefore properly designated YE2/36 HLK (Fig. 2).

YE2/36 HLK was not cytotoxic in a conventional ^{51}Cr release assay using guinea-pig serum as a source of complement (results not shown).

Tissue distribution of the antigen recognized by YE2/36 HLK in man

The reactivity of YE2/36 HLK with a number of human cell lines was determined by indirect radioactive binding assay (Fig. 3) and by indirect immunofluorescence (Table 1) on cell suspensions.

YE2/36 HLK bound to lines of B lymphocyte origin, including the (SmIg) negative 117 line, but not to those of T lymphocyte or non-lymphoid origin. The monoclonal antibody did not react with red cells coated with human IgM or human IgG. The pattern of reactivity on normal and abnormal human cells also suggested that YE2/36 HLK recognises an Ia antigen.

YE2/36 HLK recognizes a monomorphic determinant in man

YE2/36 HLK was tested for binding to a panel of chronic lymphocytic leukaemia (CLL) patients' cells of known HLA-DR type (provided and typed by Dr V. Joysey, Tissue Typing Laboratory, Old Addenbrooke's Hospital, Cambridge) by indirect immunofluorescent (Table 2). Positive immunofluorescence was detected with all HLA-DR types tested, indicating

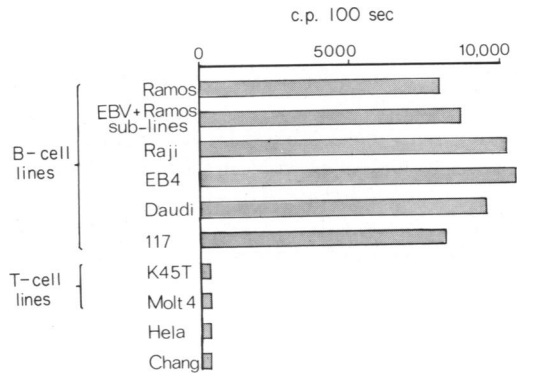

Figure 3. Reactivity of YE2/36 HLK with human cell lines in indirect radioactivity binding assay.

Table 2. Reactivity of YE2/36 HLK on a panel of cells from CLL patients of known HLA-DR type, by indirect immunofluorescence

Patient number	HLA-DR type	% Immunofluorescence positive cells	
		YE2/36 HLK	Y3-Ag 1.2.3
33	4	76	0
34	2/3	100	0
38	1	76	0
43	1/2	78	0
44	3/4 × 7 (?)	84	0
48	5/6	77	0
49	2/6	88	0
50	5/6, 7 (?)	84	0
53	3/4	94	0
57	1/5	74	0
63	3/4	80	0
64	2/4	76	0
65	4/6 (?)	88	0
66	1/2	75	0
69	7	95	0
70	1/2	94	0

that YE2/36 HLK recognizes a monomorphic determinant in man.

Biochemical nature of the antigen recognized by YE2/36 HLK

YE2/36 HLK ascitic fluid specifically precipitated three polypeptides from Raji cells, with molecular weights 34,000, 28,000 and 25,000 Daltons (Fig. 4). The 25,000 Dalton polypeptide appeared as a discrete band or as a broadening of the 28,000 Dalton band in different gel runs.

Mapping of locus coding for the antigen recognized by YE2/36 HLK in mice

As shown in Table 3, YE2/36 HLK recognizes a determinant which is polymorphic in the mouse. This determinant is present in mice of independent haplotypes b, d and q but not f, k or s, (rows 1 to 6) and, since C3H/HE (row 1), CSW & CWB (row 4) are congenic strains, it must be coded for within the H-2 region. The pattern of reactivity with B10A (4 R) (row 7) and D2GD (row 8) mice further shows that the determinant is coded for by genes within H-2 K or H-2 I-A, whilst the pattern of reactivity with AQR (row 9) & B10 T (6 R) (row 10) shows that these genes must lie within H-2 I. Taking these results together, the genes coding for the determinant recognized by YE2/36 HLK in the mouse map to the H-2 I-A region.

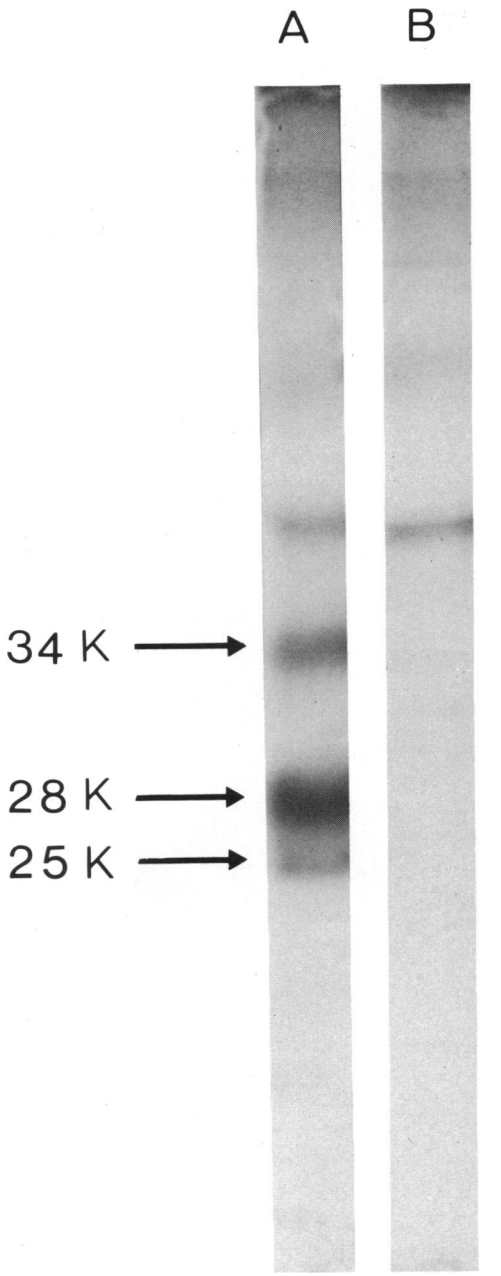

Figure 4. Immunoprecipitation of labelled protein from surface-iodinated Raji cells with (A) YE2/36 HLK ascites fluid and (B) normal rat serum.

Table 3. Strain distribution of the antigen recognised by YE2/36 HLK in mice. Indirect radioactive binding assay on crude spleen cell suspension

	H-2 haplotype									Mean c.p. 10 sec bound		
	K	I				C	S	G	D			
Strain		A	B	J	E					YE2/36 HLK	YE2 28.8	Y3-Ag 1.2.3
1. AKR B10-BR C3H/HE CBA/Ca	k	k	k	k	k	k	k	k	k	460	360	360
2. SJL	s	s	s	s	s	s	s	s	s	340	340	340
3. BALB/c B10 D2 DBA/2	d	d	d	d	d	d	d	d	d	1840	380	475
4. C57 BL CSW CWB	b	b	b	b	b	b	b	b	b	1220	420	335
5. B10 M	f	f	f	f	f	f	f	f	f	430	380	340
6. B10 G DBA/1	q	q	q	q	q	q	q	q	q	1820	620	440
7. B10A (4R)	k	k |	b	b	b	b	b	b	b	500	520	435
8. D2GD	d	d |	b	b	b	b	b	b	b	1860	NT*	600
9. AQR	q |	k	k	k	k |	d	d	d	d	600	485	390
10. B10T (6R)	q |	d	d	d	d |	d	d	d	d	2300	NT	600
11. B10A (5R)	b	b	b |	k	k |	d	d	d	d	1200	675	450
12. B10A (3R)	b	b	b	b	?	?	d	d	d	1200	NT	400

* NT, not tried.

The level of binding of YE2/36 HLK to spleen cells in the indirect radioactive binding assay was consistently lower for H-2 I-A b strains than for H-2 I-A d or q strains. In BALB/c mice (H-2 d) YE2/36 HLK stained approximately 36% of splenocytes, approximately 14% of lymphocytes from peripheral and mesenteric lymph nodes and was negative on thymocytes, which correlates with the expected distribution of mouse Ia antigen.

Reaction of YE2/36 HLK with sheep lymphocytes

YE2/36 HLK reacted with 15%–20% of peripheral blood lymphocytes in sheep of all breeds tested, but appeared to be polymorphic in the Finnish Landrace and Clun breeds. Further investigations are in progress.

DISCUSSION

This paper describes the first rat monoclonal antibody (YE2/36 HLK) capable of precipitating the human Ia molecule. It was raised by immunization of AO rats with B-lymphoma cells followed by fusion of their spleen cells to the rat myeloma Y3-Ag 1.2.3.

A number of groups have raised mouse monoclonal antibodies against human Ia antigens (Charron & McDevitt, 1980; Lampson & Levy, 1980; Quaranta, Walker, Pellegrino & Ferrone, 1980: Accolla, Gross, Carrel & Corte, unpublished). Another rat monoclonal antibody which recognizes an antigen with the cellular distribution of human Ia antigen has also been described (Ziegler, Heinrichs, Secher, Wright & Milstein, 1980).

The specificity of YE2/36 HLK was established by

studies of the tissue distribution of its target and by immunoprecipitation. YE2/36 HLK does not react with the majority of peripheral blood T lymphocytes or with platelets, but reacts strongly with all B lymphocytes and 40% of peripheral blood monocytes. It also reacts with a large proportion of T lymphocytes activated either *in vitro* or *in vivo* (Crawford, Brickell, Tidman, McConnell, Hoffbrand & Janossy, 1981) and has been used to distinguish between common acute lymphoblastic leukaemia, in which Ia-positive blasts predominate, and thy-acute lymphoblastic leukaemia, in which the abnormal cells are Ia-negative (Janossy, Tidman, Crawford, Papageorgiou, Francis & Bradstock, 1980).

In addition to the expected 34,000 and 28,000 Dalton chains, YE2/36 HLK precipitates a 25,000 Dalton polypeptide from surface-iodinated Raji cells. The origin of this polypeptide is not known. It may represent a post-translational modification or an unglycosylated form of the 28,000 (β) Dalton chain. Alternatively, YE2/36 HLK may be precipitating two distinct species of β chain. Accolla *et al.* (unpublished) have recently described two mouse monoclonal antibodies which define two distinct subsets or isotypes of human Ia-like α and β chains. Quaranta *et al.* (1980) have also used a mouse monoclonal antibody to separate subsets of human Ia-like antigens which have β chains of differing molecular weights. In addition, Lampson & Levy (1980) have described work with two mouse monoclonal antibodies directed against human Ia-like antigens, one of which precipitates 34,000 and 28,000 Dalton chains from Raji cells whilst the other also precipitates a third polypeptide of molecular weight 25,000 Daltons.

One drawback to these analyses, discussed by Accolla *et al.* (unpublished), is that the antigens recognized by the monoclonal antibodies have not been rigorously mapped either within or outside the HLA-DR region. The observation that YE2/36 HLK cross-reacts polymorphically in mice and recognizes an antigen which maps to the H-2 I-A sub-region is good evidence that the target antigen in man is encoded within HLA-DR, and YE2/36 HLK and other monoclonal antibodies showing such fortuitous cross-reactions may help to overcome some of these difficulties.

Although monomorphic in man, the determinant recognized by YE2/36 HLK is polymorphic in mice, mapping to the H-2 I-A sub-region and coded for by strains with H-2 I-A haplotypes b, d and q. Although H-2 I-A haplotypes b and d both code for public specificity Ia8, this specificity is not encoded by H-2 I-A q and no other Ia specificities are shared by these haplotypes. Thus, YE2/36 HLK appears to recognize a previously unspecified determinant on the mouse Ia molecule. Other workers have also raised monoclonal antibodies against major histocompatibility antigens which cross-react with a number of different species. Thus, McMaster & Williams (1979) have described two monoclonal antibodies raised against rat Ia antigens, one of which recognizes a determinant which is monomorphic in the rat but which cross-reacts polymorphically with mouse Ia antigen, mapping to the H-2 I-A or I-B regions. The other reacts with a determinant which is polymorphic in the rat, the mouse and in man and which may be coded for in the mouse by the H-2 I-A, I-B or I-C regions. In addition, Brodsky, Parham, Barnstable, Crumpton & Bodmer (1979) review a number of monoclonal antibodies, raised by them against determinants on the HLA-A, -B and -C molecules, which cross-react with non-human primates. The cross species reactivity of monoclonal antibodies to the HLA-DR region product raises the possibility that other fortuitous cross-reactions may be found to identify molecules encoded within other H-2 I subloci. This would be useful, especially in the case of H-2 I-J which is thought to be involved in suppressor activity, (Tada, Taniguchi & David, 1976) and against the product of which there are, as yet, no reliable antisera.

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