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Production and characterization of monoclonal antibodies recognizing the α-chain subunits of human Ia alloantigens*

T. E. ADAMS, JULIA G. BODMER & W. F. BODMER *Imperial Cancer Research Fund, Lincoln's Inn Fields, London*

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Summary. Two monoclonal antibodies, TAL-1B5 and TAL-3C3, specific for human Ia α-chain subunits have been produced by fusing P3/NSI/1-Ag4-1 mouse myeloma cells with spleen cells from a BALB/c mouse immunized with purified α-chains. Specificity for the α-chain subunits was initially established using a solid-phase radioimmunoassay. Indirect binding assays demonstrated that TAL-1B5 bound strongly to all human B lymphoblastoid lines tested and to CLLs, but only weakly to PBL-B cells and not to PBL-T cells or the T-cell lines Molt 4 and HSB-2. TAL-3C3 bound only weakly to B lymphoblastoid lines and not to CLLs or PBL-B cells.

From ^{125}I cell surface-labelled lysates TAL-1B5 immunoprecipitated a 33,000(α):28,000(β) Ia dimer, but TAL-3C3 failed to immunoprecipitate cell surface molecules. Under denaturing conditions, however, both TAL-1B5 and TAL-3C3 immunoprecipitated the 33,000 α-chain subunit. Competitive inhibition studies demonstrated that both monoclonal antibodies recognize the same or spatially related α-chain antigenic determinants with some slight cross-reactivity against β-chains. 2D-NEPHGE/SDS-PAGE analysis of TAL-1B5 immunoprecipitates from [^{35}S]-methionine biosynthetically labelled cells revealed the presence of a number of α-chain spots in association with β-chain products of three previously described loci (β-1, β-2, β-3) suggesting that this antibody recognizes an antigenic site common to those human Ia α-chains so far identified.

* The term Ia is used to describe those cell-surface molecules encoded by the HLA-D region having a heterodimer structure similar to the murine Ia antigens which are coded for in the homologous part of the H-2 region.

Abbreviations: 2-D, two-dimensional; NEPHGE, non-equilibrium pH gradient electrophoresis; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; NS1, P3/NS1/1-Ag4-1 BALB/c mouse myeloma cells; FCS, foetal calf serum; HAT, hypoxanthine, aminopterin (methotrexate) and thymidine; CLL, chronic lymphatic leukaemia; DOC, sodium deoxycholate; PBS, phosphate-buffered saline; K, molecular weight $\times 10^{-3}$; PLT, primed lymphocyte test; IEF, isoelectric focusing; PBL, peripheral-blood lymphocytes; RAM, rabbit $F(ab')_2$ anti-mouse IgG antibody.

Correspondence: Dr Julia G. Bodmer, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX.

INTRODUCTION

The human homologues of the mouse H-2 Ia alloantigens are those associated with HLA-DR and coded for by the HLA-D region, whose serological complexity is not yet fully understood. At present the original serologically defined locus, HLA-DR, is well established (Bodmer, 1978). However, the identification of at least one related series of polymorphic alloantigens, the DC (Tosi *et al.*, 1978), MB (Duguesnoy, Marrani & Annen, 1979), or LB (Termijtelen *et al.*, 1980) series, in linkage disequilibrium with HLA-DR products, and the partial biochemical identification of these

products using monoclonal antibodies (Shackelford *et al.*, 1981b; de Kretser *et al.*, 1982b) establishes the existence of a second locus coding for HLA-DR related alloantigens (for review see Dick, 1982). In another study performed in this laboratory, a biochemical analysis of human Ia antigens on a variety of HLA-DR homozygous cell lines using monoclonal antibodies against monomorphic Ia antigenic determinants, provides evidence for the existence of a third polymorphic Ia 33K(α):28K(β) chain polypeptide complex (de Kretser *et al.*, 1982a). Thus as many as three loci may contribute to the expression of serologically detectable human Ia alloantigens.

Recent studies using rabbit antisera to the separated human Ia α and β subunits have suggested a specific role for the α-chain in T-cell interactions *in vitro* (Palacios *et al.*, 1982; Palacios, 1981). Monoclonal antibodies to the separated subunits should therefore be useful in clarifying further the functional role of Ia α- and β-chains. Using a variety of immunochemical techniques a number of monoclonal antibodies have been characterized as specific for antigenic determinants on human Ia β-chains (Quaranta, Tanigaki & Ferrone, 1981; Shackelford, Lampson & Strominger, 1981a; Johnson *et al.*, 1982) and on subsets of α-chains (Guy *et al.*, 1982). In the present study we describe the production and characterization of two monoclonal antibodies, TAL-1B5 and TAL-3C3, that bind to epitopes carried on α-chain subunits of human Ia antigens. We have used one of these, TAL-1B5, to analyse the heterogeneity of Ia α-chain subunits on B lymphoblastoid cell lines.

MATERIALS AND METHODS

Cell lines and media

The cell line used for fusion was P3/NS1/1-Ag4-1, an 8-azaguanine BALB/cMOPC21-derived myeloma (Kohler, Howe & Milstein, 1976). Maja, MST, WT49, Priess, IDF, WT46, Mann, IBW9, Madura T, Koz and LL ICRF are HLA-DR homozygous B lymphoblastoid lines (de Kretser *et al.*, 1982a) maintained in the Tissue Antigens Laboratory, ICRF. Bristol 8 is an HLA-DR heterozygous (DR2,5) B lymphoblastoid cell line (Brodsky *et al.*, 1979). Molt 4 (Minowada, Ohnuma & Moore, 1972) and HSB-2 (Adams, Flowers & Davis, 1968) are T-cell lines. Cells were grown in hydrogen carbonate-buffered RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), penicillin (100 units/ml) and streptomycin (100 units/ml). The hybrids TAL-IB5 and TAL-3C3 were initially grown in the same medium supplemented with 20% FCS, and HAT constituents (10^{-4} M hypoxanthine, $1{\cdot}6 \times 10^{-5}$ M thymidine, and 10^{-5} M methotrexate).

Human peripheral-blood lymphocytes were prepared by defibrination and separation on Ficoll-Triosil, as described (Bodmer & Bodmer, 1979). Separation of lymphocytes into T- and B-cell subpopulations was performed according to de Kretser, Bodmer & Bodmer (1980). Isolation of lymphocytes from patients with chronic lymphatic leukaemia (CLL) was performed as above.

Purification of human Ia α-chains

The preparation and purification of human Ia α-chains were performed according to a protocol developed in the laboratory of Dr M. J. Crumpton, ICRF. Briefly, a glycoprotein fraction (5–10 mg) was prepared from a pooled sodium deoxycholate (DOC)-solubilized plasma membrane–microsome preparation, obtained from 4×10^{10} Bristol 8 lymphoblastoid cells, by affinity chromatography using a *Lens culinaris* lentil lectin–Sepharose 4B column (Pharmacia, Uppsala, Sweden) as described by Snary *et al.* (1977). The glycoproteins were precipitated with 4 volumes of $-20°$ ethanol and incubation at $-20°$ for at least 48 h. The precipitated glycoproteins were pelleted by centrifugation for 15 min at 3000 r.p.m. and were redissolved in 500 μl of Laemmli SDS-PAGE sample buffer (Laemmli, 1970), containing 0·1 M dithiothreitol. The sample was then boiled for 5 min at 100°, and allowed to cool prior to the addition of fluorescamine (4-phenyl spiro [furan-2 (3H), 1-phthalan]-3,3′-dione, in *NN′* dimethyl formamide, 6 mg/ml w/v; Sigma, St. Louis, MO) at a final concentration of 0·3 mg fluorescamine/mg of glycoprotein. Fluorescamine binds to primary amines to yield fluorescent products (Ragland, Pace & Kemper, 1974) and thus allows the visualization of proteins under u.v. light after electrophoretic separation.

The glycoprotein sample was layered onto a single large slot (25 cm in length) at the top of a preparative acrylamide slab gel (32 cm × 24 cm × 0·5 cm) containing a 5% (w/v) acrylamide stacking gel and a 10% (w/v) running gel. Electrophoresis was performed in SDS-Tris/glycine buffer (Laemmli, 1970); the gels were run at 100 V until the tracking dye reached the bottom of the gel (16–20 hr). Upon completion of electrophoresis the smaller gel plate was removed, and the separated glycoproteins visualized under u.v. light. The band

corresponding to the 33K α-chain subunit was then cut from the gel. The excised gel slices were then macerated by being forced through a 5-ml plastic syringe and the glycoprotein eluted by placing the acrylamide in a flask containing 15–20 ml of 0·5% DOC in 10 mM Tris-HCl buffer, pH 8·2, and allowing further disruption by means of a magnetic stirrer for 24–48 hr at 4°. The eluted glycoprotein was recovered by centrifuging the acrylamide suspension at 10,000 r.p.m. for 20 min, Millipore filtration of the supernatant, and concentration by pressure dialysis. The purified subunit was then stored frozen at −70° until required. The yield of purified α chains varied somewhat, with 300–1000 μg being obtained by this means from 4×10^{10} cells. The purity of the antigen preparation was established by iodination and re-electrophoresis of a small sample of protein. No cross-contamination of the α-chain preparation by β-chains could be observed upon autoradiography.

Immunization

Immunization of BALB/c mice with α-chain preparations was performed in a similar manner to that described by Stahli *et al.* (1980). Mice received a primary i.p. injection of 50 μg of antigen in Freund's complete adjuvant, followed by a boost 28 days later of 50 μg of antigen delivered i.p. in Freund's incomplete adjuvant. The mice were then 'rested' for 10 weeks, after which they received a further 50 μg of antigen i.p. in incomplete adjuvant. Four weeks later they received, on successive days, 50 μg of antigen i.p., 50 μg of antigen i.v., and 50 μg antigen i.p. and i.v. Spleens were removed for the following day and teased into a single-cell suspension for immediate fusion with NS1.

Cell fusion, cloning and tumour growth of hybrids

Cell fusion was performed using a slight modification of the technique described by Galfre *et al.* (1977) using polyethylene glycol 4000 (Merck, Darmstadt, W. Germany). Suspensions of fused cells (10^7 NS1/spleen preparation) in HAT-containing medium were plated into 24-well tissue culture trays (Linbro, New Haven, CT), each well containing 2×10^4 peritoneal macrophages as a feeder layer. Cells from wells with active supernatants were transferred to tissue culture flasks and maintained on medium containing HAT constituents prior to cloning.

Positive hybrids were either cloned by limiting dilution (Goding, 1980) or by single-cell cloning. Ascites tumours were propagated by the inoculation of pristane-primed BALB/c mice with $1–3 \times 10^6$ cloned hybrid cells that had been weaned off HAT constituents.

Indirect trace binding assays

A solid-phase radioimmunoassay to detect the binding of monoclonal antibodies to purified α-chain preparations was performed in 96-well polyvinyl microtitre plates (Dynatech Laboratories Inc., Alexandria, VA). Alpha-chain preparations in 0·5% DOC, Tris-HCl buffer, pH 8·2, were dialysed against 0·1 M Tris-HCl buffer, pH 8·2 for 3–4 hr prior to use to reduce the concentration of DOC. Preliminary studies had demonstrated that DOC inhibited the binding of antigen to the microtitre wells. Antigen (25 μl of 40 μg/ml pure α-chain) was added to each well of a microtitre plate and incubated for 1 hr at 37°. The protein solution was then removed and 100 μl of 10% FCS in phosphate-buffered saline (PBS) added to each well followed by a further incubation for 1 hr at 37°. The wells were then washed three times with PBS and 25 μl of hybridoma supernatant added to each well. The wells were then incubated overnight at room temperature, after which they were washed three times with PBS prior to the addition of 25 μl of 10% FCS-PBS containing 2×10^5 c.p.m. of ^{125}I-labelled rabbit $F(ab')_2$ anti-mouse IgG antibody (RAM) to each well. After 90 min the plates were washed five times, dried, the wells cut out and counted for bound radioactivity. All screening assays were performed in duplicate. Titration of active supernatants and ascites fluid were performed using 10% FCS-PBS as diluent. As a positive control, antiserum from the immunized mice was used in each assay.

An indirect trace binding assay to detect the binding of monoclonal antibodies to cell-surface determinants was performed essentially as described (Stocker & Heusser, 1979) using glutaraldehyde-fixed cells (2×10^5 cells/well). Titration studies revealed that the monoclonal antibody supernatants used contained saturating levels of antibody.

Characterization and purification of the Ig secreted by hybridomas

Subclass characterization of TAL-1B5 and TAL-3C3 was performed using immunodiffusion analysis (Ouchterlony, 1969). Rabbit anti-mouse IgM, IgG and IgG subclass-specific antisera were obtained from Miles Laboratories Ltd., Slough, U.K.

Monoclonal antibodies were purified from 10 times concentrated culture supernatants or ascites fluid on

protein A-Sepharose 4B columns (Pharmacia) (Ey, Prowse & Jenkins, 1978).

Biosynthetic and ^{125}I-labelling of cells, and preparation of cell lysates

Biosynthetic labelling of lymphoblastoid cell line with [^{35}S]-methionine (The Radiochemical Centre, Amersham, U.K.) was performed as described by Whitehead, Sim & Bodmer (1981). Cell surface proteins were labelled with ^{125}I (The Radiochemical Centre) by the lactoperoxidase technique (Hubbard & Cohn, 1975). Following labelling cell suspensions (routinely 2×10^7 cells) were washed twice in cold methionine-free RPMI-1640 and were solubilized in 1% (w/v) Nonidet P-40 in 10 mM Tris-HCl buffer, pH 7·4, containing 0·15 M NaCl, 1 mM EDTA, 0·1 mM phenyl methyl sulphonyl fluoride (PMSF), and 1 mg/ml bovine serum albumin (BSA), at a concentration of 10^7 cells/ml for 1 hr on ice. The lysate was centrifuged for 5 min at 2,000 *g* and the supernatant passed through a Millipore filter (0·22 μm: Millipore Ltd., London, U.K.). Immunoprecipitation studies were routinely performed immediately on labelled cell lysates.

Immunoprecipitations and one-dimensional electrophoresis

Immunoprecipitations from labelled cell lysates were performed as described by Owen, Kissonerghis & Lodish (1980). Immunoprecipitations under denaturing conditions were accomplished by adding SDS to a final concentration of 1% to each lysate and boiling for 5 minutes prior to the addition of antibody. Routinely 10–20 μg of purified monoclonal antibody was used per immunoprecipitation. As TAL-3C3 does not bind to *S. aureus* Cowan I strain bacteria the additional step of incubating antibody–antigen complexes with 2–3 μl of a rabbit anti-mouse Ig antiserum for 15 min at room temperature was included prior to the addition of fixed *S. aureus* bacteria.

SDS-PAGE was performed according to Laemmli (1970) in 0·1% SDS-Tris/glycine buffer on 10% (w/v) acrylamide slab gels using a 5% (w/v) stacking gel. All samples were reduced with 2-mercaptoethanol prior to electrophoresis. After fixing, Coomassie Blue staining, destaining and drying gels as described by Fairbanks, Steck & Wallach (1971), autoradiographs were produced by exposure to Kodak SB-5 film (^{35}S) or XAR-5 film (^{125}I).

Two-dimensional (2-D) NEPHGE/SDS-PAGE

Immunoprecipitates were analysed by 2-D gel electrophoresis as described by O'Farrell, Goodman & O'Farrell (1977) using a non-equilibrium pH gradient in the first dimension. Immunoprecipitates representing 5×10^6 cell equivalents were routinely analysed. Labelled proteins were revealed by fluorography.

Competitive inhibition studies using radiolabelled monoclonal antibodies

Protein-A-purified monoclonal antibodies were iodinated using the chloramine-T method (Greenwood, Hunter & Glover, 1963). The absorption of purified α-chain onto the wells of microtitre trays was performed as described above.

Twenty-five microlitre serial dilutions of purified, unlabelled antibody (starting concentration 50 μg/ml) were added to each antigen-coated well, followed immediately by the addition of 1×10^5 c.p.m. (10 μl) of ^{125}I-labelled competing antibody. The wells were incubated for 3–4 hr at room temperature, washed five times with PBS and the bound radioactivity determined. The positive control for competitive binding was an unlabelled antibody competing against itself. As a negative control, the monoclonal antibody W6/32, which recognizes a common determinant shared by the HLA-A,B,C cell surface glycoproteins (Barnstable *et al.*, 1978) was used.

RESULTS

Identification of cell lines secreting antibody against Ia α chains

From a single fusion double cloned, antibody-secreting hybrid cell lines, TAL-1B5 and TAL-3C3, were established in culture. The specificity of TAL-1B5 and TAL-3C3 for the α-chain subunit of human Ia antigens was initially demonstrated using a solid-phase radioimmunoassay. Figure 1 illustrates the binding of protein A-Sepharose purified antibodies to the α-chain when compared to the negative control antigen, the 43K HLA-ABC heavy chain. In Table 1, some evidence of cross-reactivity against the β-chain subunit can be seen with respect to both TAL-1B5 and TAL-3C3. This is unlikely to be the result of contaminating subunits as the purity of both preparations could be demonstrated by SDS-PAGE analysis of iodinated chain preparations (data not shown). Such cross-reactivity may be due to homologies between the membrane proximal external domains of the DR and α-chain subunits, as demonstrated by DNA sequencing (Lee *et al.*, 1982).

Ouchterlony analysis of concentrated supernatants identified both TAL-1B5 and TAL-3C3 antibodies at IgGl, although TAL-1B5 bound *S. aureus* Cowan I strain protein A under normal assay conditions.

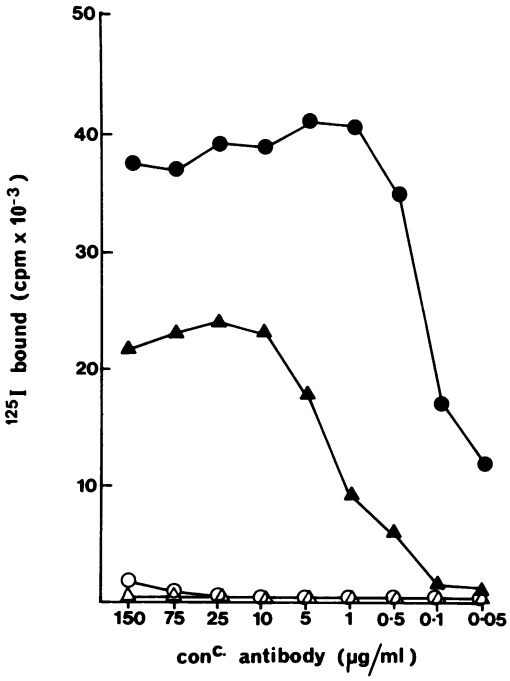

Figure 1. Binding of TAL-1B5 (●,○) and TAL-3C3 (▲,△) to Ia α-chains (●,▲) and the HLA-ABC (43K) subunit (○,△) detected by trace-binding of ^{125}I-labelled rabbit F(ab')$_2$ anti-mouse fragments as described in 'Materials and Methods'.

Table 1. Binding of TAL-1B5 and TAL-3C3 supernatant to human Ia α- and β-chains, and HLA-ABC (43K) chain

Monoclonal supernatant	α-chain subunit	β-chain subunit	HLA-ABC subunit
TAL-1B5	35·2*	3·2	0·7
TAL-3C3	23·2	4·1	1·9
W6/32†	1·2	1·0	1·1

* Indirect solid-phase radioimmunoassay performed as described in 'Materials and Methods'. Results expressed as c.p.m. x 10^{-3} of ^{125}I-labelled rabbit F(ab')$_2$ anti-mouse fragments bound.

† Negative control antibody.

Activity of TAL-1B5 and TAL-3C3 with human cells and cell lines

To investigate whether the antigenic determinant(s) recognized by TAL-1B5 and TAL-3C3 on denatured Ia α-chains were also present on Ia molecules expressed at the cell surface, both antibodies were screened against a variety of Ia$^+$ and Ia$^-$ cells of human origin. As can be seen from Table 2, TAL-1B5 bound strongly to all B lymphoblastoid cell lines tested, and to CLLs, but only weakly to PBL-B cells and not to Molt 4 and HSB-2 (human T-cell lines), or peripheral-blood T cells. In contrast, TAL-3C3 bound only weakly to B lymphoblastoid cell lines, but not to B cells, CLLs or cells of T-cell lineage. Thus the ability of TAL-1B5 to recognize an α-chain epotope in the denatured conformation remained unchanged, although the ability of TAL-3C3 to bind to an α chain epitope on the cell surface was significantly reduced.

Table 2. Binding of TAL-1B5, TAL-3C3 and DA2 monoclonal antibodies to cells and cell lines of human origin

		^{125}I-labelled RAM* (c.p.m. x 10^{-3})		
Cells		DA2†	TAL-1B5	TAL-3C3
Dr-homozygous B-cell lines				
MAJA	(DR1)	28·4	14·3	0·6
MST	(DR2)	23·6	12·7	3·5
WT49	(DR3)	35·7	16·5	3·9
PRIESS	(DR4)	24·1	20·2	3·2
IDF	(DR5)	26·4	13·9	2·9
WT46	(DRw6)	32·3	26·9	3·9
MANN	(DR7)	21·0	12·9	2·0
MADURA T	(DRw8)	29·8	13·3	0·9
COS	(DRw9)	15·1	10·1	2·7
LL ICRF	(DRw10)	25·2	13·5	3·3
T-cell lines				
HSB-2		0·8	1·2	1·5
MOLT.4		0·5	0·6	1·2
Peripheral blood				
CLLs‡		7·8	7·6	0·9
PBL.B‡		6·8	1·7	0·6
PBL.T‡		0·8	0·1	0·2

* Values expressed as mean of duplicate after background subtracted.

† DA2 is a monoclonal antibody against a monomorphic antigenic determinant expressed on all human Ia antigens (Brodsky *et al.*, 1979).

‡ Mean of three results.

Competitive inhibition studies

Competitive inhibition studies were performed in order to indicate whether the two monoclonal antibodies were recognizing the same antigenic determinants or possibly distinct determinants that might be carried on the same or different populations of human Ia α chains. Increasing concentrations of unlabelled TAL-1B5 successfully inhibited the binding of ^{125}I-labelled TAL-1B5 and TAL-3C3 to Ia α-chains in a solid-phase system (Fig. 2); in contrast TAL-3C3 failed to inhibit the binding of ^{125}I-labelled TAL-1B5 to Ia α-chains, although it successfully inhibited binding of ^{125}I-labelled TAL-3C3. This data may be interpreted two ways. It may be that TAL-1B5 and TAL-3C3 recognize spatially related epitopes on the same molecule, and that the binding of TAL-1B5 results in steric hindrance with respect to the binding of TAL-3C3 to its epitope but not vice versa. Alternatively both antibodies might recognize the same epitope, and then the ability of TAL-1B5 to inhibit the binding of TAL-3C3 to this epitope and not vice versa simply reflects a difference in affinity that the two antibodies have for the antigenic site in question. A third possibility is that TAL-1B5 recognizes additional subsets of Ia α-chains not detected by TAL-3C3.

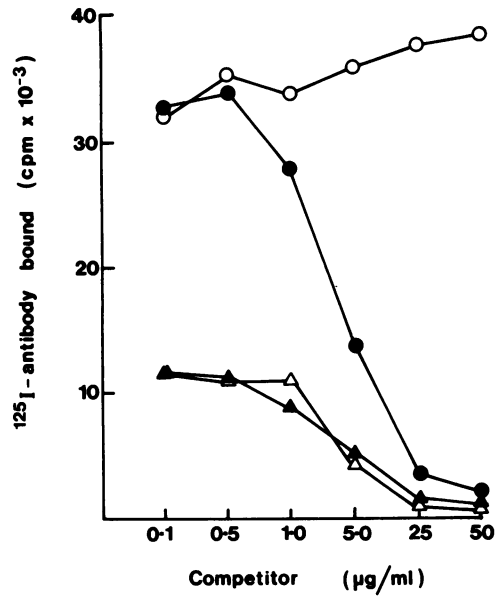

Figure 2. Competitive inhibition of binding of radiolabelled monoclonal antibodies TAL-1B5 and TAL-3C3 to human Ia α-chains. Binding is expressed as c.p.m. of ^{125}I-labelled monoclonal antibody bound to Ia α-chains in the presence of various concentrations of a competing, unlabelled antibody. (●—●) TAL-1B5 *vs* ^{125}I-TAL-1B5; (○—○) TAL-3C3 *vs* ^{125}I-TAL-1B5; (▲—▲) TAL-3C3 *vs* ^{125}I-TAL-3C3; (△—△) TAL-1B5 *vs* ^{125}I-TAL-3C3.

Immunoprecipitation studies

The antigenic specificity of both TAL-1B5 and TAL-3C3 was further demonstrated by immunoprecipitations performed using lysates from ^{125}I cell surface-labelled B lymphoblastoid lines under normal and denaturing conditions. Under normal conditions TAL-1B5 immunoprecipitated from radiolabelled Bristol 8 cells a characteristic Ia antigen doublet with both 33K(α) and 28K(β) subunits being observed (Fig. 3, track a). Under conditions whereby radiolabelled proteins were denatured by boiling in 1% SDS prior to immunoprecipitation, only the 33K α-chain subunit was precipitated by TAL-1B5 (Fig. 3, track b). It

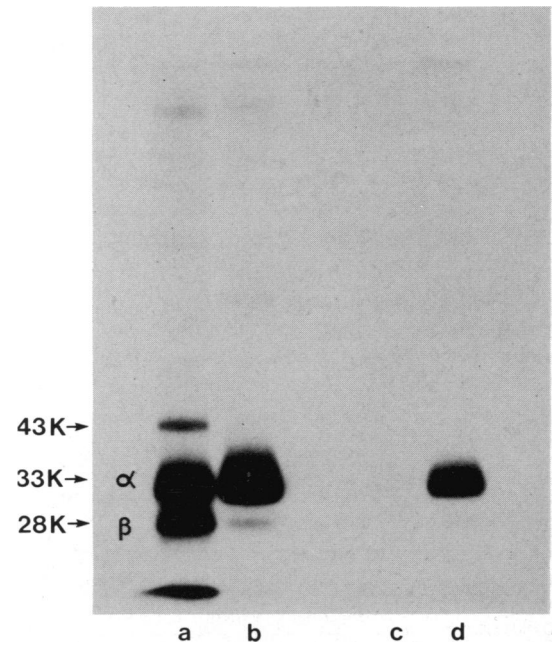

Figure 3. SDS-PAGE analysis of immunoprecipitates of TAL-1B5 and TAL-3C3 from Bristol 8 ^{125}I-labelled cell-surface proteins under normal and SDS-denaturing conditions. Track a: material precipitated with TAL-1B5 from untreated lysate. Track b: material precipitated with TAL-1B5 from lysate denatured by boiling for 5 min in 1% SDS. Track c: material precipitated with TAL-3c3 from untreated lysate. Track d: material precipitated with TAL-3C3 from SDS-denatured lysate.

seems likely that the trace amounts of 28K β-chains observed in Fig. 3b may represent some reassociation of the complex during the immunoprecipitation procedure. TAL-3C3 failed to precipitate any Ia antigens under normal conditions (Fig. 3, track c); however, after SDS-denaturation of labelled lysates, this antibody clearly immunoprecipitated the 33K α-chain subunit (Fig. 3, track d).

Interestingly, associated with the Ia α:β chain complex immunoprecipitated with TAL-1B5 was a protein with molecular weight of approximately 43K. A similar association has been noted when immunoprecipitations are performed using other monoclonal antibodies against human Ia antigens (unpublished observations). Other studies suggest that this protein is in fact the HLA-ABC chain (H. Durbin & W. F. Bodmer, personal communication).

NEPHGE/SDS-PAGE analysis of immunoprecipitates of TAL-1B5

Analysis of immunoprecipitates of ^{35}S-biosynthetically labelled human Ia antigens has highlighted both the complexity of the loci assumed to code for these antigens and the complex associations that exist between these antigens and a number of other proteins during biosynthesis (de Kretser *et al.*, 1982a, b). As demonstrated from studies using ^{125}I-labelled cell surface products, TAL-1B5 immunoprecipitates Ia α-chain subunits both in association with, and independent from, β-chain subunits (Fig. 3, tracks a, b). It was therefore of obvious interest to determine whether this antibody recognized products of all the biochemically recognized Ia loci, or only a subset thereof, using NEPHGE/SDS-PAGE analysis.

Immunoprecipitates formed by TAL-1B5 with ^{35}S-labelled whole cell lysates from the Maja (DR1) cell line and analysed by NEPHGE/SDS-PAGE and shown in Fig. 4A. A complex pattern of spots characteristic of immunoprecipitates of ^{35}S-labelled Ia antigens is observed. To the left of the gel (representing the acidic region obtained in the first dimesion under NEPHGE conditions) a heterogeneous collection of α-chain spots can be found, including those previously designated DRα and ε (de Kretser *et al.*, 1982a). Up to 10 spots may be resolved upon close examination. Three 'families' of β-chain spots (β-1, β-2, β-3) may be distinguished, representing the biochemically characterized, polymorphic products of three loci (de Kretser *et al.*, 1982a, b). The series of spots observed within each β-chain 'family' probably represent different stages of glycosylation and biosynthesis prior to, and including, the expression of the final product at the cell surface. In this respect the relationship between the individual α-chain spots is less clear.

In addition, the presence of a series of major spots (Im and Ii) representing different stages in biosynthesis and processing of the so-called 'invariant polypeptide', together with other minor spots (δ-1, σ-2) and actin (Ac) are observed. The overall pattern is representative of that obtained when immunoprecipitation is performed using monoclonal antibodies directed against monomorphic Ia antigenic determinants (de Kretser *et al.*, 1982a) or rabbit antisera raised against purified Ia glycoproteins from B lymphoblastoid lines (unpublished observations).

When TAL-1B5 immunoprecipitates from SDS-denatured, biosynthetically labelled lysates are examined a strikingly simpler picture is found. As demonstrated in Fig. 4B the spots designated DRα and ε are preferentially precipitated. Absent are a number of the more acidic, higher molecular weight α-chain polypeptides; in addition none of the invariant series of polypeptides or β-chain spots are observed. Why the more basic DRα and ε polypeptides are preferentially precipitated is not clear. It may reflect the inaccessibility of the antigenic site for TAL-1B5 on some biosynthetic α-chain intermediates when immunoprecipitations are performed under denaturing conditions.

NEPHGE/SDS-PAGE analysis of TAL-1B5 immunoprecipitates from ^{35}S-labelled whole cell lysates from the IBW9 (DR7 homozygous) cell line again demonstrate the complexity of Ia products recognized by this antibody (Fig. 5A). Predominant are the DRα:β-1 series of spots (de Kretser *et al.*, 1982b). Also present, but to a much lesser extent, are ε and at least two distinct series of β-chain polypeptides, including that designated β-2. The differences in radiolabelling of the different β-chain spots are more marked in this cell line when compared to Maja (Fig. 4A), and may reflect differences in the level of expression of these antigens both within and between cell lines. Analysis of TAL-1B5 immunoprecipitates from SDS-denatured, ^{35}S-labelled lysates of IBW9 again reveal a much simpler pattern, with only the DRα chain polypeptides being observed (Fig. 5B). The ε spot is not readily detectable, although this probably reflects the low levels of labelled ε originally present (Fig. 5A; de Kretser *et al.*, 1982b) and the inability of TAL-1B5 to precipiate easily all biosynthetically-labelled material

under denaturing conditions. In Fig. 5C a NEPHGE/SDS-PAGE analysis of an immunoprecipitate from a ^{35}S labelled, SDS-denatured cell lysate of IBW9 using TAL-3C3 is presented. This antibody immunoprecipitates an identical DRα chain pattern to that obtained with TAL-1B5 under the same conditions (Fig. 5B) but of much less intensity. It is therefore unlikely that TAL-3C3 recognizes a subset of Ia α-chains. Thus the differences observed between TAL-1B5 and TAL-3C3 in their respective abilities to bind to denatured

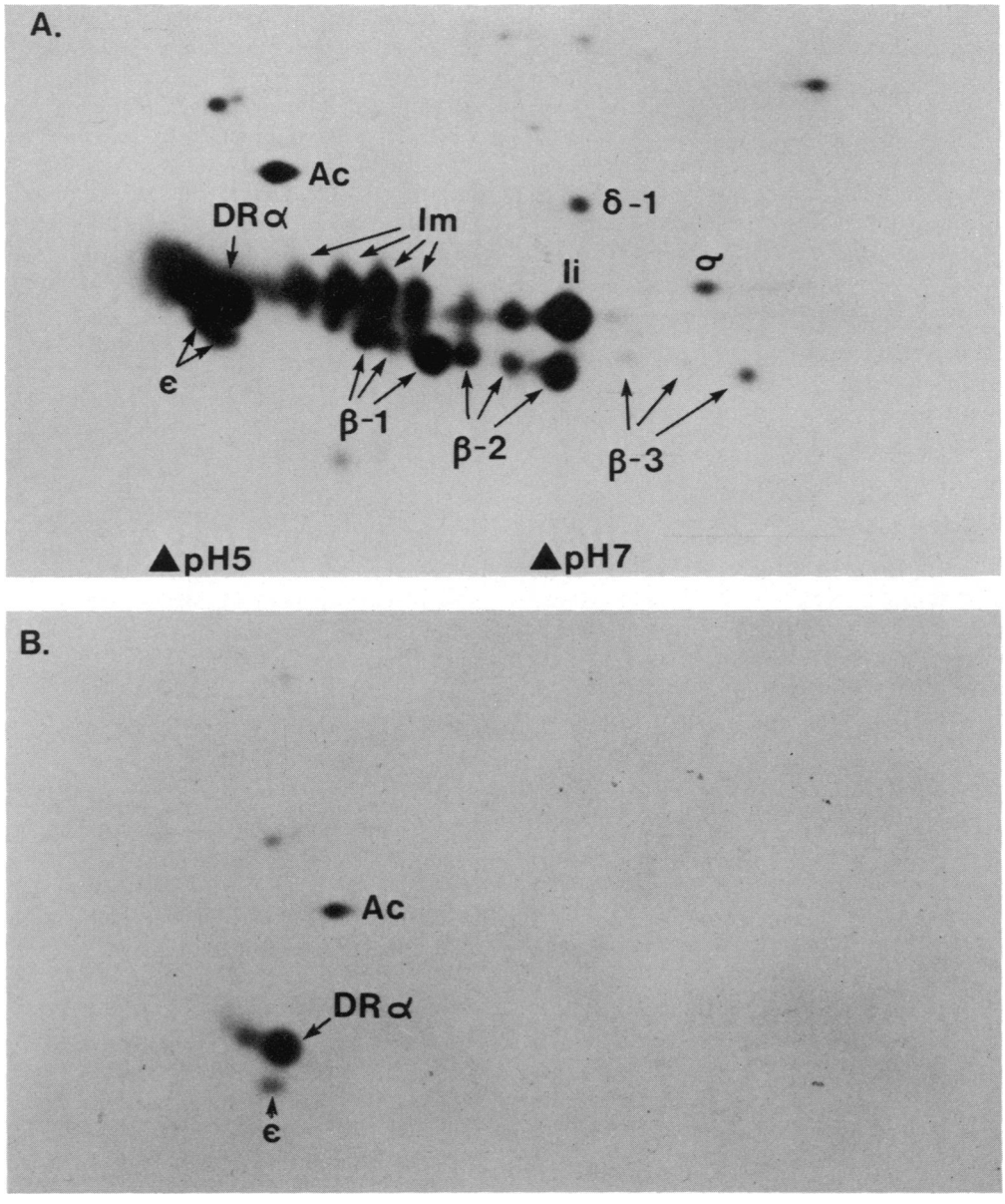

Figure 4. 2-D NEPHGE/SDS-PAGE analysis of polypeptides immunoprecipitated by TAL-1B5 from lysates of biosynthetically ^{35}S-labelled Maja (DR1) cells. Designation of polypeptide spots performed according to de Kretser *et al.* (1982a, b). Immunoprecipitations were performed using native (A) or SDS-denatured boiled cell lysates (B).

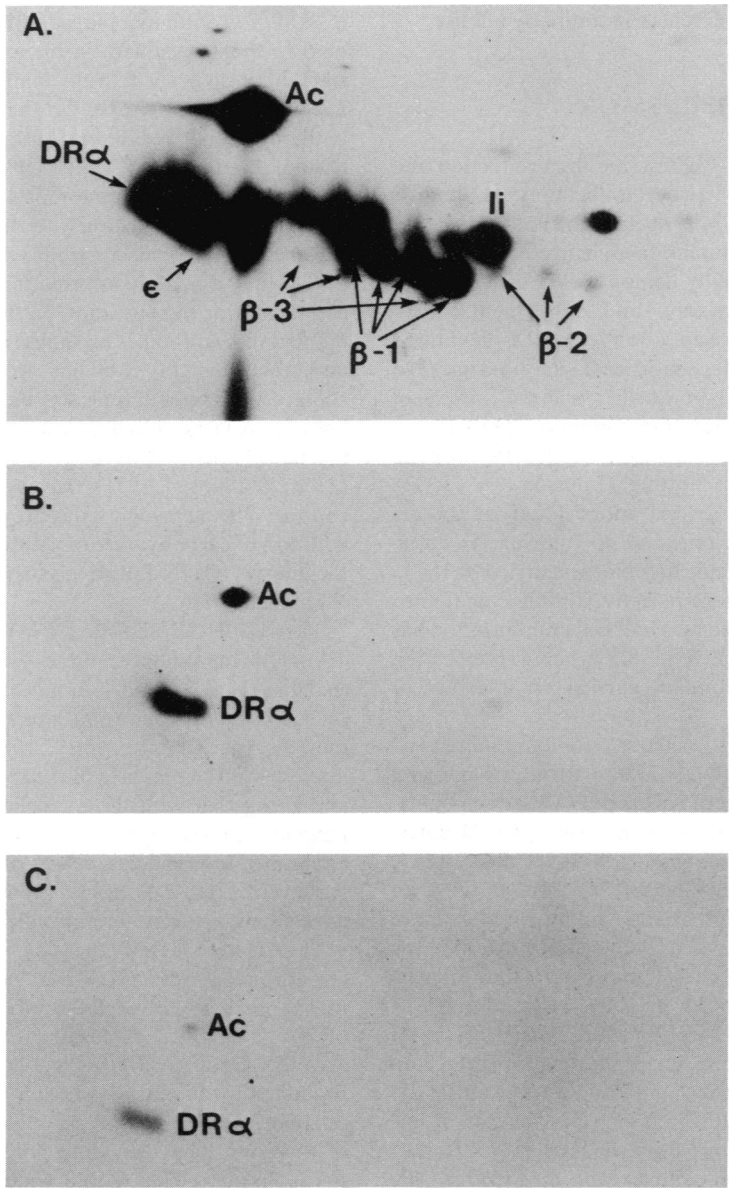

Figure 5. 2-D NEPHGE/SDS-PAGE analysis polypeptides immunoprecipitated by TAL-1B5 and TAL-3C3 from lysates of biosynthetically ^{35}S-labelled IBW9 (DR7) cells. Designation of polypeptide spots performed according to de Kretser *et al.* (1982a, b). Immunoprecipitations performed using TAL-1B5 were with native (A) or SDS-denatured, boiled cell lysates (B). Immunoprecipitations with TAL-3C3 were performed using only SDS-denatured, boiled cell lysates (C).

α-chains in solid-phase assays and to the cell surface most likely reflect differences in antibody affinity.

DISCUSSION

In the present study we describe the production and characterization of two monoclonal antibodies, TAL-1B5 and TAL-3C3, specific for antigenic determinants carried on human Ia α-chains. Specificity for the α-chain was initially demonstrated using a solid-phase radioimmunoassay, and subsequently confirmed by immunoprecipitation from SDS-denatured lysates of ^{125}I cell surface-labelled B lymphoid lines. To date, a number of laboratories, using a variety of techniques including radioimmunoassay (Quaranta *et al.*, 1981), immunoprecipitation (Shackelford *et al.*, 1981a), and 'Western blotting' (Johnson *et al.*, 1982), have characterized several monoclonal antibodies directed at monomorphic and polymorphic Ia determinants as being specific for epitopes carried on the Ia β-chain subunits. More recently, a monoclonal antibody, DA6.147, raised by whole cell immunization has been described (Guy *et al.*, 1982) as being specific for a monomorphic determinant carried on a subset of human Ia α-chains.

As revealed by cellular radioimmunoassay, TAL-1B5 bound to all B lymphoblastoid cell lines and CLLs tested in a pattern typical of DA2, a monoclonal antibody directed against a monomorphic Ia determinant (Barnstable *et al.*, 1978). Unlike DA2, TAL-1B5 bound only weakly to PBL-B cells, an interesting observation as this finding parallels those obtained by Guy *et al.* (1982) when DA6.147 was tested against PBL-B cells. It may be that the antigenic determinant recognized by TAL-1B5 is somehow 'masked' or not accessible on the surface of resting B cells. Differential expression of the determinant recognized by TAL-1B5 seems implausible as the antibody, as judged by NEPHGE/SDS-PAGE analysis of immunoprecipitates from ^{35}S-labelled cell lines, is able to bind to all Ia antigen complexes so far identified (de Kretser *et al.*, 1982a, b).

In contrast TAL-3C3 bound only weakly to B-cell lines, and not to CLLs and PBL-B cells. These observations parallel those obtained using immunoprecipitation whereby TAL-1B5 could precipitate the native Ia α:β dimer from the cell surface whereas TAL-3C3 could not. Competitive inhibition studies suggest that TAL-1B5 and TAL-3C3 recognize the same or spatially related epitopes on α chains. If both recognize the same epitope then TAL-1B5 represents a high-affinity antibody, judged by its ability not only to bind to the determinant in the native and denatured state, but also in successfully inhibiting the binding of TAL-3C3 to the epitope. On the other hand if both antibodies bind to different epitopes, then the inhibition of binding of TAL-3C3 by TAL-1B5 can be explained by steric hindrance. The inability of TAL-3C3 to bind effectively to the native Ia complex may be due to the inaccessibility of the antigenic site, or its loss due to conformational changes in the complex prior to expression on the cell surface. Both monoclonal antibodies retain the ability to precipitate unglycosylated Ia α chains from biosynthetically ^{35}S-labelled lysates of tunicamycin-treated B lymphoid lines (T. Adams, unpublished observations). Tunicamycin inhibits N-linked glycosylation of proteins (Tkacz & Lamsen, 1975), thereby suggesting that the epitope(s) being recognized is/are protein in nature, as addition of carbohydrate moieties to human Ia α- and β-chains is via N-linked glycosylation (Charron & McDevitt, 1980).

The NEPHGE/SDS-PAGE pattern obtained with TAL-1B5 immunoprecipitates was characteristic of that observed for other monoclonal antibodies directed against monomorphic human Ia antigenic determinants (de Kretser *et al.*, 1982a). Of particular interest was the presence of the spots designated DRα and ε, together with those labelled β-1, β-2 and β-3. Elegant studies employing NEPHGE/SDS-PAGE analysis of immunoprecipitates of Ia antigens from a variety of HLA-DR homozygous B lymphoid lines have allowed de Kretser and colleagues (de Kretser *et al.*, 1982a, b) to identify the glycoprotein subunits that corresponded to the HLA-DR encoded specificity, as well as biochemically-defined polymorphic Ia antigens of two other loci. According to the nomenclature adopted the DRα:β-1 polypeptides therefore correspond to the subunits of the HLA-DR specificity of the cell line analysed. The ε:β-3 polypeptides are products of a second locus encoding polymorphic Ia alloantigens, in all likelihood that which codes for the DC series of antigens (Tosi *et al.*, 1978; Duquesnoy *et al.*, 1979; Termijtelen *et al.*, 1980; Shackelford *et al.*, 1981b). In addition the presence of a third β-chain, β-2, representing a polymorphic product of an as yet serologically undefined locus, is in agreement with other biochemical studies which indicate that at least three loci code for polymorphic human Ia alloantigens (de Kretser *et al.*, 1982b). TAL-1B5 therefore appears to recognize an antigenic domain common to Ia

α-chains of all identified Ia α:β chain complexes. The identification, both functionally and biochemically of a third HLA-linked locus coding for polymorphic Ia-like antigens, the SB locus (Shaw *et al.*, 1981, 1982) suggests that the β-2 polypeptides may represent products of this locus. Amino-terminal sequence analysis of the HLA-DRα chain product has revealed it is homologous to the mouse I-E subregion α-chain (Bono & Strominger, 1982) while similar studies with the HLA-DC α-chain reveal homologies with the mouse I-A subregion heavy chain product (Allison *et al.*, 1978).

In the absence of NEPHGE/SDS-PAGE analysis of TAL-1B5 immunoprecipitates of lysates from tunicamycin-treated cells, it is difficult to comment on the relationship between the 10 spots comprising the Ia α-chains. Analysis of immunoprecipitates of ricin- and lentil lectin binding glycoproteins from B lymphoid lines indicate that the more acidic, high molecular weight spots represent the mature, cell-surface Ia α-chain products (de Kretser *et al.*, 1982b). Which product, however, is the DRα equivalent for the β-2 series of polypeptides is not known. Markert & Cresswell (1982) find three to five α-chain spots when Ia antigens from homozygous cell lines are analysed on two-dimensional gels incorporating an isoelectric focusing (IEF) protocol. A minimum of three spots are observed when precipitates from tunicamycin-treated cells are examined. Analysis of TAL-1B5 immunoprecipitates from tunicamycin-treated and untreated cell lines using IEF/SDS-PAGE, which allows a greater resolution of the α-chain region by utilizing a narrower pH gradient, should answer these questions.

The availability of monoclonal antibodies against the subunit structures of human Ia antigens offers powerful tools not only for the biochemical analysis of these antigens, but also with respect to their functional role in immunological assays *in vitro*. Such studies have already been successfully performed using specific rabbit antisera (Palacios *et al.*, 1982; Palacios, 1981). The antibody TAL-1B5 has also been shown, in preliminary tests, to react in immunoperoxidase assays on paraffin block sections (T. E. Adams, A. A. Epenetos *et al.*, personal communication) and so should be most valuable for detecting the presence of DR positive cells in different normal and abnormal tissues. In addition such reagents may prove useful in the biochemical characterization of determinants carried on the plethora of antigen-specific and non-specific helper and suppressor factors so far described and so give further insight into the mechanism by which they act in the immune response.

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