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Characterization of monoclonal antibodies specific for α- and β-chains of sheep MHC class II

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SUMMARY

In order to identify and characterize the sheep major histocompatibility complex (MHC) class II molecules, a panel of 19 monoclonal antibodies (mAb) has been raised following immunization of mice with a variety of class II antigen preparations. Antibodies were selected by ELISA using immunopurified sheep class II as antigen and further screened for the ability to react on immunoblots. Nine mAb reacted with the β-chain and four reacted with the α-chain. The chain specificity of the remainder could not be determined as they did not blot. The anti-α and anti-β mAb all reacted with deglycosylated class II. With the exception of one anti-α and one anti-β antibody, the antibodies reacted with both efferent lymph cells and peripheral blood mononuclear cells (PBMC). Three of the anti-sheep class II mAb reacted with human PBMC.

INTRODUCTION

The major histocompatibility complex (MHC) class II molecules are transmembrane glycoproteins found in all vertebrates examined. They bind peptide fragments of self- and foreign antigens and present them to T cells. Hence they have a major role in determining the immune response (Kappes & Strominger, 1988). The molecules consist of an α-chain, with a molecular weight (MW) of approximately 33,000, non-covalently associated with a β-chain, of approximately 28,000 MW. The human class II molecules consist of three distinct subtypes, DP, DQ and DR, which are encoded by three different subregions of the HLA-D locus. Molecular analysis and sequencing data have identified a further two subregions, DN (Trowsdale & Kelly, 1985; Bodmer *et al*., 1988) and DO (Tonnelle, De Mars & Long, 1985). The DN and DO subregions encode α- and β-chains, respectively, but equivalent polypeptides have not yet been detected.

The diversity of MHC class II products in the sheep is at least as complex as that in the human and requires further analysis. Sheep genes related to all the human class II genes, with the exception of the DP α gene, have been found (Scott, Choi & Brandon, 1987) and monoclonal antibody (mAb) studies have defined four subgroups of class II at the polypeptide level (Puri & Brandon, 1987). In view of the important contribution which studies on the ovine immune system can make to understanding the physiology of the immune system, there is a need to define the ovine MHC. This paper reports on the development and characterization of a panel of mAb against sheep class II molecules and identifies the chain specificity of these mAb.

Abbreviations: BSA, bovine serum albumin; EDTA, ethylene diamine tetraacetic acid; EndoF, endoglycosidase F; FITC, fluorescein isothiocyanate; FSC, forward scatter; HAT, hypoxanthine aminopterin thymidine; HBSS, Hanks' balanced salt solution; mAb, monoclonal antibody; MHC, major histocompatibility complex; NMS, normal mouse serum; NRS, normal rat serum; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate; SSC, side scatter; TCA, trichloroacetic acid; TEA/DOC, 15 mM triethanolamine, pH 8·0, 0·5% sodium deoxycholate TNT, 20 mM Tris-HCL, pH 8·0, 150 mM NaCl, 0·5% Triton X-100.

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MATERIALS AND METHODS

Production of mAb

A total of six fusions were carried out (Table 1). Mice bred in our house were immunized either with sheep concanavalin A (Con A) blasts, sheep thymocytes, sheep afferent lymph cells or immunopurified sheep class II precipitated with five volumes of ice-cold acetone overnight at −20°. Sheep cells and tissues were prepared from animals obtained from the Moredun Research Institute, Edinburgh or the local abattoir. For fusions four and six, the immunopurified class II was acid treated prior to immunization of the mice to increase the likelihood of obtaining anti-α-chain-specific mAb (Cohen *et al*., 1984). Cell fusion was performed as described by Galfré *et al*. (1977) using the NSO cell line. After HAT selection, supernatants were screened by ELISA using the immunopurified class II as antigen. In fusions four and six, ELISA positive supernatants were rescreened by immunoblotting against immunopurified sheep class II. Positive cell lines were then cloned by limiting dilution and subsequently recloned in soft agar (Cotton, Secher & Milstein, 1973).

Table 1. mAb to sheep MHC class II molecules

Antibody	Fusion no.	Immunogen	Isotype
VPM 1	1	Afferent lymph cells	IgM
VPM 3			IgM
VPM 4	2	Con A blasts	IgG2a
VPM 14	3	Purified class II	IgG2a
VPM 15			IgG3
VPM 16			IgG1
VPM 17			IgG3
VPM 36	4	Acid-treated class II	IgG1
VPM 37			IgG1
VPM 38			IgG1
VPM 39			IgG1
VPM 40			IgG1
VPM 41			IgG1
VPM 43			IgG1
VPM 44			IgG1
VPM 45	5	Sheep thymocytes	IgG2a
VPM 46			IgG2a
VPM 47	6	Acid-treated class II	IgG1
VPM 48			IgG2a

Immunopurification of sheep class II

Sheep class II was purified from sheep spleen (obtained from the local abattoir) as previously described using our rat mAb to sheep class II, SW73.2 (Hopkins, Dutia & McConnell, 1986). Briefly, spleen tissue was teased out of the capsule, homogenized in HBSS; the cells were pelleted by centrifugation at 800 *g* for 15 min and lysed in 20 mM Tris-HCl, pH 8·0, 150 mM NaCl, 0·5% Triton X-100 (TNT), containing 0·2 mM phenyl methyl sulphonyl fluoride. Nuclei were removed by centrifugation for 15 min at 1200 *g* and the lysate was further clarified by centrifugation for 90 min at 30,000 *g*. The lysate was filtered through glass fibre filters and pumped onto a normal rat Ig Affigel 10 (Bio-Rad, Hemel Hempstead) column, followed by an SW3.2 Ig Affigel 10 column. The SW73.2 column was washed with TNT followed by 15 mM triethanolamine-HCl, pH 8·0, 0·5% sodium deoxycholate (TEA-DOC) and eluted with 15 mM triethanolamine-HCl, pH 11·5, 0·5 M NaCl, 0·5% sodium deoxycholate. Eluted fractions were neutralized with respect to pH, dialysed against TEA-DOC and reapplied to the columns. After two passages through the columns the material contained only class II, as judged by silver stain on SDS–PAGE (Morrisey, 1981).

ELISA for mAb to class II

1:10 (or appropriate) dilutions of SW73.2 antigen in PBS azide were incubated on ELISA plates (Dynatech, Billinghurst, Sussex) overnight at 4°. The plates were then washed three times for 1 min in 0·1% Tween 20 in PBS, blocked for 30 min with 2% BSA in PBS, washed as before and incubated for 1 hr at room temperature in mAb supernatant. After further washing, the plates were incubated 30 min with 1:1000 goat anti-rat Ig horseradish peroxidase (Sigma, Poole, Dorset) or sheep anti-mouse Ig horseradish peroxidase (Scottish Antibody Production Unit, Law Hospital, Carluke), washed and developed with OPD (Sigma).

Polyacrylamide gel electrophoresis and immunoblotting

Purified class II and spleen lysate were either boiled in 0·125 M Tris-HCl, pH 6·8, 20% (v/v) glycerol, 2% (w/v) SDS, 0·04% bromophenol blue (non-reduced class II) or the same buffer containing 5% β-mercaptoethanol (reduced class II) and fractionated on 12% linear or 7·5–20% gradient SDS polyacrylamide gels (Laemmli, 1970). Alternatively, in order to detect intact class II αβ heterodimers, purified class II was dissolved in buffer containing 0·1% (w/v) SDS and loaded directly onto the gel without boiling. The proteins were transferred electrophoretically to nitrocellulose membrane (HyBond C, Amersham, Aylesbury, Bucks) using the Ancos semi-dry blotter. Following transfer, marker tracks were stained with 0·5% Amido black (BDH, Poole, Dorset) and the remainder of the membrane cut into strips, incubated in the appropriate mAb supernatant and developed either using horseradish peroxidase as described previously (Hopkins *et al.*, 1986) or using anti-mouse or anti-rat alkaline phosphatase conjugates (Sigma) and Bio-Rad alkaline phosphatase substrate.

Endoglycosidase F treatment of sheep class II

In order to investigate the reactivity of the mAb with deglycosylated antigen, immunopurified sheep class II was denatured by boiling for 20–30 seconds in 0·75% SDS then incubated at 37° for 18 hr in 0·2 M sodium phosphate, pH 6·7, 20 mM EDTA, 1% Triton X-100 and an appropriate amount of Endoglycosidase F (EndoF; Boehringer-Mannheim, Lewes, East Sussex). The material was trichloroacetic acid (TCA) precipitated by addition of an equal volume of 30% TCA for 30 min on ice. The precipitates were pelleted and washed three times in ice-cold acetone, air dried and analysed on 7·5–20% SDS–polyacrylamide gels.

Preparation of cells

Efferent lymph cells were obtained by the cannulation of the prefemoral efferent lymphatic (Hall, 1967). Sheep (Moredun Research Institute, Edinburgh) were allowed at least 10 days post-operative recovery before efferent lymph cells were taken for examination. Peripheral blood lymphocytes were isolated from heparinized venous blood by NH_4Cl lysis as described by Mishell & Shiigi (1980).

Flow cytometry

Cells were washed three times in 0·1% BSA/PBS/azide and resuspended to 2×10^7/ml. Twenty-five microlitres of cells were incubated with 25 μl of mAb supernatant (1/500 dilution of normal mouse or normal rat serum was used as the negative control) for 30 min. Cells were washed and then incubated for 30 min with the appropriate dilution of FITC-labelled affinity-purified anti-mouse or anti-rat Ig antibody. After washing, the cells were resuspended in 0·2 ml BSA/PBS/azide and fixed by addition of 0·2 ml 1% paraformaldehyde. The labelled cells were analysed using a FACScan flow cytometer (Becton-Dickinson, Cowley, Oxon). 10^4 resting lymphocytes were analysed by 'live gating', which eliminated dead cells and red blood cells as well as granulocytes and blast cells. The percentage of positive cells in each sample was determined by reference to the normal serum control.

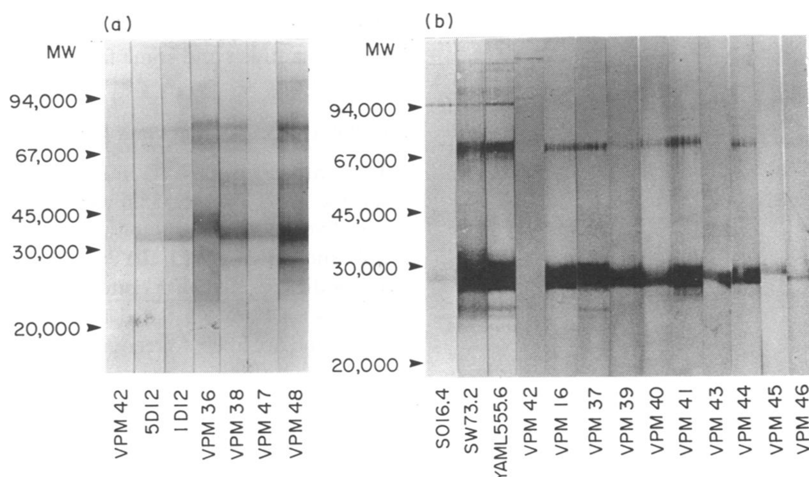

Figure 1. Immunoblot of non-reduced purified sheep class II showing reactivity with anti-sheep class II and cross-reacting anti-human class II mAb. (a) Anti-α-chain-specific antibodies. (b) Anti-β-chain-specific antibodies.

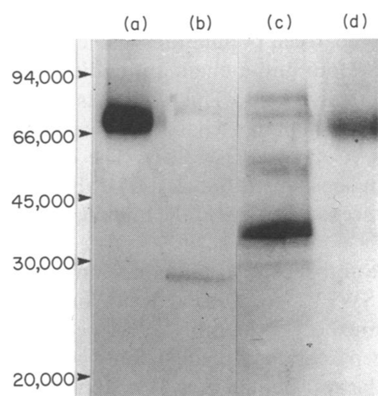

Figure 2. Immunoblot of purified sheep class II developed with the anti-β-chain-specific mAb VPM41 (a and b) and the anti-α-chain-specific mAb VPM38 (c and d). The material in (a) and (d) was diluted into 0·1% SDS and loaded immediately onto the gel. These tracks show the αβ heterodimer. The material in (b) and (c) was boiled in 2% SDS prior to loading onto the gel.

RESULTS

Screening of mAb

mAb supernatants were screened initially by ELISA for anti-class II reactivity against immunopurified SW73.2 antigen. Positive supernatants were further screened for their ability to react with class II on SDS polyacrylamide gel blots. In some fusions, the cells producing ELISA-positive supernatants that did not blot were subsequently discarded. Table 1 lists the antibodies produced as a result of six fusions. The use of a purified antigen for immunization proved to be the most effective method for producing anti-class II antibodies.

Immunoblot analysis of the mAb

Monoclonal antibody supernatants were blotted against both boiled non-reduced and boiled reduced purified class II and whole spleen lysate. Figure 1 shows non-reduced class II blotted with either mouse mAb raised to sheep class II or mouse mAb to human class II that cross-react with sheep class II. The α-chain-reactive mouse mAb, including the two anti-human class II antibodies 1D12 and 5D12 (Cohen, Crichton & Steel, 1987), are shown in Fig. 1a. The β-chain-reactive antibodies, including the previously described rat anti-sheep class II mAb SW73.2 (Hopkins *et al.*, 1986) and the rat anti-human class II antibody YAML555.6 (Hale, Clark & Waldmann, 1985), are shown in Fig. 1b. S016.4, a rat mAb to sheep erythrocytes, and VPM 42, a mouse mAb to rat Ig, were used as negative controls for the rat and mouse antibodies, respectively. Some of the mAb recognized bands with different molecular weights (e.g. VPM 36 and 38, 45 and 46), indicating that they recognize different polypeptides within the sheep class II complex.

The mAb also reacted with two to three other polypeptide bands on the blots. The anti-α and anti-β antibodies both reacted with a 69,000 MW band, and the anti-α-specific antibodies reacted with an additional 73,000 MW band. One explanation for the 69,000 MW weight band might be that it represents the αβ heterodimer. The dimer can be detected by loading samples in sample buffer containing 0·1% SDS without boiling. Figure 2a, shows the dimer detected with anti-β and anti-α antibodies, respectively. The dimer runs with an apparent MW of 66,000, which is slightly smaller than the band detected in samples boiled in 2% SDS (Fig. 2b,c). The 69,000 MW band may, however, represent the dimer which is running slightly slower in the presence of 2% SDS. The identity of the 73,000 MW band is unknown.

SW73.2 reacted with at least two bands of lower MW than the 28,000 β-chain. These bands are seen with some, e.g. VPM37, but not all, e.g. VPM16, of the mouse anti-β mAb. A similar series of lower MW bands is seen with the anti-α mAb VPM 38, 47 and 48. These lower MW bands may represent unglycosylated precursors, and the inability to detect them with some mAb may indicate that the antibodies react with sugar residues. In order to investigate this, immunopurified class II was deglycosylated with EndoF and blotted against the panel of mAb. Figure 3 shows that VPM 37, 39, 40, 41, 44, 46, SW73.2 and YAML555.6 all reacted with the deglycosylated β-chains. Furthermore, the unglycosylated β-chains are similar in size to the lower MW bands present in undigested class II.

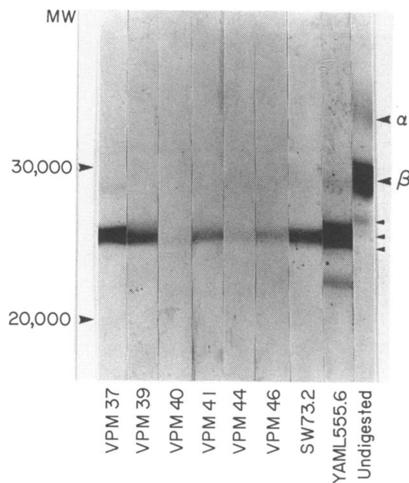

Figure 3. Immunoblot showing reactivity of EndoF-treated sheep class II with a panel of anti-β-chain-specific mAb. The track labelled undigested was developed with the anti-β-chain-specific antibody SW73.2 and the anti-α-chain-specific antibody VPM38. Arrows indicate α and β and the position of lower MW bands.

YAML555.6 consistently reacted with an extra low MW band. The identity of this band has not been investigated. All six anti-α antibodies similarly reacted with the deglycosylated α-chains (data not shown). These experiments show that the antibodies reacted with the polypeptide backbone of the class II molecules and indicate that the lower MW bands seen with the untreated material are likely to be precursors at different stages of glycosylation. The inability to detect these bands with some antibodies most probably reflects a lack of sensitivity of weakly reacting mAb.

Figure 4a, shows immunoblots of the anti-α-chain-specific and anti-β-chain-specific mAb, respectively, blotted against reduced immunopurified class II. These blots clearly show that the β-chains increase in apparent MW on reduction such that they are not separated from the α-chains as has been shown previously by immunoprecipitation studies (Puri, Mackay & Brandon, 1985; Puri, Gorrell & Brandon, 1987). It is also clear that most of the antibodies reacted more weakly with the reduced antigen and some failed to react.

Cellular expression of class II

The anti-sheep class II mAb and the anti-human β-chain-specific mAb YAML555.6 were tested for their ability to react with live cells by immunofluorescence on sheep efferent lymph cells and PBMC. With the exception of VPM 39 and 48, all the antibodies reacted with epitopes present on live cells (Table 2). VPM 40, 44 and 45 failed to react with cells from one sheep tested and are likely to recognize polymorphic determinants. All the other antibodies reacted with all sheep tested (> 15) and hence are unlikely to be directed against polymorphic determinants. Table 2 shows that for any given mAb, class II molecules are expressed by significantly more sheep PBMC than by sheep efferent lymphocytes. Peripheral blood contains a greater proportion of B cells than efferent lymph and it is likely that the result, in part, reflects this increased number of B cells. The overall percentage of class II-positive cells in both compartments is greater than the percentage of B cells, which implies that certain sheep T-cell populations also express class II.

Three of the four anti-α-chain antibodies, VPM 36, 38 and 47, reacted with live cells. This contrasts with the reported failure of the anti-human α-chain-specific antibodies to react with live cells (Cohen *et al.*, 1987). The cross-reactive anti-human α-chain-specific antibodies 1D12 and 5D12, which are immunofluorescence negative in the human, are also negative in the sheep.

Four of the anti-sheep class II monoclonal antibodies, VPM 16, 38, 46 and SW73.2, cross-reacted with human PBMC. These antibodies stained a considerably lower percentage of human cells than sheep cells, but their staining was similar to that observed for the anti-human class II mAb YAML555.6 (Table 2) and for other anti-human class II mAb (Brickell *et al.*, 1981).

DISCUSSION

This study has described the characterization of 19 mAb raised against sheep MHC class II molecules using various immunogens. Acid-treated purified class II was the most effective

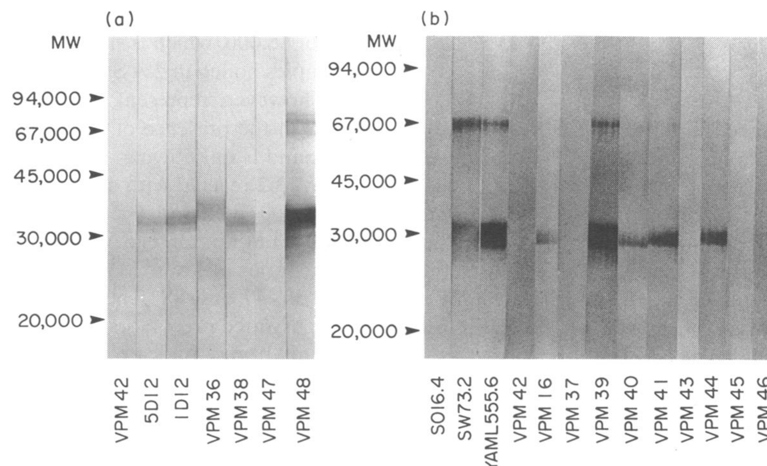

Figure 4. Immunoblot of reduced purified sheep class II showing reactivity with anti-sheep class II and cross-reacting anti-human class II mAb. (a) Anti-α-chain-specific antibodies. (b) Anti-β-chain-specific antibodies.

Table 2. Chain specificity and cell-surface reactivity of anti-sheep class II mAb

mAb	Chain specificity	PBMC ($n=17$) mean ± SD	Human PBMC ($n=2$)	Efferent lymph cells ($n=6$) mean ± SD
VPM 1	ND	42·97±5	—	23·2±3·4
VPM 3	ND	40·57±8·5	—	17·9±2·5
VPM 4	β	53·86±3·6	—	31·7±4·2
VPM14	ND	21·19±5·3	—	4·5±2·2
VPM15	ND	48·66±7·0	—	28·2±5·3
VPM16	β	59·96±3·7	14	44·1±5·2
VPM17	ND	15·02±8·6	—	7·8±3·5
VPM36	α	48·92±1·6	—	30·0±6·2
VPM37	β	50·69±3·1	—	42·3±5·0
VPM38	α	58·46±3·0	9	45·1±6·6
VPM39	β	0·72±0·47	—	0·88±0·4
VPM40	β	44·39±1·9 1·84±1·5*	—	24·67±6·3
VPM41	β	46·24±6·8	—	31·63±7·6
VPM43	β	55·2±3·5	—	43·1±4·1
VPM44	β	40·8±3·0 0·8±0·34*	—	22·7±9·6
VPM45	β	37·34±2·3 0·95±0·16*	—	16·54±4·8
VPM46	β	58·9±9·9	14	50·0±4·4
VPM47	α	56·7±5·7	—	40·5±7·7
VPM48	α	2·35±1·6	—	1·65±1·7
SW73.2	β	61·9±4·8	9·5	55·36±7·4
YAML555.6	β	56·9±6·9	13·4	39·38±7·6
1/500NMS		<1	<1	1·0
1/500NRS		<1	<1	<1

* Mean percentage cells stained in sheep negative for expression of this epitope.

immunogen. Treatment with acid partially denatures the molecule rendering it more immunogenic, and the use of a purified preparation means it is possible to immunize with a higher concentration of the molecules of interest. Furthermore, the ability to recognize blotted proteins means that some of the problems arising from co-precipitation of unrelated proteins are eliminated.

The mAb recognized a number of other bands in addition to the mature α- and β-chains. Deglycosylation of purified sheep class II, followed by blotting, has shown that the anti-β mAb recognize a polypeptide band that is close in MW to the three bands which react with SW73.2, YAML555.6 and VPM37, and it is likely that these polypeptides represent different stages of glycosylation.

The higher MW bands probably represent αβ, αα and ββ dimers, which occur as a result of disulphide interchange during the class II purification and are not dissociated in non-reduced gels. Aggregates such as these have been described previously for sheep (Puri *et al.*, 1985) as well as for human (Springer *et al.*, 1977) and rabbit (Knight, Leary & Tissot, 1980) class II molecules.

With the exception of two mAb, VPM39 and VPM48, all the mAb reacted with live cells. VPM39 reacted very weakly in ELISA (data not shown) and it is likely that this antibody is directed against an epitope not available on the native molecule. It has been suggested that anti-α mAb react poorly or not at all with live cells (Cohen *et al.*, 1987) and this has been interpreted to show that the α-chains are relatively inaccessible on the cell surface. While all the anti-α mAb used here were raised against acid-treated antigen, three out of four cross-reacted well with live cells, and there is no evidence that they were reacting with non-intact cells. One of the anti-α mAb reacted with human cells. It is possible, therefore, that the mAb all react with α epitopes that are fortuitously available or that the sheep class II molecules are relatively more accessible than the human.

Although all these mAb recognized sheep class II, they did not all stain the same proportions of cells. This suggests that some of the mAb react with subgroups of sheep class II that are differentially expressed. VPM 40, 44 and 45 appear to recognize a polymorphic determinant and are by definition subgroup specific. Preliminary experiments have shown that some of the other mAb recognize mutually exclusive subgroups of sheep class II. Studies to investigate the subgroup specificity of the antibodies and the differential expression of sheep class II are underway and will be reported in a subsequent publication.

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