Locus-specific detection of HLA-DQ and -DR antigens by antibodies against synthetic N-terminal octapeptides of the β chain

Thomas Deufel, Anne Grove, Hans Kofod and Åke Lernmark

Hagedorn Research Laboratory, Niels Steensensvej 6, DK-2820 Gentofte, Denmark

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Antibodies against synthetic peptides representing the class-II antigen HLA-DR and -DQ β chain N-terminal sequences were prepared in rabbits. The two octapeptides only share two amino acids and enzyme-linked immuno-assays showed the antisera only to bind to its own antigen. Both peptide antisera detected a 29 kDa component in immunoblots of Raji and AL-34 cell plasma membrane proteins separated by SDS gel electrophoresis. The binding of either N-terminal peptide antiserum was selectively inhibited only by the peptide used as antigen. Indirect immunofluorescence analysis by flow cytofluorometry showed specific surface immunofluorescence in 1:100–1:1000 dilutions in lymphoblastoid and blood mononucleated cells. In the latter the binding was primarily confined to monocytes and a subpopulation of lymphocytes. It is concluded that locus-specific immunological reagents to distinguish between β chains of HLA-DR and -DQ have been prepared by the preparation by the production of antibodies against the N-terminal sequences of each polypeptide

HLA class II antigen *Flow cytofluorometry* *ELISA* *Immunoblotting*

1. INTRODUCTION

HLA class II antigens are structurally closely related, highly polymorphic, heterodimeric proteins consisting of an α chain (M_r 35000) and a β chain (M_r 29000) [1,2]. The class II antigens are primarily expressed on the surface of cells important to the immune response [3]. The genes encoding for these antigens are part of the major histocompatibility complex (MHC) located on the short arm of chromosome 6. Serologic studies indicate the existence of at least 3 distinct loci, DP, DQ, and DR [3], and more recent molecular cloning studies have revealed the presence of several α and β chain genes at each locus [4].

Little is known about the differences in expression and function of these different gene loci. These problems are difficult to investigate and require immunologic reagents able to distinguish products of the different loci, as well as the subspecificities due to allelic variation within one locus. The extensive sequence homology between all the class II antigens makes it difficult to obtain monospecific antibodies [5–7]. We have therefore used antibodies against synthetic peptides representing different parts of HLA class II β chains to detect binding to the surface of immune competent cells. Antibodies to synthetic intramolecular amino acid sequences are successfully employed to obtain antibodies with a predetermined specificity against a variety of antigens [8]. Such antibodies may even detect variation in sequences contained within large homologous but polymorphic polypeptides, such as the rat thy-1 [9], and human MHC Class I [10] and Class II [11,12] antigens. The advantage of this technique

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is that antibodies can be produced against proteins not yet isolated and purified, but predicted from the nucleotide sequence of cloned genes whether genomic or complementary to mRNA molecules.

Analysis of published amino acid sequences of HLA class II β chains obtained from protein sequencing [13], or predicted from cloned genomic or cDNA [5–7,14,15] revealed the N-terminal sequences of DQ and DR β chains to be highly conserved for each locus. Since only 2 out of the first 8 amino acids are shared, we chose these 2 octapeptides to produce locus specific antisera to detect binding of HLA-DQ and -DR β chains on the surface of living cells. Our results demonstrate that rabbit antisera against either peptide bound to the surface of living cells and detected a component of M_r 29000 by immunoblotting [16] of denatured components in plasma membrane preparations of cells known to express Class II antigens.

2. MATERIALS AND METHODS

2.1. *Cells*

The lymphoblastoid cell lines Raji and AL-34, established following EBV transformation of lymphocytes from an HLA-DR 3/4 positive patient with insulin-dependent diabetes by H. Vissing, were cultured in RPMI 1640 medium containing 10% (v/v) fetal calf serum. Human peripheral blood mononuclear cells were isolated by Ficoll-Paque density centrifugation [17] from blood donated by healthy volunteers, or recently diagnosed insulin-dependent (type 1) diabetic patients in good metabolic control.

2.2. *Peptides and immunization*

The peptides DQ(1–8) and DR(1–8) (table 1) represent the N-terminal amino acid sequences shown [5–7,13–15] to be common to DQ and DR β chains, respectively. Both peptides were synthesized by Cambridge Res. Biochem. Ltd, Cambridge, England and the sequence confirmed by sequence analysis (Gas Microsequenator, Applied Biosystems, Foster City, CA). The peptides were coupled to porcine thyroglobulin (PTG) (Sigma, St. Louis, MO) using difluorodinitrobenzene [18].

New Zealand white rabbits were injected intracutaneously at multiple sites with 1 mg antigen in complete Freund's adjuvant, and then given 3 booster injections every other week with 0.5 mg in incomplete Freund's adjuvant, and then at monthly intervals. Blood was sampled 5–7 days after each booster injection.

2.3. *ELISA*

Microtiter plates (NUNC, Roskilde, Denmark) were coated with 2 μg/well of peptides DQ(1–8) and DR(1–8), respectively, and blocked with 0.01% (w/v) bovine insulin in phosphate-buffered saline (PBS). Sera were diluted (1:1000–1:50000) in PBS containing 0.01% insulin and 0.05% Tween 20. The plates were incubated at 37°C for 2 h first with rabbit serum and then with an $f(ab)_2$ goat anti-rabbit immunoglobulin-peroxidase conjugate (Tago, Burlingame, CA) second antibody, diluted 1:2500 in PBS with insulin and Tween. The plates were washed 3 times in PBS containing insulin and in distilled water, respectively, prior to and after incubation with the second antibody. Peroxidase activity was detected at room temperature with 0.4 g/l *o*-phenylenediamine (Sigma) and 0.03% hydrogen peroxide in 50 mM citrate-phosphate (pH 5.0). The absorbance at 490 nm, automatically subtracted with that at 600 nm, was read on an ELISA reader (Dynatech, Alexandria, VA). A standard antiserum for each peptide was analyzed, diluted 1:5000, on each

Table 1

HLA antigen	Carrier	Sequence	Rabbit no.
DQ(1–8)	PTG	-Arg-Asp-Ser-Pro-Glu-Asp-Phe-Val	1828,1829
DR(1–9)	PTG	-Gly-Asp-Thr-Arg-Pro-Arg-Phe-Leu	1789,1790

HLA-DQ and -DR N-terminal sequences were synthesized and coupled to porcine thyroglobulin (PTG) with the N-terminal reagent difluorodinitrobenzene

plate to correct for interassay variation. Results are expressed as absorption at 490 nm. Non-specific binding to non-coated wells was subtracted.

2.4. *Flow cytofluorometry on living cells*

Cells were washed once and resuspended in PBS containing 1% BSA and 5 mM sodium azide (PBS/BSA/NaN_3 buffer). Rabbit antisera were also diluted in this type of medium and incubated for 1 h at 4°C with 0.5×10^6 cells per tube in a final volume of 0.2 ml. The cells were washed by centrifugation ($150 \times g$, 5 min) in 2.5 ml buffer and resuspended gently in 0.1 ml FITC-labelled goat anti-rabbit immunoglobulin serum (Tago, Burlingame, CA) diluted 1:40 in PBS/BSA/NaN_3 buffer. After 45 min at 4°C in the dark the cells were washed as before and then fixed in 1% paraformaldehyde in PBS/BSA/NaN_3. Finally, the cells were washed, resuspended in 0.5 ml PBS/BSA/NaN_3 and kept at 4°C in the dark until fluorescence was analysed in a FACS IV (Becton Dickinson, Mountain View, CA) flow cytometer. Results are given as mean fluorescence intensity per cell, relative to the autofluorescence of glutaraldehyde fixed chicken red blood cells as a standard, in percent of the fluorescence obtained with normal rabbit serum, and as the increase of fluorescence peak channel number.

2.5. *Immunoblotting*

Plasma membrane enriched preparations of Raji and AL 34 cells, obtained as described [19] were denatured by boiling for 3 min in 3% SDS and 5% β-mercaptoethanol and subjected to gel electrophoresis on 10% polyacrylamide slab gels [20]. Molecular mass standards were analyzed on the same gel. The proteins were electroblotted onto nitrocellulose filters (BA 85, Schleicher and Schuell, Dassel, FRG) which were treated with 2% gelatine in 50 mM Tris-saline (pH 7.4) containing 0.05% Tween 20 and kept at −20°C until antibody binding was tested. The part of the filter containing the molecular mass standards was stained with fast green. Filter strips were incubated for 1 h at room temperature in rabbit sera or with a mouse monoclonal antibody against HLA-DR β chain (DAKO, Copenhagen), all diluted 1:100 in Tris-saline containing 0.2% gelatine and 0.05% Tween 20. The strips were washed in Tris-saline with 0.05% Tween 20 and incubated with a peroxidase-labelled second antibody, either swine anti-rabbit immunoglobulin serum (DAKO) or anti-mouse immunoglobulin serum (DAKO), followed by washing and staining with 0.5 g/l 4-chloro-1-naphthol (Merck, Darmstadt) and 0.01% hydrogen peroxide as substrates in 50 mM sodium acetate buffer (pH 5.5). Antisera were absorbed with the synthetic peptides by incubation at 4°C overnight with undiluted sera to which the respective peptides, dissolved in distilled water, had been added (1:10, v/v). Absorbed sera were diluted 1:100 in Tris-saline immediately before immunoblotting.

2.6. *Isolation of IgG on protein A-Sepharose*

Rabbit serum (1 ml) was applied to a column packed with 1 ml protein A-Sepharose 4 B (Pharmacia, Uppsala) equilibrated in PBS. The column was washed with at least 5 vols PBS, IgG eluted with 0.05 M glycine buffer (pH 3.0) and 0.5 ml fractions collected in test tubes containing 20 μl of 1 M Tris for immediate neutralization. Fractions containing protein were pooled, dialysed overnight at 4°C against PBS, and tested for anti-peptide antibodies in the ELISA test.

3. RESULTS

3.1. *Production of peptide specific antibodies*

Analysis by ELISA (fig.1) showed that immunization with the PTG coupled peptides

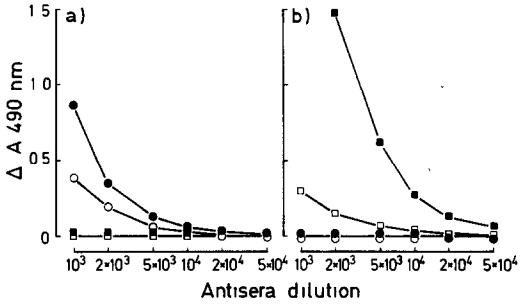
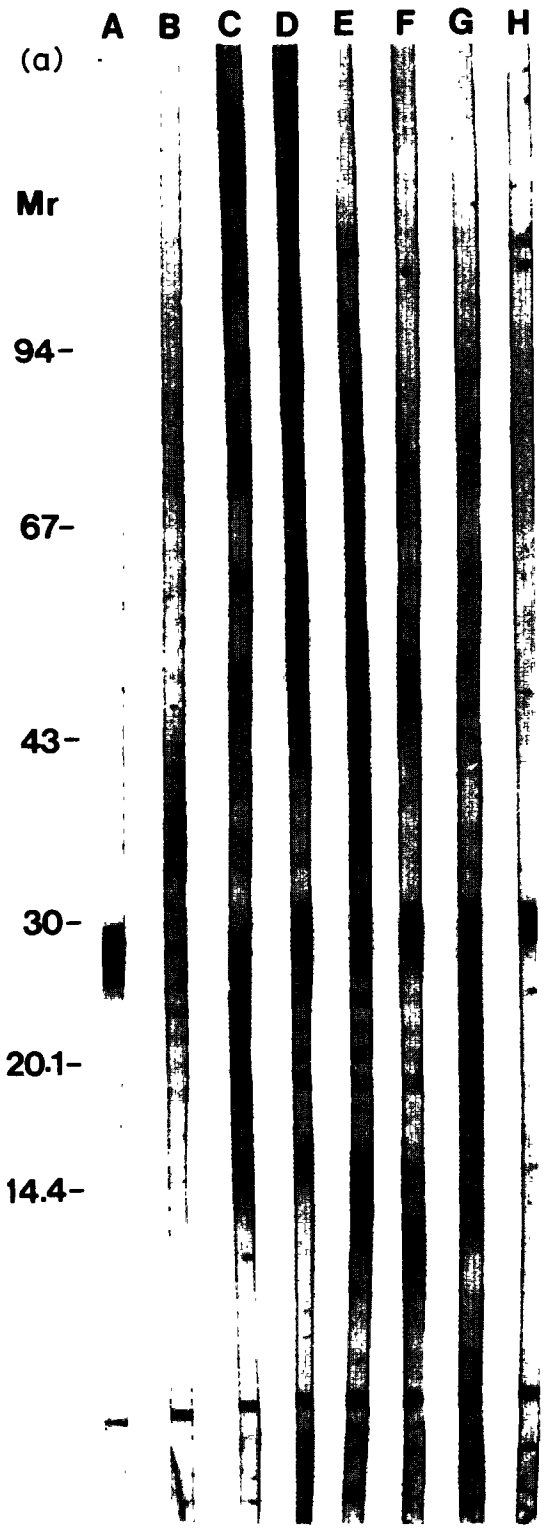
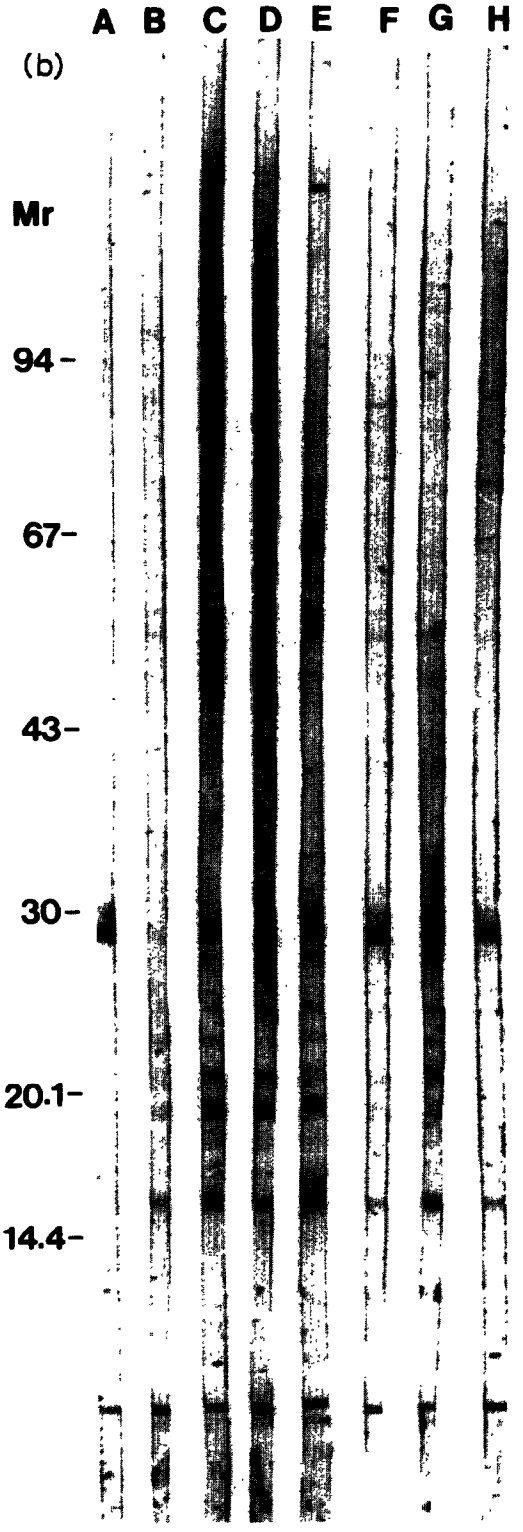


Fig.1. ELISA of rabbit sera from animals immunized with PTG-coupled peptides DQ(1–8) (no.1828, ●; no.1829, ○), and DR(1–8) (no.1789, □; no.1790, ■) respectively on microtiter plates coated with DQ(1–8) (panel a), and DR(1–8) (panel b), respectively. Sera tested were obtained after the 4th booster injection.


A B C D E F G H
(a)
Mr
94-
67-
43-
30-
20.1-
14.4-


A B C D E F G H
(b)
Mr
94-
67-
43-
30-
20.1-
14.4-

←

Fig.2. Immunoblotting of plasma membrane enriched preparations from the cell lines Raji (panel a) and AL-34 (panel b), incubated with a mouse monoclonal anti HLA-DR β chain (DAKO) (lane A), normal rabbit sera (B), anti DQ(1–8), no.1828 (C) and no.1829 (D), anti DR(1–8), no.1789 (E) and no.1790 (F), lanes G and H show incubation with IgG from sera nos 1829 and 1790, respectively, purified on protein A-Sepharose. For details see section 2.

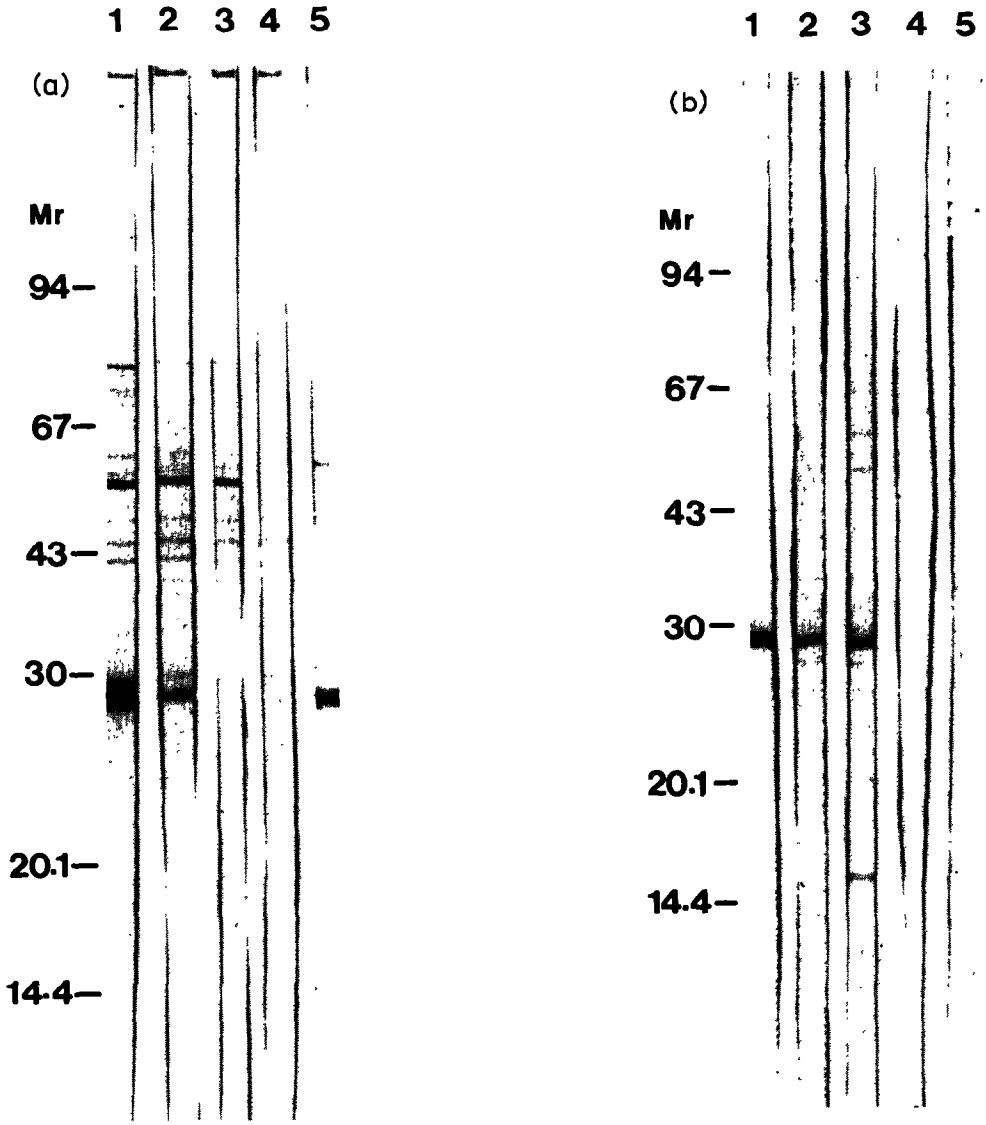


Fig.3. Inhibition of antiserum binding to AL-34 membrane preparation by preincubation with free peptide. Filter strips were incubated with antisera from rabbit no.1829 (anti DQ(1–8), panel a, pretreated overnight with saline (lane 1), peptide DQ(1–8) at a final concentration of 0.1, 1.0 and 5.0 mg/ml, respectively (lanes 2–4, respectively), and peptide DR(1–8), at 5.0 mg/ml, respectively (lane 5). Panel b shows filter strips incubated with antisera from rabbit no.1790 (anti DR(1–8)) pretreated overnight with saline (lane 1), peptide DQ(1–8) at 5 mg/ml (lane 2) and peptide DR(1–8) at 0.1, 1.0 and 5.0 mg/ml, respectively (lanes 3–5). Final dilution of rabbit sera was 1:100, for details see section 2.

resulted in antibodies recognizing specifically the amino acid sequence of the 2 different peptides. A maximal titer was usually obtained after 3 booster injections and remained constant for several months (not shown). Pre-immune sera as well as sera from animals immunized with PTG alone did not bind to peptide coated plates. There was no cross-reactivity detected when each antiserum was tested at various dilutions on plates coated with the other peptide (not shown). All antisera showed high titers, often greater than $1:10^4$, of PTG antibodies when tested on PTG coated plates (not shown).

3.2. *Detection of 29 kDa proteins*

DQ(1–8) and DR(1–8) peptide antisera detected 29 kDa components in immunoblots of Raji and AL-34 cell plasma membrane proteins separated by SDS-gel electrophoresis (fig.2). Each peptide was used to immunize 2 rabbits, and only one, no.1828, immunized with DQ(1–8), was not reactive with this band. The HLA-DR monoclonal antibody also detected a 29 kDa component (fig.2). The binding of either N-terminal peptide antiserum was selectively inhibited in a dose-dependent manner only by the peptide which was used as antigen (fig.3). Protein A-Sepharose purified IgG bound to a component of similar size allowing detection of the 29 kDa component during the condition of a reduced background (fig.2).

3.3. *Surface binding of peptide antisera*

The antisera to both N-terminal peptides bound to the surface of Raji and AL-34 cells known to express both HLA-DR and -DQ antigens (table 2). The cell surface immunofluorescence showed a ring-shaped to dotted appearance and in experiments separate from those shown in table 2, immunofluorescence was discernible by both microscopy and flow cytofluorometry in dilutions ranging from 1:100 to 1:1000. When tested on human peripheral blood mononuclear cells, the an-

Table 2

Flow cytometric analysis of rabbit antigen binding to living cells

	Expt no.	Relative fluorescence					Peak fluorescence channel number				
			Individual					Individual			
		NRS (%)	1828	1829	1789	1790	NRS	1828	1829	1789	1790
Raji	1	100	114	100	100	133	0	4	0	0	38
	2		115	147	109	308	0	3	21	4	48
	3		121	128	106	174	0	5	8	3	17
	4		108	119	126	192	0	3	7	12	16
	5		111	117	102	154	0	13	11	13	28
AL-34	1	100	250	433	375	283	0	15	35	42	24
	2		100	133	143	161	0	0	6	4	21
	3		111	106	110	143	0	21	0	0	34
Human peripheral blood MC	1	100	128	147	nd	nd	0	13	20	nd	nd
	2	100	140	139	135	176	0	16	16	12	19
	3	100	140	138	154	170	0	14	10	12	19
	4	100	151	120	129	134	0	23	13	16	21
	5	100	138	120	109	141	0	17	25	23	23

Cells (0.5×10^6/tube) were incubated with rabbit sera (final dilution 1:100) and FITC-conjugated goat anti-rabbit immunoglobulin sera, as described in section 2, and analysed in the FACS IV. Results are expressed as mean fluorescence intensity per cell, calculated based on the autofluorescence of glutaraldehyde-fixed chicken red blood cells (= 1.0), and expressed as percent of the value obtained with NRS (= 100%), or as increase in fluorescence peak channel number (1–255) based on NRS (= 0), and corrected for the fluorescence peak of chicken red blood cells (= 100). All samples were run in duplicates, and 10000 cells were analysed per sample

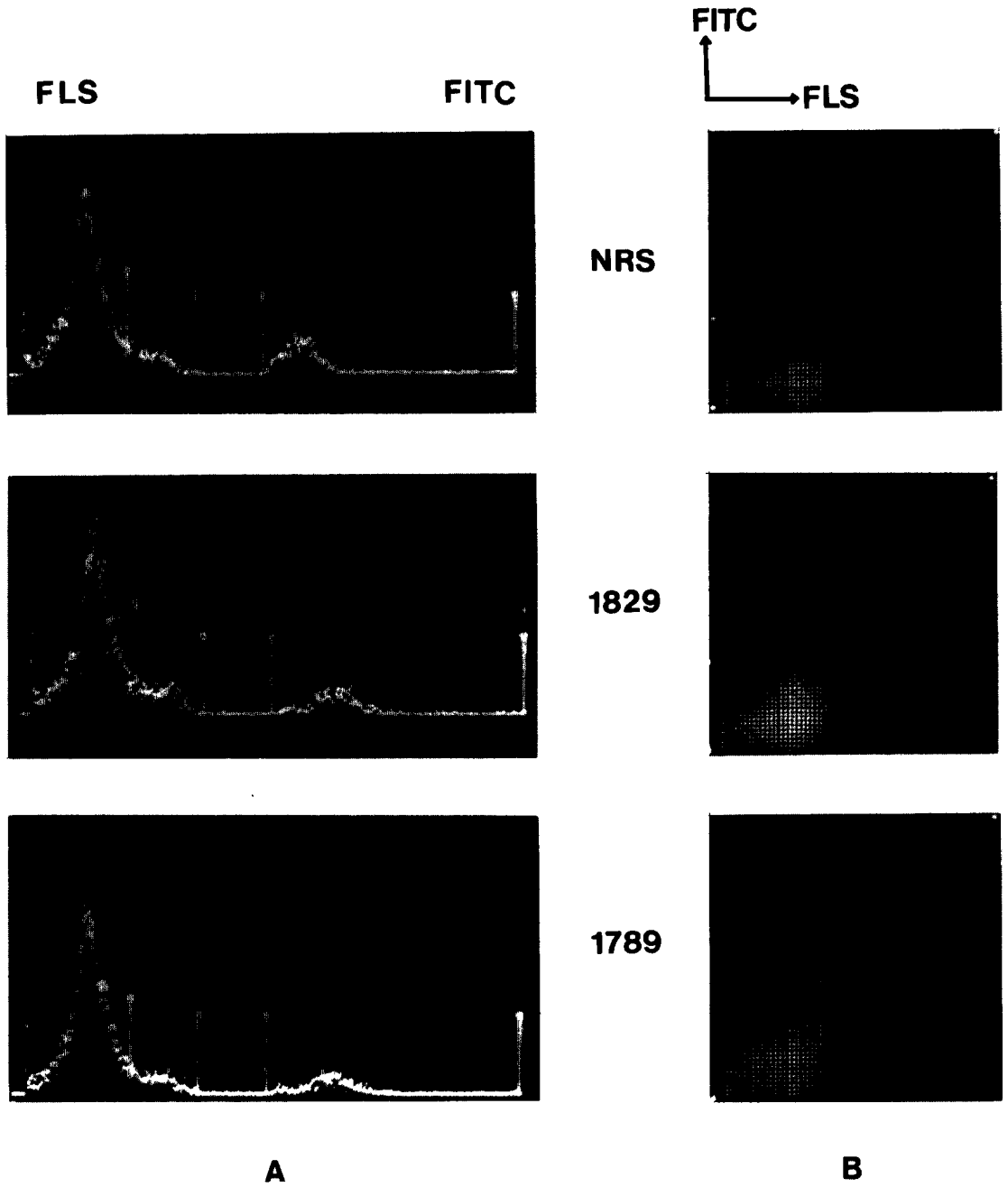


Fig.4. Flow cytometric analysis of antisera binding to human peripheral blood mononuclear cells. Panel A shows a typical result for forward light scatter (FLS) and FITC fluorescence. Pointers indicate monocyte gating in FLS. Panel B shows dual parameter analysis of the same sample. For details see section 2.

tisera bound primarily to cells defined by forward light scatter to belong to larger cells which are consistent with the population of monocytes, and a subpopulation of lymphocytes (fig.4). The binding of the peptide antisera showed a similar intensity, however, antiserum no.1828 showed little binding.

4. DISCUSSION

A variety of parameters have been claimed to be of importance when chosing a peptide sequence for antibody production. Among these, hydrophilicity and probability of certain characteristics to determine secondary structure have been claimed to determine the ability of a given peptide to elicit antibody binding to the same sequence in a protein [8,9,21,22]. Also, a peptide length of around 14 amino acids was thought to be necessary to produce specific antibodies [21]. However, the present study demonstrates that these constraints are not always applicable, nor necessary. First, we used the peptide antibody technique to make antibodies against a given part of a polypeptide sequence, determined rather by its uniqueness than its hydrophilicity or amino acid composition. The important test was to determine whether it was possible to make antibodies against the N-terminal sequences of the HLA-DQ and -DR β chains, respectively. Such antibodies would be useful as markers of a given subgroup of the family of HLA-D region proteins. In the case of these HLA class II antigens, the regions of interest are rather short, and inclusion of longer stretches of homologous sequences would increase the risk of obtaining cross-reactive antisera. The peptides chosen by us (table 1) were therefore by necessity selected primarily due to their uniqueness of sequence, and both peptides are only 8 amino acids long.

Yet, both peptides elicited production of antibodies which by the ELISA test proved to be sequence specific as they did not cross-react with each other. Antisera against both peptides also bound to a protein species of M_r 29000. The components detected corresponded to that recognized by the monoclonal HLA class II β chain antibody. Although DQ and DR β chains cannot be distinguished by one-dimensional SDS-gel electrophoretic analysis and the lack of proof that both antisera do not detect the same molecule, our finding that there was a selective inhibition of binding only by the peptide used for immunization excludes the possibility of non-specific cross-reactivity. Our results are consistent with the possibility that 2 structurally distinct proteins of the same apparent molecular mass were detected.

The antisera against the N-terminal peptide sequences also bound to the surface of living HLA class II positive transformed cells kept as continuous cell lines. In addition, and more importantly, the antisera also bound to the monocyte and only part of the lymphocyte subpopulation of human peripheral blood cells. Experiments with double fluorescence showed no binding to T-lymphocytes as defined by binding of the monoclonal antibody Leu 1 (Beckton and Dickinson), which normally do not express class II antigens (not shown). The absence of binding to T-lymphocytes is a useful negative control with respect to the specificity of our antisera.

Binding to living cells has so far been demonstrated only in peptide antibodies against the mouse thy-1 antigens [9], and more recently against HLA-DR β chains [12]. The latter peptides were, however, chosen from a hydrophilic part of the molecule showing extensive homology with other DR and DQ polypeptides. These antisera would therefore not be locus specific. The same limitation would also be true for the study using 14–16-mer peptides to produce antibodies against HLA-DR β chains [11]. These antibodies showed reactivity with denatured proteins on immunoblots, but not surface binding to living cells. In contrast to these studies, our antisera are unique since they, despite being raised against peptides of only 8 amino acids in length, bind to their respective target proteins both in the native and denatured state. We therefore conclude that the 2 sets of antisera should be useful as specific immunological reagents to detect expression of β chain genes in the HLA-DQ and -DR loci.

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