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ANTIGENIC DETERMINANTS REACTING WITH RHEUMATOID FACTOR: EPITOPES WITH DIFFERENT PRIMARY SEQUENCES SHARE SIMILAR CONFORMATION

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Abstract—Polyclonal or monoclonal human IgM rheumatoid factors (RF) react with eight antigenic sites on the CH3 IgG domain, four sites on CH2 and two on human β_2-microglobulin. All 14 of these RF-reactive epitopes are linear 7–11 amino acid peptides with different primary sequence. We questioned whether RF reactivity with such a variety of epitopes showing no obvious sequence homology might result from conformational similarities shared by various RF-reactive regions. Strong support for this concept was obtained using rabbit antisera as well as mouse mAbs to individual CH3, CH2 or β_2m RF-reactive peptides. Major cross-reactivity was demonstrated between most of the 14 different CH3, CH2, or β_2m RF-reactive peptides using individual anti-epitope antibodies. Molecular modelling studies of these peptides showed striking similarities in three-dimensional shape among many RF-reactive peptides. Main-chain atoms rather than side chains seemed to contribute most directly to conformational similarity. Molecular simulation studies on control peptides showed no conformational similarities with RF-reactive peptides. Our studies indicate that autoantibodies such as RF recognize main-chain conformations of reactive epitopes and react with a number of antigenic determinants of quite different primary sequence but similar main chain conformations. © 1997 Elsevier Science Ltd.

Key words: rheumatoid factor, antigenic epitopes, antigen conformation.

INTRODUCTION

Rheumatoid factors (RF) which react with antigenic determinants on Fc fragment (CH2 and CH3 domains of IgG) represent one of the first human autoantibodies ever described (Franklin *et al.*, 1957; Kunkel *et al.*, 1959; Fudenberg and Kunkel, 1961). Besides binding to Fc of IgG, both polyclonal and monoclonal RF also react with two major antigenic regions on β_2m—another member of the Ig gene superfamily (Williams *et al.*, 1992a, 1993; Peterson *et al.*, 1972; Bodmer, 1972). When RF-reactive linear regions of CH3, CH2 and β_2m were recently identified using an overlapping heptamer approach and pin ELISA assay in parallel with a glycine/alanine substitution strategy, eight RF-reactive regions on human IgG CH3 as well as four major reactive sites on CH2 were identified (Williams and Malone, 1994; Peterson *et al.*, 1995). All eight CH3, as well as the four CH2 and two β_2m RF-reactive antigenic determinants showed completely different primary amino acid sequences. In many instances glycine/alanine substitution for each amino acid within these diverse RF-reactive epitopes identified relative restriction of immunodominant residues to specific tyrosine, tryptophan, arginine, valine, lysine, glutamic acid, and leucine residues (Williams and Malone, 1994; Peterson *et al.*, 1995). Nevertheless, the actual primary sequences of solvent accessible RF-reactive epitopes on CH3, CH2 and β_2m showed no obvious homology. These RF-reactive CH3, CH2 and β_2m epitopes are shown in Table 1. We hypothesized that the diversity of primary amino acid sequences within these 14 RF-reactive sites might result at least in part from similarities in conformation not readily apparent from mere examination of their primary amino acid sequence. Results reported here confirm this hypothesis using both molecular modelling studies and antigenic cross reactivity data. During the last ten years there have been at least five examples from studies of crystal structure of proteins which show that proteins having very different primary structure can show similar three-dimensional conformation (Kabsch and Sanders, 1984; Kolaskar and Ramabrahmam, 1984; Laurents *et al.*, 1994; Quaratino *et al.*, 1995; Wucherpfennig and Strominger, 1995). Results presented here indicate that this may be the case in terms of epitopes reacting with RF.

MATERIALS AND METHODS

Rabbit antisera produced by immunization of New Zealand white rabbits with single CH3, CH2, or β_2m

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Table 1. Rheumatoid-factor reactive antigenic epitopes on CH3 and CH2 domains of human IgG and β_2-microglobulin

	Primary sequence of RF-reactive site	
CH3	PREPQVY[a]	residues 343–349
	PQVYTLP	residues 346–352
	TLPPSRE	residues 350–356
	DGSFFLY	residues 401–407
	WQQGNVF[b]	residues 417–423
	CSVMHEG	residues 425–431
	EGLHNHY	residues 430–436
	KSLSLSP	residues 439–445
CH2	SVFLFPP[b]	residues 239–245
	KFNWYVD	residues 274–280
	NSTYRVVSV	residues 297–305
	VLTVLHQNWL	residues 305–314
β_2m	SKDWSFY	residues 57–63
	LSQPKIVKWDR	residues 87–97

[a]Underlined residues indicate apparent immunodominant amino acids as defined by glycine/alanine substitution.
[b]RF-reactive epitopes in which glycine/alanine substitution failed to identify immunodominant residues.

peptides (Table 1) linked to BSA using four weekly subcutaneous injections and complete Freund's adjuvant provided antibodies to individual CH3, CH2 or β_2m peptides. After absorption with BSA–Sepharose these antibodies were tested for peptide reactivity using a panel of peptides linked to Falcon microtiter plates as previously described (Williams *et al.*, 1992b). Specifically, Falcon ELISA plates were first precoated with polyalanine, polylysine (Sigma) overnight in pH 9.6 carbonate buffer. The next day peptides (10μg/ml) were coated on the plate using 0.125% glutaraldehyde. Uniform coating of peptides was assayed for all β_2m, CH2 and CH3 peptides as well as many control peptides using rabbit or murine anti-peptide antisera and ELISA to monitor equal, reproducible coating of test peptides on ELISA plates. Control peptides with no obvious sequence homologies derived from melanoma (GMERVRWCATDGEG), Klebsiella (CNSRQTDREDELI), HLA B27 (AKAQTDRED) and laminin (CIKVAVS) were included in each assay. In addition a much larger panel of 37 control peptides of approximately similar length (7–12 amino acids) with no sequence homologies to any of the CH3, CH2 or β_2m peptides used here were also tested for reactivity in all assays reported here. These additional control peptides are shown in Table 2.

Since peptides linked to BSA were employed to produce polyclonal anti-peptide antisera in rabbits, we had to be certain that the BSA employed did not contain any contamination with bovine or other IgG. BSA itself assayed in ELISA by goat anti-bovine Fc of IgG or anti-human Fc of IgG was negative. Moreover, before use, all anti-peptide antisera were absorbed with BSA–Sepharose 4B.

A panel of nine murine IgG monoclonal antibodies produced against whole human β_2-microglobulin (β_2m) and previously examined for anti-β_2m epitope reactivity (Williams and Malone, 1993) were also used to study cross reactivity with RF-reactive CH2 and CH3 domain epitopes. Additional monoclonal antibodies were produced from mice immunized with two RF-reactive epitopes, CH2 peptide KFNWYVD and CH3 peptide EGLHNHY linked to BSA. Hybridoma supernatants were screened by ELISA using peptides alone coated on flat-bottom Falcon plates as previously described (Williams and Malone, 1993).

Antigenic cross-reactivity between RF-reactive β_2m, CH3 and CH2 epitopes shown in Table 1 was also tested using inhibition of polyclonal rabbit anti-peptide antisera or monoclonal mouse antibody reacting with its homologous peptide antigen coated on ELISA plates at a concentration of 10 μg/ml. Preliminary titration of antibody dilutions reacting with homologous immunogen peptide established a 50% of maximal binding concentration of anti-peptide antibody and this concentration of antibody was used with pre-incubation of other inhibiting peptides or whole protein at a broad micromolar range of concentrations before completion of the ELISA and determination of inhibition (Williams *et al.*, 1992a; Williams and Malone, 1993).

Molecular modelling on RF-reactive peptides

Since the RF-reactive peptides shown in Table 1 displayed no sequence similarity, the possibility of conformational similarity was explored using the conformational similarity index (CSI) described previously (Kolaskar and Ramabrahmam, 1981; Kolaskar and Kulkarni-Kale, 1992). Computer modelling employed Insight II, Version 2.3.0, 1993 (Biosym Technologies, San Diego, CA). These studies were performed to determine if the RF-reactive peptides displayed any consistent pattern of similarities in three-dimensional shape. All RF-reactive epitopes on CH3 and CH2 had been established as solvent accessible using the coordinates for IgG Fc previously established by Diesenhoffer and coworkers (Diesenhoffer *et al.*, 1976, 1978) and structures available through the Brookhaven Data Bank. Computer analysis utilized the SYBYL program (Tri-post Associates, St. Louis, MO). In addition, determinations of whether particular peptides or single residues were solvent accessible were checked against the epitope analyses previously reported by Novotny *et al.* (1986). To obtain further insight regarding the shape of these peptides, minimum energy conformation of each of the peptides given in Tables 1 and 4 was determined using the following procedure. Initial conformations for peptides from CH3 and CH2 of IgG or β_2m were chosen from corresponding crystal structure data of the protein. For control peptides initial conformation was determined by choosing (ϕ,ψ) values from the corresponding region of the Ramachandran plot. Energy minimization was then carried out using the steepest descent method followed by application of the Newton–Raphson method to reach the convergence up to 0.001 kcal/mol of potential energy values. In order to reach the global minimum energy conformation, the peptide was heated up to 400 K for about 20 psec and then cooled to 300 K and the minimum

Table 2. Control peptides examined for reactivity in ELISA assay systems

Peptide sequence tested	Source	Peptide sequence tested	Source
GPLHPSWV	gE[a]	CLRDIPGRLV	proteinase 3
GTPRLPPPP	gE[a]	CDGIQGIDS	proteinase 3
GLYTLSVGD	gE[a]	AHCLRDIPQR	proteinase 3
ESCLYHPQLP	gE[a]	GSNKGAIIGLM	β-amyloid
ASTWTSRLA	gE[a]	LGKNIGISGAM	β-amyloid
PTQKLLWAV	gE[a]	KARVLIEAIG	HIV-1
VEHSDLSFS	β_2M[b]	KARVIIEAIG	HIV-2
NYLAWYQ	CDR1VL[c]	KARVFFEAIG	HIV-3
SGVPDRF	CDR2VL[c]	IQDDCPKAGR	AT-1
HIWWNDN	CDR VH[c]	YRPPPNCPNC	C50
KPSQTLS	3I HC Id[c]	SMVGSWAKV	B1
FSGSGSG	3I LC Id[c]	KPIRFFRL	RS6
RASQSIN	2A4LC CDR1[c]	ARGNVILTAKP	JS1
VYYCARD	2A4HC CDR3[c]	GRGDSPC	adneston
YCQQSYS	2A4VL CDR3[c]	KPIEFFN	HIV
KYNPSLK	2A4VH CDR2[c]	KPIGFR	HIV
PPPGMRPP	Sm peptide[d]		
PPGIRGP	Sm peptide[d]		
RGYDSYA	HAG CDR1[e]		
GSHTRGD	HAG CDR2[e]		
IYSTVASS	HLA Class I		

[a]Peptide derived from HSV-1 glycoprotein gE.
[b]Non-antigenic region of β_2m.
[c]Peptides derived from CDR sequences of monoclonal anti-DNA 2A4.
[d]Sm peptide.
[e]CDR regions of lambda HAG human light chain.

energy conformation again calculated. This process of heating and cooling the peptide was carried out several times until we reached a state where the difference between two lowest energy conformers was small and also the differences in main chain conformation measured in terms of (ϕ,ψ) values were also small. The Insight II package of Biosym Tech was used to carry out energy minimization studies as well as to identify conformationally homologous regions, with respect to conformation of the reference peptide (Kolaskar and Kulkarni-Kale, 1992). In addition, all peptides studied were also soaked in water and the effect on global minimum energy conformation in the presence of water as a solvent examined. The changes in conformation of these peptides due to water were found to be negligible. It is important to note that minimum energy conformation calculations and analysis were possible due to the fact that three-dimensional structural information from X-ray crystal data was available for CH3 and CH2 of IgG and β_2m domains (Diesenhoffer *et al.*, 1976, 1978; Saper *et al.*, 1991).

RESULTS

Rabbit antisera produced against β_2m peptides SKDWSFY and LSQPKIVKWDR showed strong primary reactions with their homologous peptides but also displayed significant cross-reactivity with the opposite β_2m peptide as well as with several of the other RF-reactive peptides derived from the IgG CH2 and CH3 domains (Fig. 1A). This cross reactivity included the peptides VLTVLHQNWL and KFNWYVD from CH2 and DGSFFLY, CSVMHEG, PREPQVY, and PQVYTLP from CH3. Control experiments using rabbit antisera to two unrelated proteinase-3 (PR3) peptides ATVQLPQ and RFGAHDP showed strong ELISA reactions only with the homologous PR3 peptide employed for immunization, but no detectable cross-reaction with any of the CH3, CH2 or β_2m peptides, nor with the large panel of 37 control unrelated peptides (Table 2). Similarly, antisera produced against several of the RF-reactive CH3 or CH2 peptides also showed cross-reactions with β_2m peptides or other Fc derived RF-reacting peptides with entirely different primary sequences from those employed for primary immunization (Fig. 1B). Parallel extensive control experiments showed no significant cross-reactivity with other unrelated melanoma, Klebsiella, HLA B27, laminin peptides or extensive other control peptides (Fig. 1B and Table 2).

A total of seven polyclonal rabbit antisera produced against individual solvent accessible epitopes derived from β_2m, CH3, or CH2 RF-reactive regions were tested by ELISA against peptides representing the linear epitopes shown in Table 1. A striking panorama of cross-reactivity similar to that shown in Fig. 1A, B became apparent. Thus, in addition to the cross-reactivity of antisera produced against β_2m peptides SKDWSFY or LSQPKIVKWDR with several CH3 peptides as well as CH2 peptides shown in Fig. 1A, B, rabbit polyclonal antisera produced against several other CH3 RF-reactive

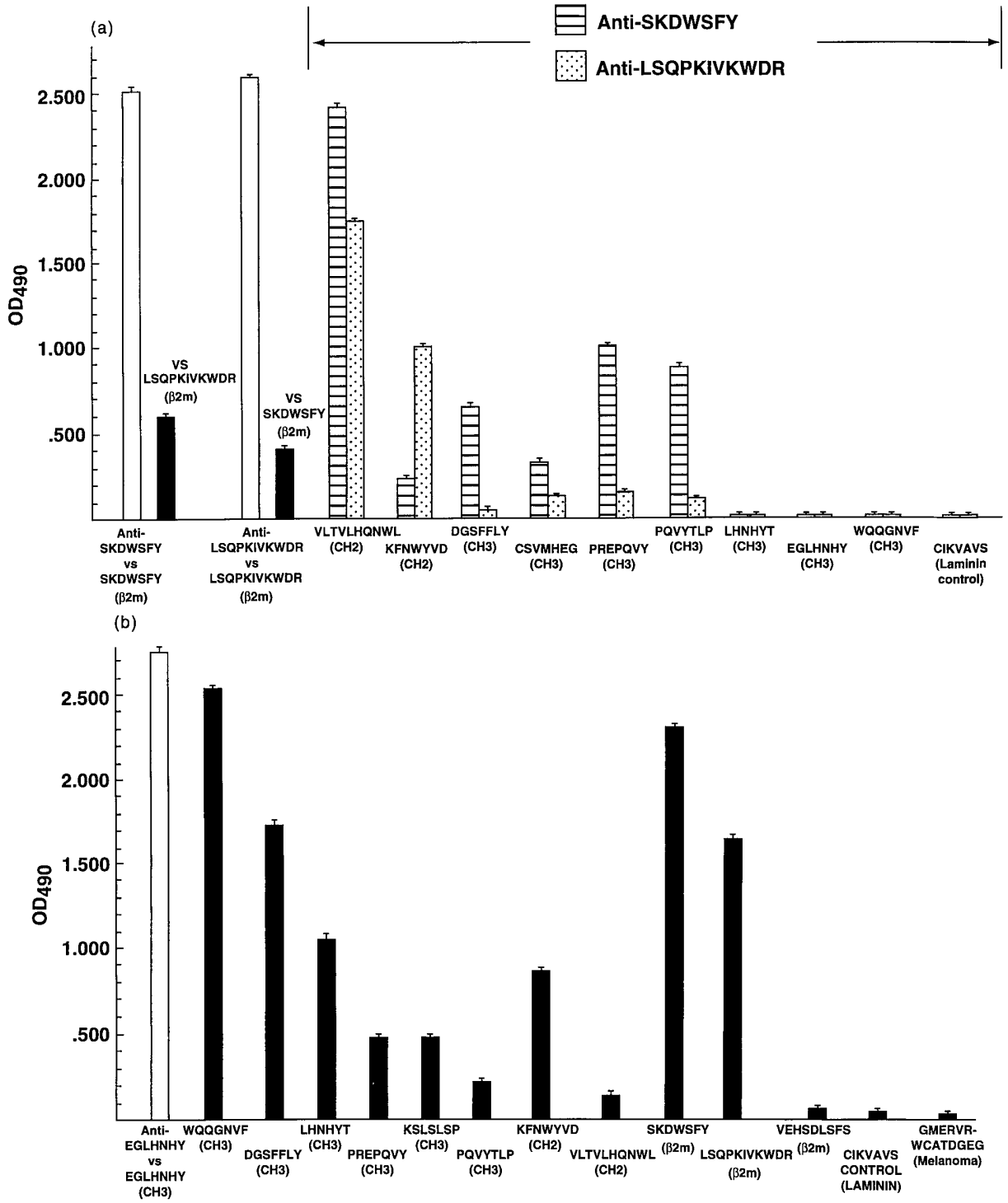

Fig. 1. (A) Rabbit antisera against RF-reactive β_2m epitopes SKDWSFY and LSQPKIVKWDR were tested in ELISA against their homologous peptides (open vertical bars) as well as the opposite β_2m peptides (solid bars) and showed significant cross-reactivity. When these same anti-β_2m peptide antisera were tested against other RF-reactive peptides derived from CH2 (VLTVLHQNWL, KFNWYVD) and CH3 (DGSFFLY, CSVMHEG, PREPQVY, and PQVYTLP) reactions were recorded with at least one of the anti-β_2m antisera (anti-SKDWSFY hatched bars and anti-LSQPKIVKWDR dotted bars). No cross-reactivity was recorded with three other RF-reactive CH3 linear peptides, nor many other control peptides (see Table 2). (B) Rabbit antiserum produced against EGLHNHY, one of the RF-reactive CH3 epitopes, not only shows strong ELISA reactivity with its homologous peptide but also reacts with a number of other CH3, CH2 and β_2m peptides, all of which represent RF-reactive epitopes. Controls include VEHSDLSFS (a non-RF reacting β_2m peptide), and laminin, as well as melanoma peptides and additional controls shown in Table 2.

epitopes (KSLSLSP, PQVYTLP, CSVMHEG, PREPQVY and DGSFFLY) or CH2 RF-reactive sites (KFNWYVD and VLTVLHQNWL) also showed strong cross-reactivity with other RF-reactive epitopes on β_2m, CH3 or CH2. A composite profile of this cross-reactivity of RF-reactive regions as identified with direct ELISA assays and polyclonal rabbit antisera made to single β_2m, CH3 or CH2 peptides is shown in Table 3. It can be seen that the seven different rabbit antisera often identified similar patterns of cross-reactivity between RF-reactive regions of β_2m, CH3 and CH2. However, cross-reactivity was not universal and some negative reactions were recorded. Thus, antisera against CH3 peptides EGLHNHY, CSVMHEG, PREPQVY all showed broad cross-reactivity with both β_2m peptides SKDWSFY and LSQPKIVKWDR as well as with the two CH2 peptides KFNWYVD and VLTVLHQNWL. However, rabbit antisera produced against CH3 epitope CSVMHEG failed to react with RF-reactive CH3 epitopes EGLHNHY, WQQGNVF and LHNHYT. Also rabbit antisera against CH3 PREPQVY failed to react with CH3 RF-reactive epitopes WQQGNVF and LHNHYT. Rabbit antisera produced against the CH2 RF-reactive regions KFNWYVD and VLTVLHQNWL showed definite cross-reactivity with the two β_2m epitopes but less in the way of cross-reactions with CH3 RF-reactive regions EGLHNHY, WQQGNVF, CSVMHEG or PREPQVY.

Moreover, some other exceptions to universal cross-reactivity were noted. Thus, rabbit antisera to CH3 peptide EGLHNHY showed strong cross-reactivity with the two β_2m peptides SKDWSFY and LSQPKIVKWDR, however, rabbit antisera produced against these two latter β_2m peptides did not react directly with EGLHNHY in ELISA assays. Similar lack of demonstrable two-way cross-reactivity was noted with β_2m SKDWSFY and CH2 VLTVLHQNWL, where rabbit antisera against VLTVLHQNWL showed only weak reactivity with SKDWSFY. A similar lack of complete two-way cross-reactivity with separate rabbit antisera to β_2m LSQPKIVKWDR and CH3 CSVMHEG was also noted. No obvious reason for these discrepancies was apparent save for the possibility that when rabbit antisera were generated, some BSA-linked peptides provided a much stronger antigenic stimulus than others. Thus, the actual coupling of peptides to BSA as a carrier may have provided a slightly different conformation to the peptides being studied than their actual shape as they are situated in their native original protein domains.

Relative degrees of antigenic cross-reactivity were also explored using competitive inhibition of reactivity between polyclonal rabbit antibody to single β_2m, CH3 or CH2 peptides and their respective homologous peptides and other RF-reactive CH3 or CH2 epitopes. Examples of such competitive inhibition reactions confirming clear-cut antigenic cross-reactivity between several distinct RF-reactive epitopes of entirely different primary amino acid sequence are shown in Fig. 2A, B. In Fig. 2A, it can be seen that the homologous peptide EGLHNHY used to produce the rabbit antibody to the RF-reactive CH3 epitope showed 100% inhibition at approximately 5 μmol concentrations of homologous EGLHNHY

Table 3. Profile of ELISA cross-reactivity of polyclonal rabbit antisera produced against β_2m, CH3, or CH2 peptides

	Peptides used for ELISA assay											
	β_2m	β_2m	CH3	CH3	CH3	CH3	CH3	CH3	CH3	CH3	CH2	CH2
		L										
		S										V
		Q										L
		P										T
	S	K	E	W	K	P	C	P	D		K	V
	K	I	G	Q	S	Q	S	R	G	L	F	L
	D	V	L	Q	L	V	V	E	S	H	N	H
	W	K	H	G	S	Y	M	P	F	N	W	Q
	S	W	N	N	L	T	H	Q	F	H	Y	N
	F	D	H	V	S	L	E	V	L	Y	V	W
Peptide used for immunization												
	Y	R	Y	F	P	P	G	Y	Y	T	D	L
SKDWSFY (β_2m)	(**4+**[a])	2+	0	0	2+	2+	1+	2+	2+	0	1+	4+
LSQPKIVKWDR(β_2m)	1+	(**4+**)	0	0	±	±	0	±	0	0	2+	3+
EGLHNHY(CH3)	4+	3+	(**4+**)	4+	1+	±	±	1+	3+	2+	2+	±
CSVMHEG(CH3)	2+	3+	0	0	1+	4+	(**4+**)	1+	1+	0	3+	1+
PREPQVY(CH3)	1+	2+	1+	0	1+	2+	1+	(**3+**)	2+	0	3+	1+
VLTVLHQNWL(CH2)	±	1+	0	0	2+	±	0	0	3+	1+	4+	(**3+**)
KFNWYVD(CH2)	3+	4+	±	±	0	0	0	0	2+	0	(**4+**)	±

[a] ± = 0.1–0.29 optical density (OD); 1+ = 0.3–0.5 OD; 2+ = 0.6–1.2 OD; 3+ = 1.3–1.9 OD; 4+ = 2.0–2.8 OD.
Reactions in parentheses indicate those with homologous peptide used for immunization.

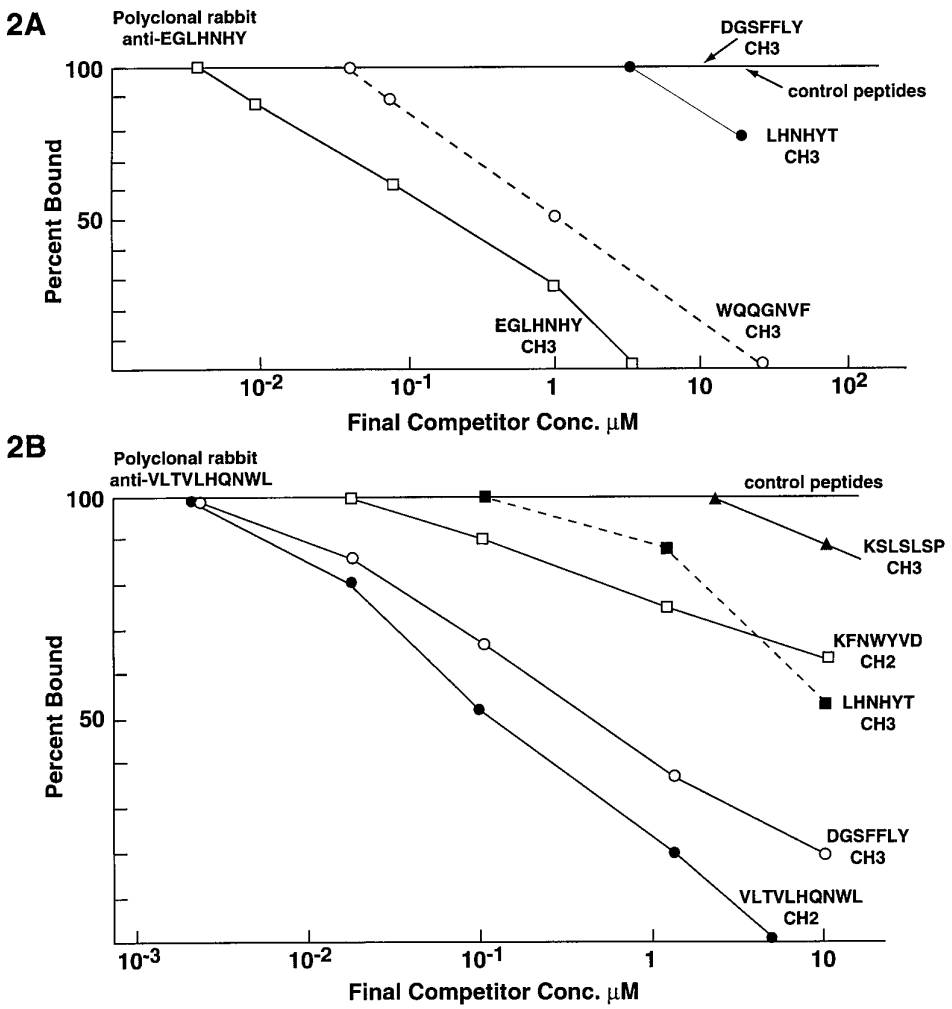

Fig. 2. (A) Competitive inhibition studies using rabbit polyclonal anti-CH3 peptide EGLHNHY reacting with EGLHNHY on the ELISA plate. Rabbit antiserum was pre-incubated with various peptides tested at a broad micromolar concentration before completion of the ELISA assay. CH3 peptides LHNHYT and WQQGNVF as well as the immunogen peptide EGLHNHY showed inhibition in the assay. (B) Competitive inhibition using rabbit polyclonal anti-CH2 peptide VLTVLHQNWL reacting with homologous VLTVLHQNWL peptide on the ELISA plate. Complete (100%) inhibition was recorded for VLTVLQHQNWL, but substantial (80%) cross-inhibition was also noted for CH3 DGSFFLY and lesser but significant inhibition for CH3 LHNHYT and CH2 KFNWYVD.

peptide. However, WQQGNVF, another CH3 RF-reactive peptide region sharing no primary amino acid sequence homology with EGLHNHY produced striking parallel inhibition at slightly higher micromolar concentrations. LHNHYT (another RF-reactive CH3 epitope) also showed slight (28%) inhibition. By contrast, a fourth RF-reactive epitope CH3 DGSFFLY and the control peptides CNSRQTDREDELI, AKAQTDRED, GMERVRWCATDGBG, and CIKVAVS produced no inhibition. Absence of inhibition by CH3 peptide DGSFFLY represented an exception to the general levels of inhibition recorded, since the original rabbit antiserum had shown considerable direct ELISA binding to this peptide. Lack of inhibition by DGSFFLY in this instance may have represented lower affinity of the primary antibody for this peptide compared with the immunogen EGLHNHY.

Another example of convincing cross-inhibition of polyclonal rabbit antibody to the CH2 RF-reactive epitope VLTVLHQNWL by both CH3 and CH2 RF-reactive peptides sharing no primary amino acid sequence homology with the peptide immunogen is shown in Fig. 2B. Here, as expected, complete inhibition was recorded with the homologous CH2 VLTVLHQNWL peptide but almost as high parallel inhibition (80%) was found with RF-reactive CH3 peptide DGSFFLY as well as lesser but substantial inhibition with LHNHYT from CH3 and CH2 peptide KFNWYVD. Again, no inhibition was observed with control peptides. These and many other similar experiments confirmed antigenic relatedness

between many of the separate RF-reactive β_2m, CH3 and CH2 regions shown in Table 1.

Studies using monoclonal antibodies

Because cross-reactivity between heterogeneous epitopes showing a diverse profile of primary sequences could theoretically possibly be ascribed to heterogeneity of affinities within the rabbit polyclonal anti-β_2m, CH3 or CH2 peptide antisera, the same question was addressed using a panel of nine different monoclonal mouse IgG antibodies produced against human β_2m and previously studied for β_2m epitope specificity (Williams and Malone, 1993). These anti-β_2m mAbs were tested against both β_2m RF-reactive peptides LSQPKIVKWDR and SKDWSFY, as well as the other CH3 and CH2 RF-reactive peptides in parallel. Two representative anti-β_2m mAb reactive profiles are shown in Fig. 3, indicating major anti-β_2m mAb cross-reactivity with CH2 KFNWYVD as well as moderate cross-reactivity with CH3 EGLHNHY and many other RF-reactive CH3 and CH2 epitopes. Particularly striking was that all nine anti-β_2m mAbs showed the same reactive profile-major cross-reactivity between β_2m antigenic regions SKDWSFY and LSQPKIVKWDR and CH2 RF-reactive peptide KFNWYVD, with lesser but significant cross-reactivity with CH2 RF-reactive peptide VLTVLHQNWL and CH3 RF-reactive EGLHNHY. This finding with the mAbs against β_2m confirmed the strong cross-reacting antigens already identified using polyclonal rabbit antisera against the CH2 peptide KFNWYVD, which gave 3+ and 4+ reactions with β_2m SKDWSFY and LSQPKIVKWDR (Table 3). Of interest were inhibition data obtained with monoclonal anti-β_2m antibody F20.11C reacting with SKDWSFY on the ELISA plate. This reaction was inhibited by 40% with CH3 EGLHNHY and by 50% with CH2 peptide VLTVLHQNWL. These cross-sinhibition results confirmed antigenic cross-reactions between SKDWSFY and EGLHNHY which had only been noted with rabbit polyclonal antisera to SKDSFY, but not with rabbit antisera to EGLHNHY.

Additional murine mAbs generated against CH2 peptide KFNWYVD and CH3 peptide EGLHNHY also confirmed that strong cross-reactivity between many RF-reactive β_2m, CH3 and CH2 epitopes was recognized by such primary mAbs generated against only one such CH2 or CH3 RF-reactive peptide. A high degree of RF-reactive cross-specificity was noted with mAbs generated against CH2 peptide KFNWYVD (Fig. 4), where strong reactivity was apparent with CH3 RF-reactive EGLHNHY, WQQGNVF, PREPQVY and CSVMHEG bearing no primary amino acid homology with the CH2

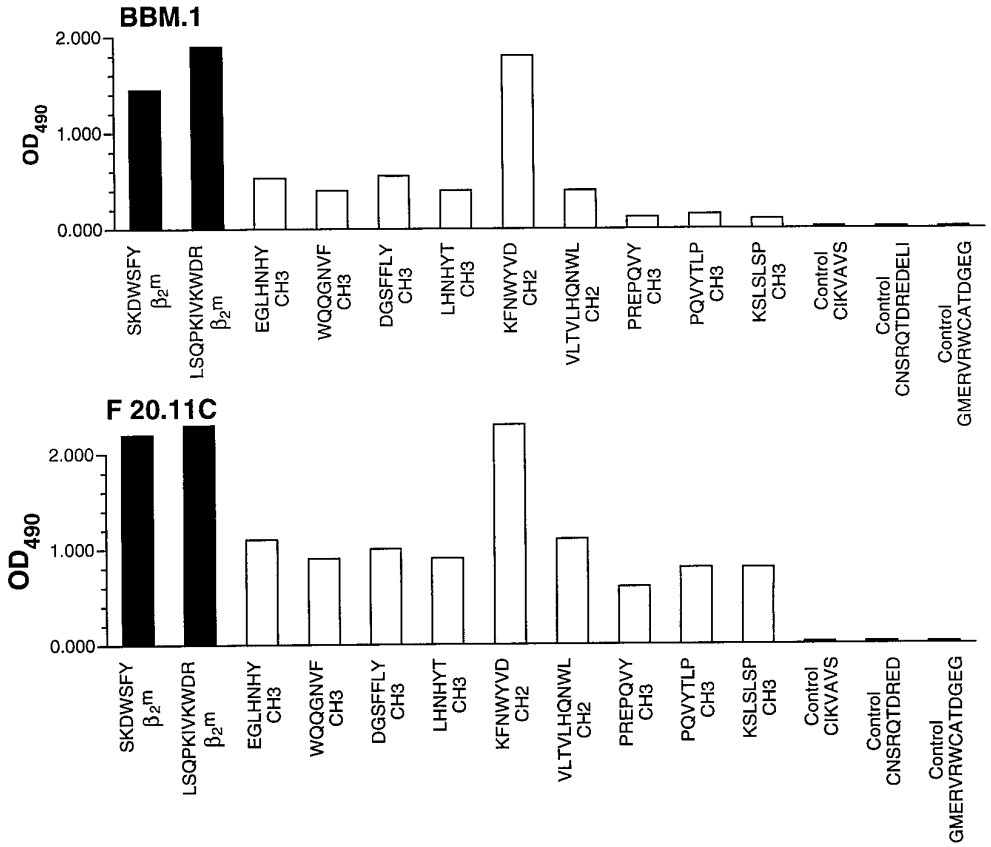

Fig. 3. Monoclonal murine IgG anti-β_2m antibodies BBM.1 and F20.11C shown reacting in ELISA with homologous β_2m peptides SKDWSFY and LSQPKIVKWDR, as well as many other RF-reactive CH3 and CH2 peptides, but not with unrelated control peptides CIKVAVS (laminin), CNSRQTDREDELI (klebsiella) or GMERVRWCATDGEG (melanoma) as well as the extended panel of control peptides shown in Table 2.

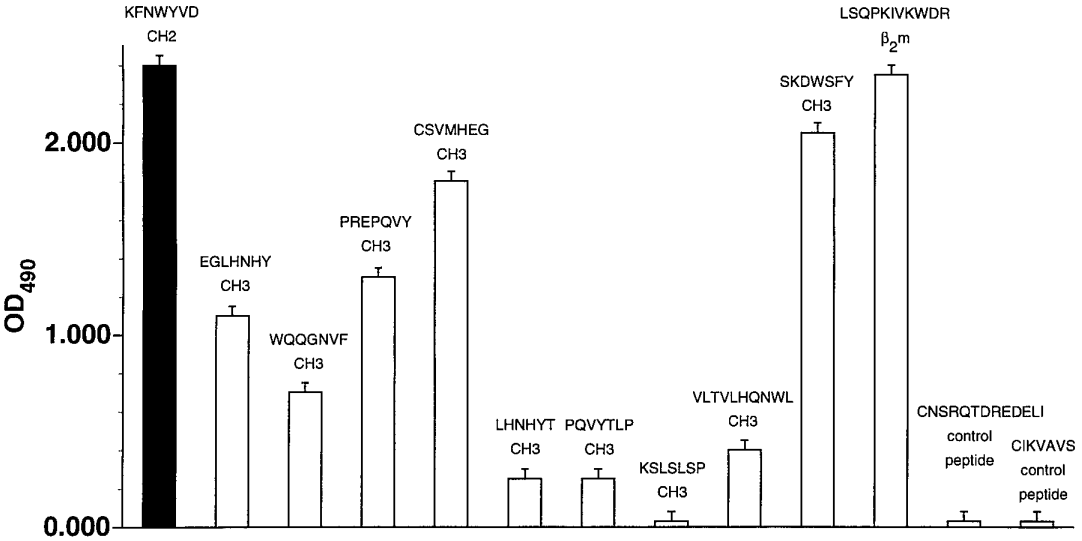

Fig. 4. Monoclonal murine IgG antibody to CH2 RF-reactive peptide KFNWYVD shown reacting in ELISA with the homologous peptide KFNWYVD immunogen (dark vertical bar) and other CH3, CH2 or β_2m RF-reactive epitopes (open bars). No significant reaction was recorded with control peptides.

immunogen KFNWYVD. Of great interest again, was the extremely strong cross-reactivity of this monoclonal antibody with both β_2m RF-reactive peptides SKDWSFY and LSQPKIVKWDR.

Inhibition studies using monoclonal murine anti-CH2 peptide KFNWYVD reacting with its homologous peptide KFNWYVD are illustrated in Fig. 5. Here as expected, KFNWYVD showed maximum (100%) inhibition; however, CH3 peptides WQQGNVF and PREPQVY showed 50% inhibition at higher concentrations and CH3 peptide EGLHNHY slight inhibition at higher concentrations. Additional inhibition results, not illustrated in the figure also showed 50% inhibition by β2m

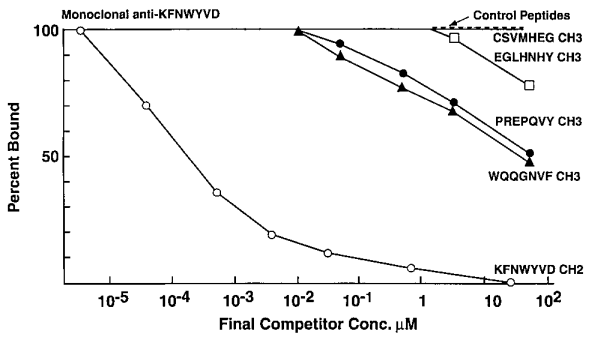

Fig. 5. Monoclonal murine antibody to KFNWYVD reacting with its homologous peptide on the ELISA plate. Significant inhibition is noted with RF-reactive CH3 peptides PREPQVY and WQQGNVF and slight inhibition with CH3 EGLHNHY but none with CSVMHEG or control peptides. Additional similar inhibition experiments not shown here revealed 50% inhibition by SKDWSFY (β_2m), LSQPKIVKWDR (β_2m) and VLTVLHQNWL (CH2). Although in the direct ELISA assay monoclonal antibody showed highest binding to β_2m peptide LSQPKIVKWDR, the inhibition experiment indicated only moderate levels of inhibition.

peptides SKDWSFY and LSQPKIVKWDR, as well as VLTVLHQNWL from CH2. Again a broad range of control peptides (fifteen) produced no inhibition. Some discrepancy was noted in these experiments between the strong direct reaction of monoclonal Ab against KFNWYVD and β_2m peptide LSQPKIVKWDR and only moderate inhibition by this β_2m peptide when mAb to KFNWYVD was tested reacting with the homologous KFNWYVD peptide. Similar differences were noted between relatively weak direct reactivity between mAb to KFNWYVD and CH3 peptides WQQGNVF and PREPQVY (Fig. 4), but strong inhibition in the mAb reacting with primary immunogen KFNWYVD system (Fig. 5). These differences which were reproducible were assumed to represent probable differences in conformation of peptides when tested on the ELISA plate in parallel with the same peptides in free solution.

Since we were examining reactivity of both polyclonal and monoclonal antibodies to various RF-reactive peptides derived from solvent accessible regions of β_2m, CH3 or CH2 structure, it was important to determine whether such antibodies actually reacted with the whole proteins containing these same designated RF-reactive epitopes. Accordingly, all polyclonal and monoclonal antibodies produced against RF-reactive epitopes were studied for ELISA reactivity with either β_2 microglobulin or whole IgG. In all such cases, strong ELISA reactivity (OD 1.5 or greater) of both polyclonal and monoclonal anti-RF-reactive peptides was demonstrated. These results emphasized that the conformations of the various peptides employed to generate either monoclonal or polyclonal anti-RF epitope antibody were similar to those present within the original parent protein structure.

Monoclonal antibody 3G9 produced against CH3 peptide EGLHNHY provided the most unique evidence for RF epitope cross-reactivity of all. This antibody showed

only weak reactivity (OD 0.5) with the original EGLHNHY immunogen but much higher optical density (2.0) with KFNWYVD (CH2) and with the SKDWSFY (OD 1.6) and LSQPKIVKWDR (OD 1.0) β_2m peptides. Reaction with all other RF-reactive CH3 and CH2 peptides was negative. Inhibition studies using mAb 3G9 reacting with homologous peptide EGLHNHY on the ELISA plate showed 50% inhibition with β_2m LSQPKIVKWDR and expected strong inhibition by EGLHNHY. Both KFNWYVD (CH2) and PREPQVY (CH3) produced moderate 30% inhibition but no inhibition was recorded with control peptides. When the same mAb 3G9 was tested reacting with the cross-reacting KFNWYVD CH2 peptide, striking inhibition (70%) was recorded with KFNWYVD, SKDWSFY and VLTVLHQNWL. In addition, moderate (30%) inhibition was found with PREPQVY and LSQPKIVKWDR. Although the monoclonal 3G9 enigmatically showed higher reactions with other RF-reactive epitopes than its homologous peptide, the general pattern of inhibition confirmed the previously noted cross-reactivity of many RF-epitopes illustrated in the previous results.

Molecular modelling studies employing three-dimensional analyses of RF-reactive epitopes based on their established crystal structures

Minimum energy conformations of each of the β_2m, CH3 and CH2 peptides were studied to examine possibly similar homologous conformational regions shared by these diverse epitopes all reacting with RF. It can be seen from Table 4, that SFY and KWD (derived from widely different portions of β_2m sequence, both of which reacted with RF) showed extremely similar conformations within the antigenic determinants SKDWSFY and LSQPKIVKWDR. Moreover, in Fig. 1A rabbit antisera raised against either SKDWSFY or LSQPKIVKWDR showed cross-reactions with the opposite β_2m peptide. In Fig. 1A, it also can be noted in that these same rabbit antisera against β_2m SKDWSFY or LSQPKIVKWDR both showed very strong reactions with the RF-reactive CH2 peptide VLTVLHQNWL, as well as RF-reactive CH2 peptide KFNWYVD and CH3 peptides DGSFFLY, CSVMHEG, PREPQVY and PQVYTLP. In parallel, Table 4 and Fig. 6A, B illustrate remarkable conformational similarities in adjacent three or four non-homologous residues of these same RF-reactive determinants showing conformationally similar regions (SFY with KWD, LTV, FNW, CSV, or WSFY with GSFF, DWS with QGN, SF with EP, HN and SL and SFY with HNH and VYT). Additional striking conformational similarities were also found for LSQPKIVKWDR (β_2m) and VLTVLHQNWL (CH2) encompassing KIVK (β_2m) and LTVL (CH2) (Table 4, middle panel). These regions were also both shown to exhibit antigenic cross-reactivity with rabbit antisera to either β_2m peptide SKDWSFY or LSQPKIVKWDR (Fig. 1A) or to mouse mAbs against whole β_2m. The KIV sequence of β_2m LSQPKIVKWDR also showed remarkable conformational similarity to KFN, CSV, NVF, EPQ and HNH of other RF-reactive peptides. When CH2 peptide KFNWYVD was used as the reference peptide (lower part of Table 4) additional extensive conformation homologies were apparent for many of the RF-reactive epitopes, particularly for FNW within the reference peptide. When conformationally similar regions (CSR) comparisons were performed with control peptides, no similarities in shape were recorded. The cross-reacting similarly shaped epitopes of β_2m SFY and KWD with FNW of CH2 peptide KFNWYVD (Table 4) were confirmed by the strong ELISA cross-reactions with monoclonal anti-KFNWYVD (Figs 4 and 5) as well as by the ELISA profile of mAb 3G9 against EGLHNHY. Of particular interest was the finding that two separate regions of β_2m (KIVK and KWD) showed conformational similarity to LTVL and LTV of CH2, respectively (Table 4). A composite visual presentation of all conformational similarities noted between CH3, CH2 and β_2m RF-reactive peptides is presented in Fig. 6B.

DISCUSSION

Results presented here indicate that antigenic epitopes which show no clear-cut similarity in primary amino acid sequence may actually exhibit very similar conformations. That antigenic regions on molecules of biologic interest such as proteins, polypeptides, complex sugars or nucleic acid components often present nonlinear or composite antigenic determinants made up of several loops or contiguous folds of tertiary structure is widely accepted (Amit *et al.*, 1986; Padlan *et al.*, 1989). Such conformationally dependent antigenic determinants probably represent a substantial proportion of all antigenic elements. Similarly, sequential or linear epitopes on proteins are also well documented (Appel *et al.*, 1990; Das and Lindstrom, 1991). However, to our knowledge, conformational epitopes in oligopeptides or linear protein sequence are not reported. The results presented here support the notion that a number of discrete regions both on CH3 and CH2, as well as β_2m may share important similar elements of antigenicity on the basis of unexpected homologies of shape or conformation. These conformational similarities among the β_2m, CH2 or CH3 RF-reactive antigenic determinants with little sequence similarity suggest that the antibodies produced may bind determinants structurally dependent on the main chain atoms of these determinants which are exposed (Fig. 6A, B). ELISA results with polyclonal rabbit antisera produced against many of the other RF-reactive epitopes from CH2, CH3 and β_2m confirmed the results obtained from molecular modelling studies. Rabbit antisera produced against RF-reactive epitopes on CH2 and CH3 showed reactivity against whole IgG, indicating that at least part of the conformation recognized in the antiserum against the peptide conjugated to BSA as original immunogen reacted with similar conformations in the native protein. Moreover, monoclonal antibodies originally produced against whole $\beta_2$microglobulin showed striking and consistent cross-reactivity with several RF-

Table 4. Conformationally similar regions (CSRs) between reference and test peptides

Reference peptide →	S	K	D	W	S	F	Y
Test peptide ↓							
LSQPKIVKWDR [β_2m]					K	W	D
VLTVLHQNWL [CH2]					L	T	V
KFNWYVD [CH2]					F	N	W
CSVMHEG [CH3]					C	S	V
DGSFFLY [CH3]				G	S	F	F
WQQGNVF [CH3]			Q	G	N		
PREPQVY [CH3]					E	P	
EGLHNHY [CH3]					H	N	
LHNHYT [CH3]					H	N	H
PQVYTLP [CH3]					V	Y	T
KSLSLSP [CH3]					S	L	
CNSRQTDREDELI[a] [Control]	-	-	-	-	-	-	-
CIKVAVS[a] [Control]	-	-	-	-	-	-	-
GMERVRWCATDGEG[a] [Control]	-	-	-	-	-	-	-

Reference peptide →	L	S	Q	P	K	I	V	K	W	D	R
Test peptide ↓											
SKDWSFY [B_2M]								S	F	Y	
VLTVLHQNWL [CH2]								L	T	V	
VLTVLHQNWL [CH2]					L	T	V	L			
KFNWYVD [CH2]					K	F	N				
CSVMHEG [CH3]					C	S	V				
DGSFFLY [CH3]					F	F					
WQQGNVF [CH3]					N	V	F				
WQQGNVF [CH3]								N	V	F	
PREPQVY [CH3]					E	P	Q				
EGLHNHY [CH3]						L	H	N			
LHNHYT [CH3]					H	N	H				
PQVYTLP [CH3]				V	Y	T					
KSLSLSP [CH3]					S	L					
CNSRQTDRED[a] [Control]	-		-	-	-	-	-	-	-	-	-
CIKVAS[a] [Control]	-	-	-	-	-	-	-	-	-	-	-
GMERVRWCATDGEG[a] [Control]	-	-	-	-	-	-	-	-	-	-	-

Reference peptide →	K	F	N	W	Y	V	D
Test peptide ↓							
SKDSFY [β_2m]		S	F	Y			
LSQPKIVKWDR [β_2m]		K	I	V			
VLTVLHQNWL [CH2]	L	T	V	L			
CSVMHEG [CH3]	C	S	V				
DGSFFLY [CH3]		S	F	F			
WQQGNVF [CH3]	N	V	F				
PREPQVY [CH3]	E	P	Q	V			
EGLHNHY			L	H	N		
LHNHYT [CH3]		N	H	Y			
PQVYTLP [CH3]			V	Y	T	L	
KSLSLSP [CH3]		L	S	L			
CNSRQTDREDELI[a] [Control]	-	-	-	-	-	-	-
CIKVAVS[a] [Control]	-	-	-	-	-	-	-
GMERVRWCATDGEG[a] [Control]	-	-	-	-	-	-	-

A region is called conformationally similar when it has at least two contiguous amino acids having similar conformations to the test peptide. The amino acids are said to be conformationally similar when their main chain dihedral angles (ϕ,ψ) lie within $\pm 30°$.

[a]No CSR as per the above criteria.

Fig. 6. (A) (1) Ball and stick model of β_2m SKDWSFY and LSQPKIVKWDR showing striking similarity for SFY and KWD (see inner white boxes). (2) Similar conformational similarities are shown between SFY (β_2m) and LTV (CH2); (3) SFY and FNW (CH2). (4) WSFY (β_2m) also shows conformational similarity to GSFF (CH3). (5) and (6) The interesting finding that LTVL or LTV (CH2) are conformationally similar to two different regions of β_2m LSQPKIVKWDR (KIVK and KWD) is also shown.

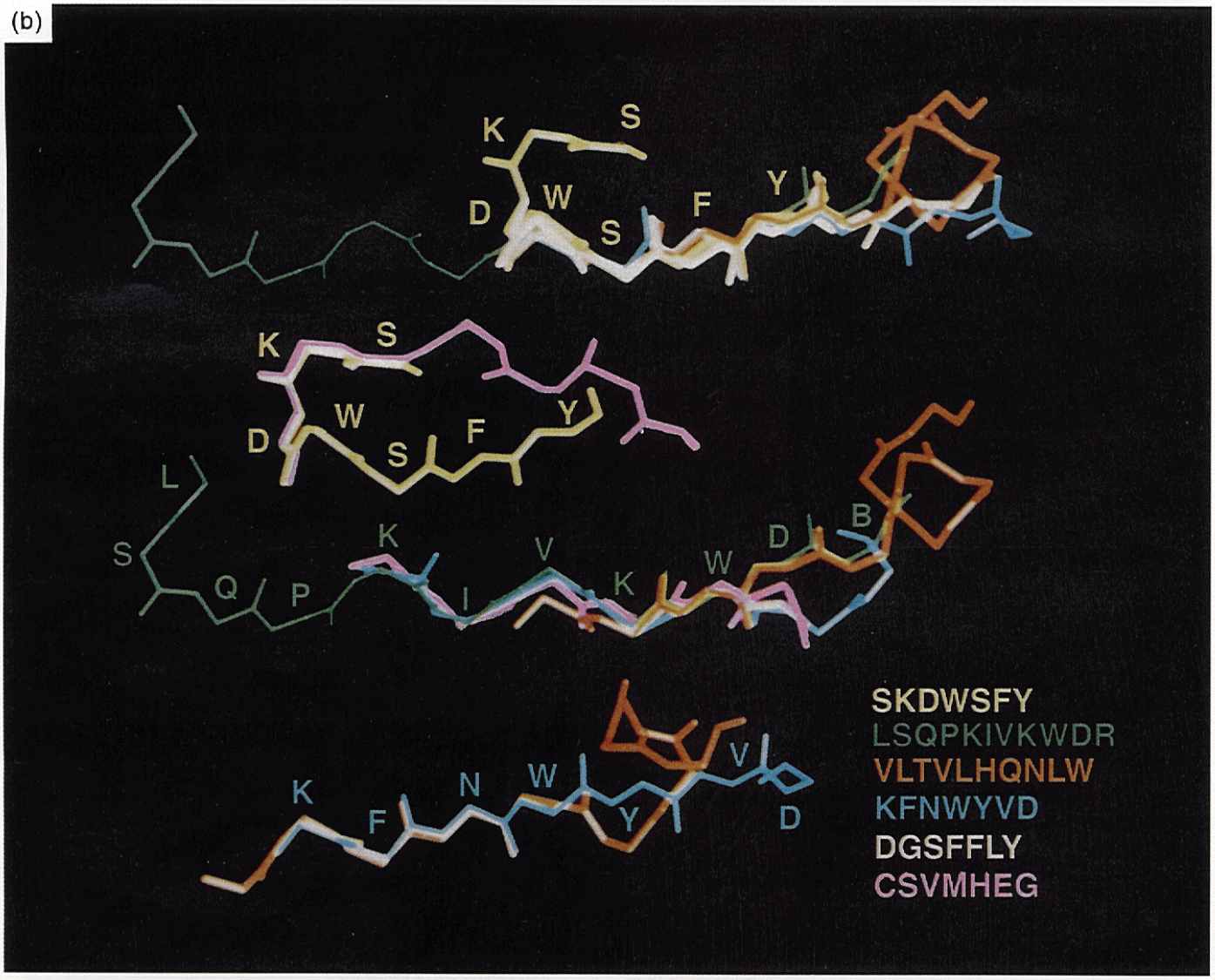

Fig. 6(B) Energy minimized conformation of antigenic determinants β_2m SKDWSFY (yellow), β_2m LSQPKIVKWDR (green), CH2 VLTVLHQNWL (red), CH2 KFNWYVD (cyan), CH3 DGSFFLY (white) and CH3 CSVMHEG (magenta). Amino acid sequence of the reference peptide is written in respective colour. Note conformationally similar regions with respect to part of SKDWSFY as reference. Similarly with LSQPKIVKWDR (green) or KFNWYVD (cyan) as reference, the main chain conformation in part is highly similar, as also shown in Table 3 with KIV, LTV, KFN and CSV. This correlates with the antigenic cross-reactivity pattern (Fig. 1A, C and Fig. 4). β_2m SKDWSFY (yellow) shows main chain conformational similarity to CSVMHEG (magenta). WSFY of β_2m (yellow) is similar to (CH3) GSFF (white).

reactive antigenic linear peptide regions on CH3 and CH2. In similar fashion, monoclonal antibodies generated against different RF-reactive epitopes on CH3 (EGLHNHY) or on CH2 (KFNWYVD) also showed striking cross-reactions with many other RF-reactive regions on β_2m, CH3 and CH2. Again monoclonal antibodies to RF-reactive CH3 and CH2 epitopes reacted with whole IgG or Fc.

Though hard experimental evidence demonstrating that three-dimensional structures of RF-reactive peptides having different sequences discussed here are conformationally similar may be lacking, X-ray crystallographic studies of many globular proteins have often shown that proteins with different sequences can have similar three-dimensional structure and support molecular modelling studies (Kolaskar and Ramabrahmam, 1981; Kolaskar and Kulkarni-Kale, 1992; Kabsch and Sanders, 1984). Further, there are many examples from crystal structures of proteins indicating that three-dimensional structures of contained oligopeptides are similar, even though the sequences are different and vice versa (Laurents *et al.*, 1994).

Data presented here indicate that well-defined solvent accessible antigenic determinants on proteins such as the RF-reactive CH3 and CH2 domains of IgG or the two RF-reactive antigenic regions of β_2m may share demonstrable similarities in conformation despite having no obvious primary amino acid sequence homology. Antigenic cross-reactions detected by polyclonal antisera in general confirmed the cross-reactivity of many diverse RF epitopes though there were several exceptions to this as a general finding. Possibly differences in relative affinities of antibodies present in respective rabbit antisera could have been responsible for such exceptions. Thus, when peptides were linked to BSA to induce antibodies by intentional immunization, it seems possible that three-dimensional shapes of the immunizing peptides could have been slightly different from those of the same regions in the native protein. Another explanation for lack of complete two-way cross-reactivity between presumably similar peptides using the various anti-peptide antibodies could relate to variations in the shapes or fit within respective anti-peptide antibody CDR3s. Thus, in some instances the anti-peptide antibodies could accommodate a number of related structures, while with other anti-peptide antibodies the shape of the paratopes was much more restrictive, leading to a more rigid or narrow range of reactivity. Such a phenomenon could apply both to polyclonal as well as to monoclonal antibodies to peptides.

The reactivity with one of the monoclonal mouse antibodies to the CH3 determinant EGLHNHY was of considerable interest and lent further support for the apparent strong antigenic cross-reactivity between several RF-reactive epitopes of different primary sequence. Thus, monoclonal anti-EGLHNHY showed maximum ELISA reactivity against KFNWYVD, SKDWSFY and LSQPKIVKWDR rather than against the peptide employed for immunization. This paradoxical reactivity profile actually provided strong support for the antigenic cross-reactions described above. That conformational homology was more important than identity of primary amino acid sequence for monoclonal antibody (mAb) binding to protein antigenic determinants was emphasized by our previous finding that two different mAbs with the same specificity for the Fcγ-binding gE protein of HSV-1 bound to six gE heptamer determinants showing no obvious similarity in primary amino acid sequence (Williams *et al.*, 1992b). Moreover, our previous attempts to identify single immunodominant residues within RF-reactive epitopes employed glycine or alanine substitution within individual RF-reactive peptides. This strategy established that certain amino acids such as tyrosine, tryptophan, arginine, valine, lysine, glutamic acid, or leucine were important for antigenic epitope integrity (Williams, 1996). However, the molecular modelling CSR comparisons presented here now identify the frequent presence of three amino acid segments of almost exact three-dimensional conformation as being key elements in antigenic similarity between the various CH3, CH2 or β_2m regions compared (Table 4 and Fig. 6A, B).

On the basis of cross reactivity data of monoclonal antibodies against peptides and proteins presented in Table 3 and Figs 3–5, as well as molecular modelling studies presented in Table 4, one can hypothesize that autoantibodies such as RF seem to recognize main-chain conformations rather than side chains of amino acid residues in the epitope. Owing to this property, these antibodies appear to recognize many epitopes having different primary sequence but similar three-dimensional shape. These antibodies thus can cross-react with different parts of the protein which are solvent-accessible and show similar main-chain conformation. We are well aware that all autoantibodies may not recognize only main-chain conformation and may, in addition, also recognize antigenic segments involving amino acid side chain conformations or nonlinear segments of widely different primary sequence. More studies in this direction are necessary and are currently in progress.

The scientific approach in much biochemical and immunological research has often been to base a number of hypotheses or strategies on identification of exact similarity of amino acid sequence. Indeed, a similar approach employing comparative analyses of primary amino acid sequence represented one of the decisive elements in analysis of some of the first recognized oncogenes and their products (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983). Data presented here indicate that similarities in shape and not only primary amino acid sequence are important determinants for a number of biologic interactions. Studies must now be directed at organizing methods whereby one might be able to predict shape similarity on the basis of minimum energy conformational analysis. As noted in the present report, additional support for presumed three-dimensional similarity could also be provided by antibody/antigen reactivity as well.

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