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The nature of the subset of MHC class II molecules carrying the CDw78 epitopes

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Abstract

A CDw78 mAb FN1 was shown to recognize DP and/or DR molecules under the conditions of Western blotting. DP molecules were specifically retarded on a column of the FN1 immunosorbent; binding of FITC-labeled FN1 to B cell lines was completely blocked by excess of mAb to DR/DP β chains, partially by several mAb to DP and weakly by some mAb to DR. The binding of two other CDw78 mAb, FN4 and MR11, to the B cell surface was most strongly inhibited by excess of different mAb to DR. Kinetics of stable binding of the CDw78 mAb indicated that their monovalent binding is of low affinity and that the stable binding to the surface is due to bivalent binding to two spatially close MHC class II molecules. FN1-based immunosorbent effectively immunoisolated complexes of MHC class II proteins with several tetraspanin molecules from a mild detergent lysate of a B cell line. It is concluded that FN1 and most likely also the other two CDw78 mAb recognize with low affinity determinants on MHC class II molecules (DP or DR) and preferentially bind in a stable fashion to dimerized or aggregated MHC class II molecules. Such dimers or aggregates may either exist as preformed on the cell surface or may be gradually formed and stabilized by bivalent interaction with mAb. These structures may be related to the previously described 'superdimers' of MHC class II and/or 'MHC–tetraspanin complexes'. CDw78 mAb may be valuable tools targeting such aggregated fraction of MHC class II molecules which can exhibit important signaling and antigen-presenting properties.

Introduction

The CDw78 antigen was defined at the 4th International Human Leukocyte Differentiation Antigen Workshop as a B cell activation marker (1,2). It remained poorly defined in molecular terms until Slack *et al*. (3) attempted expression cloning of cDNA. Surprisingly, the cloned cDNA encoded the transcription factor CIITA that controls MHC class II expression. Based on these data and on immunoprecipitation results the authors concluded that CDw78 mAb recognize epitope(s) residing on MHC class II molecules. However, the number of CDw78 epitopes on the cell surface is much lower than that of MHC class II molecules; also, the pattern of immunohistochemical staining by CDw78 mAb on tonsillar tissue sections is different from conventional MHC class II mAb (1). A recent paper examined in detail characteristic features of lymphoid tissue section staining by a CDw78 mAb (4); this study also demonstrated that a CDw78 mAb recognizes a subset of MHC class II molecules associated with the cytoskeleton which easily cap. This may explain in part previous observations on the ability of CDw78 mAb to induce membrane signals augmenting (5) or inhibiting (2,6) B cell proliferation.

In spite of this progress in elucidation of the nature of the CDw78 antigen several basic questions have remained.

The molecular identity of MHC class II and CDw78 was demonstrated only by indirect methods, leaving a possibility that the CDw78 epitope may reside either on an unidentified molecule (e.g. a glycolipid) tightly associated with (and obligatorily dependent in expression on) MHC class II or that it is an epitope composed of MHC class II and another associated molecule. In this respect, the associations of MHC class II molecules with proteins of the tetraspanin family, for example, might be relevant (7–9).

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The molecular nature of the difference between the minority of MHC class II molecules carrying (or associated with) the CDw78 epitope(s) and apparent majority of CDw78⁻ MHC class II molecules has remained unclear; the only positively identified difference was the degree of cytoskeleton association (4).

It has been unclear whether different isotypes of MHC class II molecules (DR, DQ and DP) are differentially involved in formation of the CDw78 epitopes.

The aim of the present work was to clarify these points.

Methods

Reagents, antibodies and cells

CNBr-activated Sepharose 4B and mol. wt standards were purchased from Pharmacia (Uppsala, Sweden), chemicals for buffers and SDS–PAGE, CHAPS detergent and Kodak autoradiography films from Sigma (St Louis, MO), anti-Ig-peroxidase conjugates from BioRad (Hercules, CA), NP-40 detergent from Fluka (Buchs, Switzerland), enhanced chemiluminescence Western blotting kit from Amersham Buchler (Braunschweig, Germany), nitrocellulose membrane from Schleicher & Schuell (Dassel, Germany), and Eupergit C1Z from Rohm (Weiterstadt, Germany). mAb FN1 (IgG1; CDw78), FN4 (IgG3, CDw78), 22C1 (IgG1; DP), FN81 (IgG2a; DQ) and 1C6 (IgG1; DP) were prepared and characterized in the Oslo laboratory; mAb MEM-12 (IgG1; DR), MEM-28 (IgG1; CD45), MEM-53 (IgG1; CD53), MEM-103 (IgG3; DR/DPβ), MEM-111 (IgG2a; CD54), MEM-112 (IgG1; CD54), MEM-136 (IgG1; DR/DPβ), MEM-138 (IgG2a; DR/DPβ), MEM-137 (IgG1; DRα), HL38 (IgG2a; DR/DPβ) and HL39 (IgG3; DR) were prepared and characterized in the Prague laboratory. Genox 3.53 (IgG1; DQ) and B7/21 (IgG2a; DP) was a gift from J. Gorga, Spvl3 (IgG1; DQ) from H. Spitz (15), MR11 (also known as anti-Ba or Leu21; IgM; CDw78) was kindly provided by H. Kikutani; M38 (IgG2a; CD81) and C33 (IgG2a; CD82) by O. Yoshie; S-B3 (IgG1; CD37) was from Biosys (Compiege, France). FITC-labeled mAb were prepared in our laboratory by coupling FITC to purified mAb or (in the case of FN4 which is very sensitive to any manipulations) to unseparated ascitic fluid. FITC-labeled goat $F(ab)_2$ anti-mouse Ig (FITC–GAM) used as the secondary antibody in indirect immunofluorescence measurements was from Jackson Immunoresearch (West Grove, PA). Fab fragments were prepared by papain treatment essentially as described elsewhere (16); undigested antibody and Fc fragments were removed by Protein A–Sepharose and the Fab fragments were finally purified by gel filtration HPLC (Superdex 200 HR 10/30 column; Pharmacia, Uppsala, Sweden). B cell lines Raji, Rael and Daudi were obtained from ATCC (Rockville, MD), LG2 and JY from the laboratory of J. L. Strominger, and the CEB line was provided by G. Sarmay. Paraformaldehyde-fixed cells were prepared by incubation of a suspension of 1×10^7 cells in 1 ml of 3.7% paraformaldehyde for 30 min at 20°C followed by blocking in 1% glycine + 0.2% gelatine (20 min at 4°C).

Cytofluorometric methods

After 10 min blocking of Fc receptors by 20% human AB serum, cells were incubated on ice with solutions of FITC-labeled mAb [in HBSS containing 0.2% gelatine and 0.1% NaN_3 (HBSS-GA)]. After two brief (3 min) washes and addition of 0.1 μg/ml propidium iodide (Sigma) the cells were analyzed on a FACSort cytofluorometer (Becton Dickinson, Mountain View, CA) in a standard three-color set-up. At least 5000 viable cells (propidium iodide negative) were collected for each sample. Performance of the instrument was checked on a daily basis using Rainbow beads (Spherotech, Libertyville, IL). Alternatively, in some experiments cells were incubated for 30 min with solutions of unlabeled mAb (10 μl/ml) and after washing for 30 min with a solution of FITC-labeled GAM (10 μl/ml). In experiments aimed at association kinetics measurement, 2×10^5 cells were incubated with 100 μl of HBSS-GA containing 50 μg mAb/ml on ice for different time intervals, and aliquots were washed and analyzed by cytofluorometry. To follow the kinetics of dissociation, following mAb binding the cells were washed twice (3 min each), incubated for different time intervals on ice in 200 μl of HBSS-GA with gentle stirring and measured by cytofluorometry. To follow the concentration dependence of mAb binding, the cells were incubated under the same conditions with different concentrations of the FITC-labeled mAb for 60 min. List mode data were analyzed using WinMDI2.5 software (J. Trotter, The Scripps Research Institute). Debris and aggregated cells were gated out based on their scatter properties. Geometric mean values of fluorescence intensity (in arbitrary units) were determined and corrected by subtraction of background fluorescence (staining with FITC-labeled irrelevant isotype-matched mAb controls). In competition (cross-blocking) experiments, the cells were first incubated 10 min on ice in a solution of an unlabeled mAb (~0.5 mg/ml), then the tested FITC-labeled mAb was added (final concentration ~5 μg/ml), the mixture was incubated on ice for further 60 min and after washing the cells were analyzed by cytofluorometry.

Biochemical methods

Preparation of cell lysates, preparation and detergent solubilization of cell membranes, and SDS–PAGE and Western blotting (employing luminographic detection) were all performed as described in detail elsewhere (7,17). mAb were covalently bound to CNBr-activated Sepharose according to the manufacturer's instructions (5 mg mAb/ml gel). Immunoaffinity chromatography was performed on minicolumns of the immunosorbents (42 mm long, total volume 300 μl) washed thoroughly with the lysis buffer (0.15 M NaCl, 0.1 M Tris–HCl buffer, pH 8.2, 1% NP-40, and protease inhibitors 5 mM iodoacetamide and 1 mM Pefabloc). Then 25 μl of detergent lysate of Raji cells (5×10^7/ml lysis buffer) was applied at the top of the column which was then eluted with the lysis buffer (at the rate of 15 μl/min) and 11 fractions of 50 μl were collected. Then elution with alkaline buffer (0.1 M glycine NaOH, pH 11.5, containing 0.1% NP-40) followed and 200 μl fractions were collected. All fractions were mixed 1:1 with 2 times concentrated non-reducing SDS–PAGE sample buffer and non-boiled samples were analyzed by SDS–PAGE followed by Western blotting.

Immunoisolation from CHAPS detergent lysates was performed, using the Protein A-purified mAb covalently attached to the non-porous Eupergit C1Z microparticles (18), as described before (7). After washing, the antigens bound to

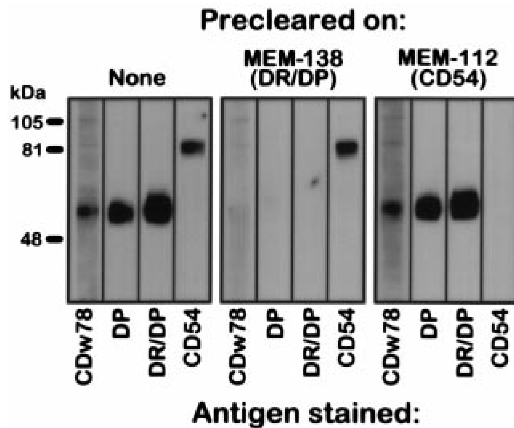

Fig. 1. Western blotting. Raji cell membranes were detergent solubilized and non-reduced, non-boiled samples subjected to SDS–PAGE, electroblotting and immunostaining with mAb to the antigens indicated at the bottom (FN1 to CDw78, 22C1 to DP, MEM-138 to DR/DP and MEM-111 to CD54). The samples used were either not precleared or precleared by passage through the immunosorbent columns indicated at the top.

these immunosorbents were eluted with non-reducing sample buffer and analyzed by SDS–PAGE and Western blotting. It should be noted that in this method antibodies are covalently immobilized only on the surface of non-porous particles and these are fully available even for binding of the respective antigen present in very large complexes that would not penetrate into the pores of gel-based immunosorbents. However, this method is not quantitative and to avoid unacceptable non-specific adsorption it is necessary to use excess of antigen (i.e. cell lysate) over the relatively small amounts of low-capacity immunosorbent (7).

Results

CDw78 mAb FN1 recognizes MHC class II-like molecules under the conditions of Western blotting

Although immunoprecipitation of MHC class II-like molecules by CDw78 mAb was reported in two previous studies (3,4), a possibility remained that these molecules were actually co-precipitated due to their tight association with another poorly detectable molecule carrying the CDw78 epitope. Therefore, we sought conditions under which CDw78 mAb might bind to MHC class II glycoproteins unassociated with other molecules. mAb FN1 bound to a zone of mol. wt ~55 kDa on nitrocellulose blots obtained from non-reduced, non-heated samples of B cell line membranes after SDS–PAGE and blotting onto nitrocellulose (Fig. 1). It was necessary to use cell membranes for solubilization; when whole cells were used instead, specific staining of the zone was poorly reproducible and weak. Two other CDw78 mAb studied here (MR11 and FN4) did not stain reproducibly any zone under these conditions (not shown). The results reported here for the samples obtained from Raji cell membranes were very similar to those obtained with Rael cell membranes (not shown). The position of this zone was identical to that of intact $\alpha\beta$ dimers recognized by mAb to conventional MHC class II mAb (Fig. 1); the zone reactive with FN1 (as well as with conventional mAb to MHC class II) was lost after passing the membrane lysate through a column of an immunosorbent made of immobilized mAb to a determinant common to DR and DP β chains (Fig. 1). The reactivity was also lost if the sample was boiled before electrophoresis (i.e. under the conditions when $\alpha\beta$ heterodimers of class II molecules dissociate) (not shown). These results indicated that mAb FN1 directly binds to DR and/or DP molecules.

Immunoaffinity chromatography on an FN1 immunosorbent

A small volume of NP-40 lysate of Raji cells was subjected to immunoaffinity chromatography on a minicolumn of an immunosorbent prepared by coupling of the FN1 mAb to CNBr-activated Sepharose 4B, and the fractions obtained were analyzed by SDS–PAGE/Western blotting for the presence of DR, DQ and DP molecules as well as CD54 (negative control). CD54 and DQ were not retarded at all, DR was possibly slightly retarded but DP was markedly retarded and a significant fraction of it was released from the immunosorbent only by high pH buffer while none of the MHC class II isotypes was retarded on a control column (immobilized CD45 mAb) (Fig. 2); only the proper MHC class II isotypes were selectively strongly and quantitatively bound to the respective control immunosorbents (anti-DR, anti-DQ and anti-DP); no retardation of any of the MHC isotypes was observed on immunosorbents made of two other CDw78 mAb, MR11 and FN4, under the conditions used (not shown).

Competition of FITC-labeled CDw78 mAb with other mAb

As shown in Fig. 3, the binding of FITC-labeled FN1 to Raji cells was strongly blocked by the mAb to DR/DPβ MEM-136 (similar strong blockers were four other mAb of similar epitope specificity, MEM-138, MEM-103 and HL38; not shown), less strongly by three DP mAb (22C1, B7/21 and 1C6; not shown), and weakly by a DR mAb MEM-12 (a similarly weak blocker was another DR mAb, HL39; not shown). The blocking effect of the DR mAb MEM-12 and DP mAb 22C1 was additive as their mixture completely inhibited the binding of FITC–FN1. FITC-labeled FN1 did not compete with a DQ mAb FN81 (and two other DQ mAb, Spvl3 and Genox 3.53; not shown), mAb to DRα chain MEM-137 and two other CDw78 mAb, MR11 and FN4. A very similar binding inhibition pattern was observed also when cell lines Rael, CEB, Daudi, JY and LG2 were used (not shown). It should be noted that mAb MEM-12, MEM-136 and MEM-137 react with spatially distinct epitopes as they do not cross-block each other (K. Drbal, unpublished).

On the other hand, the binding of FITC–MR11 to Raji cells was completely blocked by the DR mAb MEM-12 (and also by another DR mAb, HL39; not shown), partially by a DRα mAb MEM-137 but not by mAb to DR/DPβ (MEM-136 or four other mAb of similar specificity; not shown), two DP mAb (22C1 and B7/21) and mAb to DQ (FN81; similarly also two other DQ mAb, Genox 3.53 and Spvl3; not shown). FITC–MR11 also did not compete with the two other CDw78 mAb, FN1 and FN4 (Fig. 3). The last CDw78 mAb, FN4, was difficult to label with FITC (the binding activity was largely lost after purification and/or labeling) and therefore it could not be used in the cross-blocking experiments. It should be noted

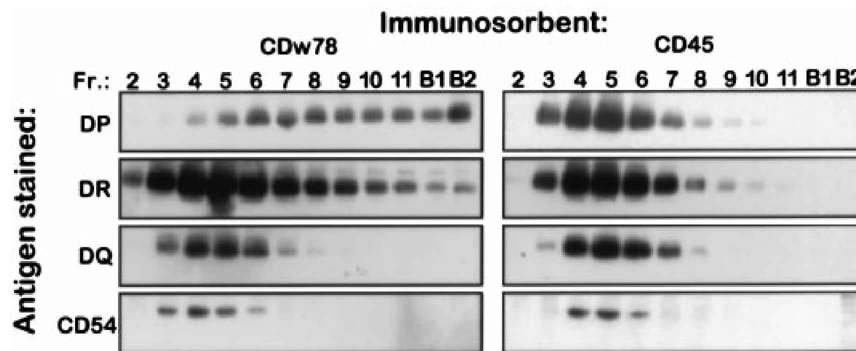

Fig. 2. Immunoaffinity chromatography on the FN1 column. A Raji cell detergent (1% NP-40) lysate was used for immunoaffinity chromatography on the FN1 (CDw78) or MEM-28 (CD45; negative control) columns as described in Methods. Fractions collected (numbered at the top) were subjected to SDS–PAGE and electroblotting onto nitrocellulose and immunostained by mAb to DP (22C1), DR (MEM-12), DQ (FN81) and CD54 (MEM-112; negative control). Only the relevant parts of the blots (the range ~40–70 kDa for MHC class II and 70–110 kDa for CD54) are shown. The last two fractions (B1 and B2) are those obtained by elution with the alkaline buffer (pH 11.5).

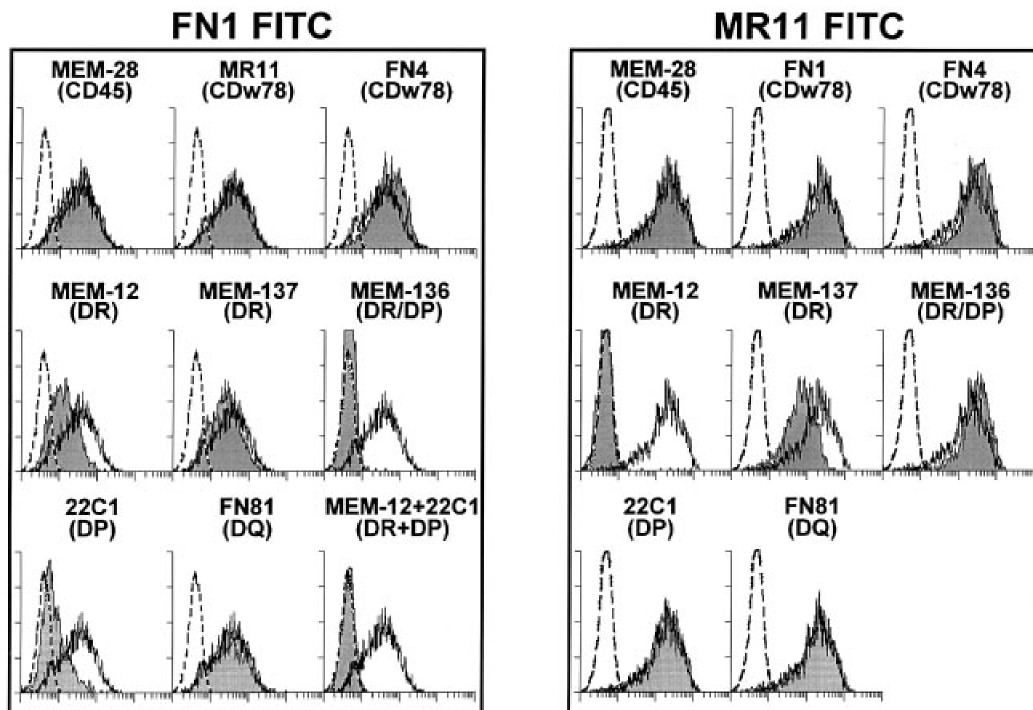

Fig. 3. Competition (cross-blocking) of FITC-labeled CDw78 mAb FN1 (left panel) and MR11 (right panel) with other unlabeled mAb. The unlabeled mAb used for blocking are given in each window. The cytofluorometric histograms of Raji cells with the FITC-labeled CDw78 mAb are given as the solid lines; negative controls (staining with FITC-labeled irrelevant isotype-matched mAb) as dotted lines. The shaded peaks correspond to the histograms obtained under the conditions of competition with the respective unlabeled mAb.

that the number of CDw78 mAb molecules bound to the cell lines was always much lower (<10%) than in case of conventional mAb to MHC class II molecules, either if direct staining with FITC–mAb or indirect staining with mAb + FITC–anti-mouse Ig was used.

The simplest interpretation of these data is that mAb FN1 reacts predominantly with a fraction of DP molecules, while MR11 reacts with a subset of DR molecules (see Discussion on the interpretation of some of the complexities of the antibody-blocking data).

The CDw78 mAb are of low affinity

The observed characteristics of the interaction of the CDw78 mAb with their target antigens, MHC class II molecules, are compatible with the possibility that they are low-affinity mAb that can bind in a sufficiently stable manner only bivalently.

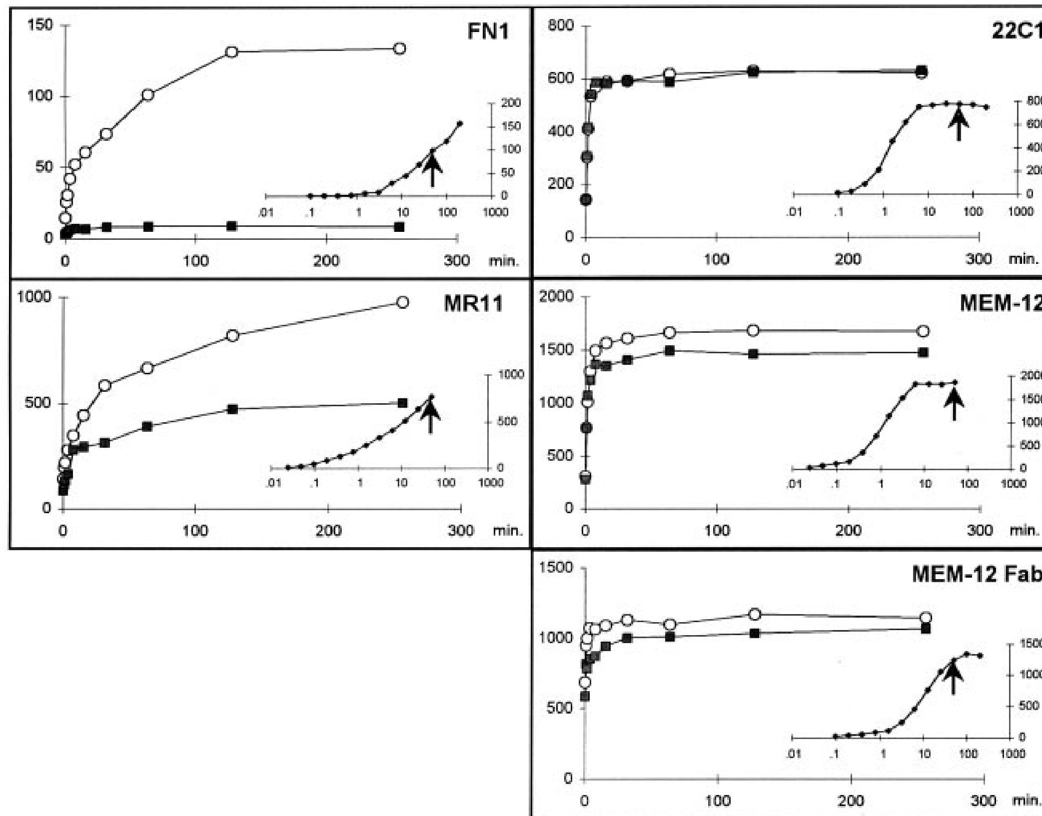

Fig. 4. Kinetics of mAb binding to the Raji cells. The cells were incubated with the FITC-labeled mAb for different time intervals (0–256 min), washed and fluorescence intensity was determined by cytofluorometry as described in Methods. Corrected fluorescence intensities (arbitrary units) are plotted (*y*-axis) against the incubation time (min) (*x*-axis). Geometric mean values of three independent experiments were used for construction of the plots. Open circles: binding to unfixed cells; closed squares: binding to paraformaldehyde-fixed cells. Note different scales on the *y*-axis reflecting mainly differences in expression of the respective molecules. Insets: concentration dependence of the respective mAb binding to the unfixed cells. The arrows point to the concentrations used for the kinetic (time dependence) experiments.

Actually, previous direct measurements of the FN1 mAb (whole molecule and monovalent Fab fragments) indicated that the equilibrium (association) constant (K_a) of binding of the intact (bivalent) FN1 mAb binding to the cell surface is 1.5×10^8 M^{-1}; the K_a value corresponding to the interaction of the monovalent Fab fragment was too low to be measured by the method used ($<10^5$ M^{-1}) (4). The kinetics of stable binding of FITC–FN1 and FITC–MR11 to the cell surface was slow (as compared to standard mAb to MHC class II) (Fig. 4). It should be noted that the assay used obviously detected only relatively stable binding to the cell surface (the cells were washed before the measurement). Indeed, the kinetics of dissociation of the FN1 and MR11 mAb once bound to the cell surface was very slow and qualitatively comparable to the standard (high-affinity) mAb to MHC class II molecules: >98% of MEM-12, 22C1, FN1 and MR11 remained associated with the cell surface after thorough washing and incubation in the absence of the mAb at 0°C for 20 h (data not shown). Binding of FITC-labeled FN1 Fab fragments to cell surface could not be measured, obviously because the rate of dissociation of the monovalently bound fragment was too rapid, causing its loss during the washing step. The time course of the binding of these low-affinity mAb to the cell surface (Fig. 4) is compatible with the idea that these mAb bind to suitably oriented dimers (or higher aggregates) of the relevant MHC class II molecules which exist on the cell surface either as pre-formed or are gradually formed (stabilized) by the interaction with the bivalent mAb. A prediction of this model is that fixation of the cell surface, e.g. by paraformaldehyde, should stabilize the dimeric or otherwise aggregated forms of MHC class II molecules and prevent gradual formation (stabilization) of additional dimers by interaction with the mAb. Indeed, the kinetics of FN1 and MR11 binding to paraformaldehyde-fixed cells corresponds to this prediction (Fig. 4): the mAb rapidly bind to a low number of binding sites (presumably the performed dimers) and with time they essentially do not bind to additional sites presumably formed by diffusion-based transient dimerization. In contrast, conventional mAb to MHC class II molecules, 22C1 and MEM-12, and also Fab fragments of MEM-12, bound similarly to unfixed and paraformaldehyde-fixed cells (Fig. 4). The difference in the absolute number of the binding sites available for FN1 and MR11 on the fixed cells may reflect different isotype specificity (DP versus DR) and different valency of these mAb (IgG versus IgM respectively). Also, the concentration dependence of the mAb binding was markedly different for

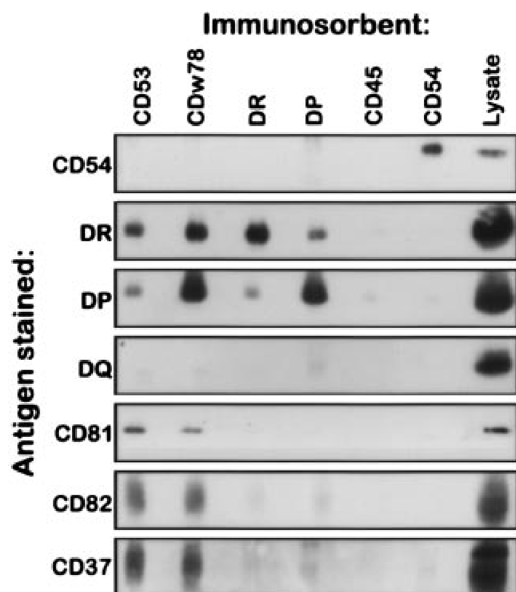

Fig. 5. Western blotting of the materials eluted from various immunosorbents (the indicated mAb bound to Eupergit C1Z), using mAb against the indicated molecules. Non-reduced samples were used. The CD45 and CD54 immunosorbents were used as negative specificity controls. Only the relevant parts of the blots are shown (corresponding to the molecular mass of the proteins).

the CDw78 mAb FN1 and MR11 as compared to conventional mAb to DR (MEM-12) or DP (22C1): while saturation of binding was achieved for the latter (and even for the MEM-12 Fab) at low concentrations, no saturation could be achieved for the CDw78 mAb even at the highest concentrations used (insets of Fig. 4).

mAb FN1 preferentially binds MHC class II molecules associated with tetraspanin proteins

A fraction of MHC class II molecules was previously found to be associated with multiple proteins of the tetraspanin family (7); another study demonstrated that MHC class II molecules present in these 'tetraspanin complexes' on the cell surface are clustered (8). Therefore, we tested whether the FN1 mAb-based immunosorbent is able to co-isolate specifically tetraspanin proteins from mild detergent lysates of B cell line membranes. Indeed, tetraspanin proteins CD37, CD81 and CD82 were effectively co-isolated on the FN1 immunosorbent. The FN1 immunosorbent immunoprecipitated the MHC class II–tetraspanin complexes (containing tetraspanin proteins CD37, CD81 and CD82) with an efficiency similar to that of an immunosorbent made of a mAb to CD53, another tetraspanin protein (Fig. 5). CD53, an additional tetraspanin protein obviously present in these complexes (see the results of the immunoisolation on the CD53 immunosorbent), could not be directly demonstrated as the mAb does not react well under the conditions of Western blotting. In contrast, very little tetraspanin molecules were co-isolated with the respective MHC class II molecules on immunosorbents made of mAb to DR (MEM-12) or DP (22C1) (Fig. 5). The simplest interpretation of these results is that FN1 mAb preferentially binds the MHC class II molecules present in the tetraspanin complexes, presumably because these complexes contain suitably oriented dimers or clusters of MHC class II proteins; the monomeric MHC class II proteins outside these complexes may bind to the immunosorbent with too low affinity. On the other hand, the conventional MHC class II immunosorbents (MEM-12 and 22C1) bind avidly the monomeric DR or DP molecules, which prevent efficient isolation of the less abundant tetraspanin–MHC class II complexes. It should be noted that the immunoisolation method used here is not quantitative; the immunosorbents (mAb-coated Eupergit particles) have to be used under the conditions of large excess of antigen (cell lysate). This is obviously why, in contrast to the CDw78 immunosorbent, very little tetraspanin molecules are co-isolated under these conditions on the DR and DP immunosorbents (these immunosorbents bind much more the free, dissociated DR or DP molecules rather than the multicomponent complexes preferentially bound by the CDw78 immunosorbent).

Discussion

We demonstrate that two CDw78 mAb actually bind with low affinity to epitopes on MHC class II molecules. This was most directly proven in the case of mAb FN1 which (i) reacted with molecules of correct size under the conditions of Western blotting, (ii) specifically interacted with DP molecules under the conditions of immunoaffinity chromatography, and (iii) competed for cell surface binding with mAb to a determinant shared by β chains of DR and DP and with a mAb to DP and less well with mAb to DR. Thus the conclusion is that FN1 recognizes an epitope present mainly on DP molecules. Furthermore, our results explain why the number of binding sites for FN1 on the cell surface is much lower than the total number of DP: monovalent affinity of FN1 to the target antigen is low so that sufficiently stable binding can be achieved (in contrast to other mAb of higher affinity) only if the mAb can bind bivalently. Thus, FN1 obviously rapidly binds to dimers of DP or to larger complexes containing several suitably oriented DP molecules. The existence of MHC class II 'superdimers' was suggested (10) and apparently even demonstrated (11–13) on the cell surface. Such structures loaded with identical antigenic peptides could play an important role in effective cross-linking (dimerization) of TCR and thus in the initiation of T cell activation. On the other hand they could be involved in MHC class II-triggered signaling in the APC (reviewed in 14). At least some such 'superdimers' might be kinetically rather stable complexes, formed as a result of biosynthetic mechanisms and stabilized by non-covalent interactions with other molecules, e.g. tetraspanin proteins. Interestingly, immunosorbents based on the mAb FN1 efficiently bound the previously described (7–9) complexes of several tetraspanins and MHC class II molecules present in mild detergent (CHAPS) lysates of a B cell line (Fig. 5). Solubilization by means of a more stringent detergent (1% NP-40) disrupts such supramolecular assemblies and MHC class II molecules are present in the lysates mainly unassociated with other components. Alternatively, MHC class II dimers or oligomers may be formed transiently and reversibly under physiological conditions; such structures could be obviously stabilized by interactions with suitable mAb; low-affinity mAb

should be efficient in this respect as they highly prefer bivalent binding to such dimers.

We have less direct data on another CDw78 mAb MR11; based only on the cross-blocking studies with other mAb we suggest it is a low-affinity mAb to a DR epitope. Unfortunately, this mAb did not measurably interact with its target molecules under the conditions of Western blotting or immunoaffinity chromatography. The reason could be an even lower monovalent affinity to the monomeric DR molecules solubilized by the detergent. The last CDw78 mAb available to us, FN4 (IgG3), was sensitive to the conditions used for its purification and FITC labeling and therefore it could not be used in most experiments. However, its binding to the cell surface could be readily observed when using diluted ascitic fluid containing the mAb and secondary anti-mouse Ig antibodies as second-step reagents. When this second-step reagent was FITC-labeled anti-mouse IgG3, cross-blocking experiments with mAb of non-IgG3 isotypes could be performed; under these conditions, an anti-DRα mAb MEM-137 blocked completely and CDw78 mAb FN1 and MR11 partially the FN4 binding indicating that this mAb reacts with a DR epitope (K. Drbal, unpublished data). The instability of FN4 obviously also precluded preparation of good immunosorbents.

Several points of this study require further brief discussion. First, how is it possible that FN1 detectably binds to the zone corresponding to MHC class II monomers (55 kDa) electroblotted onto the nitrocellulose membrane though according to our interpretation it should strongly bind only to DP–DP (or possibly also DR–DR) dimers? (It should be noted that we never observed binding of FN1 to a minor zone of 110–120 kDa that could be reproducibly detected on the blots, although with rather low intensity, by means of several conventional mAb to MHC class II and that may correspond to a sort of SDS-resistant MHC class II dimers of dimers.) We believe that the concentration of the MHC class II (presumably mainly DP and DR) monomers (i.e. $\alpha\beta$ dimers) in the 55 kDa zone on the nitrocellulose was under suitable conditions (high loading of the sample prepared from cell membranes under relatively gentle temperature and detergent conditions) so high that sufficient number of these molecules were close enough and properly oriented to each other that the bivalent binding of the mAb could occur. In contrast, the conformation of the putative SDS-resistant class II dimers (observable as a minor 110–120 kDa zone) could be affected by exposure to SDS such that the mAb could not bind in a bivalent fashion to them. We believe that our demonstration of direct binding of FN1 to the 55 kDa zone (which disappears after preclearing the membrane detergent lysate by means of an anti-DR/DPβ immunosorbent) definitively rules out the possibility that CDw78 is a molecule tightly associated but physically different from MHC class II (except for the possibility that the associated molecules would have exactly the same size as MHC class II). The lack of reactivity of MR11 and FN4 under these conditions probably reflects that the epitopes recognized by these mAb are more sensitive to the conditions of SDS–PAGE and Western blotting even if the sample is prepared in a relatively gentle way.

The immunoaffinity chromatography procedure used here (Fig. 2) should be in principle suitable to detect even relatively low-affinity interactions, as it can reveal also mere retardation of the antigen on the immunosorbent, not necessarily only firm complete binding. It can be seen that most of the DP molecules were more or less retarded on the FN1–Sepharose column and a fraction was eluted only by the high pH buffer. It is possible that the fraction of DP sticking to the immunosorbent and released by high pH buffer might actually represent DP dimers resistant even to 1% NP-40 that could bind more strongly to the immobilized mAb molecules in the immunosorbent. However, we have no direct evidence on what fraction of DP (or other MHC class II molecules) is associated within homodimers (or in complexes with other molecules) under the solubilization conditions used. The DP molecules which were only retarded (and not tightly bound to the immunosorbent) were presumably monomers interacting with the immunosorbent more weakly.

The antibody cross-blocking experiments essentially support the idea that FN1 mAb recognizes an epitope residing mainly on DP molecules: its binding to the cell surface is completely blocked by mAb that recognize an epitope present in DR and DP (but nor DQ) β chains and less strongly by two DP-specific mAb. However, weak competition is also observed with two anti-DR mAb; notably, cross-blocking by anti-DP and anti-DR mAb was additive (Fig. 3). At least two possible explanations can be offered for the partial cross-blocking of FN1 by mAb to DR: (i) either the specificities of the mAb are not so strict (e.g. the anti-DR mAb may weakly react also with DP, the reactivity being stronger with the DP dimers, or, reversally, FN1 reacts weakly also with some DR molecules on the cell surface); or (ii) some of the cross-blocking may reflect the existence of supramolecular complexes in which, for example, a fraction of DR molecules is very close to DP and thus some mAb to DR may sterically interfere with binding of some anti-DP mAb (such as FN1). Both these possibilities are plausible: we observed very weak but detectable binding of, for example, the anti-DR mAb used here to DR^-, DP^+ or DR^-, DQ^+ mutant cell lines (K. Drbal, unpublished) and DR was weakly retarded on FN1 immunosorbent (Fig. 2); on the other hand, proximity of a fraction of DR to DQ was demonstrated in fluorescence energy transfer experiments (8).

The CDw78 mAb, i.e. low-affinity mAb to MHC class II molecules, may be a special case of a more general phenomenon—specific recognition by such low-affinity mAb of a fraction of cell surface molecules (receptors) organized within multicomponent surface complexes. Ligation of such a structurally distinct, usually minor fraction of surface receptors may result in signaling qualitatively or quantitatively different from that observed after ligation of essentially all such receptor molecules by high-affinity mAb which may preferentially bind (especially at relatively high concentrations) in a monovalent fashion. Thus, low-affinity mAb may be in some cases valuable reagents more or less specifically recognizing oligomerized, functionally distinct fractions of surface receptors.

The clustered fraction of MHC class II molecules recognized preferentially by CDw78 mAb appears to be identical to that present in the multicomponent 'tetraspanin complexes' (7–9) (Fig. 5). The existence of such structures in intact membrane was confirmed by fluorescence energy transfer experiments (8); they may contain also additional components such as β_1 integrins (9) and small amounts of MHC class I and CD20 (8; P. Angelisova, unpublished data) but their stoichiometry and

even details of qualitative composition are not known at present. Because of the presence of clustered MHC class II proteins in them, they could be either functionally important in antigen presentation and TCR aggregation and/or in signal transduction in the APC. In this respect it may be relevant that the fraction of MHC class II molecules preferentially recognized by the FN1 mAb is associated with cytoskeleton and easily caps (4), and that CDw78 mAb are able to induce in B cells signals resulting in marked physiological effects (2,5,6). Interestingly, complexes of MHC class II molecules with multiple tetraspanin proteins may be essential structural components of externalized vesicles, termed exosomes, enriched also in the co-stimulatory molecule CD86 and efficiently stimulating T cells *in vitro* (19); we would predict that exosomes are $CDw78^{+}$. In addition, the tetraspanin–MHC class II complexes may be involved in cell adhesion as they (or a subpopulation of them) contain β_1 integrins (9). The FN1 immunoprecipitates of CHAPS lysates did contain the β_1 integrin subunit (CD29) as detected by Western blotting (P. Angelisova, unpublished data). Therefore, CDw78 mAb can be useful tools to study these potentially functionally important multicomponent membrane assemblies.

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Abbreviations

GAM	goat anti-mouse Ig
HBSS-GA	HBSS containing 0.2% gelatine and 0.1% NaN_3

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