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Mouse CD4 binds MHC class II with extremely low affinity

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Abstract

Interaction of CD4 with MHC class II molecules plays a crucial role during thymic development and activation of single-positive CD4 T lymphocytes. The quantitation of this interaction is, therefore, important for understanding the role of CD4 during these events. To this end, we have developed a rosette assay, which enabled us to study this molecular interaction. By coupling soluble mouse CD4 onto beads, we could show specific binding of CD4 to MHC class II molecules on A20 B lymphoma cells. These binding studies revealed an extremely low affinity ($K_a \leq 10^4$ M^{-1}) between CD4 and MHC class II molecules in mouse.

The CD4 co-receptor is expressed on T cells which are MHC class II restricted and mostly of T helper (T_h) cell type (1,2). CD4 is known to bind to a non-polymorphic determinant on MHC class II (2 – 6). Interaction of CD4 with MHC class II is crucial during thymic ontogeny (for review see 7) and plays a dual role in T cell activation. First, by strengthening the overall interaction between T_h cells and antigen presenting cells (APCs) (8 – 10), and, second, by transducing signals to T cells (for review see 11).

The quantitation of this interaction would provide further insights into the complex interactions occuring in a TCR – CD3 – CD4 – MHC plus peptide complex during antigen recognition by T cells and is of specific interest since the affinity of mouse TCR to MHC class II plus peptide was recently resolved to be about 10^5 M^{-1} (12,13).

The direct binding of CD4 and MHC class II has been studied in the human system (3,6), but no such studies are available for the corresponding murine molecules. Here, we report the development of an *in vitro* assay, with which direct and specific binding of mouse soluble CD4 (sCD4) to mouse MHC class II on the cell surface is demonstrated. This assay enabled us to estimate the affinity of mouse CD4 for MHC class II to be lower than 10^4 M^{-1}. This further supports the hypothesis that the role of CD4 as a co-receptor probably is more important for signal transduction than adhesion.

To generate soluble CD4 molecules, i.e. CD4-hCμ and CD4-Cκ, Ig expression vectors containing the cDNA for the extracellular part of the mouse CD4 gene and the $C_2 - C_4$ exons of human μ heavy chain gene or the mouse Cκ exon of light chain genes were transfected into the myeloma cell line J558/L by protoplast fusion (18,19). Stable transfectants were used for mass culture. CD4-hCμ was purified from concentrated supernatants by gel filtration, as described in Fig. 1, and CD4-Cκ by using affinity chromatography (19).

SDS – PAGE analysis of the materials used in this study showed that chimeric sCD4s of predicted molecular nature were produced. Non-reducing SDS – PAGE analysis revealed that CD4-hCμ assembled into a typical pentameric structure of IgM (Fig. 1), corresponding to a valency of 10 for the CD4 part, while CD4-Cκ behaved as a monomer.

In addition, all tested mAbs (GK1.5 (20); H129 (21); and RM4-4 and RM4-5, both Pharmingen, San Diego, CA) against different CD4 epitopes recognized the CD4-Cμ as well as the CD4-hCκ protein. Reciprocally, antibodies raised against CD4-hCμ and CD4-Cκ stained $CD4^+$ T cells (data not shown). These results suggest that antigenically native sCD4s are produced. However, the attempts to detect direct binding of the CD4-hCμ and CD4-Cκ to MHC class II positive cells failed consistently, presumably because of low affinity.

To test this hypothesis, we further increased the valency of sCD4 by coupling CD4-hCμ onto beads and performed a rosette assay by incubating CD4-hCμ – beads with A20 cells, which are known to express high levels of MHC class II molecules (17). Subsequently, rosette formation was analysed by microscopy (Fig. 2). Most of the rosetted cells were completely covered by CD4-hCμ – beads. To demonstrate that the 'bead-berries' are rosetted cells and not aggregated beads, cells were stained with acridine orange, as shown in Fig. 2(A and B).

The rosette formation was specific for the CD4 – MHC class II interaction, since it was completely inhibited by anti-MHC class II mAb M5/114 (22) (Fig. 2 and Table 1). Irrelevant rat anti-mouse mAb (53-6.7) as well as an anti-MHC class I mAb (15.5.5s) did not inhibit rosette formation (summarized in Table 1). M5/114

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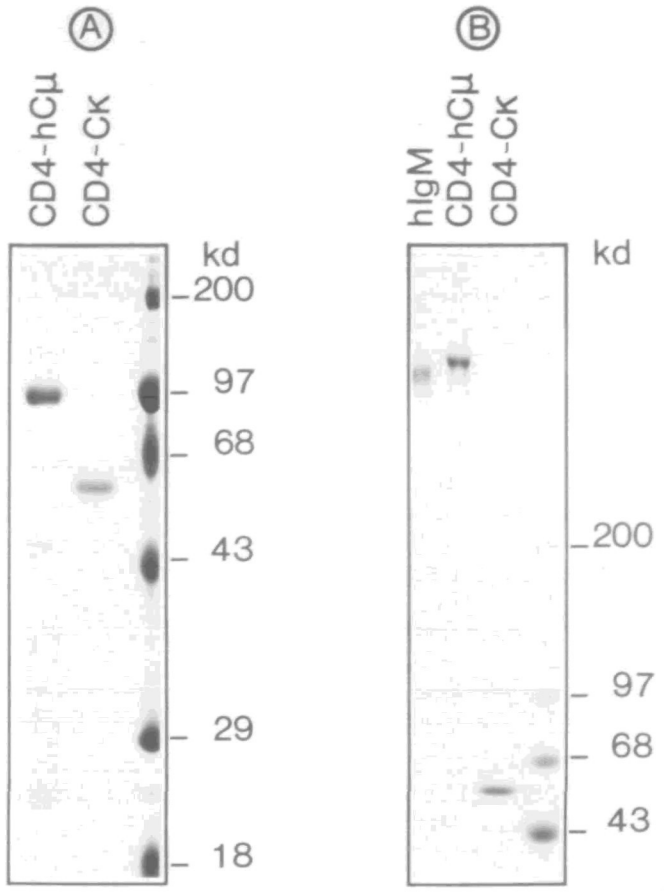

Fig. 1. SDS – PAGE analysis of CD4-hCμ and CD4-Cκ. Purified CD4-hCμ, CD4-Cκ, and, as a control, human IgM (hIgM) protein were analyzed by SDS – PAGE. The gels were subsequently stained with coomassie blue: (A) 7% PA-gel, reducing conditions; (B) 3 – 10% PA-gradient gel; non-reducing conditions. Standard molecular weight markers are indicated on the sides. Method: the part of the mouse CD4 (L3T4) cDNA sequence (14) coding for the extracellular region of the protein was engineered into Ig expression vectors (15,18) upstream of the $C_2 - C_4$ exons of the human μ heavy chain gene or the C exons of the mouse κ light chain gene. The resulting constructs pL3T4-hCμ and pL3T4-Cκ (18,19) were introduced into the myeloma cell line J558/L by protoplast fusion as described (16). Stable transfectants, secreting 2 – 6 mg/l CD4-hCμ were adapted to serum free medium (IMDM, 5 mg/l insulin, 5 mg/l transferrin, 1 × non-essential amino acids, 0.03% primatone). CD4-Cκ was purified from supernatant with an anti-mouse Cκ affinity column and CD4-hCμ was purified from concentrated supernatant by gel-filtration with a FPLC superose 6 column (Pharmacia, Uppsala, Sweden).

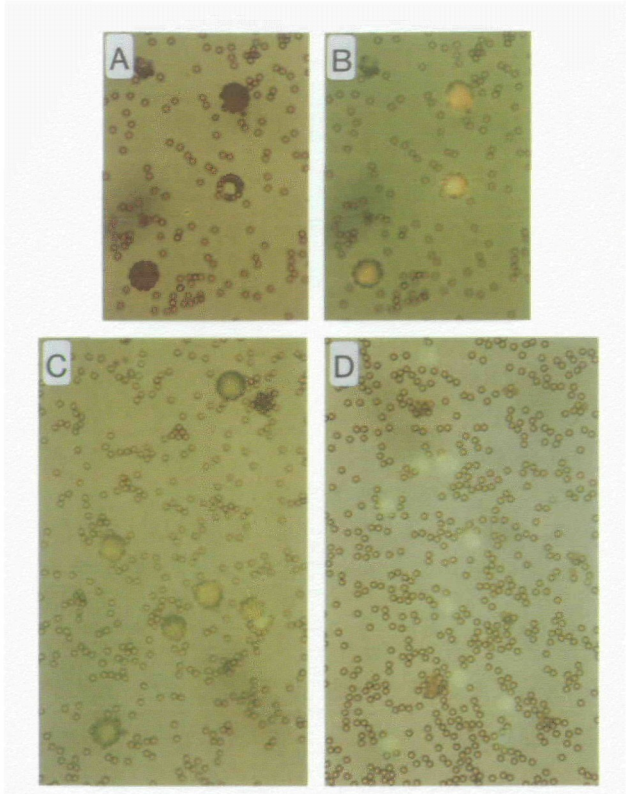

Fig. 2. Rosette formation. A20 cells were incubated with CD4-hCμ – beads; subsequently, the cells were stained with acridine orange. The comparison of (A) light micrograph only versus (B) light micrograph overlaid by fluorescence micrograph reveals that most of the rosetted A20 cells are completely covered by the beads. (C) Rosette formation of A20 cells with CD4-hCμ – beads. (D) Complete inhibition of rosette formation by anti-MHC class II mAb (M5/114, 20 μg/ml) (× 400). Method. CD4-hCμ – beads were prepared by coupling polyclonal anti-human IgM antibodies (Jackson Immunoresearch, West Grove, PA) onto tosylactivated Dynabeads M-450 (Dynal, Oslo, Norway) overnight, in 0.5 M borate buffer pH 9.5 at 4°C. Washed Dynabeads were then incubated with purified CD4-hCμ overnight in PBS/0.1% BSA at 4°C. The beads were washed and resuspended in medium (IMDM/5% FCS) to a concentration of 4×10^8 beads/ml. The resulting beads (CD4-hCμ – beads) have thus a layer of CD4, which is oriented to the outside and has a high density of CD4. For negative controls, anti-human IgM – beads were loaded the same way with human IgM (Chemicon, Temecula, CA). The rosette formation assay was performed by mixing 10 μl of CD4-hCμ – bead suspension (4×10^8/ml) with 50 μl A20 cell (17) suspension (2×10^6/ml, IMDM/5% FCS), followed by incubation at room temperature for 1 – 2 h with continuous shaking. To distinguish between bead-covered cells and aggregated beads, the cells were stained with acridine orange. Analysis of rosette formation was performed in a hematocytometer by simultaneous light and fluorescence microscopy. Cells with more than five attached beads were counted as rosetted cells. The percentage of rosetted cells varied from 40 to 90% of total cells between assays. Anti-human IgM-coupled beads and beads loaded with human IgM protein were included in each assay as negative controls.

recognizes an epitope on MHC class II involving β_1 and β_2 domains (R. König, personal communication) and is conserved in both MHC class II antigens, I-E and I-A. Consistent with this is the recent finding that part of the CD4 binding site to MHC class II molecules is located on the β_2 domain and that this binding site must also be conserved in I-E and I-A antigens. We then tried to carry out further epitope mapping by using a panel of additional anti-MHC class II mAbs. The mAb MKD6 reacting with I-A β_1 domain (23) and mAb 14.4.4s, recognizing I-Eα as well as two other mAbs, AMS-16 (anti-I-E) and AMS-32-1 (anti-I-A), with undefined epitopes were used. None of the I-E- or I-A-specific mAbs, nor the combination of I-E- and I-A-specific mAbs had inhibitory effects on rosette formation at concentrations up to 250 nM (40 μg/ml). In contrast, M5/114 inhibited rosette formation completely at concentrations of 15 nM (2 μg/ml). Thus, it seems that only mAb M5/114 binds MHC class II at an epitope interferring with the interaction of CD4.

Interestingly, although we failed to show direct binding,

Table 1. Specific inhibition of the CD4 – MHC class II interaction

Addition of	Inhibition of rosette formation	Reference
None	no	—
Rat anti-I-E/I-A	completely	M5/114 (22)
Rat anti-CD8	no	53-5.7 (30)
Mouse anti-H-2^K	no	15.5.5s (31)
Mouse anti-I-E	no	14.4.4s (30) and AMS-16 (Pharmingen)
Mouse anti-I-A	no	MKD6 (32) and AMS-32.1 (Pharmingen)
Mouse anti-I-E plus Mouse anti-I-A	no	as above
CD4-hCμ	completely	—
CD4-Cκ	no	—
Human IgM	no	(Chemicon)

A20 cells (H-2^d) were preincubated with different antibodies and sCD4 before performing rosette formation assay. Non-blocking antibodies had concentrations up to 250 nM (40 μg/ml), except human IgM and CD4-Cκ, where concentrations up to 2 mg/ml respectively did not inhibit rosette formation.

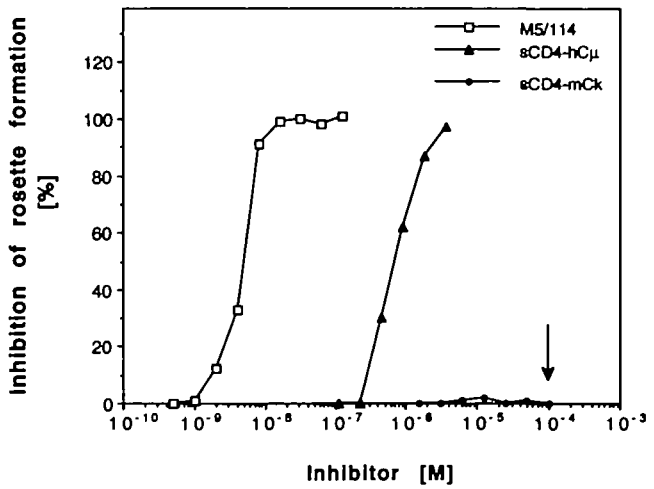

Fig. 3. Inhibition assay of rosette formation. A20 cells were preincubated with serial dilutions of anti-MHC class II mAbs (M5/114), CD4-hCμ or CD4-Cκ before addition of CD4-hCμ – beads for the rosette formation assay. The arrow indicates the endpoint of CD4-Cκ titration. Inhibition was standardized to rosette formation without any inhibitor (0%). A typical example of such an inhibition assay is presented here. Variation between assays was very small.

CD4-hCμ completely inhibited rosette formation, most likely because no washing steps are needed for specific rosette formation. Control human IgM did not inhibit rosette formation, indicating that binding of CD4-hCμ – beads to A20 cells is mediated by the CD4 part of the chimeric molecule. Complete inhibition of rosette formation by CD4-hCμ was obtained at concentrations of about 4 μM. Importantly, a monovalent form of CD4, CD4-Cκ, was not observed to inhibit the rosette formation at up to 100 μM (4 mg/ml). We attribute these findings to the increased avidity of the pentameric CD4-hCμ, due to its multivalency compared with monovalent CD4-Cκ. In fact, the pioneering work of Karush has shown that multivalency of IgM can increase the avidity up to 10^7-fold compared with monovalent affinity (24).

The concentration values needed for 50% inhibition of the rosette formation can be used to estimate the affinity or avidity of an inhibitor (Fig. 3). This is illustrated by the fact that bivalent M5/114 mAb inhibits 50% of the rosette formation at about 5×10^{-9} M (Fig. 3), the value, which corresponds exactly to the avidity of the bivalent antibody measured by classical Scatchard analyses, $K_a \sim 5 \times 10^9$ M^{-1} (21 and our unpublished results). Thus, the avidity of CD4-hCμ is estimated to be about 7×10^7 M^{-1}, since a 100-fold higher concentration of CD4-hCμ is needed for inhibition of rosette formation compared with M5/114. Since extrapolation of affinities from avidity values is not possible (24), we can confidently estimate the upper limit for K_a to be 10^4 M^{-1} or below from the fact that the monovalent form of sCD4 did not inhibit rosette formation at concentrations of 100 μM (10^{-4} M). This value is in agreement with more indirect estimates of Hussey *et al.* (24) in the human system, but much lower than the affinity ($K_a = 3 \times 10^6$ M^{-1}) measured by Cammarota *et al.* (6), again for the human CD4 – MHC class II interaction. The latter authors used both sCD4 and soluble MHC class II molecules to derive their affinity values, and it is conceivable that soluble MHC class II molecules are more accessible to sCD4 than MHC class II molecules embedded in the membrane. This may also explain why human sCD4 has never been shown directly to bind MHC class II positive cells.

CD4 has been proposed to have two different roles in antigen-specific interaction between T_h cells and APCs: firstly, CD4 as an accessory molecule is thought to contribute to the general adhesion between T_h cells and APCs (8 – 10), and, secondly, CD4 as a co-receptor for TCR has been suggested to be essential for optimal T_h cell activation, due to its signal transducing capabilities (for review, see 11). Based on our results, we think that CD4 makes only a very minor contribution to the general adhesion, since the other adhesion systems used by T_h cells, like CD2/LFA-3 and LFA-1/ICAM, have affinities of at least two orders of magnitude higher than that of CD4/MHC class II molecules (26). Therefore, we reason that the co-receptor function is most likely physiologically more significant. Interestingly, it has been shown that CD4 can associate with TCR complexes (27 – 29) and thus, presumably, it has some affinity to the TCR complex as well. Therefore, due to this dual binding specificity of CD4, it may exhibit much higher affinity to the TCR – CD3 – MHC plus peptide complex, as opposed to its affinities to either TCR – CD3 or MHC class II molecules alone. This would also explain why the CD4, as a signalling molecule during T cell activation, can find its relevant partner in the presence of an excess of potentially irrelevant MHC class II and TCR molecules.

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Abbreviations

APC	antigen presenting cell
sCD4	soluble CD4
T_h	T helper cell

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