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Quantitative Immunofluorescent Assay of Full-Length, Recombinant CD4 in Solution and Mapping of Its Epitopes

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A specific, rapid, and sensitive method for the detection of CD4 in solution was developed using pairs of fluorescently stained monoclonal antibodies which do not cross-compete. The assay is quantitated by flow cytometry using Simply Cellular microbeads (SC beads) as the primary support for the first anti-CD4 mAb. This method uses the standard conditions for anti-CD4 monoclonal antibody binding, washing, detection, and quantitation by flow cytometry of the CD4 antigen either bound to the SC beads or expressed on the cell surface. The monoclonal antibody used (Leu 3a PE) is the standard reference used to evaluate the CD4 concentration. This method differs from ELISA techniques, which need an antigen standard curve and thus can be influenced by the quality and source of the antigen. This type of assay is also a procedure which enables determination of the level of oligomerization of the bound antigen. It can be used for any antigen to which monoclonal antibodies recognizing at least two distinct epitopes are available. The use of soluble or full-length CD4 derivatives as potential therapeutic agents against AIDS, would benefit from a precise quantitation of the CD4 molecules which still have their proper tertiary structure.

KEY WORDS: Flow cytometry; immunofluorescent assay; antigen quantitation; recombinant CD4; epitope mapping.

INTRODUCTION

CD4 is a phenotypic marker for helper T-lymphocyte populations. This cell surface glycoprotein, together with the T-cell receptor, plays an essential role in helper T-lymphocyte recognition of the antigen in association with the class II major histocompatibility complex [1,2]. The CD4 molecule is also the major receptor for the gp120 envelope glycoprotein of HIV-1 [3,4]. Soluble CD4 (sCD4)[3] (i.e., without hydrophobic transmembrane and cytoplasmic domains) has been used as a competitor in SIV-infected monkeys, where it showed significant therapeutic activity [5]. Two of the main disadvantages of this therapeutic approach, at least in the monkey, are the short *in vivo* half-life of sCD4 and its immunogenicity. Another potential strategy is to use a CD4–erythrocyte complex, which presents the advantage of long-lived circulating cells, which are capable of interacting with both HIV- and gp120-positive cells [6]. For this purpose, recombinant full-length CD4 has been produced in baculovirus-infected insect cells [7]. The full-length protein was extracted, purified [7], and electroinserted into the membrane of red blood cells [8,9]. We report here the development of a novel quantitative flow cytometry method (QIFA), a variant of the particle concentration fluorescence immunoassay (PCFIA[10]), using two monoclonal antibodies against two distinct CD4 epitopes. This technique allows the immunological analysis of the CD4 protein (or any other antigen with at least

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[4] Abbreviations used: FITC, fluorescein isothiocyante conjugate; PE, phycoerythrin; PBS, phosphate-buffered saline; SC beads, Simply Cellular microbeads; rCD4, recombinant CD4; QIFA, quantitative immunofluorescent assay; FCM, flow cytometry; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; sCD4, soluble CD4; PCFIA, particle concentration fluorescence immunoassay.

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two distinct epitopes recognized by mAbs) in solution in the presence of detergent. The selection of two anti-CD4 monoclonal antibodies directed to nonoverlapping epitopes was a prerequisite and yielded new information about the CD4 epitope map.

MATERIALS AND METHODS

Lymphocytes and Cell Line

Human acute lymphoblastic leukemia cells of the line CEM-CM3 (American Type Culture Collection, Rockville, MD) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C with 5% carbon dioxide. Human peripheral blood lymphocytes (PBL) were obtained from healthy donors after centrifugation of buffy coat through Ficoll–Paque (Pharmacia, Uppsala, Sweden).

Monoclonal Antibodies

The following anti-CD4 monoclonal antibodies were used: OKT4, A, B, C, D, E, and F (provided by P. Rao, Ortho Diagnostics); OKT4 and OKT4A fluorescein isothiocyanate conjugate (OKT4 FITC, OKT4A FITC; Ortho Diagnostics, Raritan, NJ); 13B 8.2, BL4/10T4, phycoerythrin (PE)-conjugated 13B 8.2 (13B 8.2–PE), BL4/10T4–FITC (AMAC, Westbrook, ME); MT 151 (Boehringer Mannheim); and Leu 3a and Leu 3a-PE (Becton Dickinson, CA).

Human Recombinant Full-Length CD4

This recombinant protein was expressed, extracted, and purified as previously described [7]. Briefly, Sf9 insect cells, 2 days postinfection with a recombinant baculovirus (AcCD4) encoding full-length CD4, are extracted with Triton X-114. After temperature-induced phase separation, the detergent phase was harvested and an equal volume of PBS was added. The resulting mixture was centrifuged for 1 h at 100,000*g* and the supernatant was used for immunoaffinity or for quantitation. Further purification was achieved by immunoaffinity chromatography using immobilized 13B 8.2 mAb [7]. The final product was lyophylized and stored at −70°C.

Immunofluorescent Assays

The surface immunofluorescence of cells or beads was quantified by flow cytometry (FCM) using an Epics Profile (Coulter) with an argon laser (448-nm excitation). Two-color fluorescence emissions were measured by filtering the photons through dichroic mirrors narrow-band pass filters (500 to 525 nm for green, 575 ± 10 nm for red).

mAb saturating conditions were determined by flow cytometry (10^4 cells counted). Typically, 10^6 CEM cells were incubated for 30 min on ice with a range of primary mAb concentrations (usually from 0.1 to 10 μg mAb in a final volume of 50 μl PBS) and then washed with PBS (0.15 *M* NaCl, 10 m*M* sodium phosphate-pH 7.4). Control cells were stained with mouse isotypic standards containing IgG1, IgG2a, IgG2b, IgG3, or IgM (Coulter). All antibody-treated cells were washed twice with PBS. The unlabeled primary mAb were detected by 10 μg PE-conjugated goat anti-mouse antibodies (Molecular Probes, Eugene, OR). We estimated saturating conditions to be achieved when a doubling of primary mAb concentrations did not result in any change (±5%) in the linear mean peak channel fluorescence.

Cross-Competition Between mAb's to CD4

CEM cells were labeled for 30 min on ice with a saturating concentration of primary mAb. The cells were washed twice and then labeled with a saturating concentration of a second mAb (Leu 3a-PE, OKT4A-FITC, BL4/10T4-FITC, 13B 8.2-PE). After washing, flow cytometry histograms were generated from 10^4 cells.

The percentage of inhibition (I) of binding was calculated from the formula:

$$I = [(F_1 - F_2)/F_1] \times 100$$

where F_1 is the linear mean peak channel fluorescence of the second mAb, without primary mAb; and F_2 is the linear mean peak channel fuorescence of the second mAb, with primary mAb.

CD4 Quantitative Immunofluorescent Assay (QIFA)

Flow Cytometry Standards Corporation (Research Triangle Park, NC) produces Simply Cellular microbeads, with a quantitated number of mouse IgG binding sites per bead surface (8.4-μm diameter with 0.5 to 1.5 × 10^5 sites/bead, depending on the lot).

The beads' IgG sites were conjugated with BL4/10T4-FITC or OKT4-FITC as the first mAb. Typically, 10^6 beads were incubated for 20 min at 22°C with a saturating concentration of primary mAb (2μg BL4/10T4-FITC) and washed twice with PBS. For the assay, 10^5 beads labeled with BL4/10T4-FITC were incubated for 20 min at 22°C with CD4 samples serially diluted in PBS–Triton X-114, 1%. The total volume of incubation

was between 50 and 150 μl. Control beads were incubated under the same conditions but without CD4. To avoid nonspecific binding of the second mAb, the beads were incubated for 10 min at 22°C in the presence of 1 μl of normal mouse serum. The beads were then washed with PBS and resuspended for 20 min at 22°C in 50 μl PBS containing 0.5 μg Leu 3A-PE. After further washing, two-color fluorescence histograms were generated from data collected from 5×10^3 beads.

RESULTS AND DISCUSSION

In general, immune responses are directed against a small number of antigenic determinants confined to a few loci on the molecule [11]. In the case of CD4, a large number of anti-CD4 mAbs recognize epitopes located in the first N-terminal domain [12]. In order to identify the two mAbs with the weakest cross-reactivity against human recombinant CD4, we test the ability of a panel of mAbs to inhibit the binding of Leu 3a, OKT4A, BL4/10T4, and 13B 8.2 to CEM cells (Table I). These mAbs have been previously shown to bind to human CD4 expressed on the cell surface of recombinant baculovirus-infected insect cells [7]. OKT4A, OKT4D, and 13B 8.2 produced a strong inhibition (≥80%) of Leu 3a binding. OKT4B, OKT4F, and Leu 3a strongly (≥80%) cross-blocked OKT4A. BL4/10T4 binding to CEM cells was clearly inhibited by OKT4B and MT151. Finally, OKT4B, C, D, and E, Leu 3a, and MT151 inhibited 13B 8.2 binding to CD4 molecules. It is evident that Leu 3a coupled with BL4/10T4 results in one of the best combinations for CD4 quantitation. Indeed, other mAbs which strongly inhibited Leu 3a binding failed to block binding of BL4/10T4. This is, however, not true for the other combinations of the four mAbs tested. The cross-reactivity of different mAbs couples (OKT4A–Leu3a, BL4/10T4–Leu 3a, 13B 8.2–Leu 3a, 13B 8.2–OKT4A) was verified on human PBL. The inhibition obtained was similar to that found for CEM cells (respectively, >80, <20, 80, 20%).

To develop an effective and quantitative assay to measure human recombinant CD4, we used Simply Cellular microbeads (SC beads) with a predetermined number of mouse IgG antibody binding sites on the surface. The IgG antibody binding sites were first saturated with BL4/10T4–FITC (0.2 μg/10^5 beads) and then incubated with recombinant CD4 as previously described. The bound CD4 was then quantified by flow cytometry using a second mAb: Leu 3a PE (Fig. 1). The CD4 concentration was calculated using the number of Leu 3a–PE mAb bound to CD4 per bead. For these calculations, we estimated as 100 the linear mean peak channel fluorescence of SC beads saturated with Leu 3a–PE ($1.5 \cdot 10^5$ sites/beads). A saturating amount of isolated CD4, either from 100,000*g* extract or fully purified, gave a fluorescent signal which, respectively, represents 80 and 120% of the saturated Leu 3a–PE control beads (Fig. 2). This demonstrates the existence of oligomeric forms of lyophylized rCD4 after reconstitution and dilution in the presence of 1% Triton X-114. It suggests that the origin of these oligomers is not via hydrophobic interaction of the transmembrane sequence. These oligomeric forms of rCD4 were confirmed by Western immunoblotting using Leu 3a as a detection mAb (data not shown). The lower threshold of this assay corresponds to 3 ng/ml of CD4 ($3 \cdot 10^{10}$ CD4 molecules detected). In these calculations,

Table I. Cross-Competition Between mAbs to CD4[a]

Monoclonal antibody	Anti-CD4 mouse isotype	% competition			
		Leu 3a	OKT4A	BL4/10T4	13B 8.2
OKT4	IgG2 b	20	<20	20	<20
OKT4A	IgG2 a	>80	>80	<20	<20
OKT4B	Ig M	20	>80	>80	80
OKT4C	IgG2 a	20	<20	<20	>80
OKT4D	IgG1	80	<20	<20	80
OKT4E	IgG1	<20	<20	<20	>80
OKT4F	IgG1	<20	80	<20	<20
MT151	IgG2 a	20	<20	>80	80
13B 8.2	IgG1	80	<20	<20	>80
BL4/10T4	IgG2 a	20	<20	>80	<20
Leu 3a	IgG1	>80	>80	20	80

[a]CEM cells, after saturation by the first mAb, are incubated with the fluorescently labeled second mAb. The numbers represent the percentage of inhibition of binding of the second mAb (see Materials and Methods). Each percentage is the average of three experiments.

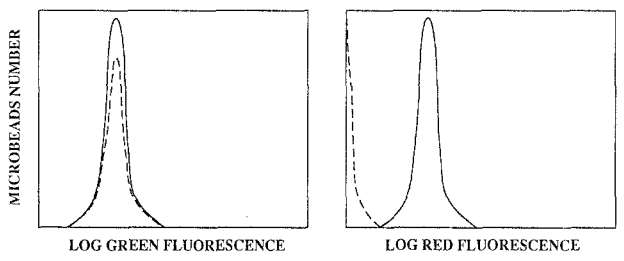

Fig. 1. Flow cytometry of SC beads saturated by rCD4. SC beads labeled with BL4/10T4–FITC (green fluorescence) were incubated with saturating rCD4 (50 ng) or without rCD4. The red fluorescence indicates the binding of Leu 3a-PE to saturated rCD4 beads ($\frac{2}{m}$) or control beads (----).

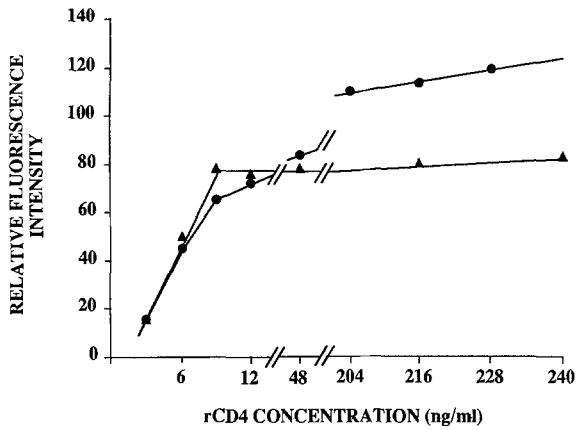

Fig. 2. rCD4 quantitation by QIFA and demonstration of eventual oligomerization of the bound antigen. Serial dilutions in PBS–Triton X-114, 1%, of rCD4 were incubated with SC beads labeled with BL4/10T4–FITC. The rCD4 samples either were from 100,000*g* supernatant (▲) or were purified lyophilized (•). The relative red fluorescence intensity was scaled using Leu 3a–PE-saturated SC beads as a standard (100). The rCD4 concentration was estimated by reference to this scale, where 100 represents $1.4 \cdot 10^5$ rCD4 molecules bound per bead. The total number of microbeads used per incubation was 10^5. Every point is the average of three measurements (average SD is 8%).

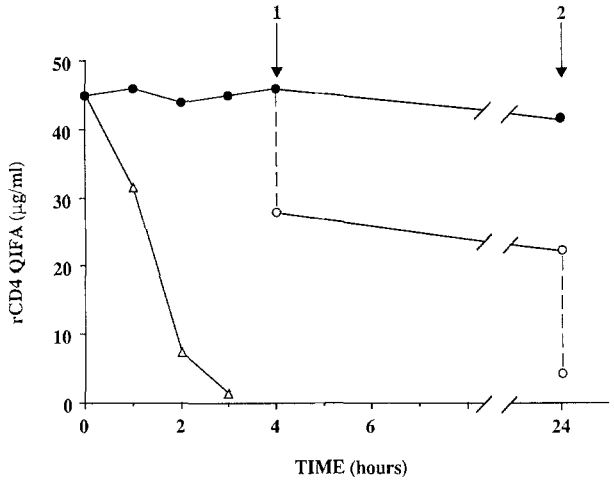

Fig. 3. Stability of rCD4 monitored by QIFA. rCD4 was incubated in PBS, pH 7.4, at 4°C. At different incubation times, aliquots were assayed by QIFA (•). Other aliquots were subjected to two cycles of freezing (−70°C) and thawing (air, 22°C): arrows 1 and 2 (○). Between the two cycles, the samples were kept at 4°C. rCD4 was incubated in Tris buffer, pH 8.9, 20 m*M*, at 4°C. After 1, 2, and 3 h, samples were diluted in PBS, pH 7.4, and the CD4 epitopes quantified (△).

the control beads incubated with no CD4 were used as a reference for zero fluorescence (Fig. 2).

Using this method, it was possible to monitor the stability of rCD4 under different conditions: PBS, pH 7.4, 4°C, Tris buffer, pH 8.9, 20 m*M*, and cycles of freezing and thawing (Fig. 3). These data show the sensitivity of the tertiary structure of rCD4 to alkaline treatment and to cycles of freezing and thawing.

In this report we describe a specific, sensitive, and rapid assay for the characterization and quantification of the human CD4 protein. For this purpose, two CD4-specific mAbs, Leu 3a and BL4/10T4, have been selected because they exhibit the least degree of cross-reactivity, as determined in FCM experiments (Table I and Fig. 4), similar results were obtained using OKT4–FITC and Leu3a (data not shown). This capture assay presented the following advantages.

(i) It is a rapid (<2-hr) one-step method for the detection of the bound antigen. This method uses the standard conditions for mAb binding, washing, and detection and FCM quantitation of rCD4. Thus, the antibody–antigen complex is exposed to similar stresses whether the CD4 is bound to SC beads or naturally exposed on the cell surface. Furthermore, the rCD4 concentration is calculated by taking as a reference the SC beads saturated by the detection mAb. Thus, in comparison to ELISA methods, we do not need an antigen standard curve, which can be influenced by the quality and source of the antigen.

(ii) It is not affected by the presence of detergent normally used for membrane protein extraction and solubilization.

(iii) It allows us to study oligomeric forms of the bound antigen during extraction and purification. It can also be used to bind and analyze membrane vesicles or reconstituted liposomes with the antigen in their membrane (data not shown).

(iv) It presents a flexible way of binding antigen to different mAbs and then a means to study both epitope mapping and steric hindrance due to the interaction of multiple ligands (e.g., HLA II or gp120 and CD4).

In addition, our results provide new information on the epitopes of the CD4 antigen. Sattentau *et al.* have proposed an anti-CD4 mAbs classification based on interactions with HIV and cross-competition assays between mAbs [13]. Accordingly, OKT4 and OKT4C were defined as non-HIV-blocking mAbs, whereas all the others studied either partially or totally inhibited HIV binding [13,14].

In our study, Leu 3a and OKT4A were tested for

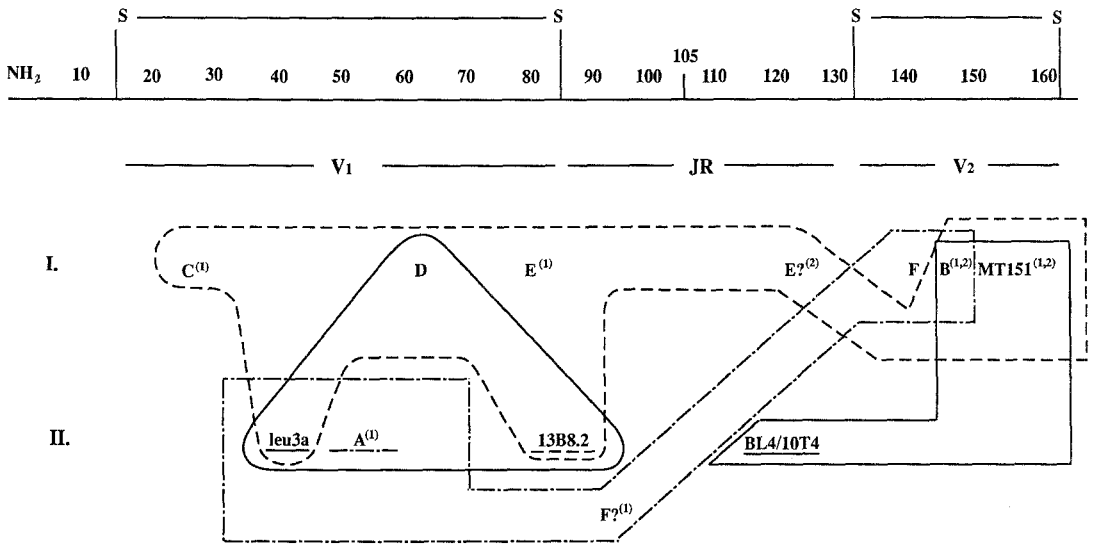

Fig. 4. Cross-competition between mAbs to human CD4 and their putative epitope locations. The upper line represents the first 160 amino acids of CD4 with the two disulfide bonds, the V1 and V2 domain positions, and the junctional region (JR) [25,26]. Lines I and II locate the different mAbs putative epitope sites according, respectively, to Refs. 26 and 28. When location is in agreement with other results, it is indicated by (1) for Ref. 16 and (2) for Ref. 28. The four groups represent mAb cross-reactions. MAbs were judged to be in the same cluster when 80% or more inhibition of binding was observed using Leu 3a ($\frac{a}{m}$), OKT4A (--), 13B 8.2 (----), and BL4/10T4 ($\frac{a}{m}$) versus each of the other respective mAbs.

competitive binding on CEM cells against the other anti-CD4 mAbs (Fig. 4). In agreement with Sattentau *et al.* [13], our data confirm the strong cross-inhibition between OKT4A and Leu 3a for CD4 epitopes, as well as the strong competition between OKT4A and OKT4B and F, respectively, as previously observed [15]. However, it does not provide any information on the location of the OKT4F epitope, which could be on either the first [13] or the second domain [15] of the CD4 molecule (Fig. 4).

Likewise, because BL4/10 T4 strongly interacts with MT151 and OKT4B, we propose that OKT4B and BL4/10T4 reside in the MT151 cluster [i.e., their epitopes can be placed in the second loop (V2 domain) of the CD4 protein]. Indeed, using the OKT4 antibody panel and MT151 in conjunction with a panel of CD4-derived synthetic peptides, it has been proposed that the epitope location for OKT4B and MT151 resides in the second loop of the CD4 protein [15]. This is confirmed by MT151 and OKT4B binding to cells expressing mouse–human chimeric CD4 [16].

The binding of 13B8.2 to CD4 was strongly inhibited by OKT4C, D, and E, which suggests that their epitopes are close together (Fig. 4). However, we cannot resolve the ambiguity about the locations of the OKT4E and F epitopes, which have been assigned to both the first [16] and the second domain [15]. Several qualitative studies demonstrate that the V1 domain is critical for the recognition of HIV envelope glycoprotein gp120. A recent quantitative analysis shows that the high–affinity binding site for gp120 is localized to a region encompassing amino acid residues 40 to about 55 in the first immunoglobulin-like VJ region [17]. The high reciprocal steric hindrance of the different mAbs, e.g., OKT4A against OKT4B or F and 13B 8.2 against OKT4B or MT151 (Table I), and the strong inhibition of gp120 binding by OKT4A and F and MT151 [13,14] are consistent with the idea that the tridimensional structure of the CD4 protein is such that regions of the V1 and V2 domain must be close to each other [15].

Previous studies [15,18] have demonstrated that the seven OKT4 mAbs recognize distinct epitopes of CD4. Our data from cross-competition confirm that none of the 11 mAbs used have exactly the same antigen combining site. None of these 11 mAbs are able to react with denatured rCD4 (Western immunoblotting of SDS-PAGE gel; data not shown). Leu 3a and BL4/10T4 or Leu 3a and OKT4 appear to be the best combination of mAbs to use in QIFA. Leu 3a is of importance since it seems to be one of the mAbs which binds to CD4 with sequence requirements similar to gp120 [16,19].

The QIFA method can be used also for quantification of all forms of sCD4, since they contain V1 and V2 domains [20–24]. Recent studies [25] indicate that

rCD4 proteins that will react with an affinity equivalent to that of intact cell surface CD4 must have a correct tertiary structure. This method might also be used to quantify other antigens to which two distinct mAbs are available. The use of two nonoverlapping mAbs can increase specificity and limit the risk of cross-reaction from other proteins in biological fluids. Finally, the method appears to be unaffected by detergent concentrations in the 1.5 n*M* to 50 m*M* range (data not shown) and so can provide a good estimate of membrane antigens solubilized by detergents.

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