Epitopes of the CD4 Antigen and HIV Infection

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The CD4 (or T4) surface antigen of human T lymphocytes is an important part of the receptor for the human immunodeficiency virus (HIV). After binding to the receptor, the HIV may enter the T cell and induce the formation of syncytia. In an attempt to identify the receptor site more closely, monoclonal antibodies (Mab's) to CD4 were tested for their ability to block HIV infection in a syncytium formation assay, and the CD4 epitopes so identified were mapped by antibody cross-blocking. The antibodies that showed strong inhibition of HIV fell into two main families while a third group of Mab's blocked syncytia formation weakly or not at all. Several different isolates of HIV as well as the laboratory strain CBL1 grown in CEM cells were used to induce the syncytia. The data indicate that only some epitopes of CD4 are important for virus binding and imply that the virus-binding site for CD4 is conserved in different isolates of HIV with substantially divergent *env* gene sequences. Preliminary studies of patients suggest that polymorphism of these epitopes does not play a role in determining susceptibility to infection.

THE CD4 (T4) ANTIGEN DEFINES A subset of mature T lymphocytes known as helper/inducer T cells that have both regulatory and antigen recognition functions. This molecule is the receptor for the AIDS virus [human immunodeficiency virus or HIV (*1*)], as shown by inhibition of syncytium formation and pseudotype infection of CD4-positive cell lines (*2*) and by blocking of virus infection of peripheral blood mononuclear cells (*3*). McDougal *et al.* (*4*) have shown by immunoprecipitation studies that the viral glycoprotein gp110 binds directly to the CD4 molecule, and that no cell surface structures other than CD4 appear to be involved in the virus-receptor complex. These authors also demonstrated that OKT4a, a monoclonal antibody (Mab) to CD4 is unable to bind in the presence of virus. Similarly, Popovic *et al.* (*5*) showed that binding of OKT4a, OKT4f, and Leu-3a is inhibited by preincubation of CD4-bearing cells with concentrated HIV. To date, however, the relationship of CD4 Mab's to each other, and the importance of the epitopes they recognize in terms of virus binding have not been elucidated.

The OKT4 series of Mab's recognize distinct noncompeting epitopes of the CD4 molecule, and are known as OKT4, OKT4a, 4b, 4c, 4d, 4e, and 4f (*6*). These antibodies have been used to study the epitopes of the CD4 molecule that are involved in the induction of T-helper function (*7*) and T-proliferative responses to antigen stimulation (*8*). In this study we used the OKT4 series a to e, and other Mab's to CD4 (*9*) grouped into clusters by cross-competition studies, to determine which epitopes of CD4 as defined by these clusters are of importance in binding virus.

An inherited variant of the CD4 molecule has been demonstrated in Japanese and black African individuals, in which the OKT4-defined epitope is not expressed, whereas other antigenic sites are expressed normally (*10*). Because the AIDS retrovirus may have originated in Africa and because OKT4 itself does not block syncytium formation, we thought it of interest to examine the expression of other CD4 epitopes on African T cells. We were unable to demonstrate the reported OKT4 polymorphism in a small group of black African subjects, but report on a quantitative variation in expression of certain epitopes of CD4.

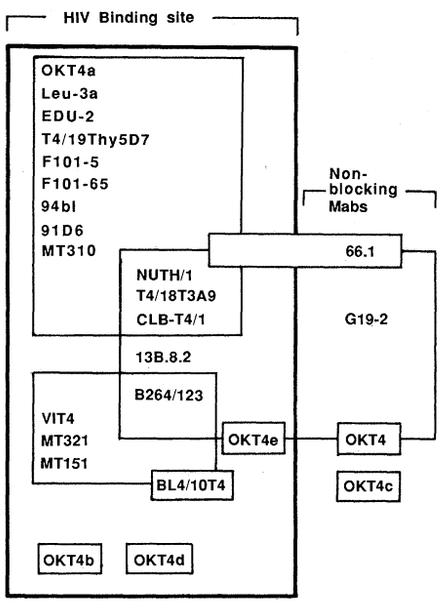

Fig. 1. Cross-competition between Mab's to CD4 and blocking of syncytium formation. The results from Table 2 were assimilated into a map of the various clusters of Mab's in relation to their HIV blocking activity (Table 1). Mab's within the thick black line block the HIV (CEM/CBL1 isolate) receptor interaction, those outside do not. Where boxes containing clusters of Mab's cross, this represents cross-competition between the clusters.

We tested the ability of the CD4 Mab's to block the formation of syncytia between an HIV-producing cell line (CEM/CBL1) and CD4-bearing target cells. This technique has been used previously to inhibit the virus CD4 interaction (*2*) and is an alternative to infection with pseudotypes or virus supernatants for investigating the presence of HIV receptors on the surface of uninfected cells. With the exception of four Mab's (OKT4, 4c, G19-2, and 66.1) all blocked syncytium induction with the CBL1 isolate, although with varying efficacy. Table 1 shows that of the OKT4 series of antibodies, OKT4a blocks most efficiently and titers out to a similar concentration to MT151. OKT4b, 4d, and 4e block less efficiently, and OKT4 and OKT4c are unable to inhibit syncytium formation at any concentration tested. Table 1 also lists the highest dilutions at which the workshop Mab's inhibit syncytia. As with the OKT4 series of antibodies, these Mab's can be classified into three groups: those that block strongly, those that inhibit at medium to high concentration only, and those that are unable to block.

There are a number of well-defined isolates of HIV that are known to differ considerably in nucleotide sequence. This genomic diversity, particularly in the *env* region, may result in alteration of the virus-binding site so that different isolates might utilize different epitopes of CD4 for binding and infection. To study this, we performed syncytium inhibition assays with one isolate very similar to CEM/CBL1, H9/III_B, and with three isolates that differ substantially, H9/III_{RF}, H9/ARV-2, and K12/Z129. It is interesting that CEM/CBL1, which was not blocked by Mab's 66.1 and G19-2, is very similar in sequence to H9/III_B, which was inhibited by these Mab's, although only when used at the highest concentration tested (Table 2). It is possible that this variation reflects differences in the cell lines in which these isolates are propagated, such as relative ability to form syncytia, rather than differences in the virus-receptor interaction. The three other isolates all shared the same pattern of inhibition, but differed from H9/III_B in that they were not blocked by 66.1 and CLB-T4/1 whereas H9/III_B was blocked. It seems unlikely that these differences between iso-

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lates are important in terms of receptor binding, since G19-2, 66.1, and CLB-T4/1 are at best only weak blockers of syncytia.

For the cross-competition studies, MT151 was labeled with ^{125}I by a method based on that described by Greenwood *et al.* (*11*) and tested for competitive binding against the other Mab's to CD4. Only two Mab's gave >80% inhibition of MT151 binding—VIT4 and MT321. A second group of Mab's, which included some blockers and some nonblockers of syncytia, gave intermediate inhibition, and a third group including Leu-3a and OKT4a did not compete. Leu-3a and OKT4a were labeled, and in addition to identifying the same three groups as before, they were found to cross-block each other. Finally, G19-2 was labeled and confirmed the existence of three main clusters of Mab's to CD4. Table 3 shows these data, which are presented in the context of the syncytium inhibition data in Fig. 1. It is evident that there are two distinct epitopes of CD4 that are important in virus binding, and a third site that partially overlaps the other two and is defined by Mab's that are less efficient or unable to inhibit syncytia. Other Mab's either partially cross-reacted with well-defined clusters (OKT4, OKT4e, BL4/10T4, and 66.1) or did not cross-react at all (OKT4b, 4d, and 4c).

In drawing Fig. 1 we assumed that if one Mab from an epitope cluster competes with an antibody from another cluster, then all the Mab's in these groups compete. This may not necessarily follow, and there are a few examples in which Mab's within the same group may compete differently with Mab's from another group. To determine every cross-reaction, however, we would need to have done pair-wise cross-competition studies between all 25 Mab's, which was not practical. We therefore propose that Fig. 1 be taken as a broad guideline for epitopes of CD4 involved in HIV binding, rather than as a definitive cross-competition study.

Comparison of CD4 staining of $H9/III_B$ and CEM/CBL1 with their uninfected counterparts confirmed the finding of Dalgleish *et al.* (*2*) that CD4 expression is reduced in infected cells. Staining by all the OKT4 series Mab's decreased from between 40 to 85% on uninfected cells to less than 10% on the infected cells, suggesting either that the whole molecule is down-regulated or that all CD4 epitopes are masked by virus glycoproteins in infected cells.

We also examined peripheral blood lymphocytes of several groups of subjects in which noncoordinate expression of CD4 epitopes might be important. Peripheral blood lymphocytes from 28 black African and 12 Caucasian subjects did not show the absence of any CD4 epitope but did exhibit a quantitative variation in the expression of certain epitopes (Fig. 2), particularly those recognized by OKT4 and OKT4c; it is interesting that antibodies to the same epitopes do not block virus binding (Table 1). There was no difference between African and Caucasians with respect to expression of CD4 epitopes. We also examined the lymphocytes of a small sample of HIV-susceptible individuals [five patients with persistent generalized lymphadenopathy (PGL)] and individuals who might be resistant to the

Table 1. Inhibition of HIV-induced syncytium formation by Mab's to CD4. This assay was based on the method of Dalgleish *et al.* (*2*). One hundred microliters of a suspension of 2×10^5 CEM/CBL1 cells per milliliter in RPMI 1640 containing 10% FCS was mixed with 100 μl of JM cells at 10^5 per milliliter. Monoclonal antibody was added immediately, and syncytium formation was evaluated after overnight incubation at 37°C. Titrations of purified immunoglobulin (Ig) were by 50% end points, where wells containing no obvious syncytia were negative and one or more syncytia were positive. There were four replicate wells per dilution of Ig. A total of 25 Mab's to CD4 were tested for their efficiency at inhibiting syncytium formation between the HIV-producing cell line (CEM/CBL1) and the CD4-bearing indicator line (JM). Antibodies were titrated as micrograms per milliliter of purified Ig, or fourfold dilutions from 1:50 to 1:3200 of ascitic fluid.

Mab	Highest inhibitory dilution of ascites	Mab	50% Inhibitory Ig concentration (μg/ml)
	Strong blocking		
Leu-3a		OKT4a	0.12
MT151	>1:3200	MT151	0.16
VIT4			
	Intermediate blocking		
91d6		OKT4b	0.59
T4/18T3A9		OKT4e	0.5
T4/19Thy5D7			
13B8.2			
94b1			
F101-69			
F101-5	1:200 to 1:3200		
NUTH/1			
EDU-2			
MT310		OKT4d	0.9
MT321			
CLB-T4/1			
BL4/10T4			
	Nonblocking		
66.1		OKT4	>20
G19-2	<1:50	OKT4c	>20

Table 2. Variation in syncytium blocking in different HIV isolates by Mab's to CD4. The assay was done as described for Table 1. Of the panel of 25 Mab's, only the three we show in the table varied in their blocking activity. A plus sign indicates that the antibody was able to block syncytia and a minus sign represents an inability of the Mab to block, even at the highest concentration tested.

HIV isolate	Divergence of *env* nucleotide sequence from $H9/III_B$ (%)	Mab's to CD4 with variant syncytium inhibition pattern		
		66.1	G19-2	CLB-T4/1
CEM/CBL1	2.2	−	−	+
$H9/III_B$	−	+	+	+
$H9/III_{RF}$	~15.0	−	+	−
H9/ARV-2	~15.0	−	+	−
K12/Z129	>20.0	−	+	−

Table 3. Cross-competition between Mab's to CD4. Fifty microliters of Mab at 20 μg/ml or 50 μl of 1/50 ascites was mixed with 50 μl of CD4-bearing CEM cells at 5×10^6 per milliliter in RPMI 1640 with 10% FCS and 0.1% sodium azide. After 30 minutes on ice the cells were washed three times and resuspended in 50 μl of medium containing I^{125}-labeled Mab at a concentration that gave between 2000 to 5000 specific counts per minute. They were then incubated and washed as before. The activity of bound, labeled Mab was measured as counts per minute on an LKB 1280 Ultrogamma. The percentage of inhibition (I) of binding was calculated by the formula: $I = 100 - [(T - C/M - C) \times 100]$ where C = counts bound in the presence of excess of the identical unlabeled Mab, M = counts bound in the presence of excess of a Mab unrelated to CD4, and T = counts bound in the presence of other Mab's to CD4. Symbols: ++, 80 to 100% inhibition of binding of labeled Mab; +, 20 to 80% inhibition; no symbol, <20% inhibition.

Mab's to CD4	^{125}I-labeled Mab's			
	MT151	Leu-3a	OKT4a	G19-2
OKT4				+
OKT4a		++	++	+
OKT4b				
OKT4c				
OKT4d				
OKT4e				+
BL4/10T4	+			
Leu-3a		++	++	
VIT4	++			+
B264/123	+			++
G19-2	+			++
66.1		+	+	+
13B8.2				++
F101-5		++	++	
F101-69		++	++	
T4/18T3A9		++	++	++
T4/19Thy5D7		++	++	
MT151	++			+
MT310		++	++	
MT321	++			+
91d6		++	++	
CLB-T4/1	+	+		++
EDU-2		++	++	
94b1		++	++	
NUTH/1		++	++	++

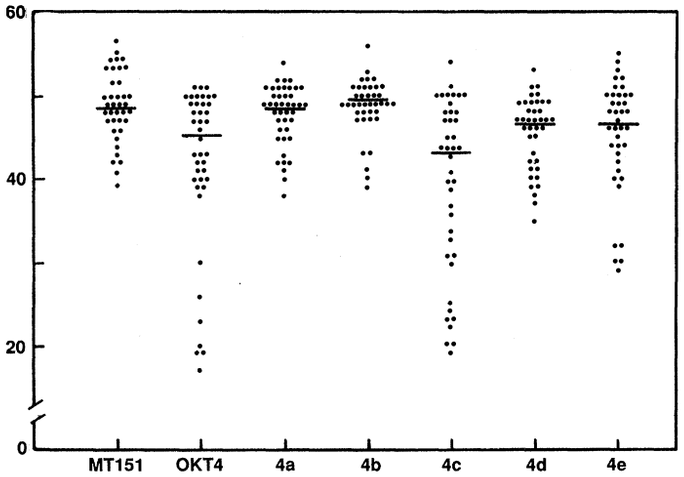

Fig. 2. Variation in the expression of CD4 epitopes in 40 normal subjects. Peripheral blood mononuclear cells (PBM) were fractionated from heparinized whole blood by centrifugation through Ficol-Hypaque (Pharmacia) for 20 minutes at 2200 rev/min. Fifty microliters of cells (5×10^6 per milliliter) were incubated with 50 μl of Mab at 5 μg/ml or 50 μl of 1:100 of ascitic fluid for 30 minutes at 4°C. The cells were washed three times in RPMI 1640 with 2% FCS and 0.1% sodium azide, and resuspended in 50 μl of goat antibody to mouse immunoglobulin conjugated to FITC (Nordic Laboratories). After 30 minutes at 4°C the cells were washed as before and resuspended in PBS "A" containing 1% formaldehyde. The percentage and peak channel fluorescence were analyzed on a FACS IV (Becton Dickinson) at least 24 hours after fixation of the cells. Percentage fluorescence of PBM from each subject stained with each Mab is indicated by a single point. Horizontal bars represent the median.

virus or its effects (five active partners for at least 1 year of HIV seropositive patients who had remained seronegative). In the PGL patients there was a coordinate decrease in the percentage of cells expressing all CD4 epitopes and no evidence for expression of a single epitope without others. In the seronegative partners, staining with antibodies to all the OKT4 epitopes was within the normal range.

We extended the observations of Dalgleish *et al.* (*2*) by demonstrating that whereas most Mab's to CD4 block syncytium formation in CD4-bearing, HIV-susceptible target cell lines, a small number of Mab's do not. Among the Mab's that block there is a quantitative variation in efficacy, some antibodies requiring only very low concentrations to block completely, others requiring high concentrations for any blocking (Table 1). Some of this variability may be due to differences in antibody affinity, but a major factor must be the epitopes to which the antibodies are directed since some antibodies do not block even at high concentration. A similar conclusion was reached by both Popovic *et al.* (*5*) and McDougal *et al.* (*4*) who showed in competition binding experiments between HIV and Mab's, that OKT4a, OKT4f, and Leu-3a block binding whereas OKT4 does not.

The syncytium blocking data for different isolates of HIV show that a large divergence in *env* sequence (up to at least 20%) results in only small differences in syncytium blocking, and suggest that these differences are unlikely to be of importance in the virus-receptor interaction. This implies that the virus-binding site for CD4 may be highly conserved despite variation in the rest of the *env* gene product.

Since many Mab's to CD4 block syncytium formation and several CD4 epitopes have been defined, we conducted competition binding experiments to determine how many separate epitopes are important in virus binding. Our results show that there are two major families of Mab's that do not block each other but can block virus binding. A third family of antibodies partially competes with both of the first two families but is itself heterogeneous in that some antibodies of this family block syncytium formation while others do not. In addition, there are other Mab's that only partially compete with any of the major groups (OKT4e and B264/123) and block syncytia, as well as Mab's that partially compete (OKT4 and 66.1), or do not compete (OKT4c) and do not inhibit syncytia. Interpretation of these data is not straightforward since inhibition of virus-CD4 or antibody-CD4 binding could be due not only to direct competition for the same or adjacent epitopes, but to conformational changes in the molecule induced by antibody binding at a distant site. However, we tentatively conclude that the most effective inhibition is probably due to direct competition for adjacent epitopes. This suggests that the virus-binding site may be relatively large, because antibodies from two noncompeting families, exemplified by OKT4a and MT151, can inhibit syncytia extremely effectively. The three Mab's OKT4d, 4e, and 4b, which compete only partially or not at all with these groups, also inhibit effectively, again suggesting a relatively large virus-binding site.

Epitopes detected by OKT4 or OKT4c seem to be irrelevant for virus binding. The OKT4 epitope is trypsin resistant (*12*) and may therefore be close to the cell membrane. It is interesting that the OKT4 epitope shows low expression in infected cells; epitopes detected by virus inhibitory Mab's also show low expression in infected cells, even though the CD4 molecule can be surface-labeled with ^{125}I by the lactoperoxidase method in infected cells and immunoprecipitated by Mab's to CD4 after the cells are lysed with detergent (*4*). Presumably, CD4 is present in the membrane but the epitopes detected by existing Mab's are masked by viral glycoprotein.

It is well established that polymorphisms occur in the expression of the OKT4 epitope of CD4 (*10*) and that low or null expression of this epitope has little effect on the function of CD4 (*7, 8, 12*) or the binding of or infection with HIV (*5, 13*). In both black African and Caucasian subjects we found variation in OKT4a, 4b, 4d, and MT151, but more substantial variation in OKT4e and particularly OKT4 and 4c. The OKT4 and 4c Mab's are also unable to block class II restricted T-cell (CD4-bearing) function (*7, 8*). Thus the epitopes of CD4 involved in T-cell helper proliferative functions may be the same as, or highly related to, those acting as the receptor for HIV, and appear to be particularly conserved.

According to this argument one would expect susceptible (seropositive) individuals to express at least the epitopes defined by syncytium-blocking Mab's. In a small number of PGL patients this was found to be the case, since although there were low numbers of CD4-positive cells, these showed coordinate expression of the OKT4 series and MT151 epitopes. More interesting was the finding that the same was true for seronegative sexual partners of seropositive individuals, implying that if these subjects are relatively resistant to HIV this is not likely to be due to a polymorphism of CD4.

We have provided evidence that not all epitopes of CD4 defined by Mab's are equally important in HIV-induced syncytium formation and that irrelevant epitopes show more quantitative variability than relevant ones. Competition binding experiments show that antibodies to at least four distinct epitopes can interfere with virus infection. While these studies do not reveal the actual structure of the virus receptor, they provide a map of it and suggest that it represents a conserved gene region. It will be of interest to compare the map for HIV (represented

by CEM/CBL1) with those of newer isolates of virus having apparently very different properties (*14*).

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Alterations in T4 (CD4) Protein and mRNA Synthesis in Cells Infected with HIV

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Cells infected with the human immunodeficiency virus (HIV) show decreased expression of the 58-kilodalton T4 (CD4) antigen on their surface. In this study, the effect of HIV infection on the synthesis of T4 messenger RNA (mRNA) and protein products was evaluated in T-cell lines. Metabolically labeled lysates from the $T4^+$ cell line Sup-T1 were immunoprecipitated with monoclonal antibodies to T4. Compared with uninfected cells, HIV-infected Sup-T1 cells showed decreased amounts of T4 that coprecipitated with both the 120-kilodalton viral envelope and the 150-kilodalton envelope precursor molecules. In four of five HIV-producing T-cell lines studied, the steady-state levels of T4 mRNA were also reduced. Thus, the decreased T4 antigen on HIV-infected cells is due to at least three factors: reduced steady-state levels of T4-specific mRNA, reduced amounts of immunoprecipitable T4 antigen, and the complexing of available T4 antigen with viral envelope gene products. The data suggested that the T4 protein produced after infection may be complexed with viral envelope gene products within infected cells. Retroviral envelope-receptor complexes may thus participate in a general mechanism by which receptors for retroviruses are downmodulated and alterations in cellular function develop after infection.

THE HUMAN IMMUNODEFICIENCY viruses (HIV) selectively infect T lymphocytes that express the 58- to 62-kilodalton (kD) glycoprotein T4 (CD4) (*1–5*) and produce characteristic cytopathic effects (*1, 2*). The acquired immune deficiency syndrome (AIDS), which may result from HIV infection, is characterized clinically by a progressive depletion of T4 cells (*6*). The tropism of HIV for T4 cells results, at least in part, from a direct interaction between the virion and the T4 molecule itself (*3, 7–9*). Monoclonal antibodies (Mab's) reactive with T4 can inhibit syncytia formation by HIV and neutralize infection of T-cell lines and peripheral blood T cells (*3*). Moreover, studies with concentrated preparations of HIV have demonstrated that Mab's to particular epitopes on the T4 molecule compete directly with the virus for binding to the cell (*7, 8*). McDougal *et al.* (*9*) have demonstrated that during the binding of HIV to the cell surface, a complex forms between the T4 molecule and the 110- to 120-kD glycoprotein that forms the externalized portion of the viral envelope (*9*). Because the T4 molecule appears to be important in determining the susceptibility of cells to infection, and because T4 expression may be modulated by virus after infection, we have studied the internal processing of T4 in normal and HIV-infected cells.

The alterations in expression of T4 that occur during viral binding to the cell surface are distinct from changes that occur after the production of viral antigens by infected T cells and cell lines (*3, 7, 8*). During the initial attachment of HIV to cells, the binding of monoclonal antibodies OKT4A, OKT4D, and Leu-3a is inhibited whereas the binding of OKT4 is not; thus, while some regions on T4 are required for viral binding and infection, the epitope defined by OKT4 is not (*7, 9–11*). However, after the production of viral antigens by infected cells the expression of all epitopes on T4, including those that are not involved in viral binding, are markedly diminished or absent, suggesting the loss of T4 from the cell surface (*2, 3, 7, 12*). This change in T4 expression could result from modulation of the T4 molecule at the cell surface or from the direct effects of viral products on the synthesis of T4 or its transport to the cell surface. We have evaluated both the T4 protein and T4 messenger RNA (mRNA) in infected T-cell lines. In some lines we found that the decreased expression of T4 on the cell surface is associated with reduced amounts of both the T4 protein and mRNA. We also found that the T4 molecule produced by these infected cells occurs as a complex with viral envelope and envelope precursor proteins.

In the first experiment, virus obtained from supernatants of productively infected H9 cells was concentrated 100-fold by ultracentrifugation and added to MOLT-4 cultures. At various times after the addition of virus the reactivity of Mab's OKT4 and OKT4A with the MOLT-4 cell surface was determined by flow cytometry. Control MOLT-4 cells were cultured with supernatant prepared from uninfected H9 cells. As early as 1 hour after the addition of viral concentrate the binding of OKT4A was reduced compared to control cells as indicated by the mean level of fluorescence intensity (Fig. 1) or by the percentage of cells considered positive. Inhibition of OKT4A binding was maximal 24 hours after the addition of virus, when the fluorescence intensity was 45% of that of control cells.

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