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Phil. Trans. R. Soc. Lond. B 1993 **342**, 75-81
doi: 10.1098/rstb.1993.0138

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The recognition of chimeras of rat and human CD4 by HIV-1 gp120 and by monoclonal antibodies

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SUMMARY

The use of chimeras of rat and human CD4 to probe the HIV-1 gp120 and antibody binding properties of CD4 is reviewed. Short segments of human CD4 sequence were substituted for the equivalent regions of rat CD4 which does not bind gp120, and analysis of the properties of these chimeras established: (i) that residues 33–58 of the NH_2-terminal domain of human CD4 encompass the high-affinity gp120 binding site; and (ii) that chimeras containing residues 33–62 mediate HIV-1 infection. The chimera-binding specificities of gp120 and a large panel of anti-CD4 antibodies were also determined. This allowed a critical test of the popular notion that receptor mimics appear at high frequency among antibodies elicited by immunization with receptor ligands and that anti-idiotypic antibodies can be used to identify novel receptors. The data suggest that such mimics appear infrequently, if at all, a result which is consistent with the failure of the anti-idiotype approach to identify new genes encoding receptors with prescribed functions.

1. INTRODUCTION

The T-lymphocyte glycoprotein CD4 is the principal receptor for the human immunodeficiency virus (HIV-1) (Dalgleish *et al.* 1984; Maddon *et al.* 1986). The interaction of the virus with CD4 is mediated by the viral glycoprotein gp120 (McDougal *et al.* 1986). The extracellular region of CD4 was predicted to consist of four immunoglobulin-related domains (Clark *et al.* 1987), a proposal that has now been confirmed by crystallographic analyses of gp120-binding recombinant forms of human CD4 consisting of domains 1 and 2 (Ryu *et al.* 1990; Wang *et al.* 1990), and recombinant forms of rat CD4 consisting of domains 3 and 4 (Brady *et al.* 1993).

The NH_2-terminal domain of human CD4 expressed in bacteria was shown to bind gp120 with high affinity (Arthos *et al.* 1989; Chao *et al.* 1989) and *in vitro* mutagenesis has been used in a number of attempts to delineate the extent of the gp120 binding site of this domain. Initial studies suggested that residues 42–49, which constitute the region of CD4 domain 1 equivalent to the CDR2 region of immunoglobulins, encompass the gp120 site (Peterson & Seed 1988), and the importance of this region has been confirmed in all subsequent analyses. However, some studies of gp120 binding to CD4 have implicated additional residues lying outside this region. Specifically, mutations at residues 58, 59, 67 and 68 in one study (Brodsky *et al.* 1990), and at residues 29, 59–64, 77–81 and 85 in another study (Ashkenazi *et al.* 1990) were shown to disrupt gp120 binding. In a third study mutations in domain 2 were found to disrupt the interaction of CD4 with gp120 (Clayton *et al.* 1988).

A similar level of ambiguity arose out of analyses of the regions of CD4 involved in the post-binding events leading to HIV-1 entry. It had been proposed that the gp120-binding site may not be sufficient and that regions outside the binding site might be involved in entry. For example, monoclonal antibodies that bind to the region of CD4 domain 1 equivalent to the CDR3 region of immunoglobulins were shown to block syncytium formation and viral entry while only poorly inhibiting the binding of gp120 or HIV-1 (Truneh *et al.* 1991; Corbeau *et al.* 1993). Similarly, derivatized peptides corresponding to residues 81–92 of human CD4, which encompass the CDR3-equivalent region, inhibit post-binding events (Berger *et al.* 1991). A third study implicated this region by demonstrating that chimpanzee CD4 and human CD4 with a chimpanzee CDR3-equivalent loop were unable to mediate HIV-dependent syncytium formation unlike wild-type human CD4 or chimpanzee CD4 with a human CDR3 loop (Camerini & Seed 1990). In contrast to these studies, the CDR3 equivalent region has been comprehensively mutated without significant

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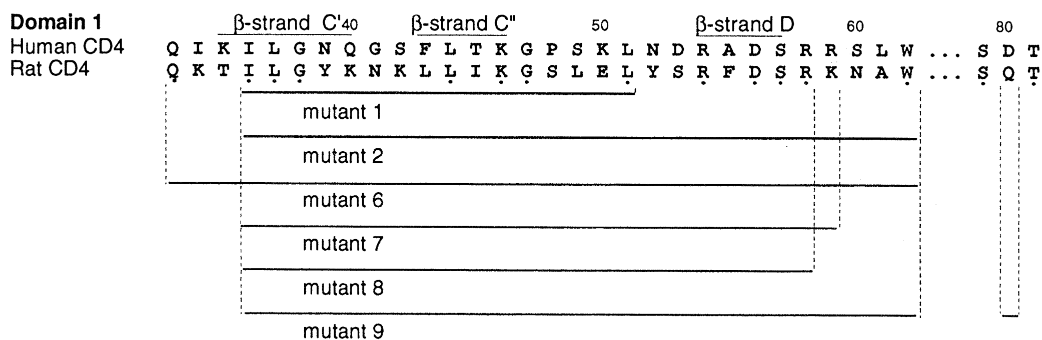

Figure 1. Human and rat CD4 domain 1 sequences (Maddon *et al.* 1985; Clark *et al.* 1987) that were exchanged in the various chimeras are underlined. The β strands are as assigned by Ryu *et al.* (1990) and by Wang *et al.* (1990) and the dots under the rat sequence indicate the residues that are identical in the two species. Reproduced from *J. exp. Med.* **175**, pp. 301–304 (1992), by copyright permission of the Rockefeller University Press.

effect on syncytium formation (Broder & Berger 1992), and peptides corresponding to this region have been shown to also interfere with the entry of the human T-lymphotrophic virus-I (HTLV-1), which, as far as is known, does not interact with CD4 (Repke *et al.* 1992).

The extracellular domains of human and rat CD4 share only 54% amino acid identity and rat CD4 does not bind HIV-1 nor mediate HIV-1 infection. In studies reviewed here, a series of CD4 chimeras in which short segments of human CD4 sequence replaced the equivalent segments of rat CD4 sequence, were used to probe the HIV-1 gp120 and antibody binding properties and HIV-1 infection-mediating function of CD4, and to resolve some of the ambiguities described above. The chimeras of rat and human CD4 also allowed a critical test of the popular notion that receptor mimics appear at high frequency among antibodies elicited by immunization with receptor ligands and that anti-idiotypic antibodies can be used to identify novel receptors.

2. ANALYSIS OF THE HIV-1 gp120 BINDING SITE OF CD4

The regions of rat CD4 domain 1 that were exchanged in the chimeras in order to probe the gp120 binding site are shown below the rat and human CD4 protein sequences in figure 1 (Schockmel *et al.* 1992). The first mutant (mutant 1) contained human CD4 sequence from residues 36 to 51 which are centred on β-strands C′ and C″ and encompass the region first proposed as the gp120 binding site (Peterson & Seed 1988). However, when expressed in a soluble form this mutant did not bind gp120 nor did it mediate HIV-1 infection when expressed on the surface of HeLa cells which have been shown elsewhere to be highly permissive for HIV-1 infection after transfection with the human CD4 gene (Maddon *et al.* 1986). In a second round of mutagenesis this region was therefore extended to include residues 36–62 on the basis of a study by Arthos *et al.* (1989) which suggested that residues beyond Leu-51 in β-strand D were part of the gp120 binding site. The first of these mutants (mutant 2) bound gp120 when expressed transiently on the surface of COS-1 cells (data not shown).

An inhibition binding assay was developed using ^{125}I-labelled human soluble CD4 and with this assay it was shown that the affinity of a soluble form of mutant 2 for gp120 was approximately 70-fold lower than that of human CD4 (figure 2). This result indicated that although a gp120 binding site had been generated in mutant 2 the gp120 binding site had not been fully reconstituted so a final round of mutagenesis, yielding mutants 6–9 (figure 1), was undertaken after inspection of the human CD4 structure (Ryu *et al.* 1990; Wang *et al.* 1990) and in the light of current analyses of the binding site by *in vitro* mutagenesis (Ashkenazi *et al.* 1990).

The affinities of mutants 6–9 for gp120 were determined using the inhibition binding assay (figure 2) and it was found that mutant 6 had an affinity only twofold less than that of human CD4 indicating that the gp120 binding site is encompassed by residues 33–62 and that the C, C′ turn has a key role in forming the gp120 binding site. However, it was not clear whether residues 33–36 have a conformational role, or directly contact gp120, or both. A recent kinetic study of CD4 mutants by Moebius *et al.* (1992) suggests that Lys-35 is a contact residue. The improvements in affinity wrought by the changes in mutants 7 and 8 relative to mutant 2 further delimits the gp120 binding site to residue 58 at the COOH-terminal end. The modest improvement in the affinity of mutant 9 relative to mutant 2 suggests that Asp-80 does not play a key role in binding.

Whereas in previous studies residues outside this region have been mutated to disrupt gp120 binding the new data argued against the possibility that these regions are part of the binding site. The study by Moebius *et al.* (1992), of largely single mutants of the gp120 binding site, prompted the conclusion that the binding site consists of the exposed hydrophobic Phe at position 43 which is surrounded by the four positively charged residues Lys-29, Lys-35, Lys-46 and Arg-59. This conclusion is in good agreement with the analysis of the chimeras given that the Lys at position 29 is conserved between human and rat CD4. It is of interest that both Lys and Arg residues are tolerated at position 59; this would imply that either these residues do not directly contact gp120 or that it is only charge that is important at this position.

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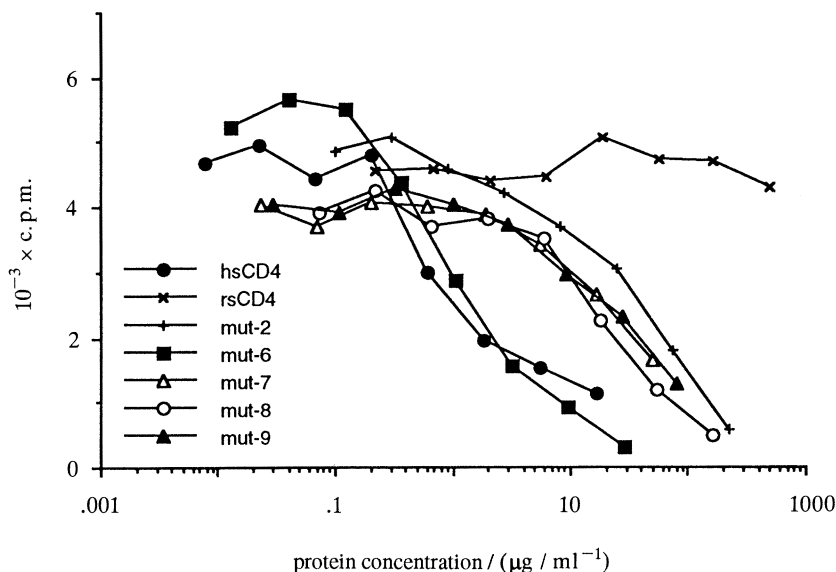

Figure 2. The binding of gp120 to soluble forms of the chimeras of rat and human CD4. The various CD4 mutants (mut-2 to mut-6), expressed in soluble form in Chinese hamster ovary cells (Davis *et al.* 1990), were serially diluted and their binding to gp120 assayed by their ability to inhibit the binding of ^{125}I-labelled human sCD4 to gp120 that had been immobilized to plastic with rabbit anti-mouse IgG and an anti-gp120 antibody (mAb 108). The relative affinities of these interactions were compared with those involving rat soluble CD4 (rsCD4)and human soluble CD4 (hsCD4). Reproduced from *J. exp. Med.* **175**, pp. 301–304 (1992), by copyright permission of the Rockefeller University Press.

3. ANALYSIS OF HIV-1 INFECTION MEDIATED BY THE CD4 CHIMERAS

Having established that a gp120 binding site could be generated by replacing residues 33–62 in rat CD4 with the equivalent residues of human CD4, the same approach was used to determine whether regions of CD4 beyond the gp120 binding site are essential for the post-binding events involved in HIV-1 infection. The affinities of mutants 2 and 6 for gp120 are 70-fold and twofold lower than that of human CD4, respectively, thus allowing a preliminary assessment of the importance of affinity in determining the kinetics of infection.

HeLa cells were transfected with constructs encoding either human or rat CD4 or mutants 2 or 6 and the cells were then challenged with HIV-1. Assays of p24 and reverse transcriptase release (figure 3) indicated that similar levels of HIV-1 replication occurred in the cultures of HeLa cells expressing hCD4 and mutant 6, although the kinetics of infection were slightly slower in the latter. In contrast, there was no replication in the cultures of HeLa cells expressing mutant 2 or rat CD4. Additional co-cultivation assays of syncytium formation and polymerase chain reaction-based assays of entry indicated that the slight delay in replication kinetics in cultures of mutant 6 expressing HeLa cells was the result of a three- to tenfold reduction in the efficiency of viral entry at the initial stage of infection.

The results suggest that HIV-1 infection of susceptible cells depends only on the presence of a high-affinity gp120 binding site on CD4. It is unlikely that infection and syncytium formation depends on the sequence of the CDR3 equivalent region of CD4 (i.e. residues 86–89), as had been proposed (Camerini & Seed 1990; Berger *et al.* 1991; Truneh *et al.* 1991), since this region is substantially different between the human (Val-Glu-Asp-Gln) and rat (Leu-Glu-Asn-Lys) homologues. The discordance between this result and the ability of antibodies that bind to the CDR3 equivalent region to block syncytium formation (Truneh *et al.* 1991; Corbeau *et al.* 1993) suggests that such antibodies indirectly sterically hinder the interaction between CD4 with the whole virus, and that such analyses of adjacent but non-overlapping epitopes should be interpreted cautiously. It is of interest that mutant 2 was unable to mediate infection in spite of having an affinity only 70-fold lower than that of human CD4 and that the efficiency of viral entry via mutant 6 was somewhat lower than might be expected given the high affinity of this mutant for gp120. This suggests that there will be *in vivo* selection against mutations that reduce the affinity of HIV-1 gp120 for CD4, a contention which is supported by the observation that gp120 molecules from primary isolates of HIV-1 have uniformly high affinities for CD4 (Brighty *et al.* 1991; Ashkenazi *et al.* 1991; Turner *et al.* 1992).

4. ANTIBODY RECOGNITION OF CD4: IMPLICATIONS FOR ANTIBODY MIMICRY

It has been argued that the binding sites of antibodies raised against a given ligand can mimic the receptors of that ligand and that anti-idiotypic antibodies, generated against these binding sites in a second immunization, will mimic the ligand and bind the relevant receptor (Gaulton & Greene 1986; Vaux *et al.* 1988, 1990; Kohler *et al.* 1989). The idea that such an approach would be useful for identifying novel receptors arose out of the concept that antibodies bear the 'internal image' of antigens which also lead to the

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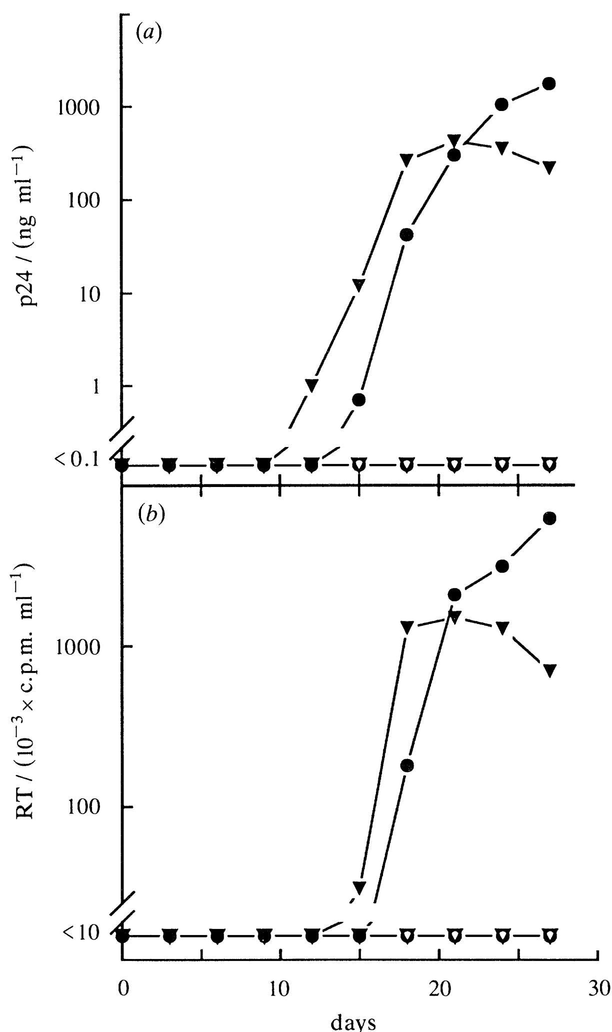

Figure 3. HIV-1 infection of HeLa cells mediated by the rat and human CD4 chimeras. HeLa cells expressing rat CD4 (open circles), mutant 2 CD4 (open triangles), mutant 6 CD4 (filled circles) and human CD4 (filled triangles) were challenged with HIV-1 and samples of cell culture supernatant were taken every three days post infection. The supernatants were analysed for the presence of HIV-1 by (*a*) ELISA assays for the p24 antigen and by (*b*) reverse transcriptase assays. Reproduced from *J. exp. Med.* **177**, 949–954 (1993), by copyright permission of the Rockefeller University Press.

proposal that the anti-idiotype approach could be used for the generation of vaccines (reviewed by Gaulton & Greene 1986; Kohler *et al.* 1989). Thus, it was suggested that an antibody against CD4 that mimics the CD4 binding function of gp120 might be useful as a vaccine against HIV-1 by stimulating the production of anti-idiotypic antibodies that recognize gp120 (Chanh *et al.* 1987; Dalgleish *et al.* 1987).

The combinatorial size of the B-cell repertoire of mice has been estimated to be larger than 10^{11} different antibodies (Davis 1990); somatic mutation will considerably increase the repertoire. Thus, the key issue that will determine the likelihood of success of the anti-idiotype approach is the frequency with which mimics will appear rather than whether mimicry is possible at all. Published experiments involving the anti-idiotype approach suggest that workers in this field expect receptor mimics to appear at very high frequency after immunization with the ligand. Thus, in some studies single anti-ligand monoclonal antibodies have been used to generate anti-idiotypic antibodies without rigorous justification for the choice of the particular anti-ligand antibody (e.g. Bjercke & Langone 1989; Kouklis *et al.* 1991). Others have used entire pools of purified first-round polyclonal antibodies to generate anti-idiotypic responses in the apparent expectation that the relevant mimics will be highly represented in such pools (Barel *et al.* 1988; Pain *et al.* 1988, 1990). However, the most ambitious approach is 'auto-anti-idiotypic' and relies on both rounds of antibody formation occurring within a single animal immunized with the ligand (Jennings & Cotton 1990; Lombes *et al.* 1990; Djabali *et al.* 1991).

Until recently, the frequency with which antibody mimics might be found had not been determined for any given receptor/ligand pair. The interaction of CD4 with gp120 presented a favourable opportunity for examining the probability of finding antibody mimics of a receptor because the gp120 viral receptor is available for binding studies and a very large number of anti-CD4 antibodies have been raised in the course of studies of HIV-1 pathogenesis. The generation of gp120 binding sites in the chimeras of human and rat CD4 allowed the frequency of gp120 mimics within a large pool of the anti-CD4 antibodies to be estimated.

(*a*) *Initial antibody screening*

The anti-CD4 antibodies studied were derived from a total pool numbering 225 and these were kindly provided by E. P. Rieber (University of Munich), D. Buck (BioTech Resources Inc., San Antonio), F. Emmrich (Max Planck Institute, Erlangen), and the MRC AIDS Directed Programme. All the antibodies were raised by immunizing mice with human CD4 in soluble or cell-associated form. The antibodies were first screened by binding analyses of COS-1 cells transiently expressing mutant 2, the first mutant found to bind gp120. It was argued that any anti-CD4 antibody that was a potential mimic of gp120 should also bind to mutant 2 but only 10 of the 225 antibodies bound to this mutant (Davis *et al.* 1992). The antibody Leu3A, which has been proposed as a gp120 mimic (Chanh *et al.* 1987; Dalgleish *et al.* 1987), was among those that did not bind, a result that is consistent with reports that immunizations with Leu3A fail to induce anti-HIV activity (Healey *et al.* 1991; Reeves *et al.* 1991; Wilks *et al.* 1991).

(*b*) *Comparison of the fine specificities of gp120 and the antibodies*

To identify putative gp120 mimics within this group of ten antibodies their fine specificities were compared with that of gp120. This was done by determining their relative affinities for soluble forms of the chimeric CD4 molecules shown in figure 1 using the inhibition binding assay. The results are shown in

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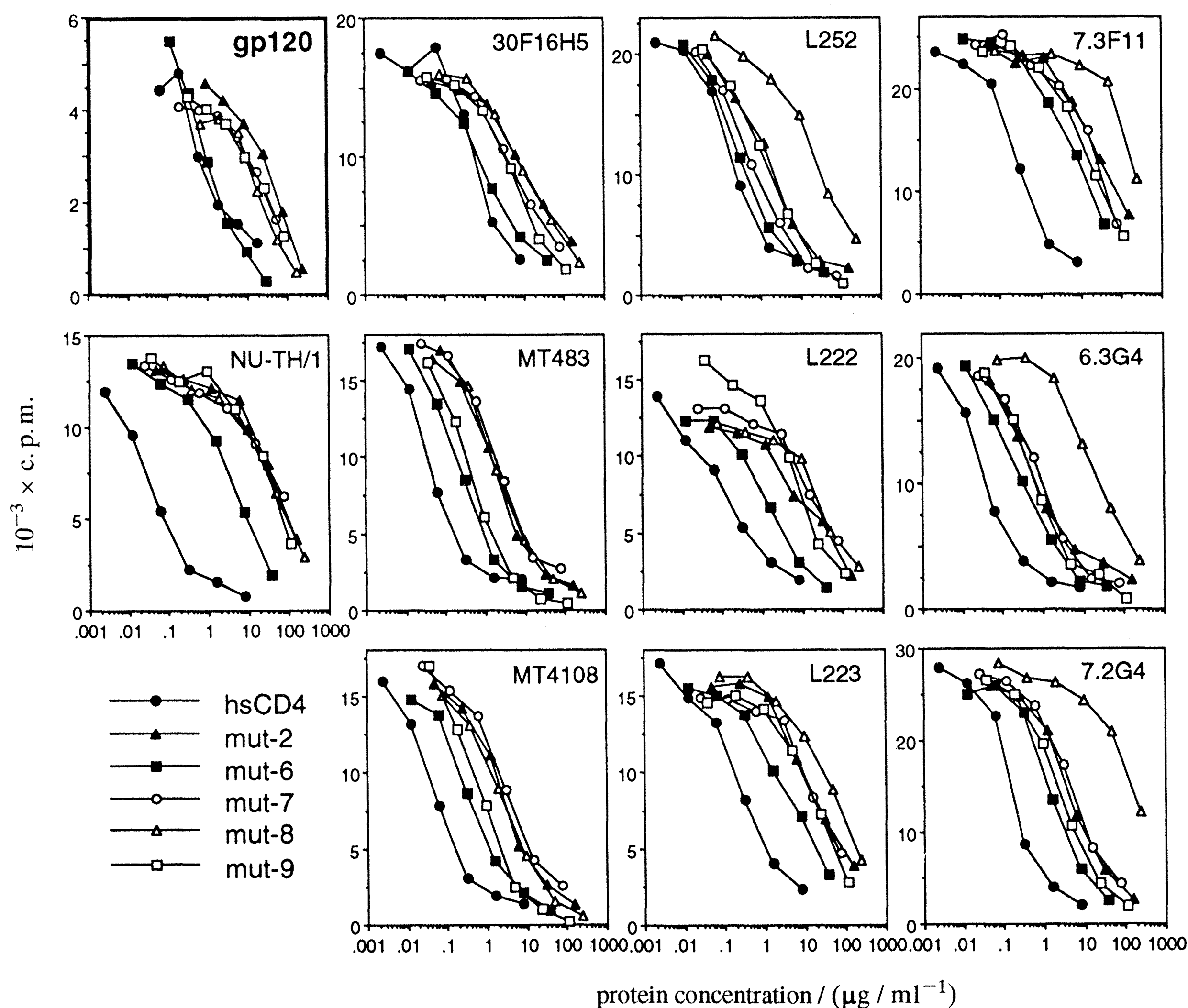

Figure 4. The fine specificities of the antibodies and gp120 for the chimeras of human and rat CD4. The CD4 mutants (mut-2 to mut-6) were serially diluted and their binding to gp120 or the anti-CD4 antibodies assayed by their ability to inhibit the interaction of ^{125}I-labelled human sCD4 with immobilized gp120 or antibody. The relative affinities of these interactions were compared with those involving human soluble CD4 (hsCD4). Reprinted with permission from *Nature, Lond.* **358**, pp. 76–79. Copyright (1992) Macmillan Magazines Limited.

figure 4. It was found that none of the antibodies had the same specificities as gp120 but it was clear that, were mimics to be present, these would be detected using this approach since the antibodies MT4108 and MT483, L222 and L223, 6.3G4 and 7.2G4, which had been assumed to be independent, had identical binding profiles. The antibody 30F16H5 was most similar to gp120 according to this assay, but this antibody differed from gp120 in that unlike gp120 it bound mutant 5, a mutant 2-based chimera which has six residues of human CD4 sequence substituted in domain 2 and that also has the rat CD4 glycosylation signal removed from this domain (Schockmel *et al.* 1992). A kinetic analysis of CD4 binding by the antibodies and gp120 provided additional confirmation that none of the ten antibodies mimic gp120 and that the antibodies are more similar as a group than they are similar to gp120.

These experiments established that less than five percent of antibodies raised by immunization with CD4 bind to the same region as gp120. Moreover, the fine specificities and kinetic properties of the antibodies indicated that none of the 10 antibodies that bind this region bind in the same way as gp120. Thus, the overall probability of finding a mimic of gp120 is less than 1/225. This result was compatible with the work of Bentley *et al.* (1990) who have determined the three-dimensional structure of complexes of an anti-lysozyme antibody with lysozyme and with an anti-idiotypic antibody. Although the binding sites of the anti-idiotypic antibody and lysozyme overlapped on the surface of the anti-lysozyme antibody, the nature of the contacts in this region were significantly different. The number of different antibodies that can be generated against a given immunogen has been studied by Kreth & Williamson (1973) who estimated the number to be 8000 for the hapten dinitrophenol, and by Staudt & Gerhard (1983) who estimated the minimal repertoire of anti-haemagglutinin murine antibodies to number 1500. In the light of the comparison of gp120 and the anti-CD4 antibodies it is not unreasonable to conclude that the fine specificities

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of each of these anti-dinitrophenol and anti-haemagglutinin antibodies would differ in the manner of lysozyme and the anti-anti-lysozyme anti-idiotypic antibody. The frequency of mimics of a given immunizing ligand that are suitable for receptor discovery is therefore likely to be considerably less than $(1/225)^2$.

Given the low probability of finding an anti-idiotypic mimic of an immunizing ligand, the generation of an anti-anti-idiotypic antibody by Budisavljevic *et al.* (1988) that binds the original immunogen, angiotensin II, is a surprising result. The crystal structure of the complex of the anti-anti-idiotypic antibody and angiotensin II has now been solved (Garcia *et al.* 1992*a*) and the gene sequences of the original antibody (Ab1) to angiotensin II and the anti-anti-idiotypic antibody (Ab3) determined (Garcia *et al.* 1992*b*). Garcia *et al.* (1992*b*) argued that molecular mimicry of angiotensin II by the anti-idiotypic antibody accounts for the remarkable degree of identity in the sequences of the Ab1 and Ab3 antibodies proposed to bear an 'internal image' of angiotensin II. However, the greatest degree of sequence identity lies outside the regions that the crystal structure shows are in direct contact with angiotensin II. For example, the CDR2 sequences of the light chains are completely conserved even though the light chain CDR2 of the anti-anti-idiotypic antibody does not contact angiotensin II (Garcia *et al.* 1992*a*,*b*). It is not obvious why this should be so given that these highly conserved non-contacting residues are unlikely to contribute to the generation of an 'internal image' of angiotensin II. Thus, it would appear that other processes have driven antibody selection in this instance and claims that it represents a *bona fide* case of antibody mimicry may be premature.

Since 1978 and the first attempt to use the anti-idiotypic approach for receptor discovery, a relatively large section of the medical and biological literature has documented the discovery of numerous antibodies purported to be specific for a variety of new and important receptors. In spite of this it would appear that none of these antibodies has subsequently been useful for the isolation of the genes encoding the relevant receptors which must be the obvious next stage of receptor characterization. As of October 1992, the SWISSPROT database contained 105 entries for receptor sequences, none of which were isolated with the anti-idiotypic approach and shown to have the requisite function. Thus, considerably more successful alternative approaches to receptor characterization do exist. The analysis of the anti-CD4 antibodies described above provided an explanation for the failure of the anti-idiotypic approach to receptor discovery.

All of the work reviewed in this paper was inspired by the thinking of the late Professor Alan F. Williams, F.R.S. The authors are also grateful to Dr Susan Daenke, Dr A. Neil Barclay and Dr Don W. Mason for critical reading of the manuscript.

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