Antibody Raised against Soluble CD4-rgp120 Complex Recognizes the CD4 Moiety and Blocks Membrane Fusion without Inhibiting CD4-gp120 Binding

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Summary
We studied the humoral response of mice immunized with soluble CD4-rgp120 complex, testing polyclonal and monoclonal antibodies (mAbs) with the aim of identifying molecular changes that take place after the first interaction between human immunodeficiency virus and the cell surface. The antisera had a paradoxically high syncytia-blocking titer associated with anti-CD4 specificity, while their capacity to inhibit CD4-gp120 binding was relatively modest. One of the mAbs produced from these responders blocks syncytia formation but does not inhibit CD4 interaction with gp120. Apparently, this mAb interacts with the CD4 moiety of CD4-gp120 complex and prevents a post-binding event necessary for membrane fusion and viral infection.

The protein CD4 on the surface of Th cells and macrophages was identified as the receptor for HIV (1, 2), and its capacity to bind the viral envelope protein gp120 with high affinity was subsequently demonstrated (3). While this interaction is essential for the virus to eventually infect the target cells, there are indications that the CD4-gp120 binding initiates a chain of events, some of which may be equally important for virus penetration and infection. This is supported by claims that some antibodies directed against determinants positioned far from the CD4-binding site on gp120 (4) or against gp41 (5, 6) exhibit neutralizing capacity. This property is analogous to that of antienvelope glycoprotein antibodies in the case of herpes and EBV (7, 8). We have observed a dissociation of neutralizing capacity and gp120 binding inhibition in anti-CD4 antibodies; this indicates a symmetrical relationship suggesting that events and/or signals occurring at the level of CD4 may represent such post-binding steps on the side of the cell membrane. For example, after binding to gp120, changes in conformation could reshuffle the fine antigenic structure, and new or modified epitopes associated with distinct functional states of the molecule may come into existence. Antibody binding to such induced or unmasked determinants could result in interruption of the chain of events leading to viral penetration of the cell.

With the aim of defining post-binding markers, we have immunized mice with complexes of recombinant gp120 and CD4, and have obtained high titers of syncytia-blocking antibodies mainly directed at the CD4 moiety. In this report, we describe specificity and function of polyclonal and monoclonal antibodies resulting from these experiments, notably of those mAbs that inhibit syncytia formation by a mechanism different from blocking gp120-CD4 binding.

Materials and Methods

Recombinant Molecules. Soluble rCD4, CD4 immunoadhesin (containing V1 and V2 domains of CD4 spliced to CH2 and CH3 domains of human IgG) (9), and rgp120 were produced by Genentech Inc. (South San Francisco, CA).

Animals. BALB-c female mice 10-15 wk old (The Jackson Laboratory, Bar Harbor, ME) were used both for immunization and for production of mAbs.

Immunizations. Mice were injected intraperitoneally with antigen emulsified in CFA (Difco Laboratories Inc., Detroit, MI). The antigens and doses were CD4, 12.8 μg/mouse; gp120, 10 μg/mouse; and CD4-rgp120, 12.8 μg CD4 and 10 μg gp120, thoroughly mixed and incubated for 20 min, and then emulsified in CFA. The mice were bled before immunization and every week after, for 13 wk. Serum samples were stored at −20°C.

Enzymes and Substrates. Alkaline phosphatase and glutaraldehyde, used to label antibodies for ELISAs, and substrate paranitro-
were used for each determination. Serum diluted 1:10

Abbreviations used in this paper: PNPP, paranitrophophosphate; RT, room temperature; sCD4, soluble CD4.

Results

Polyclonal Responses to Injection of Soluble CD4 (sCD4)-gp120 Complexes in Mice. Three groups of four mice were injected once, either with rCD4 alone (Fig. 1, A, D, and G), with sCD4-rgp120 complex (Fig. 1, B, E, and H), or with rgp120 alone (Fig. 1, C, F, and I). The weekly bleedings were titrated over a 3-mo period for capacity to bind CD4 (Fig. 1, D–F) and gp120 (Fig. 1, A–C), and for capacity to inhibit the formation of syncytia (Fig. 1, G–I). The overall results were strikingly different among the three groups. Fig. 1 displays the individual titers for each parameter and for each group. There were often multiple peaks during the response (D), and mice of a single group showed different timings for their peaks (D and H). When the timing and magnitude of the binding and neutralizing responses were examined: (a) there was no clear correlation between the titers of gp120 binding and syncytia blocking (B vs. H); (b) there was an inverse relationship between sCD4- and CD4-rgp120-immunized groups when sCD4 binding titers and syncytia blocking were compared (D vs. G and E vs. H); or (c) there was a small CD4-binding response in the group immunized with rgp120, which could be attributed to antiidiotypes (F).

Polyclonal Syncytia-blocking Responses by Mice Receiving CD4-gp120 Complexes Are Not Type Specific. The antisera from mice injected with CD4-gp120 efficiently block syncytia formation caused by such widely different HIV isolates as HTLV3-IIIB and HTLV3-RF. Fig. 2 shows individual titrations of the sera from these mice, revealing a similar rank order of the individual responses against the two isolates (93 > 94 > 91 > 92).

The Syncytia-blocking Capacity of CD4-gp120 Responders Is Absorbed by CD4 and not by gp120. To determine whether the high capacity of sera from complex-immunized mice to inhibit syncytia formation was due to anti-CD4 or anti-gp120 antibodies (or to a cooperative action of the two), a series of absorption experiments was performed using soluble and solid-phase bound antigens. The samples were subsequently monitored for changes in binding or syncytia-blocking titers. The results unequivocally attributed the syncytia-blocking capacity to antibodies that recognize the CD4 moiety of the complex rather than anti-gp120, since absorbed anti-gp120 serum shows unaltered syncytia blocking, while the decrease of CD4 binding is accompanied by a significant decrease of syncytia blocking with both isolates tested (Table 1).

The Capacity to Inhibit CD4-gp120 Binding In Vitro Correlates with the CD4-binding Titer and not with the Syncytia-blocking Titer. Since the syncytia-blocking capacity observed is mediated by anti-CD4, the inverse relationship of binding and syncytia-blocking titers in mice receiving CD4 vs. CD4-gp120 had to be attributed to a difference in the fine specificity of
the anti-CD4 antibodies in the two groups. To further characterize the anti-CD4 antibodies, one obvious test was to compare their relative capacity to inhibit gp120 binding to CD4. The results (Table 2) showed high inhibition titers in all mice immunized with CD4 alone and low titers in those immunized with the CD4-gp120 complex, indicating that the blocking of syncytia by the latter appears to be mediated by a mechanism other than prevention of binding of HIV to its receptor on the cell surface.

Study of Anti-sCD4-gp120 Responses Using mAbs. To dissect the polyclonal response of mice immunized with CD4-gp120 complex, hybridomas were produced from one of these animals, and the resulting antibodies were characterized for capacity to bind CD4, to bind gp120, and to block syncytia formation. Table 3 shows an early test of 170 wells with hybridoma clones, ordered according to their CD4 binding capacity. 30 (the majority) of the positive clones produced anti-CD4; only four produced anti-gp120, and the remainder were negative for both. Three of the anti-CD4 clones (and none of the anti-gp120) exhibited capacity to block syncytia. All borderline positives (i.e., those showing <0.100 in the gp120 binding) and those with partial blocking of syncytia (clones

Figure 1. The time course of an experiment with three groups of mice injected with CD4 (A, D, and G), CD4-gp120 complex (B, E, and H), and gp120 (G, H, and I). The weekly serum samples were assayed individually for gp120-binding antibodies (A, B, and C), CD4-binding antibodies (D, E, and F), and syncytia-blocking capacity (G, H, and I). The mice immunized with the complex showed a somewhat higher anti-gp120 response than those immunized with gp120 alone (B vs. C); a markedly lower titer of CD4 binding as compared with those receiving CD4 alone (E vs. D); and a significantly higher syncytia-blocking response (H vs. G).

Figure 2. Titration of 11-wk serum from four mice injected with CD4-gp120 complex for III_b and RF syncytia-blocking capacity. The parallel behavior of individual sera in the two tests suggests that the antibodies are directed at group-specific determinants.
Table 1. Absorption of Pooled Sera from Mice Immunized with CD4-gp120, Demonstrating that Syncytia Blocking Is Associated with anti-CD4

| Absorbant | CD4 binding | gp120 binding | syncytia
|------------|-------------|----------------|------------------|
| OD* (%) | OD* (%) | IIb | RF |
| None | 1.216 (100) | 0.940 (100) | 0 | 3 |
| gp120 | 1.180 (97) | 0.188 (20) | 0 | 5 |
| CD4 | 0.420 (34) | 0.936 (99) | 11 | 24 |
| No antiserum | - | - | 52 | 60 |

* OD450/60 min (color developed by phosphatase hydrolysis of PNPP).
† Syncytia blocking assay as described in Materials and Methods.

116, 95, and 32) became negative after subcloning. Table 4 shows a further characterization of the hybridomas when the inhibition of gp120-CD4 binding test was performed. The anti-CD4 mAbs can be divided into three categories: (a) those that do not inhibit gp120 binding and do not block syncytia; (b) those that do not inhibit gp120 binding and block syncytia; and (c) those that inhibit gp120 binding and block syncytia. mAbs 55 and 94 were further studied as representatives of the latter two categories, respectively. Both were of IgG1 isotype.

Preliminary Mapping Experiments. The binding site of both mAbs 55 and 94 was localized within the first two domains (V1-V2) of CD4 by binding experiments using the CD4 IgG immunoadhesin (Genentech), which contains the two external domains of CD4 spliced to an IgC region (data not shown). A crossinhibition experiment using labeled mAbs 94 and 55 was performed. The binding of these mAbs to solid-phase CD4 was tested in the presence of a series of anti-CD4 mAbs whose epitopes and binding characteristics are known or partially known from the literature. The two mAbs were not inhibited by any of the tested antibodies with the exception of L83, which showed a partial competition with mAb 55 (Table 5). These results do not provide a precise mapping of the binding sites of the two mAbs. OKT4A and anti-Leu-3a bind in the first domain of CD4, and the test shows that neither binding site overlaps with mAbs 55 and 94. L83, according to unpublished data (David Buck, personal communication), recognizes a conformational determinant that is affected by mutations both in region 8–40 (V1) and region 119–188 (V2). These data, taken together, indicate that the fine specificity of mAbs 55 and 94 are different from most studied antibodies and different from each other.

Interference of gp120 with CD4 Binding by mAbs. Preliminary experiments had shown that mAb 94 blocked gp120 binding to CD4, while mAb 55 did not. To be able to detect both inhibition and possible cooperation between antibody and gp120, we set up a series of three ELISAs by which the ternary interaction of CD4, antibody, and gp120 was examined by keeping in turn one of the reactants in solid phase, and varying the concentrations of the other two. The results are shown in Figs. 3, 4, and 5, which also include curves obtained with reference antibodies OKT4A (which competitively interferes with CD4-gp120 binding) and OKT4 (which does not). In Fig. 3, the binding of labeled CD4 to gp120 is slightly enhanced or nonsignificantly changed in the presence of increasing concentrations of 55, while it is progressively inhibited by 94. In Fig. 4, the binding of labeled CD4 to solid-phase captured antibody is increased 40% by gp120 in the case of 55 at the highest concentration of CD4, but is unaffected or slightly decreased at lower concentrations of CD4. In the case of 94, there is progressive decrease of CD4 binding in the presence of increased gp120 concentrations, more evident when CD4 is limiting. In Fig. 5, the binding of labeled 55 to solid-phase CD4 is moderately enhanced in the presence of 0.1 or 1.0 μg/ml gp120, while the binding of 94 is depressed in these conditions.

The conclusions of this series of experiments are that: (a) mAb 94 behaves always as an inhibitor/competitor of the CD4-gp120 binding; and (b) mAb 55 in certain conditions does not interfere with the binding in a way similar to OKT4, while in other conditions, it shows a degree of cooperativity with CD4-gp120 binding.

Discussion

It is known that the HIV infection of CD4+ cells can be prevented by antibodies specific for the gp120-binding site on CD4, defined as the V1 domain of CD4 involved in the initial CD4-gp120-binding event, i.e., the region homologous to CDR-2, amino acids 41–52 (11–14), and in part CDR-3, amino acids 83–92 (15, 16). These antibodies prevent infection by sterically interfering with the binding site.

We have obtained polyclonal and monoclonal antibodies from mice immunized with CD4 complexed to gp120 and, in this paper, we have described their binding characteristics

| Pool | CD4 binding | Blocking gp120 binding | Fusion blocking |
|------|-------------|------------------------|----------------|
| 9 (immunized CD4-gp120) | 2.4 | 80.0 | 45.0 |
| 18 (immunized CD4) | 31.0 | 890.0 | 7.0 |

* One blocking U50 is the amount of antibody that reduces to 50% the amount of gp120 bound to CD4-coated wells or, respectively, the number of syncytia in the fusion test. U50/ml was calculated by multiplying 1 U50 by the dilution factor for each test.

Table 2. Difference in Fine Specificity Distribution among Polyclonal Anti-CD4 Antibodies
Table 3. List of Hybridomas Obtained from Fusion 91, Hierarchically Ordered According to their Supernatant’s Capacity to Bind CD4, and Tested for Binding gp120 and Blocking Syncytia Formation

| Hybridoma designation | Binding CD4 | Binding gp120 | No. of Syncytia blocking |
|-----------------------|------------|---------------|-------------------------|
| Clone                  | at 1/2 dilution |              |                         |
| 1                      | 135        | 1.363         | 0                       |
|                        | 144        | 1.208         | 0                       |
|                        | 148        | 1.149         | 0.007                   |
|                        | 75         | 1.059         | 0                       |
|                        | 215        | 1.049         | 0                       |
|                        | 145        | 0.982         | 0                       |
|                        | 84         | 0.932         | 0                       |
|                        | 142        | 0.930         | 0.012                   |
|                        | 140        | 0.837         | 0                       |
| 10                     | 210        | 0.724         | 0.015                   |
|                        | 185        | 0.672         | 0                       |
|                        | 143        | 0.599         | 0.009                   |
|                        | 201        | 0.482         | 0                       |
|                        | J          | 0.437         | 0.006                   |
|                        | 203        | 0.398         | 0.006                   |
|                        | 15         | 0.385         | 0.010                   |
|                        | 156        | 0.372         | 0.028                   |
|                        | 58         | 0.313         | 0.011                   |
|                        | 224        | 0.308         | 0                       |
| 20                     | 146        | 0.255         | 0                       |
|                        | 55         | 0.182         | 0.09                    |
|                        | 165        | 0.157         | 0                       |
|                        | 36         | 0.153         | 0.041                   |
|                        | 172        | 0.145         | 0.006                   |
|                        | 94         | 0.136         | 0                       |
|                        | 223        | 0.125         | 0                       |
|                        | 59         | 0.121         | 0                       |
|                        | V          | 0.109         | 0                       |
|                        | R          | 0.106         | 0.012                   |
| 30                     | 48         | 0.106         | 0                       |
|                        | 35         | 0             | 0.361                   |
|                        | 40         | 0             | 0.358                   |
|                        | M (0.023)  | 0.176         |                          |
|                        | 68         | 0             | 0.116                   |
|                        | 116        | 0.012         | 0                       |
|                        | 95         | 0             | 0                       |
|                        | 32         | 0             | 0                       |

Table 4. Classification of mAbs from Mice Immunized with CD4-gp120 Complex

| mAbs                   | Binding Binding CD4-gp120 | Group-specific syncitia blocking |
|------------------------|---------------------------|---------------------------------|
|                        | binding                   |                                 |
| 48, 35, 40             | +                         | -                               |
| 68                     |                           |                                 |
| 135, 144, 148          | -                         | +                               |
| 75, 215, 145           |                           |                                 |
| 210, 185, 143          |                           |                                 |
| 94, 36                 | -                         | +                               |
| 55                     | -                         | +                               |

* OD$_{40}$/120 min, when the supernatant was tested in ELISA on plates coated with CD4 (respectively, gp120) (see Materials and Methods).
† Only numbers lower than the control are shown.

and capacity to prevent the formation of HIV-dependent syncytia, while the study of their effect on cell-free virus infection is in progress. The data presented here, and also results in other laboratories (Q. Sattentau, personal communication), demonstrate that there are epitopes on CD4, unrelated to the binding site of gp120, which antibodies can recognize, thus affecting post-virus-binding events that usually lead to infection. Molecular transitions can be relatively slow, and it is conceivable that several successive steps, subsequent to binding, may be susceptible to interruption. The finding that anti-CD4 mAbs with different fine specificity show similar behavior is consistent with this scenario.

Antibodies that recognize viral envelope proteins have been shown to sometimes inhibit viral infection at a post-binding step both in the case of HIV (4–6) and herpes viruses (7, 8). Several of these antibodies are directed at proteins that mediate fusion of the viral envelope with the cell membrane, which normally results in virus penetration into the cytoplasm (9). The antibodies apparently bind to the fusogenic protein at a site that prevents its catalysis of the fusion event.

In the present case of HIV infection, it is not clear what post-binding step is being blocked. The bound antibody could possibly prevent lateral migration of the CD4 receptor in the plasma membrane preventing endocytosis, or block fusion of the viral envelope with either the cell plasma membrane or an endocytic vesicle membrane.

An alternative possibility is that the antibody may stabilize an intermediate conformation of CD4 and prevent it from reaching a state conducive to membrane fusion. In general, antibodies can stabilize a conformation, and thus stabilize (or “freeze”) the antigen in a state that provides the highest affinity/lowest free energy to the paratope-epitope interaction. This mechanism has been studied in the case of antibody-mediated enzyme activation and stabilization, particularly in the Escherichia coli β-galactosidase system. The enzyme's tetra-
Table 5. Crossinhibition of CD4 Binding by mAbs 55 and 94

|              | OKT4A | Leu-3A | L83 | L88 | L92 | L120 | OKT4 | F91-55 | F91-94 | F91-36 |
|--------------|-------|--------|-----|-----|-----|------|------|--------|--------|--------|
| mAb 55      | -     | -      | ±   | -   | -   | -    | -    | +      | -      | -      |
| mAb 94      | -     | -      | -   | -   | -   | -    | -    | -      | +      | -      |

* Cut-off point for positivity was 25% or larger decrease in binding when the inhibitor was six times more concentrated than the test mAb.

The second, more direct possibility is an effect or combination of effects of the ligand on the B epitopes of the rCD4 molecule. For instance, the lower binding titers in mice injected with sCD4-rgp120 could be attributed to a “blanketing” effect of the large gp120 molecule over the relatively small CD4, many of whose epitopes would be hidden and thus unable to interact with the B cell surface Igs. In addition, the qualitative differences could be attributed to the appearance (caused by the binding of rgp120) of new or modified conformational epitopes that are absent or less represented in free molecules.

One aspect of the present results that should be noted is that the polyclonal response showed greater relative efficiency of syncyta blocking than the mAbs. This means that the small mAb population does not faithfully represent the clones active in the animals. It also supports the prediction that more varieties of antibodies recognizing intermediate epitopes, and thus possessing a greater ability to discriminate critical steps in the infection process, will be obtained by enlarging the number of immunized mice and the number of mAbs produced from them.
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