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# Human MoAbs produced from normal, HIV-1-negative donors and specific for glycoprotein gp120 of the HIV-1 envelope

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## SUMMARY

Human MoAbs of IgM class were developed against three regions of the HIV-1 envelope. Uninfected donor lymphocytes were immunized *in vitro* with recombinant protein pB1. Four out of five antibodies were directed to different parts of the V3 region, which contains a major neutralizing site. Two out of these antibodies were directed to more than one amino acid sequence, indicating reactivity to discontinuous sites. Two of the human MoAbs inhibited viral spread between cells in tissue culture, interpreted as reactivities to conserved amino acid sequences exposed during viral maturation. No MoAb neutralized virus, which may be explained by the relatively low avidity of the antibodies. One MoAb was directed to a region containing amino acids participating in CD4 binding. This technique appears to allow formation of antibodies with fine specificities other than those obtained in infected hosts.

**Keywords** human MoAbs V3 region *in vitro* immunization HIV-1 gp120

## INTRODUCTION

HIV-1 has previously been shown to be the agent that causes AIDS in humans [1,2]. After transmission, this virus infects the $CD4^+$ subset of human T lymphocytes and macrophages and establishes a permanent infection with little or no initial clinical symptoms. Following the latent infections, AIDS may ultimately develop, causing death of the infected subject. During primary infection, an immunological response to the virus develops, including both cell-mediated virus-specific reactivities as well as antibody responses. The *in vivo* elicited antibodies have been shown to mediate neutralization of virus particles [3,4] as well as antibody-dependent cellular cytotoxicity (ADCC) of infected targets *in vitro* [5].

Mouse MoAbs specific for HIV-1 proteins have been developed by several groups. Neutralizing specificities raised against either variable or conserved structures have been shown to mediate type- or group-specific neutralization [6–9]. Human MoAbs specific for these and other HIV proteins have been described [10–18]. These antibodies were obtained from infected or immunized individuals.

A possible route to establish specificities other than those obtained after *in vivo* immunization involves *in vitro* immunization of lymphocytes from uninfected donors [19,20]. Such a technique also eliminates the risks involved in the use of infected material. This approach was taken to obtain antibodies to the transmembrane protein gp41 of HIV-1 [21,22].

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In a further development of the strategy employed for the *in vitro* immunization, and in view of the knowledge of the major virus neutralizing sites, we now report on a number of cell lines producing human IgM MoAbs specific for the recombinant protein pB1 [23]. This antigen covers most of the C-terminal part of gp120 of HIV-1 strain $III_B$ and includes the major neutralizing-inducing epitope of this protein [24,25].

## MATERIALS AND METHODS

### *Antigens, antibodies and peptides*

Recombinant pB1 covering amino acids 286–467 of HIV-1 gp120 was kindly supplied by S. Putney and J. Rusche (Repligen Corp., Cambridge, MA), while peptides, synthesized by solid phase techniques, were donated by J. Rosen (Johnson Pharm. Research, La Jolla, CA). The amino acid (aa) numbers were according to Myers [26]. Native gp120 was kindly provided by G. Gilljam (National Bacterial Laboratory, Stockholm, Sweden) [27]. Native proteins and nucleic acids used for studies of multireactivity were obtained from Sigma Chemical Company (St Louis, MO). Digoxin was coupled to human serum albumin and transferrin by the periodate oxidation technique [28], while dinitrophenylated human serum albumin was prepared as previously described [29]. Human MoAb MO6, specificially recognizing digoxin [30], was used as negative control in specificity tests.

**Table 1.** Reactivities of anti-pB1 monoclonals to HIV peptides and infected cells

| MoAb/location | pB1* titre | gp160 titre | IFL, % positive IIIB | IFL, % positive MN | Peptide reactivity | | Cell-to-cell viral spread $IC_{50}$ (μg) IIIB | Cell-to-cell viral spread $IC_{50}$ (μg) MN | Avidity index | Inhibition with linear V3 peptides |
|---|---|---|---|---|---|---|---|---|---|---|
| MO99/V3 | 2400 | 195 | 80 | 80 | 294–308 | INCTRPNNNTRKSIR | 4·3 | 8·6 | 108 | No inhibition |
| | | | | | 299–313 | PNNNTRKSIRIQRGP | | | 100 | |
| | | | | | 304–318 | RKSIRIQRGPGRAFV | | | 72 | |
| MO97/V3 | 7800 | 95 | 0 | 0 | 294–308 | INCTRPNNNTRKSIR | >25 | >25 | 48 | 294–308 |
| | | | | | 299–313 | PNNNTRKSIRIQRGP | | | <10 | 299–313 |
| | | | | | | | | | | 304–318 |
| | | | | | | | | | | 296–331 |
| MO96/V3+V3 | 760 | 170 | 10 | 10 | 304–318 | RKSIRIQRGPGRAFV | >16 | 16·6 | <10 | No inhibition |
| | | | | | 309–323 | IQRGPGRAFVTIGKI | | | <10 | |
| | | | | | 324–338 | GNMRQAHCNISRAKW | | | 149 | |
| | | | | | 329–343 | AHCNISRAKWNNTLK | | | 99 | |
| MO101/V3+C4 | 690 | 94 | 20 | 30 | 304–318 | RKSIRIQRGPGRAFV | 0·3 | 2·2 | 43 | |
| | | | | | 309–323 | IQRGPGRAFVTIGKI | | | 26 | 296–331 |
| | | | | | 314–328 | GRAFVTIGKIGNMRQ | | | 18 | 308–328 |
| | | | | | 319–333 | TIGKIGNMRQAHCNI | | | 13 | 320–339 |
| | | | | | 489–503 | VKIEPLGVAPTKAKR | | | 12 | 484—498 |
| | | | | | 494–508 | LGVAPTKAKRRVVQR | | | 17 | 489–503 |
| MO86/C3 | 10 | 70 | 0 | 0 | 429–443 | EVGKAMYAPPISGQI | >40 | >40 | ND | 429–443 |
| MO6 control | <10 | <10 | 0 | 0 | No binding | Anti-digoxin | >40 | >40 | | ND |

* Western blot, neutralization without and with complement, as well as syncytium inhibition and antibody-dependent cellular cytotoxicity (ADCC) were negative with all supernatants.

ND, Not determined.

*Preparation of antigen-specific hybridomas*

Techniques used to establish cell lines secreting antigen-specific human MoAbs were recently described [21,31]. Briefly, peripheral blood mononuclear cells (PBMC) were prepared by density centrifugation of buffy coats obtained from healthy, HIV-negative blood donors (Lund University Hospital Blood Bank, Lund, Sweden). Lysosome-rich cell populations were removed by treatment with L-leucyl-L-leucine methyl ester (Bachem Feinchemichalien, Budendorf, Switzerland). Following a 6 days *in vitro* immunization procedure [19] with 25–250 ng pB1/ml, B lymphocytes were immortalized by Epstein–Barr virus (EBV) transformation and seeded at $10^5$ lymphocytes/well in 96-well plates together with $10^4$ irradiated (30 Gy) PBMC/well. Lymphoblastoid cell lines producing antibodies specific for the pB1 antigen were detected by antigen-specific ELISA. Each well was coated with 100 ng pB1 in PBS. Samples were diluted in 10 mM sodium phosphate buffer, 500 mM sodium chloride, 0·1% Tween 20, pH 8·0. Antibodies bound to the immobilized antigen were detected by isotype-specific horseradish peroxidase-labelled antibodies as previously described [21]. The antigen-specific cell lines were expanded and fused to the heterohybridoma K6H6/B5 [32] using a PEG fusion technique [21]. Fused cells were selected by HAT and ouabain, and antigen-specific clones identified by ELISA. Hybridomas were cloned at least twice by limiting dilution, and antibodies produced by growing hybridomas in larger batches in RPMI 1640, supplemented with 10% fetal calf serum (FCS; Gibco Ltd, Paisley, UK), 4 mM L-glutamine, 1% non-essential amino acids and 50 μg gentamycin/ml. Hybridoma supernatants were concentrated approximately 10 times by ultrafiltration (mol. wt cut off; 30 kD) before use.

*Characterization of anti-pB1 specific human MoAbs*

The supernatants were concentrated to around 150 μg of IgM/ml. Western blot reactivities were performed on strips (Biotech/DuPont, Rockville, MD). Multireactivity of human MoAbs was evaluated by solid phase ELISAs performed as described above. Antigens were coated at 500 ng/well. Reaction of antibodies with overlapping synthetic pentadecapeptides covering the pB1 sequence including the proposed neutralizing region of HIV-1 gp120 was performed as previously described [33].

Inhibition of antibody reactivity to pB1 in ELISA by synthetic peptides mainly representing the V3 region was determined after pre-incubation of antibodies with 20 μg/ml of synthetic peptide in 10 mM sodium-phosphate, pH 8·0, 0·5 M NaCl and 0·1% Tween 20 overnight. Bound antibody was detected with horseradish peroxidase-conjugated goat anti-human IgM (Zymed Laboratories Inc., San Francisco, CA) using *o*-phenylenediamine as the chromogen. Antibody concentrations were adjusted to give an absorbance of 0·5–1·5 in the absence of inhibiting peptides. Sixty-seven per cent or more was considered to represent specific inhibition. Peptides studied in this way covered residues 294–338.

In order to evaluate the relative avidity to the various peptides, elution of antibody from antigen was performed with 8 M urea according to Hedman & Seppälä [34]. The antibodies (diluted 1:20 in PBS-Tween) were allowed to react for 2 h at

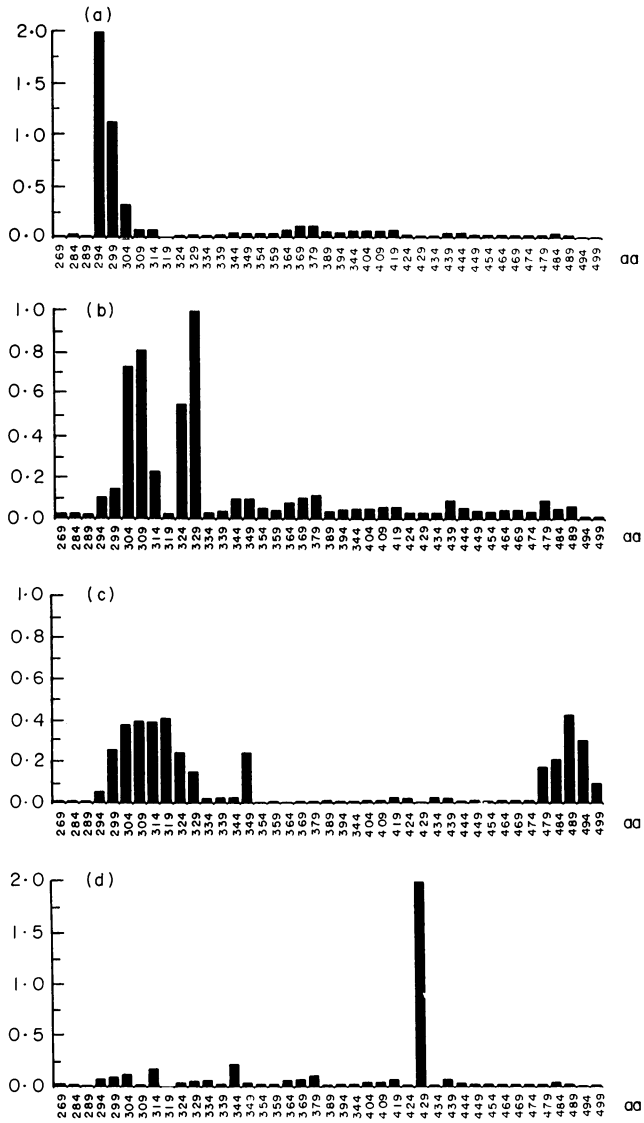


**Fig. 1.** Reactivity patterns of human MoAbs to pB1 with pentadecapeptides representing this region. The numbers indicate the first amino acid in each peptide. Most peptides overlap the proceeding one with 10 amino acids. The reactivity pattern of MO99 was similar to that of MO97. (a) MoAb MO97 (0·23 μg/ml). (b) MoAb MO96 (1·64 μg/ml). (c) MoAb MO101 (0·33 μg/ml). (d) MoAb MO86 (1·14 μg/ml).

37°C with coated peptides. The antigen–antibody complexes were washed three times for 5 min, either with PBS-Tween containing 8 M urea, or with PBS-Tween lacking urea. The residual antigen-bound IgM was quantified immuno-enzymatically by alkaline phosphatase-conjugated anti-human IgM (1 h at 37°C), followed by three washes with PBS-Tween; the substrate (*p*-nitrophenyl phosphate) reaction was terminated by NaOH and the optical density (OD) measured. An avidity index was then calculated for each specimen as follows: OD (assay with urea)/OD (assay without urea) × 100.

In an *in vitro* treatment assay the MoAbs were analysed for inhibition of viral cell-to-cell spread (CTCS). HIV-1 infected CD4$^+$ Jurkat tat cells were mixed with uninfected cells in a ratio of 1 : 10 and seeded in 96-microtitre wells. Four wells in parallel

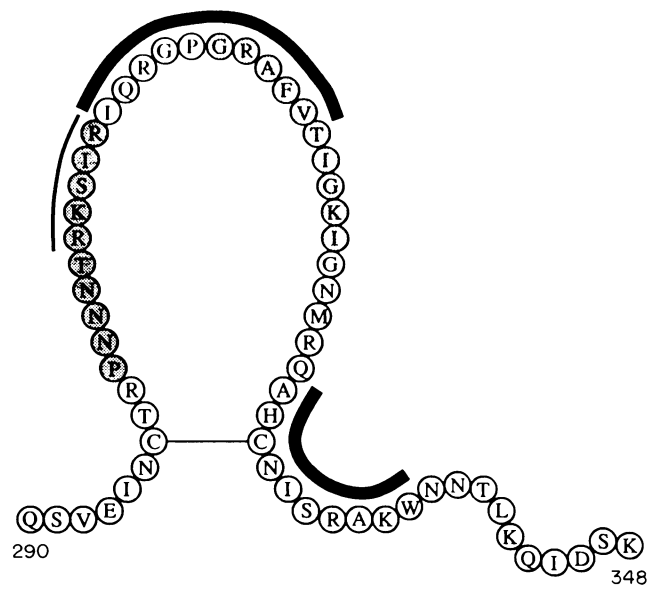


**Fig. 2.** Schematic view of MoAb reactivity to the V3 region of HIV-1 gp120 envelope protein. Dark and light grey amino acids represent the deduced minimal epitope of antibody MO97 and MO101, respectively. The complete epitope of MO101 also includes amino acids 494–503 (not shown here). The thick solid lines indicate the composite epitope recognized by antibody MO96, while the thin line shows the minimal epitope recognized by MO99.

were treated on days 0, 3, 6 and 9 with different concentrations of anti-gp120 MoAbs. One mouse MoAb, F58/H3, was used as a positive control [9]. Cultures were monitored microscopically and by analysis of p24 activity in cell culture supernatant [35]. The p24 activity was calculated in percentage relative to the untreated cell culture. After none (control) or multiple treatments with different concentrations of the five anti-gp120 MoAbs certain HIV-1 infected cell cultures showed a strong inhibition of virus spread over 12 days. Under repeated treatments a decrease of p24 activity to 50% (inhibiting concentration to 50%, $IC_{50}$) could be observed. After stopping the treatment, p24 activity increased up to control levels within 3–6 days.

Neutralization of virus infectivity on PBMC cells and ADCC were performed as described [5,8]. Immunofluorescence (IFL) of HIV-1 $III_B$ infected and non-infected H9 cells was performed on methanol-fixed cells and stained with polyvalent human antiserum followed by FITC-labelled sheep anti-human IgG (all reagents from the National Bacteriological Laboratory). The frequency of stained cells was evaluated microscopically [36]. The viral-inhibiting activity of the MoAbs was furthermore studied using the C816648 T cell line as target cells for the detection of syncytium inhibition and cell growth protection [37].

## RESULTS

Five anti-pB1 specific human MoAbs were established from healthy, HIV-1-negative blood donors. All of these antibodies were of the IgM isotype with kappa light chain. The frequency of antigen-specific lymphoblastoid cell lines (LCL) obtained was in the range of 0·06–0·3/$10^6$ cells. To exclude multireactive antibodies, they were all tested against bovine thyroglobulin,

calf thymus histone, bovine insulin, keyhole limpet haemocyanine, bovine and human serum albumin, human transferrin, acid soluble calf skin collagen, ovalbumin, haptens (dinitrophenol and digoxin) conjugated to carrier proteins (bovine and human serum albumin and human transferrin), RNA and single and double stranded DNA. No reactivity was detected with these antigens, except for a reactivity of antibodies MO99 and MO101 with calf thymus histones.

The fine specificity of the five antibodies was determined by reactivity to overlapping, synthetic pentadecapeptides adsorbed to a solid phase, as well as by inhibition of binding to the immobilized pB1 antigen by soluble synthetic peptides. The major interest in this study was focused on sequences within the region V3, containing the major neutralizing epitope of HIV-1.

Antibodies MO96, MO97, MO99 and MO101 all recognized specificities within or close to the V3 region (Table 1, Fig. 1). Avidity comparisons for MO99 showed that the higher avidity to V3 peptides was localized mainly to peptides aa 294–313, and not to the region 304–318 containing neutralization-inducing amino acids. MO96, on the other hand, had high avidity to the C-terminal part of the V3 region, aa 324–343.

Two of the MoAbs, MO99 and MO101, displayed significant inhibition of viral cell-to-cell spread (Table 1). This was manifested as a decreased amount of p24 antigen produced in cell culture after repeated addition of MoAb to a mixture of infected (5–10%) in uninfected susceptible cells. The inhibition was more pronounced with HIV strain $III_B$ than MN, and thus partially cross-reactive. Despite the V3 reactivities, none of the antibodies neutralized the virus *in vitro* or had any ADCC effect. Antibody MO96 and MO99 reacted with virus-infected cells as shown by membrane immunofluorescence. MO101 reacted specifically with nuclei of infected cells. MO86 (Fig 1, Table 1) had an interesting reactivity to a peptide sequence that is considered part of the CD4 binding region of the gp120 envelope. No immunofluorescence was shown with MO86.

Two MoAbs reacted with amino acid sequences located apart, thus potentially recognizing conformational dependent epitopes (Table 1, Fig. 1). MO96 reacted with two stretches, both within the V3 region. MO101 also reacted with an amino acid sequence in the C terminus of gp120. The putative reaction sites of the V3 region are shown in Fig. 2. Even regions to which low avidity was displayed were taken into account to deduce minimal epitopes.

## DISCUSSION

Human MoAbs were induced against three principal regions of the HIV-1 gp120 envelope protein. Two of the MoAbs appeared to react with discontinuous sites (Fig. 1).

It has previously been shown that the recombinant peptide pB1 induces HIV-1 neutralizing antibodies *in vivo* [23]. The fine reactivity of the neutralizing polyclonal response to this recombinant antigen, as well as that of the neutralizing reactivities of HIV-1 infected individuals, has been shown to be directed to the region V3 [38–42] which forms a loop through a disulphide bond between amino acid residues 296 and 331 of gp120 ([43]; Fig. 2). A human MoAb obtained by EBV transformation of B cells from an asymptomatic, infected donor [18] as well as a chimera of mouse to human IgG1 [44] both had viral neutralizing activity.

The monoclonal antibodies MO99 and MO97 reacted with linear peptides of the V3 region, with sites located at the hypervariable stretch close to the N-terminal cystein (Fig. 2). Two additional antibodies, MO96 and MO101, appeared to react with two relatively different sites each. MO96 reacted with two conserved stretches of V3 (Fig. 2), the latter related to peptides to which seroreactivity of infected mothers correlated to absence of infection in their children [45,46]. One MoAb, MO101, had an interesting reactivity, in that a reactivity to V3 was combined with reactivity to another immunodominant site, the C-terminus of gp120. The reactivity of this antibody indicates that the two regions have a propensity for folding together. The C-terminus of gp120 was previously described to be immunogenic in man [38,47,48]. It is not likely that the recognition of two spatially distant sequences is caused by oligoclonality of the cell lines. The low frequency of the LCL producing antigen-binding antibodies, repeated cloning at limiting dilution of the hybridoma cells and similar reactivity of different subclones indicate monoclonality.

Two human MoAbs displayed inhibition of viral spread and replication within a mixed culture of infected and uninfected cells. This appeared to be cross-reactive between strains $III_B$ and MN. In the sequence PNNNTRKSIR these virus strains share seven amino acids, which may permit MO99 reactivity and explain the difference in amount of MoAb needed. The sequences to which MO101 reacts share six of 10 amino acids in the V3 region, and all 10 in the C-terminal of gp120.

None of our human antibodies neutralized HIV or had any ADCC activity. The reactive sites of the human MoAbs are, however, not directed to the precise sequence GPGRAF shown to give highly active neutralizing antibodies and the epitopes seem in several cases to be substantially larger as compared with mouse IgG MoAbs obtained after *in vivo* immunization with natural antigen [9].

The absence of classical neutralizing activity *in vitro* of the human antibodies obtained from seronegative donors may furthermore be explained by several other factors. First, the affinity constants of these antibodies, resulting from a primary response, may be insufficient to mediate biological activity, at the concentrations tested. Second, it may reflect the fact that these IgM antibodies do not show a high avidity as measured by urea elution towards the conserved neutralization-inducing part of the V3 epitope. Third, since antibody MO97 does not react with infected cells by immunofluorescence and MO96 reacts only weakly, the native protein structure may not be available for binding with these antibodies, possibly as a consequence of differences in folding pattern or glycosylation of the natural antigen [43]. Only repeated dilution of MoAb as in the CTCS assay may be sufficient for viral inhibition. The reactivity may then be directed to additional proteins available during maturation process rather than those available at the surface of the intact virion. Antibody MO101 reacted with infected cells in immunofluorescence, but only with antigens localized in the nuclei. The form of the antigen has not been further studied since no recognition was evident in Western blot.

Other neutralizing epitopes have been identified in gp120 [49]. It has also been shown that soluble CD4 inhibits virus infection [50], indicating alternative targets for anti-viral therapy before initial infection. The CD4 binding site is located in a conserved region [51]. This region also contains a T cell epitope [52,53] but is usually not strongly antibody-inducing in humans.

By using an analytical technique employing modified peptides, a strong and frequent reactivity of sera from infected persons was however revealed [54]. Our MoAb MO86 reacts strongly in this region. No interference with virus infectivity or syncytium formation was however noted. A mouse MoAb to the CD4-binding domain was described that blocked binding of HIV-1 to $CD4^+$ cells when used in high doses [55]. A human MoAb directed to a similar region was also shown to be conformation-dependent and inhibited HIV infection [56].

The potential usefulness of human MoAbs specific for epitopes of HIV-1 underlines the importance of further studies of these or similarly obtained reagents. Combinations of reagents with reactivities to several functional sites should have a place in therapeutic regimens. *In vitro* immunization may also provide a technique to obtain antibodies with fine specificities other than those obtained *in vivo*. Further work is, however, necessary to improve the functional characteristics of antibodies that are obtained by this technique, including development of techniques to induce affinity maturation *in vitro*.

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