Cross-reactive neutralizing humoral immunity in HIV-1 disease: dynamics of host-pathogen interactions
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Citation for published version (APA):

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High prevalence of neutralizing activity against multiple unrelated HIV-1 subtype B variants in sera from HIV-1 subtype B infected individuals: Evidence for subtype-specific rather than strain-specific neutralizing activity
ABSTRACT

It is assumed that an effective HIV-1 vaccine should be capable of eliciting neutralizing antibodies. However, even the best antibodies known to date lack neutralizing ability against a significant proportion of primary HIV-1 variants and despite great efforts, still no immunogen is available that can elicit humoral immunity that can protect against infection or disease progression.

We tested sera from 35 participants from the Amsterdam Cohort Studies on HIV-1 infection, who were all infected with HIV-1 subtype B and therapy naïve at the time of sampling, for neutralizing activity against a panel of 23 tier 2-3 HIV-1 variants, with a minimum of 5 HIV-1 variants per subtype A, B, C and D. Strong cross-clade neutralizing activity was detected in sera from 7 individuals. Strikingly, sera from 22 out of 35 individuals (63%) neutralized 3 or more of the 6 tier 2-3 HIV-1 subtype B viruses in the panel. There was a strong correlation between neutralization titer and breadth in serum. Indeed, the IC$_{50}$ of sera with strong cross-clade neutralizing activity was significantly higher than the IC$_{50}$ of sera with cross-subtype B activity, which in turn had a higher IC$_{50}$ than sera with the lowest neutralization breadth.

These results imply that humoral immunity, at least in HIV-1 subtype B infected individuals, is often subtype-specific rather than strain-specific and that the breadth of neutralization is correlated with the titer of neutralizing activity in serum. Considering the difficulties in designing a vaccine that is capable of eliciting cross-clade neutralizing activity, subtype-specific vaccines may be explored as an interesting alternative.

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Journal of General Virology 2010 91:250-258
**INTRODUCTION**

Neutralizing antibodies (NAb) are believed to be crucial for immunity against viral infections and are therefore considered an essential component of an HIV-1 vaccine elicited immune response. The development of an immunogen that is capable of eliciting NAb is however challenged by the inaccessibility of conserved epitopes and the enormous sequence diversity of the viral envelope which is the main target for NAb. Indeed, the error prone reverse transcriptase, the lack of proofreading, and the extremely rapid viral turnover rate are responsible for huge sequence variation, which can be as high as 10% already within the viral quasispecies in a single individual. This high diversity has led to a classification of HIV-1 variants into distinct clades or subtypes, which are defined as groups of viruses that more closely resemble each other than viruses from other subtypes. The main group (M-group) is subdivided into subtypes A to K and different recombinant forms, which have different geographic distributions; subtype B for instance predominates in Europe, the Americas, and Australia, whereas subtype C predominates in Sub-Saharan Africa. The viral envelope currently differs by up to 35% between subtypes and up to 20% within subtypes. The enormity of this challenge can be put into perspective by comparison with the influenza vaccine, where a diversity of less than 2% in amino acid changes can already cause failure in the cross-reactivity of the polyclonal response elicited by the vaccine. It may therefore be put into question whether a single vaccine capable of eliciting neutralizing antibodies against all HIV-1 variants is feasible.

In addition to the high sequence diversity, the humoral immune response is thwarted by the inaccessibility of the relevant (conserved) epitopes. The inaccessibility of relevant epitopes on the HIV-1 envelope is due to a high level of glycosylation, occlusion within the oligomeric structure of the viral envelope, and the fact that their formation occurs only after engagement of the viral envelope with CD4, when spatial constraints do not allow binding of the relatively large immunoglobulins. Despite the viral mechanisms for evading humoral immunity, HIV-1 does elicit neutralizing antibodies in the natural course of infection. These however are considered to be mainly strain-specific, so only capable of neutralizing autologous virus variants and their epitopes are therefore considered irrelevant for vaccine design.

Broadly neutralizing antibodies (BrNAb) may bypass the viral defense mechanisms as they have the ability to neutralize HIV-1 variants from different subtypes. Four well known BrNAbs, b12, 2G12, 2F5 and 4E10, have been isolated from HIV-1 infected individuals. One of the current vaccine strategies is to design an immunogen that mimics the epitopes of these broadly neutralizing antibodies. However, an effective vaccine would require additional epitope specificities, as a significant proportion (~15%) of primary subtype A, B, C, D, and CRF01-AE is resistant to neutralization by all 4 BrNAb mentioned above. The high sequence diversity between HIV-1 variants may underlie the incomplete coverage by BrNAb. In that light, vaccine elicited subtype specific neutralizing antibodies may be the
best alternative to BrNAb. However, the existence of HIV-1 neutralization serotypes has been questioned 16,17.

Here, we studied the breadth of serum neutralizing activity in 35 HIV-1 subtype B infected individuals. We found that sera from 7 individuals had highly cross-clade neutralizing activity, and that the majority of sera neutralized multiple unrelated subtype B HIV-1 variants, providing evidence for a HIV-1 subtype B neutralization serotype.

**MATERIALS AND METHODS**

**Patients**

The study group consisted of long-term non-progressors (LTNP; defined as HIV-1 infected individuals who have ≥10 years of asymptomatic follow-up with stable CD4+ cell counts that were still above 400 cells/µl in the 9th year of follow-up) and progressors (HIV-1 infected individuals who progressed to AIDS within 7 years after (imputed) seroconversion) who were all participating in the Amsterdam Cohort Studies on HIV and AIDS in homosexual men. All individuals were infected with HIV-1 subtype B, and were either seropositive at entry in the cohort studies (seroprevalent cases with an imputed SC date on average 18 months before entry in the cohort 18,19) or seroconverted during active follow-up in the cohort studies. None of the participants received combination anti-retroviral therapy during the sampling period; samples were obtained on average at 28 months (range 24-33 months). The Amsterdam Cohort Studies are conducted in accordance with the ethical principles set out in the declaration of Helsinki and written consent was obtained prior to data collection from each participant. The study was approved by the Academic Medical Center institutional medical ethics committee.

**Viruses**

Sera from all 35 patients were tested for neutralizing activity in a pseudovirus assay developed by Monogram Biosciences. The tier 2-3 virus panel that we used for determining cross-neutralizing activity in serum consisted of HIV-1 pseudoviruses from subtypes A (n=5), B (n=6), C (n=7), and D (n=5) and included recently transmitted isolates, and moderately neutralization sensitive and resistant primary HIV-1 variants, based on previously determined neutralization sensitivities to subtype B sera and MAbs b12, 2G12 and 4E10 11,20,21. In addition, 5 subtype B HIV-1 reference strains were included (1196, Bal, JR-CSF, NL4-3 and SF162). Pseudotyped viral particles were produced by cotransfecting HEK293 cells with an expression vector carrying the patient-derived gp160 gene (eETV) and an HIV-1 genomic vector carrying a luciferase reporter gene (pRTV1.F-luc.PCND0-ΔU3). Forty-eight hours after transfection, pseudovirus stocks were harvested and small aliquots were tested for infectivity using U87 target cells expressing CD4, CCR5, and CXCR4. Pseudovirus stocks were then diluted to titers that, as measured by relative light units, fell within a range known to yield reproducible IC₅₀/s.
Neutralization assay
A recombinant virus assay involving a single round of virus infection was used to measure neutralization. Diluted pseudoviruses were incubated for 1 hour at 37 °C with serial dilutions of serum after which the U87 target cells were added. The ability of patient sera to neutralize viral infection was assessed by measuring luciferase activity 72 hours after viral inoculation in comparison to a control infection with a virus pseudotyped with the murine leukemia virus envelope (aMLV).
Neutralization titers are expressed as the reciprocal of the plasma dilution that inhibited virus infection by 50% (IC\textsubscript{50}). Neutralization titers were considered positive if they were 3 times greater than the negative aMLV control.

Statistical analyses
Statistical analyses were performed using the SPSS 16 software package. Neutralization titers, expressed as the reciprocal of the plasma dilution that inhibited virus infection by 50% (IC\textsubscript{50}), and the number of viruses that were neutralized were not normally distributed. Therefore the non-parametric Kruskal-Wallis test and Mann-Whitney U test were used to compare the neutralization titers between sera that had strong cross-clade neutralizing activity, only cross-subtype B specific neutralizing activity, or no cross-reactive neutralizing activity at all. For the calculation of IC\textsubscript{50} values for viruses that were not inhibited by the 1:40 serum dilution we assumed that 50% inhibition would have occurred at a 1:20 serum dilution. A result was considered significant when the P value was <0.05.

RESULTS
Prevalence of strong cross-clade HIV-1 specific neutralizing activity in patient sera
We studied sera from 35 participants from the ACS for the breadth and titer of HIV-1 specific neutralizing activity. Serum samples were obtained between 24 and 33 months after the estimated day of seroconversion and all participants were therapy naïve at this point. HIV-1 specific neutralizing antibody activity was measured in a cell-based infectivity assay using recombinant viruses that carried a luciferase reporter gene and that were pseudotyped with envelope proteins from tier 2-3 HIV-1 subtype A, B, C, and D. For comparison, 5 HIV-1 subtype B reference strains were additionally tested. To monitor neutralizing activity not mediated by antibodies directed against HIV-1 Env-specific antibodies, each plasma sample was also tested against a recombinant virus stock that was pseudotyped with amphotropic murine leukemia virus envelope proteins (gp70SU and p15TM). Typically, neutralization titers, expressed as the reciprocal dilution of plasma that established 50% inhibition (IC\textsubscript{50}) of virus infection, were <40 for amphotropic murine leukemia virus (aMLV) controls. No differences in neutralizing activity were observed between sera from LTNP and progressors (cross-clade neutralizing activity in 3/20 LTNP and 4/15 progressors) (van Gils et al. AIDS in press). In all sera, neutralizing activity against the reference strains was observed (Figure 2.1).
Neutralizing activity in sera from 35 participants of the ACS was measured against 23 pseudoviruses HIV-1 subtypes A, B, C and D, 5 HIV-1 reference strains and aMLV. The patient sera were grouped based on neutralizing activity against the tier 2-3 viruses, excluding the reference strains: strong cross-reactive neutralizing activity (≥50% of viruses per subtype with IC_{50} ≥ 100 for ≥3 subtypes) \( n = 7 \), cross-reactive neutralizing activity against multiple subtype B variants but minimal neutralizing activity against other subtypes (≥50% of subtype B viruses neutralized) \( n = 15 \), or absent cross-reactive neutralizing activity) \( n = 13 \).

Within each group, sera were ranked based on the number of viruses that were neutralized and the geometric mean titer of the neutralization activity. Neutralization titers are expressed as the reciprocal of the plasma dilution that inhibited virus infection by 50% (IC_{50}). Neutralization titers that are 3 times greater than the negative control (aMLV) are indicated in gray. IC_{50} < 40 are indicated with a stripe. LTNP patient ID numbers are gray. n.d. not done.

### Figure 2.1: Breadth and titer of HIV-1 specific neutralizing activity in sera

| Patient ID | MB_pA1 | MB_pA2 | MB_pA3 | 94UG103 | 92RW020 | APV-16 | APV-20 | APV-9 | 92BR020 | MB_pB1 | MB_pB2 | MB_C1 | 93IN905 | IAVI_C22 | MBC6 | MBC3 | 94IN11246-3 | 93MW960 | MB_pD1 | MB_pD2 | MB_pD3 | 92UG001 | 93UG070 | 1196BaL | JRCSF | NL4-3 | SF162 | aMLV |
|------------|--------|--------|--------|---------|---------|--------|--------|--------|---------|--------|--------|------|--------|--------|------|------|---------|---------|--------|--------|--------|---------|---------|--------|--------|--------|
|            |        |        |        |         |         |        |        |        |         |        |        |      |        |        |      |      |         |         |        |        |        |         |         |        |        |        |       |
|            |        |        |        |         |         |        |        |        |         |        |        |      |        |        |      |      |         |         |        |        |        |         |         |        |        |        |       |
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Strong HIV-specific cross-clade neutralizing activity, defined as an IC\textsubscript{50} ≥ 100 for at least 50% of the tier 2-3 viruses from at least 3 different subtypes (so excluding the reference strains), was observed in sera from 7 of 35 individuals (20%) (Figure 2.1). Interestingly, sera from three of these individuals neutralized >80% of all the tier 2-3 viruses in the panel with an IC\textsubscript{50} ≥ 100 (Figure 2.1; patients 19298, 19642 and 19708).

Prevalence of sera with cross-reactive neutralizing activity against multiple HIV-1 subtype B variants but less to viruses from other subtypes

The sera from 7 individuals with strong cross-clade neutralizing activity also neutralized 5 to 6 out of the 6 tier 2-3 subtype B HIV-1 variants in the panel. Sera from the other 28 of 35 HIV-1 subtype B infected individuals studied here lacked strong cross-clade neutralizing activity against HIV-1 variants from multiple subtypes, according to the definition described above. Interestingly, while sequence diversity between the envelope genes of the tier 2-3 HIV-1 subtype B variants in the panel, so again excluding the reference strains, varied by up to 12%, and while phylogenetic analysis did not reveal clustering of the viruses from this panel with autologous viruses of the different patients studied here (data not shown), sera from 26 of these 28 patients (93%) who lacked strong cross-clade neutralizing activity, showed neutralizing activity against at least 1 of the 6 unrelated tier 2-3 HIV-1 subtype B variants in the panel (Figure 2.1). Strikingly, sera from 15 of these 28 patients (54%) neutralized even 3 or more of the 6 unrelated tier 2-3 HIV-1 subtype B variants in the panel (Figure 2.1). Interestingly, 4 of these patients (19250, 19559, 19663, 19768) and also 2 patients with strong cross-clade neutralizing activity (18969, 19829) showed the same breadth of neutralization against subtype B and subtype C viruses with even higher neutralizing titers against the subtype C variants than against the subtype B variants.

The breadth of neutralizing activity against viruses from the other 3 subtypes was significantly lower, in agreement with the fact that these sera did not have strong cross-clade neutralizing activity. These data show that apart from the 7 sera with strong cross-clade neutralizing activity, the majority of sera had neutralizing activity against multiple and diverse subtype B HIV-1 variants. Indeed, of the total of 35 individuals, 22 individuals (63%) had neutralizing activity against at least 3 of the tier 2-3 subtype B viruses in the panel.

Correlation between titer and breadth of HIV-1 specific neutralizing activity in serum

Characteristics of heterologous HIV-1 specific neutralizing serum reactivity are not known in great detail. Here, we observed a strong correlation between the titer of neutralizing activity and the number of different viruses that were neutralized by a serum (Figure 2.2). Indeed, for neutralization of each individual virus in the panel of tier 2-3 HIV-1 subtype B viruses, the mean IC\textsubscript{50} values were significantly higher for sera that had strong cross-reactive neutralizing activity against viruses from different subtypes (Figure 2.2A, white bars) as compared to sera with cross-reactive neutralizing activity against multiple subtype B variants.
but not against viruses from other subtypes (Figure 2.2A, grey bars). Additionally, sera from the latter group had in turn a significantly higher mean neutralizing titer against 4 of the 6 tier 2-3 subtype B HIV-1 variants in the panel (92BR020, APV-16, APV-20, and MB_pB1) as compared to the mean neutralizing titers in the 13 patient sera that neutralized ≤ 2 of the HIV-1 subtype B viruses in the panel (Figure 2.2A, dashed bars).

The mean neutralizing titers in the patient sera studied here were higher for some of the reference viruses that were used in this study (1196, Bal, JR-CSF, NL4-3 and SF162; Figure 2.2B), in agreement with the generally higher neutralization sensitivity of these viruses. Interestingly, also for these reference strains, we observed the same pattern between neutralization breadth and titer. Indeed, the mean neutralizing titer of the 7 sera with strong cross-clade neutralizing activity (Figure 2.2B, white bars) was significantly higher for each individual reference virus than the mean neutralizing titer in the 15 sera with subtype B specific cross-reactive neutralizing activity (Figure 2.2B, grey bars) while the mean neutralizing titers in these sera were again higher than the mean neutralizing titer in the 13

Figure 2.2: Correlation between titer and breadth of HIV-1 specific neutralizing humoral immunity in sera of HIV-1 infected individuals

Mean neutralizing titer of sera in defined groups, according to their ability to neutralize the tier 2-3 viruses from the panel, against 6 unrelated tier 2-3 subtype B HIV-1 variants (A) and 5 subtype B reference strains (B). The patient sera were grouped based on neutralizing activity against the tier 2-3 viruses, excluding the neutralizing activity against the reference strains: strong cross-clade neutralizing activity, Figure 2.1 (≥50% of viruses per subtype with IC$_{50}$ ≥ 100 for ≥3 subtypes, n=7), cross-reactive neutralizing activity against multiple subtype B variants but minimal neutralizing activity against other subtypes (≥50% of subtype B viruses neutralized, n=15), or absent cross-reactive neutralizing activity (n=13). Serum neutralizing titers required for 50% inhibition of the tier 2-3 HIV-1 subtype B virus variants in the panel were calculated. White bars: sera with strong cross-clade neutralizing activity (n=7); grey bars: sera with cross-reactive neutralizing activity against multiple subtype B variants but minimal reactivity against viruses from other subtypes (n=15); dashed bars: sera that lack cross-reactive neutralizing activity (n=13). Neutralizing titers are expressed as the reciprocal of the plasma dilution that inhibited virus infection by 50%. Significant difference between the three groups were indicated; * P < 0.05, ** P < 0.01, *** P < 0.001 (Mann-Whitney U test).
sera that lacked cross-reactivity (Figure 2.2B, dashed bars). For JRCSF, a tier 2 reference strain with a known neutralization resistant phenotype 23, the mean neutralizing titer in the 15 sera with cross-subtype B activity was similar to the mean titer in the 13 sera that lacked cross-reactive neutralizing activity (Figure 2.2B).

The neutralizing titers against viruses of subtype A, C and D also showed a correlation with the neutralization breadth against these viruses, albeit that the differences in titers between groups of sera with strong cross-clade neutralizing activity, cross-subtype B neutralizing activity or almost absent neutralizing activity were less strong (data not shown).

**DISCUSSION**

All vaccines that provide protection against viral infections elicit at least a potent humoral immune response 24. In line, HIV-1 vaccine research is aiming for an immunogen in which epitopes for broadly neutralizing antibodies are present 12. This is a challenging task as the HIV-1 envelope has evolved towards a structure in which the relevant epitopes are absent in the native protein, occluded in the oligomeric structure, and/or covered by N-linked glycosylation sites. In addition, the HIV-1 envelope gene is highly variable. This variation, which can be up to 35% between different subtypes 5,7,8 makes it unlikely that a single vaccine will be capable of eliciting a humoral immune response that would cover protection against all possible variants. Indeed, even the best broadly neutralizing antibodies known to date do not neutralize all of the circulating HIV-1 variants 11,13-15,16. Most HIV-1 infected individuals mount an HIV-1 specific humoral immune response but these antibodies are considered strain-specific as neutralizing activity is assumed to be limited to the autologous virus strain. Indeed, the majority of HIV-1 infected individuals do not develop cross-clade neutralizing activity that is capable of neutralizing HIV-1 variants from different subtypes 25-27. However, cross-reactive neutralization of different HIV-1 variants of the same subtype has received only little attention.

The findings of our present study suggest that subtype-specific differences in HIV-1 neutralization may exist, similar to what is known for influenza virus 26,29. Overall, we observed that sera from HIV-1 subtype B infected individuals had stronger neutralizing activity against multiple unrelated HIV-1 subtype B variants with substantial sequence diversity in their envelopes, than against HIV-1 variants from subtype A, C and D. However sera from 4 patients with neutralizing activity against multiple subtype B variants and from 2 patients with strong cross-clade neutralizing activity, had higher neutralizing titers against the subtype C variants in our panel than against the variants from the other subtypes, including subtype B. This may suggest that at least some of the epitopes on the envelope of subtype B variants that elicited cross-clade neutralizing activity may be even better exposed on subtype C variants.

Obviously, it remains to be established whether this observation holds also true for sera from individuals infected with other HIV-1 subtypes. Other studies have not provided
evidence for HIV-1 subtype-specific differences in HIV-1 neutralizing activity in serum. However, these studies were performed with only a limited number of HIV-1 variants and sometimes with a pool of patient sera in which different neutralizing epitope specificities may have been mixed. Moreover, these studies strongly focused on broadly neutralizing antibodies that by definition neutralize HIV-1 variants from different subtypes. Although not specifically emphasized by the authors, some previous reports do include data that show that neutralizing activity in patient sera was stronger against viruses that were from the same subtype as the autologous virus.

The exact nature of the epitopes at which cross-clade neutralizing activity and subtype-specific cross-reactive neutralizing activity is directed remains to be established. It was recently reported that cross-clade neutralizing activity is not only directed against the conserved regions of the envelope, such as the CD4 binding site or the V3 loop. It is likely that epitopes that are less well conserved between subtypes but conserved within a subtype are capable of eliciting subtype-specific cross-reactive neutralizing activity. Alternatively, the neutralizing activity is mediated by antibodies directed against the V3 loop, similar to the HIV-1 subtype B specific neutralizing activity of the well characterized monoclonal antibody 446-52D. This NAb recognizes a GPxR motive which is very well conserved in the V3 loop of subtype B HIV-1 variants.

The observation that subtype-specific neutralizing activity in serum may exist can provide a new lead in HIV-1 vaccine development. Indeed, the high sequence diversity between HIV-1 variants of different subtypes may stand in the way of the development of a single vaccine capable of eliciting neutralizing humoral immunity against all circulating HIV-1 variants. Obviously, this approach may be considered once a successful protein vaccine has been developed, which is a major challenge by itself.

Interestingly, we observed relatively strong cross-reactive neutralizing activity against multiple subtype B variants in sera from 63% of subtype B-infected individuals studied here, suggesting that the epitopes that have elicited these humoral responses are present and accessible on natural HIV-1 variants. Although HIV-1 may rapidly escape from this antibody pressure, escape may be prevented if a vaccine elicits sterilizing immunity which is capable of completely preventing viral replication.

We have also observed that the ability of serum to neutralize different viruses is directly related to the neutralization titer in serum (modeled in Figure 2.3). Although this finding does not exclude that highly potent antibody specificities may exist at an average concentration in serum, as was recently reported for 2 novel cross-clade neutralizing antibodies PG9 and PG16, it may imply that sera with highly cross-clade neutralizing ability in general harbor multiple epitope specificities or that a high quantity of a single antibody specificity is more potent, even against unrelated HIV-1 variants. This observation indicates that in general, optimal boosting during vaccination, to increase the antibody titer elicited by a future vaccine may also significantly increase the breadth of the neutralizing activity.
In conclusion we have found evidence for subtype-specific neutralizing activity and a positive correlation between the titer and breadth of neutralizing activity in patient sera. The design of improved adjuvants that can optimize humoral immune responses, in combination with potentially subtype-specific epitopes, may thus provide new leads on the way to a potent HIV-1 vaccine. Developing and administering multiple HIV vaccines is far less ideal than having a single vaccine that would cover all circulating HIV variants. However, design and delivery of a single vaccine that is capable of eliciting potent and cross-clade neutralizing immunity against HIV-1 have not yet been successful. Although we realize that probably any vaccine approach will have to deal with the complexity of the HIV-1 envelope molecule and the difficulty to mimic it as an immunogen, based on our data we suggest that the approach of subtype-specific vaccines may be worthwhile to consider in current strategies.

ACKNOWLEDGEMENTS

The Amsterdam Cohort Studies on HIV infection and AIDS, a collaboration between the Amsterdam Health Service, the Academic Medical Center of the University of Amsterdam, Sanquin Blood Supply Foundation, and the University Medical Center Utrecht, are part of the Netherlands HIV Monitoring Foundation and financially supported by the Netherlands National Institute for Public Health and the Environment. This work is financially supported by the Netherlands Organisation for Scientific research (NWO), grant 918.66.628, the European Community’s Seventh Framework Programme NGIN (FP7/2007-2013) under grant agreement nº 201433, the European Community’s Six Framework Programme Europrise (FP6/2007-2012) under grant number 037611 and also partially funded by an NIH Small Business Innovation Research (SBIR) grant (5R44AI062522) awarded to Monogram Biosciences. The funding organisations had no role in study design, data collection and
analysis, decision to publish, or preparation of the manuscript.
We are grateful to Jan Albert for his valuable suggestions and Evelien Bunnik and Andrea Rachinger for their help with the sequence analysis.

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