RESEARCH ARTICLE

Assessment of Antibody Interference of Enfuvirtide (T20) Function Shows Assay Dependent Variability

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Abstract: *Background*: During HIV infection, fusion of the viral and cellular membranes is dependent on folding of the gp41 trimer into a six-helix bundle. Fusion inhibitors, such as the antiretroviral Enfuvirtide (T20), interfere with the formation of the gp41 six-helix bundle. Recent *in vitro* studies reveal that the gp41 immunodominant region one targeting antibody 3D6 can block T20 interference, but the clinical and pathophysiologic significance of this finding is unclear.

Objective/Method: We have previously characterized a number of antibodies that target conformational epitopes on gp41and herein characterized their ability to interfere with T20 in multiple assays and assess their prevalence in HIV infected subjects.

Results: The T20 interference by antibody 3D6 was confirmed in a CHO-HXB2 envelope/ HeLaT4+ cell culture assay. Antibodies that target an immunodominant region one epitope, as well as a gp41 discontinuous epitope, also interfered in this assay, however, not all antibodies that targeted these epitopes showed T20 interference. This response was not due to the direct binding of T20 by the antibodies and could not be replicated utilizing TZM-bl and HL2/3 cells. Notably, serum competition studies on a panel of HIV subjects demonstrate that these conformational targeting antibodies are common in the HIV population.

Conclusion: The relatively common nature of antibodies targeting these epitopes, the disparate *in vitro* results, and lack of reported clinical failures ascribed to such antibodies leads us to conclude that antibody interference of T20 is likely not clinically relevant. However, this warrants continued consideration with the advancement of other fusion inhibitors.

**Keywords:** HIV, T20, enfuvirtide, antibody, gp41, fusion.

1. INTRODUCTION

The viral envelope glycoprotein expressed on the surface of Human Immunodeficiency Virus (HIV) mediates fusion and subsequent infection of CD4 expressing T cells, macrophages, and monocytes [1-4]. This functional glycoprotein (Fig. **1A**) is a trimeric structure composed of three heterodimers of the surface subunit, gp120, and the transmembrane domain-containing protein, gp41 (Fig. **1B**) [5-11]. During infection, the interaction of the gp120 receptor-binding domain with CD4 cell receptors induces a series of conformational changes in the envelope protein, particularly within gp41 (Fig. **1C** and **D**) [12, 13]. After fusion peptide insertion into the targeted cell membrane, gp41 folds onto itself causing the heptad repeat (HR) 1 and HR2 domains of each gp41 monomer to form a six-helix bundle (Fig. **1E**) [14-16]. The formation of the six-helix bundle brings the viral and cellular membranes together, which allows subsequent fusion to occur [17-20].

Fusion inhibitors, such as enfuvirtide (T20), are one of the newest classes of drugs used in HIV treatment strategies [21-29]. T20 is a 36 amino acid analog of the gp41 HR2 region [30, 31], from amino acids 638-673 of HXB2 reference sequence [32, 33]. It is thought to function by targeting the HR1 sequence (540-583 (gp41 numbering 29-72) [34] of a structural intermediate in the fusion process that prevents interaction between the HR1 and HR2 regions [35, 36]. This interference prevents the formation of the gp41 six-helix bundle, which is necessary for membrane fusion (Fig. **1E**) [37]. Resistance to T20 is known to develop while on

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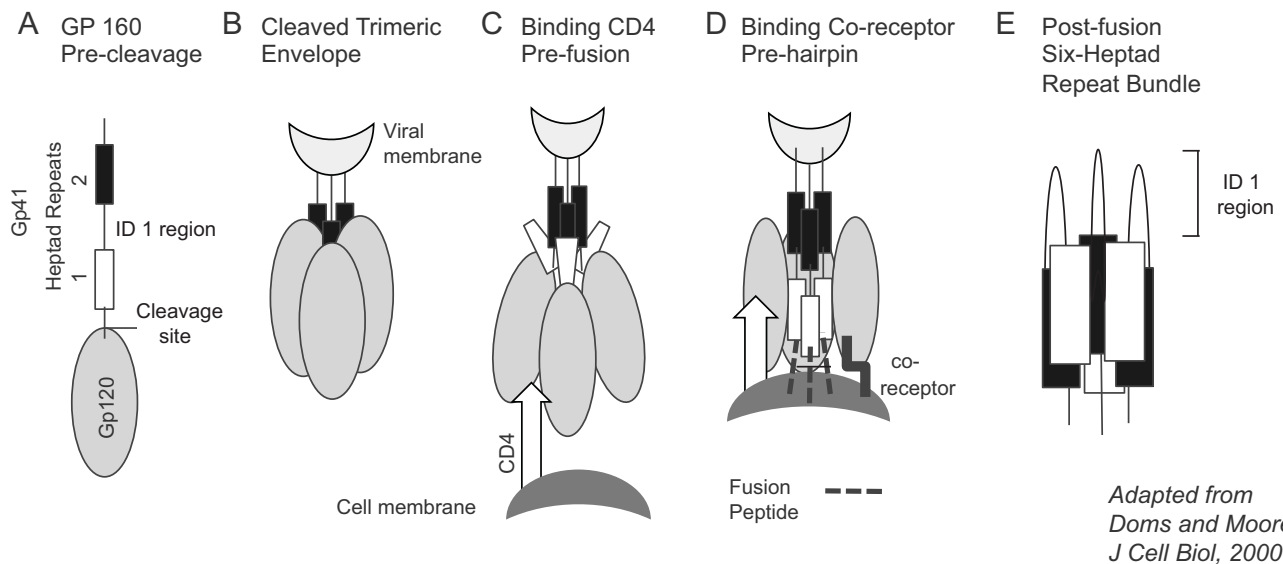
Fig. (1). HIV envelope undergoes dynamic change. A) Gp160 is first cleaved into gp41 and gp120. **B**) Gp41 and gp120 then non-covalently associate into heterodimers that then trimerize to form the functional trimeric envelope. **C**) CD4 binding increases accessibility of some gp41 epitopes and opens up co-receptor binding sites on gp120. **D**) Gp41 unfolds and inserts the fusion peptide into the cell membrane. **E**) Soon after, the HR regions form a six-helix bundle. It is thought that the six-helix bundle remains in this conformation after fusion without dissociating.

therapy due to escape mutations in gp120 and particularly within the HR1 region of gp41 [29, 35, 38-41]. Known T20 viral resistance mutations within HR1 are at G547, V549, Q551, N553 and N554 (on gp160 HXB2 reference sequence, or gp41 36, 38, 40, 42, and 43) [22, 39, 42-44].

Antibody interference has been a recently proposed additional mechanism of T20 resistance. Previous studies have suggested that antibodies synthesized by HIV-infected individuals may bind T20 to form complexes that render T20 ineffective in preventing fusion [45]. Competition for T20's target within HR1 by the antibody IG12 has also been proposed and shown *in vitro*. Intriguingly, in that study, 3D6, an antibody against the immunodominant 1 (ID1) region of gp41 [46], which neither overlaps with the T20 sequence nor its targeted sequence, also showed interference [47]. Results of five other gp41 targeting antibodies, including the ID1 targeting antibody T32 [48], did not show interference. These results support that the ID1 region likely contains a number of unique structurally influenced conformational epitopes. How this region would interfere with T20, that binds to the HR1 region remains unclear.

Our laboratory previously isolated a panel of antibodies [49, 50] known to target four conformational epitopes on the HIV glycoprotein [51, 52]. Utilizing alanine-scanning mutagenesis, two of these epitopes have recently been mapped within gp41 [52]. Four of our antibodies (group B epitope) were mapped to amino acids 596-604 (gp160 HXB2 reference sequence) in the immunodominant I hinge region of gp41. This overlaps the epitope of human monoclonal antibody 3D6 (599-613; gp160 HXB2 reference sequence) that showed interference with T20 function [47]. The epitope of our group C antibodies is characterized as a novel discontinuous epitope on gp41 (mapped to R557, E654 and E657 on HXB2 reference sequence). A number of antibodies binding to similar discontinuous epitopes are thought to target

only post-fusion forms of gp41 [52], and of similar antibodies previously tested, none interfered with T20 function [47]. Notably, mutations at the R557 site, whose mutant affected epitope C antibody 6F5 binding, have not been described to confer T20 resistance [35].

Since there are a number of new fusion inhibitors being developed [21, 53-55], we were intrigued by how clinically relevant antibody interference may be. Since there was variability shown by antibodies targeting ID1 in the original CHO-HXB2 envelope/ HeLaT4+ cell-based culture assay, we thought it would be intriguing to see if our conformational targeting antibodies would show interference in fusion assays. One of our antibodies that overlapped the epitope of 3D6 demonstrated T20 interference. Unlike HR1/HR2 antibodies tested previously, a number of our group C antibodies (6F5 and 4E4) that target a discontinuous gp41 epitope did show interference in this assay. The interference of antibodies binding to Epitope C implies epitope C is present on prefusion gp41, which makes these antibodies unique amongst the HR1/2 complex targeting antibodies. Some antibodies that target epitope B or C did not show T20 interference in this assay, so we performed a global sequence analysis to record specific amino acid mutations that may correlate with differences in their interference phenotypes. This interference was not simply from sequestration of T20, as group B and C antibodies failed to bind both overlapping short peptides and the peptide T20 itself. However, using a TZM-bl cell-based assay, all antibodies tested, including 3D6, failed to show interference. To further explore the clinical significance of this phenomenon, we performed serum competition studies against biotinylated forms of these antibodies and showed that group B and C antibodies are common amongst HIV infected individuals. The commonality of the epitope targeting and the inability of interference to be replicated in different assays makes this type of interference unlikely to be

clinically relevant. However, this assay may assist in further defining the nature of the conformational binding antibodies that target gp41.

2. MATERIALS AND METHODS

2.1. Fusion Assay CHO/HeLaT4+

Similar to recent studies [47], CHO-WT cells ($4x10^4$ in 100μl of culture medium) that stably express the surface HIV-1 HXB2 envelope glycoprotein [56] were incubated with purified antibodies (2μg/mL) for 2 hours at 37°C in a 96-well culture plate. 50 μl of T20 solution was added to the plates to reach a final concentration of 100nM. The plate was incubated for 2 hours at 37°C before adding HeLaT4+ cells ($4x10^4$ in 50μl on medium) [57] expressing CD4 and CXCR4 to every well. The cell mixture was incubated for 24 hours at 37°C. Cells were then fixed with 5% formaldehyde, stained with Giemsa dye and syncytia were counted. Each plate contained positive controls of cells without T20 (syncytia) and negative controls of cells with 100nM T20 alone and no antibodies (minimal syncytia formation). Internal control antibodies (*i.e.* T-32, 3D6, *etc.*) were in agreement with previous findings [47]. As in the previous study, we defined syncytia if six or greater nuclei were notably included. Positive interference was defined as having more than 50% of the internal positive control's average number of syncytia. Positive cutoffs for each experiment and raw data from each are included in supplemental Table **1**. Each test antibody was tested in duplicate within each experiment and the experiment was repeated minimally twice, with the majority of test antibodies used in four separate experiments or more. If disparate results were noted, experiments were repeated minimally twice further for clarification.

2.2. Sequence Analysis

Antibody gene sequences previously published [51] were analyzed to identify mutations that correlate with specific phenotype. The web-based antibody analysis software at IMGT [58] was used to compare amino acid sequences of the heavy and light chains (see Appendix for GenBank accession numbers). Comparative mutational analysis was performed by visual inspection of the IMGT database outputs.

2.3. Peptide Binding Assays

Group M consensus peptides (HIV-1 Consensus Group M Envelope Peptide Set cat# 9487) and Clade B MN sequence peptides (catalog # 6451) that are known to overlap with the T20 sequence, were dissolved in 10% DMSO in PBS. Both peptide sets were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID. ELISA plates were coated with 50 μg per well of peptide and incubated overnight at 4°C on a rocking platform. An excess peptide was washed off, and plates were blocked with 10% BSA in PBS. Antibodies (100 ng/mL) were then added and incubated at 37°C for 1 hour. 2F5 was included as a positive control since its epitope is included within the T20 sequence while 2G12 was included as a negative control. After washing, secondary goat anti human IgG (H+L) (Southern Biotech, Birmingham, AL) was added and this was incubated for 1 hour at room temperature. After washing, TMB substrate

(Pierce, Loves Park, IL) was added and color development was halted with 2N sulfuric acid. Optical density was read at 450 nm absorbance, and data were analyzed with Prism Software (GraphPad, La Jolla, CA).

2.4. T20 Binding Assay

Stock T20 (1mg/mL) was diluted 500X in bicarbonate buffer and coated on a 95 well plate. The plate was incubated overnight at 4°C. The plate was blocked with 10% fetal bovine serum in PBS. Antibodies were added in 2-fold dilutions with end concentrations from 0.5 ng/mL to 500 ng/mL. After blocking, antibodies were added to their respective wells. Next, 100μl of (1:2000) HRP-conjugated goat anti-human IgG(H+L) was added. Plates with antibodies were left at room temperature for one hour. The plate was washed three times with washing buffer. 100μl of TMB substrate was added and incubated for an additional 10-15 minutes at room temperature on an orbital shaker. 100μl of 2N sulfuric acid was added to stop color development. Optical density was read at 450 nm absorbance and data was analyzed with Prism Software (GraphPad, La Jolla, CA).

2.5. Serum Studies from HIV Infected Subjects and Controls

To determine the prevalence of these antibodies in the HIV population, serum competition studies were performed from serum collected from HIV-infected subjects. Serum samples were obtained from a previous research study. Subjects were recruited from the Immunodeficiency Services Clinic of the Erie County Medical Center with IRB approval. Binding to trimerized gp140 HIV strain BaL construct was assessed by ELISA. Serum samples at 1:80 titers (physiologic range) competed against 100ng/mL of biotinylated antibodies representing epitope B (8F6) and epitope C (6F11). Eleven samples including 3 control subjects and eight individuals infected with HIV were included. After initial incubation, plate was washed three times with washing buffer and incubated with Streptavidin-HRP (Southern Biotechnology, Birmingham Al). After another wash step, 100μl of TMB substrate was added to each well and incubated for an additional 10-15 minutes at room temperature on an orbital shaker. 100μl of 2N sulfuric acid was added to stop color development. Optical density was read at 450 nm absorbance, and data were analyzed with Prism Software (GraphPad, La Jolla, CA).

2.6. Fusion Assay TZM-bl Based

To better quantify fusion levels, cell lines TZM-bl (NIH AIDS Research and Reference Reagent Program Cat. Number 8129) and HL2/3 (NIH AIDS Research and Reference Reagent Program Catalog Number #1294 [59]) were used in a second assay. The TZM-bl cell line stably expresses CD4 and CCR5, as well as expressing luciferase genes under control of the HIV-1 promoter. The HL2/3 cell line contains the plasmids pHXB2/3gpt and pSVneo, expressing high levels of Gag, Env, Tat, Rev, and Nef [60]. Briefly, $5x10^4$ HL2/3 cells were incubated with 2 μg/ml antibody in 50 μl media in a 96 well T/C white bottom culture plate. After 2 hours at 37°C, 5 μl of fusion inhibitor T20 was added to a final concentration of 50 nM and incubated for an additional 2 hours
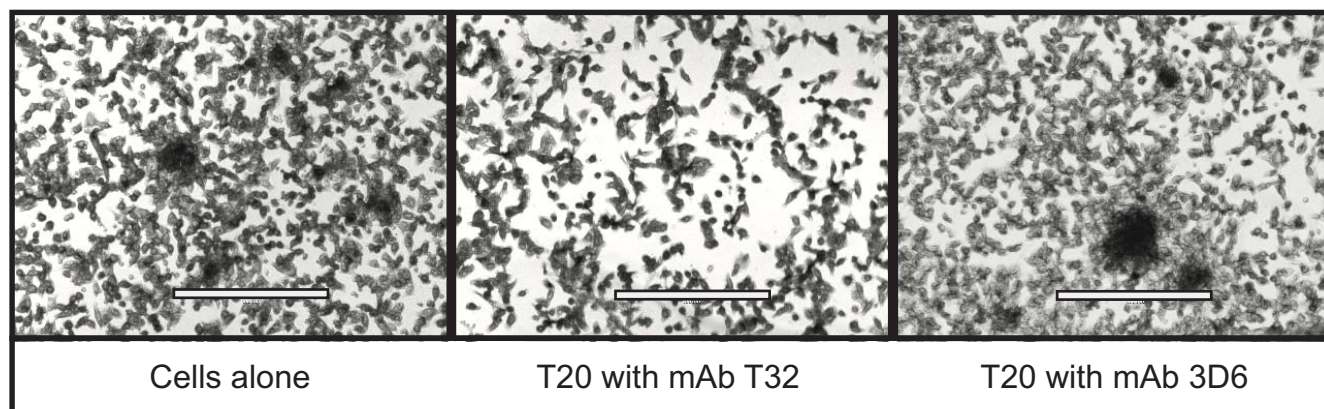
Fig. (2). Antibodies block the T20 fusion interference phenotype. Examples of assay are shown with magnification at 4X and embedded white bars representing 400 micrometers. **A**) HeLa cells expressing CD4+ (HeLaT4+) and CHO-WT cells expressing HIV-1 HBX2 fuse to form syncytia. **B**) Addition of T20 interferes with cell fusion and syncytia formation, even in the presence of certain antibodies such as T32. **C**) Antibodies that interfere with T20 function, such as 3D6, show syncytia. Tabulated results are shown in Table **1** and supplemental Table **1**.

Table 1.   Qualitative summary of T20 fusion inhibition antibody interference assays. Syncytia implies interference with T20 fusion inhibition.

Antibodies Tested	Epitope Mapping Reference	Binding Site on gp41	Results
T32^	Earl *et al.,* 1997 [48]	ID1	Minimal
3D6*^	Stigler *et al.,* 1995 [46]	ID1	**Syncytia**
240-D^	Robinson *et al.,* 1991 [61]	ID1	Minimal
7B2	Santra *et al.,* 2015 [62]	ID1	Minimal
126-7^	Vincent *et al.,* 2008 [63]	HR1/HR2 complex (Gp160/gp41)	Minimal
50-69^	Xu *et al.,* 1991 [64]	HR1/HR2 complex	Minimal
98-6	Poumbourios *et al.,* 1992 [65]	Post-fusion	Minimal
2F5	Muster *et al.,* 1993 [66]	Membrane Proximal Region (MPER)	Minimal
2C6	Sojar *et al.,* submitted	Epitope A- other gp41	Minimal
5C2	Hicar *et al.,* 2016 [52]	Epitope B - hinge	Minimal
8F6*	Hicar *et al.,* 2016 [52]	Epitope B - hinge	**Syncytia**
4E4*	Hicar *et al.,* 2016 [52]	Epitope C – HR1/HR2 complex	**Syncytia**
6F5*	Hicar *et al.,* 2016 [52]	Epitope C – HR1/HR2 complex	**Syncytia**
6F11	Hicar *et al.,* 2016 [52]	Epitope C – HR1/HR2 complex	Minimal
7C6	Hicar *et al.,* 2016 [52]	Epitope C – HR1/HR2 complex	Minimal
8B10	unmapped	Epitope D - unmapped	Minimal

* and bolded- Showed syncytia implying antibody interferes with T20 inhibitor
^T20 fusion interference previously tested (Vincent and Malvoison, 2012) [47]

at 37°C. After this incubation, 5×10^4 TZM-bl cells were added to a final well volume of 100 µl. Wells containing no antibody, no T20, only TZM-bl cells, or only HL2/3 cells were included as controls. The plate was then left to incubate overnight at 37°C in a 5% CO_2 incubator. The next day, fusion levels were measured by a luciferase assay (Bright-glo, Promega) following the manufacturer's protocol. Plates were read on an FL_X800 Microplate Fluorescence Reader (Bio-Tek Instruments, Inc.) at either 100 or 135 sensitivity. Ex-

periments were run in triplicate on each plate and relative lumens were normalized to control fusion levels with no antibodies or T20.

A titration of T20 with antibody 3D6 was also performed. The same protocol as above was followed, keeping the concentration of 3D6 at 2 µg/ml in each well, but using a serial dilution of T20 with a maximum of 50 nM diluting down to 0.39 nM. Each concentration was done in triplicate, and relative lumens were normalized to control fusion levels.

3. RESULTS

3.1. Antibodies Interfere with T20 Fusion Inhibition

Recently, we isolated a panel of novel antibodies expressed in an HIV-infected subject [51]. From these antibodies, two conformational binding epitopes have since been further delineated within the gp41 protein [52]. Since previous studies [47] described great diversity in the ability of gp41-targeting antibodies to interfere with fusion inhibitors, we desired to investigate whether our antibodies targeting these newly described epitopes on gp41 could also interfere with T20. To test this hypothesis, we adopted a fusion assay consisting of CHO-WT cells expressing HIV-1 HXB2 envelope glycoprotein mixed with HeLaT4+ cells expressing CD4 [47]. When T20 was not present in solution, fusion occurred and syncytia were seen (Fig. **2A**). The addition of T20 alone effectively prevented fusion and a minimal number of syncytia were seen (Supplemental Table **1**).

In agreement with previously published studies, addition of antibody T32 (ID1 targeting) [48] did not interfere with T20 (failed to induce syncytia) (Fig. **2B**) while addition of 3D6 (ID1 targeting) [46] revealed syncytia induction (Fig. **2C**) (Table **1**) [47]. The ID1 targeting antibodies 240-D [61] (negative for interference in a previous study [47]), and 7B2 (recognizes trimeric and monomeric gp41 surface stumps [62]) were tested and showed minimal syncytia. The HR1/2 complex-targeting antibodies included in the previous study, 126-7 [63] and 50-69 [64], and the post-fusion targeting antibody 98-6 [65], also were negative for T20 interference (showed minimal syncytia). The broadly neutralizing antibody 2F5 that targets the membrane proximal region [66] also failed to interfere with T20 function (Table **1**).

A total of eight newly characterized structurally influenced antibodies [51, 52], targeting four epitopes (Epitopes A, B, C, and D) were tested in the fusion assay and the results can be seen in Table **1**. Antibodies targeting unmapped epitopes (Group A antibody 2C6 and Group D antibody 8B10, showed no interference. Three of the other six antibodies consistently induced syncytia formation suggesting interference with T20. For illustrative purposes, antibodies that interfered with T20 fusion are further designated throughout this manuscript with (*) following their name (for instance, 6F5*).

The group B antibody 8F6* showed interference with T20 while 5C2 did not (Table **1**). The group B epitope overlaps the epitope of 3D6* and T32 within ID1, which also showed disparate results in our assay and in a previous publication [47]. Two group C antibodies (4E4* and 6F5*) exhibited syncytia consistent with T20 interference, while 6F11 and 7C6 did not (Table **1**). Previously, mapping of epitope C revealed that residues crucial for group C binding are separated on the linear sequence but in close proximity to one another on post-fusion structures [52]. Since this supports the notion that epitope C is formed after fusion we were surprised to see members of this group C showing T20 interference.

3.2. Antibodies Binding to the Same Epitope Differ in their Interference Pattern

Antibodies 3D6* and T32 are known to bind overlapping epitopes, yet show opposite interference profiles [47] (Table
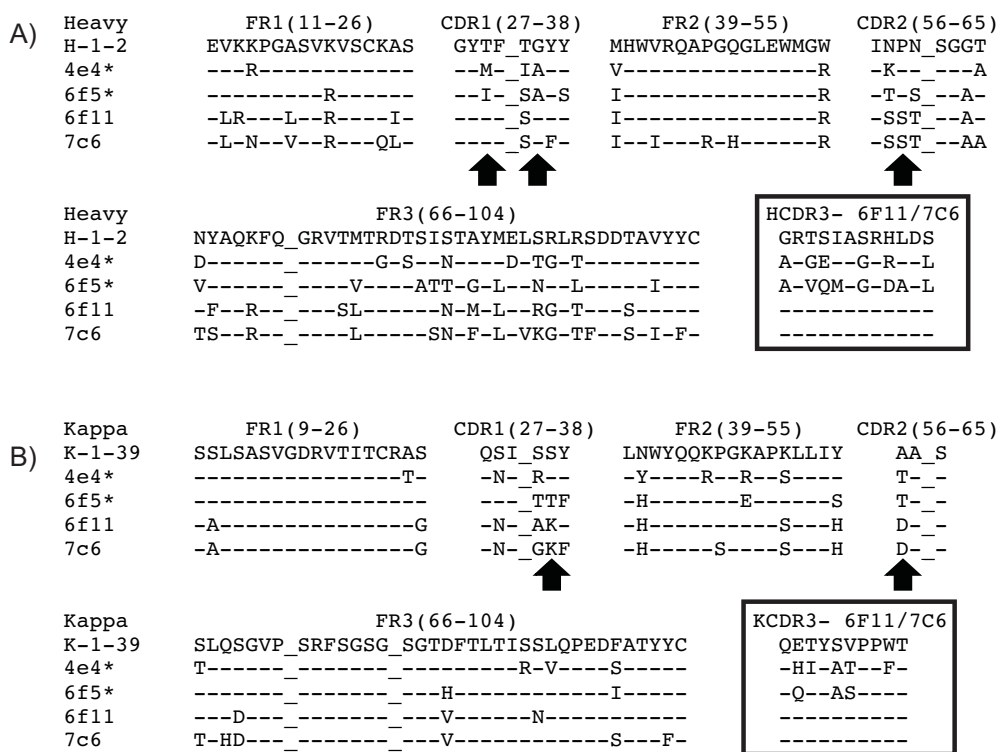
1). Therefore, it was not unexpected in our study to see different interference phenotypes for antibodies targeting similar epitopes. However, our group B and C antibodies were originally cloned from a single subject implying potential clonal relationships between members of each group [51]. To evaluate clonal relationships as well as to analyze amino acid mutations that may correlate with interference phenotypes, we used the web-based antibody sequence software of the IMGT database to review the sequences [58].

Group B antibodies (8F6* and 5C2) were previously clonally grouped by sequence homology [51]. On analysis, the heavy and light chains are predicted to have been derived from the same germline and they have identical CDR3 regions. Complete genetic analysis of the heavy and light chain variable regions revealed numerous shared mutations from germline (heavy-24; light-six), with only two unique mutations. In the 8F6* heavy chain, at position 29 within CDR1, a threonine to asparagine mutation is shown. In the 8F6* light chain, at position 52 in framework two, a leucine to valine mutation is shown.

During their initial cloning and description, group C antibodies were divided into three genetically defined clonal groups [51] with 6F11 and 7C6 divided into the same clonal group and 4E4* and 6F5* being independent clonal groups. On analysis, these four group C clones are all predicted to arise from the same germline sequences in both heavy and light chains which supports that all four may have arisen from a single progenitor B cell. Analysis of the Complementarity Determining Region (CDR) 3 of the heavy and light chains revealed that 6F11 and 7C6 share identical CDR3s in both chains; this implies a close, clonal relationship between the two (Fig. **3**). Conversely, when compared to the 6F11/7C6 CDR3, 4E4* and 6F5* only had homologous residues in the minority (three of eight in the heavy chain CDR3; one of five in the light chain CDR3) of the differences from 6F11/7C6 (Fig. **3**). If 4E4* and 6F5* are from the same lineage, this implies a much more distant relationship, however, they do share the same T20 interference phenotype. The binding of many antibodies is heavily influenced by CDR3 interactions [67], so the shared homology in 4E4* and 6F5* within the CDR3s may explain the phenotypic patterns observed.

We then analyzed all residues throughout the variable sequences of both heavy and light chains to highlight other areas of potential correlation with interference phenotype (Fig. **3**; Table **2**). The sequences were analyzed by dividing mutations that may be important for phenotype into four groups: synonymous mutations between a phenotypic group, non-synonymous mutations between a phenotypic group, synonymous mutations between a phenotypic group while at least one member of the other phenotypic group displays a mutation at the same site but to a different residue, and finally mixed mutations where multiple antibodies across phenotypic groups are mutated to different residues at the same site (Table **2**). Mutations, such as heavy chain site 39 or light chain site 66 (Fig. **3**) that have shared amino acids across the phenotypes were thought not to contribute to phenotype specificity, and are not included in Table **2**.

Within the CDR1 and CDR2 regions, five mutations were noted in the heavy chains while two were seen in the

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A)  Heavy           FR1(11-26)            CDR1(27-38)     FR2(39-55)            CDR2(56-65)
    H-1-2     EVKKPGASVKVSCKAS            GYTF_TGYY    MHWVRQAPGQGLEWMGW         INPN_SGGT
    4e4*      ---R------------            --M-_IA--    V---------------R         -K--_---A
    6f5*      ---------R------            --I-_SA-S    I---------------R         -T-S_--A-
    6f11      -LR---L--R----I-            ----_S---    I---------------R         -SST_--A-
    7c6       -L-N--V--R---QL-            ----_S-F-    I--I---R-H------R         -SST_--AA
                                            ↑      ↑                              ↑

    Heavy                    FR3(66-104)                        ┌─────────────────────┐
    H-1-2     NYAQKFQ_GRVTMTRDTSISTAYMELSRLRSDDTAVYYC           │ HCDR3- 6F11/7C6     │
    4e4*      D-------_------G-S--N----D-TG-T---------           │ GRTSIASRHLDS        │
    6f5*      V-------_----V---ATT-G-L--N--L------I---           │ A-GE--G-R--L        │
    6f11      -F--R--_---SL------N-M-L--RG-T---S-----            │ A-VQM-G-DA-L        │
    7c6       TS--R--_----L------SN-F-L-VKG-TF--S-I-F-           │ ------------        │
                                                                 └─────────────────────┘

B)  Kappa          FR1(9-26)             CDR1(27-38)      FR2(39-55)           CDR2(56-65)
    K-1-39    SSLSASVGDRVTITCRAS          QSI_SSY      LNWYQQKPGKAPKLLIY        AA_S
    4e4*      ----------------T-          -N-_R--      -Y----R--R--S----        T-_-
    6f5*      ------------------          ---_TTF      -H-------E------S        T-_-
    6f11      -A--------------G           -N-_AK-      -H----------S---H        D-_-
    7c6       -A--------------G           -N-_GKF      -H-----S----S---H        D-_-
                                            ↑                                    ↑

    Kappa                   FR3(66-104)                        ┌─────────────────────┐
    K-1-39    SLQSGVP_SRFSGSG_SGTDFTLTISSLQPEDFATYYC           │ KCDR3- 6F11/7C6     │
    4e4*      T------_-------_--------R-V----S-----             │ QETYSVPPWT          │
    6f5*      -------_-------_---H------------I-----            │ -HI-AT--F-          │
    6f11      ---D---_-------_---V------N-----------            │ -Q--AS----          │
    7c6       T-HD---_-------_---V-----------S---F-             │ ----------          │
                                                                 └─────────────────────┘
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Fig. (3). Group C antibody mutations that potentially correlate to phenotype. Heavy (**A**) and Light (**B**) variable chains of the four antibodies of group C are shown in comparison to germline sequence predicted from IMGT, H-1-2 and K-1-39 (FR-Framework Region, CDR-Complementarity determine region). Comparisons were made to allele *01F for both chains. Underlines represent gaps in numbering system. Mutations in the CDR3 (boxed) are compared to the homologous shared sequence of 6F11/7C6. Dashes represent homology to comparative sequence in each individual antibody. Amino acid positions within CDR1 and CDR2 regions that may correlate with phenotype are highlighted by arrows.

light chains (Table **2**; Fig. **3**, **black arrows**). Within heavy chain CDR1, site 29 is mutated from threonine to methionine in 4E4* and to isoleucine in 6F5*, while the non-interference group remains at germline. At site 36 within CDR1, the interference group is uniquely mutated from glycine to alanine while the non-interference group remains at germline. Notably, a cluster of three successive mutations in amino acids 57-59 was observed within the heavy chain CDR2. At site 58, the non-interference group is uniquely mutated from proline to serine while the interference group remains at germline. The adjacent sites (57 and 59) both have synonymous mutations in the non-interference group with other mixed mutations in at least one of the interference pair. In the light chains, one site at position 37 within CDR1 is notable where the non-interference group is mutated from serine to lysine while 6F5 is mutated from serine to threonine. The other mutation in the light chain, at position 56, also adds threonines in the interfering antibodies, in place of alanine at this location. A total of nine heavy chain and five light chain sites in framework regions were also notable for their potential phenotypic correlation and are further categorized in Table **2**.

3.3. Antibodies Show Little Binding to Peptides with Overlapping Sequences to T20

Antibodies interfering with T20 may do so by a variety of proposed mechanisms including by direct competition to the HR1 site which has previously been shown [47], or by sequestering/binding T20 itself which has also been proposed [45]. Through alanine-scanning mutagenesis studies, it is known that group C antibodies map to a discontinuous epitope (HXB2 reference, R557, E654, E657) [52], a portion of which is included within the T20 sequence (HXB2 reference, 638-673) [68] (Fig. **4A**). It has been previously shown that group M consensus peptides of gp41, which includes peptides overlapping T20 sequence, do not present the epitopes of group B or C antibodies [52]. To explore this possibility with improved specificity, eleven consecutive peptides (NIH AIDS reagents catalog number 6541, peptide numbers 154 -165) of 15 amino acid length with 11 overlapping amino acids from Clade B MN sequence were obtained and studied (Fig. **4A**). Despite previous alanine scanning mutagenesis data mapping residues 654 and 657, the Group C antibody 6F5 did not reveal specific binding to peptides that include these sites, further supporting a conformational epitope in this region. Group B antibodies are targeted to the immunodominant 1 region, and as predicted, the group B antibody 8F6 did not react specifically to any peptide in the region overlapping T20 sequence. 2F5 binds the ELDKWA motif in the membrane-proximal region (MPER) and binds three consecutive peptides (162-164) and is included as a positive control (Fig. **4B**).

Since smaller peptides may not present the same epitopes as a longer oligomer despite containing the same sequences,

Table 2.   Mutations by IMGT numbering of Variable regions of Group C Abs (6F11, 7C6, 4E4*, 6F5*).

Mutation Group	Heavy Chains			Light Chains		
	Position	Section	Change	Position	Section	Change
Synonymous mutations between phenotypic pairs	12	FR1	V → L (6F11, 7C6)	10	FR1	S → A (6F11, 7C6)
	36	CDR1	G → A (4E4*, 6F5*)	26	FR1	S → G (6F11, 7C6)
	58	CDR2	P → S (6F11, 7C6)	69	FR3	S → D (6F11, 7C6)
	70	FR3	K → R (6F11, 7C6)			
	99	FR3	T → S (6F11, 7C6)			
Non-synonymous mutations between phenotypic pairs	17	FR1	A → L (6F11) A → V (7C6)	48	FR2	K → R (4E4*) K → E (6F5*)
	25	FR1	A → I (6F11) A → L (7C6)			
	29	CDR1	T → M (4E4*) T → I (6F5*)			
Synonymous mutations in one phenotypic pair with differing mutations in other phenotype pair	57	CDR2	N → S (6F11, 7C6) N → K (4E4*) N → T (6F5*)	37	CDR1	S → K (6F11, 7C6) S → T (6F5*)
	59	CDR2	N → T (6F11, 7C6) N → S (6F5*)	55	FR2	Y → H (6F11, 7C6) Y → S (6F5*)
	78	FR3	S → L (6F11, 7C6) S → M (4E4) S → V (6F5)	56	CDR2	A → D (6F11, 7C6) A → T (6F5*, 4E4*)
				86	FR3	D → V (6F11, 7C6) D → H (6F5*)
Sites with mixed mutations but no shared mutations across phenotype	66	FR3	N → T (7C6) N → D (4E4*) N → V (6F5*)			None
	87	FR3	A → M (6F11) A → F (4E4*) A → G (6F5*)			
	92	FR3	S → R (6F11) S → K (7C6) S → T (4E4*) S → N (6F5*)			

we next assessed a panel of antibodies, including group A-D antibodies, for direct binding to T20. We included the T20 interfering antibody 3D6 and the MPER antibody 2F5 as controls. Overall, our antibodies demonstrated very minimal binding to T20. As expected, 2F5 showed a high level of T20 binding since the T20 sequence contains the known 2F5 epitope (ELDKWA) (Fig. **4C**). Ab 3D6, which targets ID1 similar to group B Abs, showed no binding to T20. Both epitope C antibodies, 6F5* and 7C6 (not shown) did not bind

T20 despite having amino acids mapped to this area previously by alanine scanning mutagenesis [52].

3.4. Group B and C Antibodies are Common in HIV Patients

Although we show that antibodies targeting B and C epitopes can interfere with T20 fusion inhibition, these antibodies were isolated from only one individual. This individual
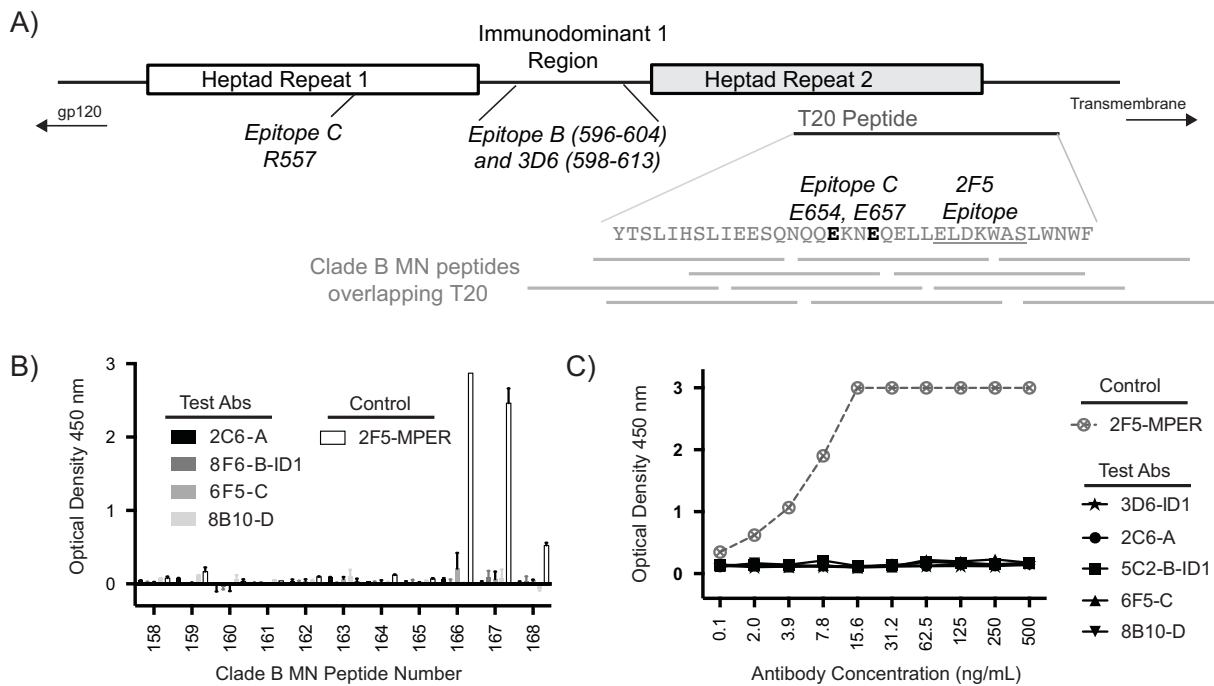
Fig. (4). Antibodies interfering with T20 function lack direct binding to T20 sequence. A) Ultrastructure of gp41 with T20 region and relative positioning of epitopes B, C and 2F5 antibody. 15-mer peptides with 11-mer overlaps were obtained that completely encompassed the T20 sequence. **B**) Antibodies (noted by Abs) shown were tested for binding to peptides that overlap T20. Only 2F5 whose epitope is included in peptides 162-164, showed specific binding. **C**) Antibodies to epitopes A-D as well as antibodies used in previous studies (2F5, 3D6) were tested for binding against T20. Note that only 2F5 demonstrated binding to T20.

was never exposed to T20, so this is unlikely induced by therapy. Due to this, we wished to assess the prevalence of antibodies targeting this epitope within a group of HIV-infected individuals. Repository stored serum from eight HIV positive subjects and three controls were obtained. Binding to the BaL gp140 trimerized envelope protein was assessed by ELISA. Binding competition of representative biotinylated antibodies of epitope B (8F6*) and C (6F11) at 100 ng/mL and serum samples at 1:80 titers (physiologic range) were performed. As shown (Fig. **5**), although not universal, antibody responses against these epitopes are common in this cohort (25% for epitope B and 50% for epitope C). Notably, subjects showing responses against these epitopes did not have a clear immunologic phenotype; some had undetectable viral loads and some had high (> 20,000 copies/mL) viral loads (Supplemental Table **2**). Notably, all three of the eight HIV infected subjects who had low + T cell numbers (< 250/mL) failed to show serum competition in this assay.

3.5. No T20 Interference Observed in Luciferase Reporter Fusion Assay

The effects of antibody interference of T20 in this specific cell-cell fusion assay could not corroborate the data obtained in the CHO-WT/HeLaT4+ fusion assay. In this assay using TZM-bl and HL2/3 cells, fusion is detected as luminescence due to the post-fusion induced expression of luciferase. Control wells containing no antibody or T20 displayed high levels of fusion, as seen by a high amount of relative luminescence, while control wells containing T20 had luminescence levels comparable to background wells containing only TZM-bl cells or HL2/3 cells. However, no antibodies, including the control antibody 3D6*, interfered

with T20 (Fig. **6A**). All wells containing T20 exhibited fusion inhibition regardless of any antibodies present. To assess the possibility that the concentration of T20 to antibody ratio was too high to see any observable interference, we also conducted a titration of T20 with a constant 3D6* concentration (Fig. **6B**). However, there was still no apparent interference of T20 by 3D6* even at lower concentrations.

4. DISCUSSION

4.1. Significance of Circulating Antibodies as Resistance Mechanism to Fusion Inhibitors

As there are numerous fusion inhibitors being developed [23, 69, 70], we felt this potential antibody interference was a significant question to study. Although we did replicate antibody interference in the CHO-based assay, [47] the TZM-bl based assay did not show interference. Additionally, these types of antibody responses are apparently not rare (Fig. **5**). Presumably, more initial failures would occur with such therapy if 25-50% of HIV infected persons had significant antibody interference *in vivo*. These latter findings support that likely, this is solely an *in vitro* phenomenon with little clinical significance. Resistance to T20 during therapy has been described in terms of mutations within the HR1 sequence, specifically between residues 36-45, as well as in other locations within gp41 [36, 71]. There are limited clinical samples available of patients who have failed T20 and who don't have a known resistant mutation [35]. This small group of failures not attributable to genetic mutations also supports that antibody interference is likely clinically irrelevant or rare.
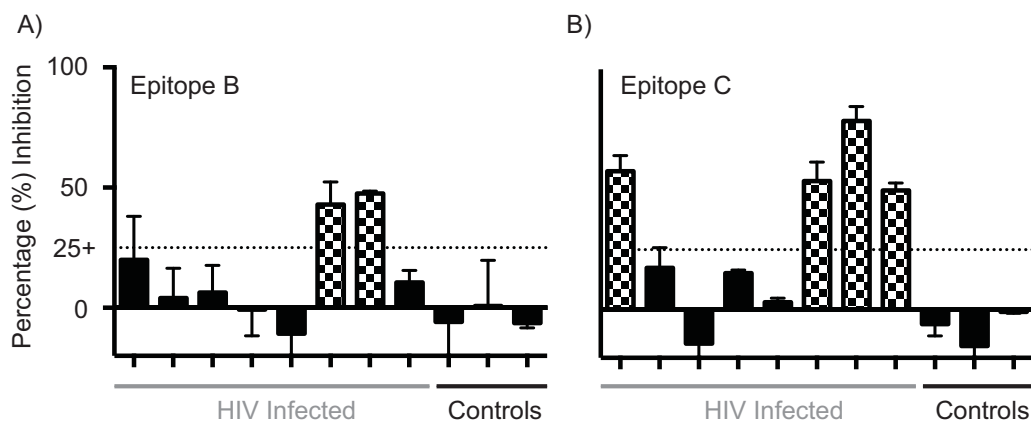
Fig. (5). Conformational targeting antibodies that have been shown to interfere with T20 fusion are not uncommon during an HIV infection. Serum competition at 1:80 titers (physiologic range) against biotinylated conformational targeting antibodies show that these immune responses against each of these novel epitopes are not rare. 25% inhibition was set as the positive cutoff as this was twice background variation. Subject details are in supplemental Table **2**.
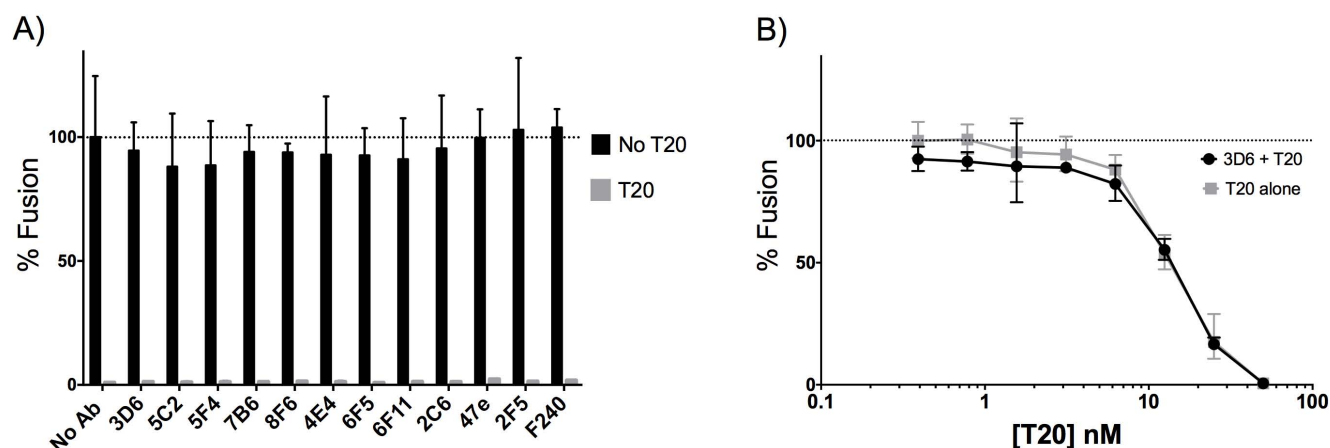
Fig. (6). Antibodies do not interfere with T20 fusion inhibition in TZM-bl luciferase assay. A) Fusion levels of TZM-bl and HL2/3 cells showing the effect of various antibodies alone and those antibodies with T20. Relative lumens (indicating fusion) were normalized to control wells containing no antibodies and no T20 (no inhibition and 100% fusion; dashed line). The average and 95% confidence intervals of triplicate wells are shown. **B)** Fusion levels of TZM-bl and HL2/3 cells with a titration of T20 against a constant concentration of antibody 3D6* (2 µg/ml). At 50 nM T20, total inhibition of fusion was observed despite the presence of 3D6*.

4.2. What are the Differences in these Assays?

Generally, fusion interference studies utilize the TZM-bl based assay likely due to ease of readout (luminescence) rather than microscopic scoring required in the CHO-based assay. It is well known that TZM-bl neutralization assays do not completely mirror PBMC based neutralization assays. HIV *in vitro* assays differences are well known, particularly for neutralization assays [72], and in some assays has completely disparate results [73]. A generally unexplored area is binding kinetics of different sequences. The helices themselves have been shown kinetically to be targeted in an equilibrium fashion for N-HR, or in-dependent on k-on for the C-HR [74]. This has not been thoroughly explored in multiple systems, so the kinetics of these processes in these cell lines may play a role in these differences. Notably, the CHO-based assay does not have a recombinant overexpression of the CXCR5 co-receptor. This may add enough 'inefficiency' to the assay to allow a biologic effect difference that we are seeing. Recent findings suggest virion-to-cell fusion is distinct from cell-to-cell fusion [18]. The later, also

termed 'fusion from without' can be assessed by HeLa based assays and interfered with by V3 targeting antibodies [17]. Notably, elimination of the gp41 tail increased this fusion from without. It's possible the disparity we show with these gp41 targeting antibodies is related to these differences.

An obvious way to interfere is direct competition for the same target on HR1 as T20, such as IG12 [47]. Targeting of this would not explain the disparity seen in our assays. 6F5* could possibly interfere in this manner, however, this has not been elucidated here. 6F5 does not bind overlapping peptides in this area but does seem dependent on trimerization, so lack of peptide binding is not unexpected [52]. It has been proposed that direct binding to T20 may interfere with its function [45]. Our group B and other group C antibodies do not bind linear peptides that overlap T20 nor do they bind T20 directly. It is interesting to note that group B antibodies, which overlap 3D6, target an area removed from either T20 or areas targeted by T20 on linear sequence (Fig. **4**) [52]. It possible that the final or transitional conformation brings regions into close enough contact with one another that these

antibodies can block T20 sterically. Similarly, binding of antibodies may affect remote folding of the protein and cause secondary interference.

4.3. Mapping the Binding Site of Antibodies Cannot Predict Interference Profiles

Despite the lack of clinical significance, the CHO-based assay may prove fruitful in exploring the nature of conformational antibodies to gp41. Our study looked at antibodies known to bind to the immunodominant I hinge region of gp41 (group B) as well as a discontinuous epitope spanning gp41 (group C). It was not surprising that we found interfering antibodies from group B (8F6*) since this group is known to have an overlapping epitope with 3D6, an antibody known to interfere with T20 [47]. Although functionally similar, direct antibody competition studies with 3D6 and this group B antibody (8F6*) show limited competition, implying differential epitope targeting [52]. Taken together, these results imply that the immunodominant 1 region presents multiple structurally influenced epitopes prior to the post-fusion form of gp41. This is consistent with the dynamic changes known to occur in this region during HIV infection [75, 76]. This data allows us to postulate that the simple linear sequence of the immunodominant 1 epitope, in fact, represents a broad range and varying types of epitopes, with some of them being structurally influenced.

In regard to group C, two of four antibodies were able to effectively interfere with T20 action. It was previously thought that epitope C is formed on a post-fusion form on gp41 since the residues crucial for binding (R557, E654, and E657) are known to be in very close proximity to each other on post-fusion structures [52]. Our data provide evidence against this notion, and suggest that epitope C must be immunologically present in a pre-fusion state since two of our antibodies effectively prevented T20 fusion inhibition. This is supported by the finding that post-fusion targeting antibodies (98-6, 126-7 and 50-69), failed to interfere with T20 in our study (Table **1**; Supplemental Table **1**) [14, 77]. Therefore, we suggest that our antibodies target this epitope in a more complex manner than by targeting solely a post-fusion form. In total, our findings from both the group B and C antibodies show that simply mapping the binding epitope cannot predict interference profiles since intra-epitope phenotypic diversity was displayed in both groups.

4.4. Specific Amino Acid Charges and Sizes may Greatly Alter an Antibody's Interference Phenotype

The near-complete homology, but variable interference of group B antibodies (8F6* and 5C2) was striking. The light chain mutation is within the framework so its significance is unclear. Within the heavy chain CDR1 at position 29, a threonine was mutated to asparagine in 8F6* while 5C2 remained at germline. Notably, mutation of this threonine at heavy chain position 29 was also shown in group C 4E4* and 6F5*. We postulate that the loss of methionine alone was sufficient to induce a change in interference phenotype. Since 8F6* and 5C2 bind and compete against one another for epitope B, and their binding has near identical EC_{50s} (2.6 and 2.5 ng/mL respectively) against trimer presented on VLPs [51] this difference in CDR1 must not be inherent to

primary epitope targeting. However, the CDR1 may further stabilize the envelope in a confirmation that affects access to T20. More formalized binding kinetic differences and structural studies of these antibodies and mutants of these antibodies will be pursued to explain this finding.

CONCLUSION

The CHO-based assay may be useful to explore functional interference of certain conformational epitope targeting antibodies. The relatively common nature of antibodies targeting these epitopes, the disparate *in vitro* results, and lack of reported clinical failures ascribed to such antibodies leads us to conclude that antibody interference of T20 is likely not clinically relevant. As new fusion inhibitors are advanced, further exploration of this phenomenon should be considered, particularly once reagents are advanced to clinical trials.

LIST OF ABBREVIATIONS

CDR	=	Complementarity Determining Region
HIV	=	Human Immunodeficiency Virus
HR	=	Heptad Repeat
MPER	=	Membrane Proximal Region
T20	=	Enfuvirtide

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Deidentified samples were obtained to the laboratory group from a previous study. IRB approved study of Dr. Hsiao (UBIRB MED6040609E).

HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All humans research procedures were in accordance with the standards set forth in the Declaration of Helsinki principles of 1975, as revised in 2008 (http://www.wma.net/en/20 activities/10 ethics/10helsinki/).

CONSENT FOR PUBLICATION

Serum samples were obtained from a previous research study. Subjects were recruited from the Immunodeficiency Services Clinic of the Erie County Medical Center with IRB approval.

CONFLICT OF INTEREST

ACKNOWLEDGEMENTS

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers web site along with the published article.

APPENDIX: SEQUENCE DATA

Nucleotide accession numbers, heavy and light chains: 2C6- JQ433108, JQ433074

5C2- JQ433100, JQ433079; 8F6*- JQ433104, JQ433091; 4E4*- JQ433117; 6F5*-JQ433118, JQ433084; 6F11-JQ433110, JQ433085; 7C6- JQ433111, JQ433087; 8B10-JQ433125, JQ433089.

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