

HIV-1 gp41 heptad repeat 2 (HR2) possesses an amino acid domain that resembles the allergen domain in *Aspergillus fumigatus* *Asp fl* protein: review, hypothesis and implications

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Abstract Enfuvirtide (ENF, T-20, Fuzeon) is the first synthetic peptide to be modeled according to the amino acid sequence of HIV-1 heptad repeat 2, which was used to treat cohorts of HIV-1-infected individuals who had failed to respond to treatment with the anti-HIV-1 cocktail HAART. It was reported that when injected subcutaneously, Enfuvirtide reduced viral RNA in patients' blood by 1.96 log₁₀, leading to a subsequent increase in the number of CD4⁺ T cells in the blood. The drug treatment caused adverse effects at the injection site in a small number of treated individuals, and a gradual increase in IgE in the blood during prolonged treatment. Enfuvirtide was approved for treatment of HIV-1 patients who developed resistance to HAART. The present review attempts to explain the adverse effects of Enfuvirtide at the skin site of injection, and the gradual increase in IgE in patients' blood during treatment. These phenomena were reported to resemble the effect of allergens that cause asthma in humans. It is hypothesized that since the amino acid domain of the *Asp fl* allergen from *Aspergillus fumigatus* was identified in the N-terminus of an 18 kDa protein, it may be useful to compare *Asp fl* peptide aa 7–22 from the β -hairpin sequence to the β -hairpin sequence of the heptad repeat 2 of HIV-1 gp41. The comparison revealed that the amino acid sequence resembles part of the *Asp fl* aa 7–22 allergenic domain. The heptad repeat 1 of gp41 also resembles the fungal allergen. It is suggested that the Enfuvirtide peptide be tested experimentally to

determine if ENF peptide is capable of binding to IgE antibodies from Enfuvirtide-treated, HIV-1-infected patients, and whether the HR2-derived peptide is capable of inducing basophils that were isolated from healthy individuals and from ENF-treated and untreated HIV-1 patients to release histamine and IL-4.

Keywords Synthetic HIV-1 gp41 peptides · Derivatives of HR2 · Inhibitors of HIV-1 fusion · Enfuvirtide (T-20 · Fuzeon) approved drug treatment of HIV-1 patients · Induction of IgE increase · Resemblance to *Asp fl* allergen motif.

Introduction

In October 1994 Wild et al. [1] published a paper identifying DP-178, a synthetic peptide that corresponds to the aa sequence (residues 643–678) of HIV-1 gp41 fusion protein, as having potent antiviral activity. At a concentration of <5 ng/ml the DP-178 peptide blocked 100% of HIV-1-mediated virus-cell fusion, and at 80 ng/ml it reduced the infectious titer of cell-free virus approximately 10-fold. Approximately 102–103 times more DP-178 peptide was required for the inhibition of an HIV-2 strain. They compared the aa sequence of HIV-1 Lai to 3 prototypic HIV-1 isolates: MN, RF and SF1, and to HIV-2 NIHZ and showed that a subsequence of DP-178, residues 657QQEKNEQELLEL668, is present in HIV-1 MN, RF and SF2 isolates. In HIV-2 only the residues 657QQEKN661 were present in the viral gp41. The authors concluded that “The results presented are consistent with the initial observation that DP-178 is a potent specific inhibitor of HIV-1-mediated cell–cell fusion [2] and infection by cell-free virus.”

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Ten years later, Matthews et al. [3] published a review detailing the research on the anti-HIV-1 fusion peptides that led to the development of Enfuvirtide (ENF, T-20, Fuzeon). “The first synthetic peptide mimetic (DP-107) was a 38-amino acid molecule corresponding to gp160 residues aa 558–595, the N-terminal region of gp41.” DP-107 at 6 µg/ml inhibited HIV-1 replication. Further studies examined the C-terminus of gp41, which was predicted to have an α -helical structure, and a 36-aa peptide DP-178 (later named Enfuvirtide) corresponding to gp41 residues aa 643–678 derived from HIV-1_{Lai} gp160 was synthesized. This peptide, ENF, inhibited HIV-1_{Lai}-induced syncytium formation at IC₅₀ of 1.5 ng/ml (100 times more potent than DP-107), but did not inhibit HIV-2 and SIV infection. The authors indicated that ENF was expected to be active against HIV-1 strains resistant to any of the 3 classes of anti-retroviral (ARV) drugs included in HAART.

In a 14-day trial (TRI-001) to establish dose response in HIV-1-infected individuals, ENF in the dose range 3–100 mg was administered twice daily by intravenous infusion to 16 patients as monotherapy. At the highest dose of 100 mg, median viral suppression was $-1.96 \log_{10}$ copies of HIV-1 RNA per ml serum, with a linear dose response within the entire range. Phase II studies established a longer-term safety profile of 48–96 weeks for ENF-containing regimens. The authors detailed all the clinical studies showing ENF efficacy: Phase III TORO (T-20 vs. Optimized Regimen Only) involved 1,000 anti-viral drug treatment-experienced patients, which “offered definitive proof of the therapeutic value of ENF.” TORO1 was conducted in the USA, Mexico, Brazil, and Canada and TORO2 was conducted in Europe and Australia. In both TORO trials, therapy with ENF was provided together with an “Optimized Background” (OB) anti-viral drug (ARV) regimen of 2 or 5 drugs.

The authors reported that “the lowest 10 mg and the highest (100 mg) twice daily doses of ENF did not select for ENF-resistant mutants, but virus isolated during exposure to, and rebound from, intermediate ENF doses, showed aa substitutions within HR1 region at gp41 positions 32 (Q to R/H), 36 (G to S), 38 (V to A), and 39 (Q to R).” They also reported the sequencing of virus from 177 ENF-naïve patients, with 80% showing the consensus sequence at gp41 aa 36–45, and 15% showing an N42S polymorphism. In protocol-defined virological failure, the virus from 78% of patients (31 out of 40) had substitutions associated with resistance at positions 36–45. The ENF-resistant mutants isolated after treatment with ENF conferred significant growth disadvantage in the absence of the drug compared with wild-type virus.

In 2003, the Federal Drug Administration (FDA) approved Enfuvirtide as the first HIV-1 fusion inhibitor for treatment of HIV-1 patients who had developed drug-resistance. Van den Broek et al. [4] developed a method for the quantification of 2 peptide HIV-1 fusion inhibitors (ENF and Tifuvirtide (T-1249)) and 1 metabolite (M-20) of ENF in human plasma by solid phase extraction using liquid chromatography-coupled electrospray tandem mass spectrometry. The method was capable of measuring concentrations of ENF and its metabolite in plasma samples from HIV-1-infected patients treated with the drug.

Schneider et al. [5] reported that fusion inhibitors can be designed to overcome resistance to ENF, as demonstrated with T-1249, in studies No. T-1249-102 and T1249-105, conducted in patients who had developed resistance and were failing an ENF-containing regimen [6].

The aims of the present review stem from the need to identify the properties of the aa domains in HR1 and HR2 heptad repeats, which are critical for the formation of the 6-helix bundle (6-HB) with the gp41 N-terminal heptad repeat (HR1, NHR) that prevents the formation of the fusogenic gp41 core between viral gp41 (HR1, NHR) and (HR2, CHR). Definition of the critical gp41 sequences may provide an explanation for the response to the cutaneous injection of ENF into HIV-1-infected patients who respond to the drug with a gradual increase of IgE.

Mode of inhibition of HIV-1 gp41 fusion with cellular membranes by Enfuvirtide

Bianchi et al. [7] reported that covalent stabilization of the HIV-1 gp41 N-terminal aa sequence yielded extremely potent and broad inhibitors of HIV-1 infection. The mechanism of HIV-1 fusion involves 2 helical regions of gp41: an N-terminal heptad repeat (HR1) and a C-terminal heptad repeat (HR2). HR1 and HR2 form a fusion-active “trimer of hairpins” conformation since HIV-1 gp160 glycoprotein molecule are made of gp41 and gp120 trimers connected by a non-covalent bond. The trimer of hairpins consists of a bundle of 6 α -helices contributed by the three gp41 monomeric peptides. The three C-peptides pack in an antiparallel manner against a central 3-stranded coiled-coil formed by the N-sequence [8]. Fusion progresses by formation of a “pre-hairpin” conformation that places the N-terminal fusion peptide near or in the target membrane, exposing the HR1 and HR2 regions. Addition of synthetic HR1 and HR2 peptides can inhibit viral infection by preventing formation of the

fusogenic trimer of hairpins. Indeed, Enfuvirtide is an HR2 (CHR) peptide that inhibits HIV-1 fusion.

Eckert and Kim [9] stabilized the HR1 (N) peptides with a low tendency to form trimeric coiled-coil fused peptide with the stable coiled-coil IZ peptide to yield the peptide IZN17, a nanomolar inhibitor, covalently stabilized by interchain disulfide bonds. The peptide cIZN17₃ was the most potent fusion inhibitor at IC₅₀ = 40–380 pM in a single-cycle infectivity assay against HIV_{HXB2}, HIV_{NL4-31} and HIV_{MN-1}. This inhibitor has a broad anti-HIV profile against a large panel of HIV isolates, including R5, X4, and R5/X4 strains. The authors concluded that the potency of cIZN17 was equal to, or greater than, the C-peptide inhibitor ENF, which was approved for clinical use.

Viega et al. [10] studied the putative mode of action of ENF at the molecular level, since ENF lacks the sequence of 8 amino acids, aa 628–635, thought to be essential for association with the HR1 N region. The aa sequence of ENF and its eventual arrangement is thought to be an α -helix with amphipathic segments, suggesting that it may interact with biological membranes. However, the authors concluded that “ENF remains in a random-coil conformation when inserted in membranes.” The authors studied the extent of the partition of ENF into the biological membrane, its location in the lipidic matrix, and the interplay between partition and conformation. The presence of tryptophan residues in ENF makes fluorescence techniques suitable tools for probing this molecule, with no need for chemical derivatization. The authors concluded that the tryptophan residues are located at a shallow position in the membrane and that partition of the intrinsically fluorescent HIV fusion inhibitor ENF into lipid membranes is relatively high and modulated by cholesterol. No conformational energetic barrier prevents its activity in either aqueous solution or lipidic membranes; this may play a key role in the biochemical mode of action of ENF. The authors depicted the putative action of Enfuvirtide at the molecular level in general terms: “ENF inserts into the external layer of the cell plasmalemma (non-charged lipids) and is prevented from translocation due to the repulsion caused by the negatively charged lipids of the inner layer, among other reasons. When HIV approaches the cell, the virus lipidic membrane does not remove the ENF from the cell outer surface because of its high cholesterol content; i.e., ENF concentrates at cell surfaces and tends to stay there. Therefore, cell membranes are an ENF reservoir. The partition equilibrium tends to stabilize the ENF concentration in the aqueous environment when binding to gp41 occurs (i.e., improving fusion inhibition efficacy). Moreover, the high levels of

ENF accumulated at the cell surface render the interaction with other segments of gp41 possible if they reach the cell surface (i.e., if ENF binding to the Leu-Ile-rich region fails and the fusion process proceeds). Binding of ENF and related molecules to the fusion peptide and other gp41-derived peptides has been reported by Jiang et al. [11].”

Kliger et al. [12] reported that DP-178 (ENF) inhibits phospholipid redistribution (IC₅₀ values are 43 and 335 nM, respectively), while C34, a synthetic peptide which overlaps with DP-178 (ENF), but contains the cavity binding residues, did not show this phenomenon (IC₅₀ values are 11 and 25 nM, respectively). The ability of DP-178 to inhibit virus and cell membrane fusion at a post-lipid mixing stage is due to its ability to bind and oligomerize on the surface of membranes. The authors indicated that their results are consistent with a model in which DP-178 inhibits the formation of gp41 hairpin structure at low affinity and the C34 peptide (also an HR2 peptide) inhibits its formation at high affinity. However, DP-178 binds with high affinity to a sequence domain immediately adjacent to the membrane-spanning domain, inhibiting the recruitment of several gp41-membrane complexes, and inhibiting fusion pore formation.

Liu et al. [13] compared the activity of the HR2 synthetic peptide C34 to that of ENF, since the C34 peptide is known to compete with HR2 of gp41 to form a stable 6-HB with the gp41 N-terminal heptad (HR1), and prevent the formation of the fusogenic form of gp41 core between the viral gp41 HR1 and HR2. As a result, fusion between the viral and the target cell membranes is inhibited. The authors' experimental results demonstrated that ENF cannot form stable 6-HB with the N-peptides derived from HR2 region, and it cannot inhibit the 6-HB formation of the fusogenic core. It was suggested that ENF (T-20, Fuzeon) mediated anti-HIV activity that can be abrogated by peptides derived from the membrane-spanning domain of gp41 and peptides from the coreceptor binding site in gp120. It was concluded that “These new findings imply that T-20 (ENF) inhibits HIV-1 entry by targeting multiple sites in gp41 and gp120.”

HIV-1 mutants with amino acid substitutions in gp41 are resistant to Enfuvirtide (ENF) treatment

The efficacy of ENF

In a review on ENF Matthews et al. [3] presented the results of a 14-day trial (TRI-001) carried out to establish the dose response. ENF was administered twice daily to

16 patients as a monotherapy by intravenous infusion in a dose range of 3, 10, 30 and 100 mg. The highest dose of 100 mg caused viral suppression of $-1.96 \log_{10}$ copies/ml of HIV-1 RNA and the dose response was linear within the entire range. Kilby et al. [14] reported that all 4 subjects receiving 100 mg twice daily had a decline in plasma HIV RNA to less than 500 copies/ml by bDNA assay, a sensitive RT-PCR assay (detection threshold 40 RNA copies/ml) which demonstrated that, although undetectable levels were not achieved in a 14-day dosing period, there was a $1.96 \log_{10}$ median decrease in plasma HIV RNA in these subjects. The authors concluded that “this study provides proof-of-concept that viral entry can be successfully blocked in vivo.” Short-term administration of T-20 seems safe and provides potent inhibition of HIV replication comparable to presently approved anti-retroviral regimens.

Emergence of ENF-resistant HIV-1 mutants

Sista et al. [15] identified substitutions in HIV-1 gp41 at positions 36, 38, 40, 42 and 43 in ENF-treated HIV-1 patients.

Mink et al. [16] aimed to further the investigations on HIV-1 resistance to ENF carried out during the early clinical trials, and revealed that changes in the gp41 genotype, which confer resistance to ENF may cover longer aa sequences than the gp41 aa 36–38 motif identified in early in vitro selection experiments. The authors studied the binding of ENF to recombinant maltose-binding HIV strain NL4-31 fusion proteins, either wild-type HR1 sequence aa 29–43 or a subset of aa substitutions in the aa 29–43 sequence, 29QARQLLSGIV-QQQNN43, and determined the effect on ENF binding to the peptides and on its anti-viral activity. It was reported that only substitutions in the region of aa 36–43 resulted in a >4-fold reduction in ENF binding. The largest reductions in ENF binding (>40-fold) and susceptibility (>20-fold) were seen with the Q40H and N43D substitutions. In addition, the authors studied HIV-1 isolated from ENF-treated patients and concluded that “the data presented clearly demonstrated that substitutions in the aa sequence 36–45 of gp41 are primary determinants for the development of viral resistance to ENF.”

Marcial et al. [17] developed assays for detecting ENF-resistant mutations in HIV-1 gp41 in viruses isolated from plasma samples from HIV-infected patients. The authors concluded that the use of the gp41 assay resulted in a higher success rate for generating gp41 sequences from test samples with a low copy number and from clinical samples revealing substitutions in the aa sequence 36–45.

Su et al. [18] examined full-length baseline and gp120 and gp41 sequences in isolates from Phase III studies of ENF, including 281 patients receiving ENF + OB and 88 patients receiving OB alone. It was reported that in addition to changes in gp41 aa 36–45, changes at 3 amino acid positions (126, 129 and 133) in the HR2 domain occurred more often in patients undergoing ENF treatment due to virological failure. The mutations in HR1 aa 36–45 alone account for slightly more than 90% of the variation. The authors wrote that the “study represents a statistical approach to highlight positions in HIV envelope gp120 that undergo mutations in the presence of ENF.”

Perez-Alvarez et al. [19] investigated the susceptibility to ENF and the dynamics of amino acid changes in HR1 and HR2 of gp41 of HIV-1 isolates from plasma, peripheral blood mononuclear cells (PBMC) and primary isolates (PI) in 4 highly anti-retroviral-experienced patients who received ENF plus antiretroviral regimen and were followed over a period of 40–72 weeks. In one non-responder patient an N43D substitution was detected at 12 weeks of treatment. Double mutations of N42T + N43D were found in plasma HIV RNA at 32 weeks and remained detectable for 16 weeks following withdrawal of the ENF. The S138A substitution in HR2 was found in plasma RNA at 32 weeks and mutations, V101G and E137K in HR2 were also observed. In 3 temporary responders G36D and N42T substitutions were observed at 12, 24, and 40 weeks, respectively.

Jenwitheesuk et al. [20] reported that mutations in HR2 improve structural stability of the HR1/HR2 hairpin complex and may lead to enhancement of ENF resistance when present with resistant HR1 mutations.

Subcutaneous (SC) injection of Enfuvirtide into patients causes skin reactions and IgE hyperproduction

The primary objective of Lalezari et al. [21] was to determine the steady-state pharmacokinetics and relative bioavailability of ENF following SC injections at 3 anatomical body sites: abdomen (A), thigh (B), and arm (C). The study design was a single-center, open-label, multi-dose, 3-way randomized, cross-over study. The authors recruited twelve HIV-1-infected adults from 3 ongoing Phase II ENF clinical trials and randomized them into 3 groups. Each group continued to receive SC ENF injections at a dose of 90 mg twice daily according to 3 treatment sequences, ABC, BCA or CAB, over 3 consecutive periods of 7 days each. Plasma concentrations of ENF and its metabolite (Ro 50-6343) were measured by validated liquid chromatography-tandem

mass spectrometry methods. The authors reported that although injection site reactions (ISR) were common, the overall grading (based on pain and discomfort) for all was Grade I (mild). The incidence of ISRs varied according to the site of injection, but no patient required treatment for ISRs.

Ball et al. [22] studied the ISR to Enfuvirtide and reported that the most common type of adverse event associated with ENF treatment is the occurrence of ISR in up to 98% of patients. The study investigated these reactions occurring in seven HIV patients treated with ENF. All the biopsy specimens revealed an inflammatory response consistent with a localized hypersensitivity reaction resembling granuloma annulare and the recently described interstitial granulomatous drug reaction. Staining with immunoperoxide indicated that the inflammatory and collagen changes were greatest at ENF deposition. The authors concluded that “the pathologic changes and clinicopathologic correlations associated with ISR in human ENF recipients have been described for the first time. This information also provides valuable insights into unusual pathogenic responses to foreign antigens for patients with HIV.”

Maggi et al. [23] studied cutaneous ISR to long-term therapy with ENF in 6 HIV patients. Four macroscopic patterns were noted: (a) no evidence of lesions, (b) transient infiltrative lesions that auto-resolved within 24 h, (c) transient nodular lesions that auto-resolved within 7–15 days and (d) stable lesions after more than 30 days with a scleroderma-like aspect. The histological examination of the skin biopsies showed 3 patterns: (1) an acute urticaria/vasculitis-like pattern with inflammation of the fat tissue, (2) a subacute pattern with initial dermal sclerosis and (3) a chronic scleroderma-like pattern with connective tissue disposition. Immunohistochemical study revealed the prevalence of T lymphocytes and neoangiogenesis.

Burastero et al. [24] studied the immunological basis for IgE hyper-production in ENF-treated HIV patients to define whether ENF peptide was capable of directly driving B lymphocytes in vitro to switch from IgG to IgE production and/or whether it could act through a Th2 skewing mechanism. The authors also reported that eosinophilia occurred more frequently in ENF-treated HIV patients, while clinically systemic hypersensitivity reactions were reported in less than 3% of patients. The authors reported that “the main findings of the present study is that ENF treatment is inducing a systemic immunological disequilibrium, in HIV-1-positive patients which is not interfering with efficient suppression of the viral load. This phenomenon is characterized by a production of increased IgE levels, is not dependent on a direct effect on B lymphocytes but is likely driven

by induction of Th2 cells. The clinical consequences of this side effect are limited to poorly symptomatic reactions at the injection site, likely due to the fact that these IgE are not ENF-specific.”

Harris et al. [25] assessed the use of the biojector B2000 needle-free, gas-powered injection system for SC administration of ENF to HIV-infected patients and compared the results to needle injection. The biojector was rated as being significantly easier to use. ENF levels in the plasma were not different from that of needle injection.

Miro et al. [26] reported that “switching a HAART-containing didoxy-NRTI to ENF + tenofovir and saquinavir/ritonavir minimally altered metabolic parameters and exerted beneficial effects on mitochondrial function at 24 weeks.”

HR2 peptide Enfuvirtide contains an amino acid motif that functions as an allergen

Injection of Enfuvirtide is the cause of IgE synthesis, a marker of allergy

The above-described effects caused by the SC injection of ENF into the skin of HIV-positive individuals provided information on the induction of inflammation in the skin [21–23]. In addition, the gradual increase in the level of IgE in the serum of ENF-treated patients follows the course of ENF skin injections and is an indication that ENF is an inducer of the release of Th2 cytokines from the innate system cells (basophils, mast cells, and monocytes). These cells express FcεRI⁺, the IgE receptor. The binding of peptide allergens to IgE/FcεRI⁺ cells induces the innate system cells, which are loaded with cytoplasmic granules containing large amounts of Th2 cytokines (IL-4, IL-5, IL-10, and IL-13), to release the cytokines into the bloodstream. The marked increase in the level of IL-4 induces B cells to stop IgG synthesis and switch to the synthesis of anti-allergen IgE molecules. In addition, the increased IL-4 level is inhibitory to Th1 cell cytokine synthesis and prevents the activation of anti-HIV-1 CTLs. This mechanism of skewing the Th1/Th2 cytokine balance in HIV-1-infected patients toward Th2 cytokines has been described previously for HIV-1 [27–29].

The biological activities of *Aspergillus fumigatus* ribotoxin I (*Asp fl*)

The pathogenicity to humans

Rementeria et al. [30] summarized the nature of the “Genes and molecules involved in *Aspergillus fumigatus*

virulence.” *Aspergillus fumigatus* is a saprophyte fungus with the most prevalent niche on the ground and is the most ubiquitous fungus due to the dispersion of its small conidia (2–3 μm) that can reach the human pulmonary alveoli. Immunocompetent individuals who inhale the conidia rarely have adverse effects since they are efficiently eliminated by the innate and acquired immune systems. Yet, in immunocompromised individuals, such as HIV-1-infected patients, *Aspergillus* causes mycotoxicosis, allergic reactions, invasive aspergillosis and allergic bronchopulmonary aspergillosis (ABPA), asthma, rhinosinusitis, and aspergilloma. The *A. fumigatus* genome contains 20 different allergen-coding genes. The major allergen, *Asp fl*, is an 18-kDa protein and its pathogenicity is due to its ribotoxin activity, which inhibits protein synthesis in infected cells by cleavage of the 28S ribosomal RNA in ribosomes. Ribotoxin causes type I hypersensitivity and has the properties of a cytotoxin.

Ribotoxin toxicity to cells, ribonuclease activity and allergenicity

Moser et al. [31] cloned and expressed a recombinant *A. fumigatus* allergen I/a (r*Asp fl*/a) that has the capacity to bind to IgE in the type I skin test. The authors reported that *A. fumigatus* secretes an 18 kDa non-glycosylated IgE-binding protein that is a ribotoxin like α -sarin and mitogillin. The authors cloned the 686-bp cDNA gene and expressed it in *E. coli* as a fusion protein with 6 adjacent histidines (r*Asp fl*/a). The purified recombinant protein had ribotoxin activity and was identified by a murine mAb made against 18-kDa ribotoxin. It was also reported that the *Asp fl* recombinant protein was able to bind to IgE antibodies isolated from individuals allergic to *A. fumigatus*. In addition, it was shown that this peptide elicited a positive immediate type I skin reaction in individuals allergic to *A. fumigatus* using skin prick testing or intracutaneous testing of allergens application to the forearm. The reaction was compared to two commercial and two crude extracts from *A. fumigatus*.

Arruda et al. [32] purified *Asp fl* protein and compared its aa sequence to that of mitogillin, produced by *Aspergillus restrictus*, and reported that the 2 proteins were antigenically identical in cross-inhibition radioimmunoassay using murine mAb and human IgG and IgE antibodies. The 2 proteins inhibited protein synthesis in vitro by >90%. The authors suggested that “*Asp fl* could play a dual role in pathogenesis of *A. fumigatus*-related diseases by promoting colonization through cytotoxic activity and by causing inflammatory reactions involving IgE antibodies.”

The amino acid domains in Asp fl ribotoxin for ribonuclease activity and allergenicity

Gluck and Wool [33] used systemic deletion of amino acids to determine the ribotoxin-active domain of ristricotocin that is involved in the cleavage of ribosomal 28S RNA. The authors reported that 93 out of 149 aa (62%) in ristricotocin were not essential for the action of the toxin. Glu95 is the active site residue, which is replaced by Asp90 in the *Asp fl* Δ 91–95 deletion mutant.

Garcia-Ortega et al. [34] identified the allergen domain in the aa sequence of *Asp fl* by producing a deletion mutant of *Asp fl* Δ (7–22) which displayed reduced IgE binding. The authors reported that *Asp fl* and its deletion mutant *Asp fl* Δ (7–22) have been produced as recombinant proteins, and that the deleted aa sequence corresponds to an exposed NH_2 -terminal β -hairpin in *Asp fl* protein. The NH_2 -terminal β -hairpin is highly flexible and has an exposed region, folded independently from the protein core. The authors studied the involvement of the allergen *Asp fl* and the deletion variant in which the β -hairpin (aa 7–22) was substituted by 2 Gly residues [34]:

	1	7	allergen	22
Asp fl	AVTWTCT	INQQLNPKTNKW	WEDKR	LLYNQAKA...
Asp fl Δ (7–22)	AVTWTCTG	deletion		GLLYNQAKA...

The authors compared the cytotoxic activity of *Asp fl* and its Δ (7–22) deletion mutant to rhabdomyosarcoma (RD) cells which have been shown to be a suitable target for ribotoxins. It was reported that the IC_{50} of *Asp fl* was 0.7 μM , while the deletion mutant had an IC_{50} about 10-fold higher (8 mM). The binding of *Asp fl* to human-specific IgE antibodies, which were obtained from a population of 58 patients sensitized to *Aspergillus*, revealed a 30% decrease in IgE-binding of *Asp fl* Δ (7–22) in comparison to *Asp fl*. The authors quantified the ability of *Asp fl* Δ (7–22) to inhibit *Asp fl* binding to IgE and reported that the percentages of inhibition at the highest concentration of inhibitor were 80% for *Asp fl* Δ (7–22). The authors concluded that “the results showed that the deleted NH_2 -terminal β -hairpin of ribotoxins is involved in the allergenic response to the wild-type allergens and the deleted aa sequence of *Asp fl* Δ (7–22) contains at least 1 allergenic epitope.”

Asp fl N-terminal β -hairpin interaction with cellular membranes

Garcia-Ortega et al. [35] studied the interaction of *Aspergillus* ribotoxin amino-terminal β -hairpin with

membranes. Studies on the 3-dimensional structures of 2 ribotoxins, α -sarcin and restrictocin, showed that these proteins interact with cellular phospholipid bilayers, enter into eukaryotic cells and cleave a specific phosphodiester bond in the 28S RNA in the large ribosomal subunits. The authors reported that 2 single mutants (K11L) and (T20D) and the corresponding K11L/T20D double mutant proteins were produced in *E. coli*. It was reported that “the overall native structure of the mutants was preserved, as was their toxic effect.” However, the mutant (K11L) and restrictocin displayed a lower phospholipid-interacting capability. The authors concluded that “the results obtained are interpreted in terms of the involvement of the amino-terminal β -hairpin in the interaction with membranes and polyadenylic acid.”

Hypothesis and conclusions

Hypothesis: the need to identify putative allergens in anti-fusion peptides

Subcutaneous injection of Enfuvirtide has been reported to cause both an allergic response at the site of injection and a gradual increase of the level of IgE. Both biological responses of HIV-1-treated patients indicate an allergic response to the anti-fusion drug. These responses should be noted carefully even if only the minority of treated HIV-1 patients respond adversely. Here we hypothesize that Enfuvirtide resembles *Asp fl* in the amino acid sequence of the N-terminal β -hairpin domain 7–22:

	7	22
Asp fl aa 7-20	AVTWT	INQQLNPKTNKWE-DKRLLNQA
HIV-1 gp41 (HR1)	SGIVQQQNNLLRAIE	AQQHLLQLTVWGIKQLQARIL
ENF (HR2) aa 638-673	YTSLIHSLIEES	QNQQEKNEQELLELDK WASLWN

It is interesting to note that Xu et al. [36] studied the basis for fusion inhibition by heptad repeat regions of the fusion protein from the Nipah and Hendra paramyxoviruses. The authors reported that the HR2 peptide aa 453–485 is:

453 KVDISSQISSMNQSLQQSKDYKEAQRLLDTVN 485,

suggesting resemblance to *Asp fl* allergen motif.

The above hypothesis connects the findings that the injection of HIV-1 gp41 HR2 synthetic peptide Enfuvirtide into the skin of HIV-1-infected individuals with a gradual increase of IgE, similar to the increase in the level of IgE in *A. fumigatus* infection in humans. The

mechanism by which HIV-1 gp120 induces inhibition of the adaptive and cellular immune responses in infected individuals was described in previous publications [27–29]. It was reported that the viral-shed gp120 binds to IgE/Fc ϵ R1⁺ hematopoietic cells (basophils, mast cells, and monocytes) and induces the release of large amounts of Th2 cytokines (IL-4, IL-6, IL-10, IL-13) into the blood, skewing the Th1/Th2 balance toward Th2 cytokines. The increased IL-4 and IL-10 levels inhibit Th1 cytokine production. In addition, IL-4 inhibits IgG synthesis by B cells and induces IgE synthesis.

Conclusions

HIV-1 infection was reported to induce a gradual increase in IgE, an indication of a process related to the induction of IgE increase in allergic individuals. The finding that the HR2 synthetic peptide Enfuvirtide binds to several amino acid domains in the viral gp120 in addition to sites in gp41 [12, 13] may indicate the existence of additional undiscovered functional domains.

Experimental studies of the allergenicity of HR2 synthetic peptides may lead to improvements in the drug that will not cause increased levels of IgE above those resulting from HIV-1 infection.

It was hypothesized [37, 38] that treatment of HIV-1 patients with CpG ODN may inhibit HIV-1 replication by the induction of interferon α and β release from plasmacytoid dendritic cells (PDC) that reactivate the failing adaptive immune response. In a subsequent

paper it was hypothesized that combining the IL-4 antagonist IL-4 Δ 2 with CpG ODN treatment may inhibit the decline of the adaptive and cellular immunity in HIV-1 patients in addition to interferon inhibition of HIV-1 replication [39]. Treatment with HR2 synthetic peptides together with the above treatment may prevent HIV-1 infection following exposure to the virus.

Two approaches are available to experimentally explore the properties of Enfuvirtide and other HR2 synthetic peptides: (1) binding of these peptides and control peptides to IgE molecules isolated from the serum of HIV-1-infected individuals and from HIV-1 patients treated with Enfuvirtide; and (2) isolation of basophils from healthy and treated infected individuals and testing of the effect of HR2 and control peptides

on the release of histamine and Th2 cytokines from the basophils [39].

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