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Immunological and virological characteristics of human immunodeficiency virus type 1 superinfection: implications in vaccine design

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Abstract

Superinfection is frequently detected among individuals infected by human immunodeficiency virus type I (HIV-1). Superinfection occurs at similar frequencies at acute and chronic infection stages but less frequently than primary infection. This observation indicates that the immune responses elicited by natural HIV-1 infection may play a role in curb of superinfection; however, these responses are not sufficiently strong to completely prevent superinfection. Thus, a successful HIV-1 vaccine likely needs to induce more potent and broader immune responses than those elicited by primary infection. On the other hand, potent and broad neutralization responses are more often detected after superinfection than during monoinfection. This suggests that broadly neutralizing antibodies are more likely induced by sequential immunization of multiple different immunogens than with only one form of envelope glycoprotein immunogens. Understanding why the protection from superinfection by immunity induced by primary infection is insufficient and if superinfection can lead to cross-reactive immune responses will be highly informative for HIV-1 vaccine design.

Keywords

human immunodeficiency virus type I; superinfection; incidence; immune response

Introduction

After infection by an HIV-1 strain, a person can be superinfected by a different HIV-1 strain [1–3]. Superinfection occurs in high-risk and general populations [4–10]. Frequent

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superinfection raises two important questions: (1) if primary infection induces protective immunity to reduce subsequent heterologous infection and (2) if superinfection leads to broad neutralization responses to heterologous viruses. Frequent superinfection suggests that immune responses induced by primary infection may not be strong or broad enough to prevent subsequent infections by heterologous viruses [7,11–13]. This notion was supported by a study which revealed that neutralizing antibody (nAb) responses were developed prior to superinfection during chronic infection [14]. However, a recent study showed that the polyfunctionality of CD4⁺ effector memory T cell responses contributed to the suppression of superinfection through mucosal transmission in a rhesus monkey model [15]. Thus far, no evidence was found to support the association of neutralization potency and breadth with the time lag between superinfection and primary infection, but the length of the time that an individual harbored two viruses was found to be correlated with neutralization breadth [16]. A number of other studies also showed that sequential infections led to the augmentation of the broad neutralization responses [16–18]. These observations show that studying superinfection may provide valuable insights into the potential mechanisms that contribute to development of neutralization breadth. Although immune responses can be fundamentally different between HIV-1 infection (damaged CD4⁺ T cell responses) and vaccination (intact immune system), understanding how superinfection occurs can have important implications in future HIV-1 vaccine design.

Detection of superinfection

Some HIV-1-infected individuals can be infected by two or more HIV-1 strains. Based on the timing of infection, infection with more than one HIV-1 strain can be defined as coinfection and superinfection. Coinfection is for a person who is infected by two different strains at the same time or who is infected by a second strain before development of immune responses against primary infection viruses. Superinfection is for a person who is infected by a heterologous HIV-1 strain after development of immune responses to the primary infection strain [19,20]. Superinfection can occur between two different strains from the same subtypes (intrasubtype) or between strains from different subtypes (intersubtype). The first intersubtype superinfection cases (subtype B and CRF01_AE) were found from two injection drug users (IDUs) in Thailand in 2002 [4]. The first intrasubtype superinfection case (subtype B) was reported in the USA also in 2002 [11]. Since then, superinfection has been reported from a variety of populations and modes of transmission, such as men who have sex with men (MSM) [21–25], IDUs [6,26], heterosexual transmission (HST) [10,27,28], female sex workers (FSWs) [28,29], mother-to-child transmission (MTCT) [30], and general populations [8,10]. More intersubtype superinfection had been reported than intrasubtype superinfection, possibly due to the easier detection of the intersubtype superinfection than intrasubtype superinfection. The variation of *env* sequences between different subtypes is as high as 30%, much higher than the variation among the viruses from the same subtype (less than 10%) [31]. Because some methods are not sensitive enough to distinguish different HIV-1 strains from the same subtype, intrasubtype superinfection is more likely to be underestimated.

Superinfection is determined by detecting the presence of additional distinct viral genomes in an individual who has been infected by the first virus for some time during primary

infection. The methods for the detection of superinfection have been improved significantly over the years (Table 1). In the early days, many methods with low sensitivity and low throughput were used, including restriction fragment length polymorphism (RFLP) [6], subtype-specific PCR amplification (ssPCR) [4], restriction fragment analysis, and direct sequencing [32]. Later on, population-based screening strategies were developed for superinfection detection, including the heteroduplex mobility assay (HMA) [31,33–35], the multiregion hybridization assay (MHA) [36], bulk viral sequence analysis, and selective cloning [22,36–38]. Although bulk PCR sequence analysis cannot unequivocally confirm the presence of two distinct HIV-1 strains, its results can indicate a high likelihood of superinfection [22]. Because population-based sequencing is less sensitive to the minority viral population (< 20%) than HMA, it underestimates the frequency of superinfection [36]. On the other hand, MHA can only detect intersubtype superinfection and misses the intrasubtype superinfection. HMA, which is sensitive for < 5% minority viral populations, can be used to identify intrasubtype superinfection but often causes false positive results [39]. All these methods require confirmation by cloning and sequencing.

Recently, more sensitive and accurate methods have been used to detect superinfection (Table 1). Limiting dilution PCR and single genome sequencing (SGS) methods can determine distinct viral populations in samples [40–43]. However, both methods are costly and labor intensive due to the amount of sequences required [39–41]. The next-generation sequencing (NGS) method is very sensitive for the detection of minority variants, as low as 0.25% of the population in samples. The use of sequence identification tags has significantly reduced the cost and increased the throughput of the NGS method [44–46]. Thus, it has been widely used to detect superinfection in a large number of samples by analyzing multiple genome regions to determine the incidence of superinfection [47]. Moreover, NGS can detect different subtypes or different strains from the same subtype present in 1%–5% of the viral populations [8]. In an MSM acute HIV-1 infection cohort, 15.6% of the cases were found to be superinfected by NGS. Among them, 60% of the superinfected cases were between CRF01_AE and CRF07_BC or subtype B, whereas the other 40% were intrasubtype superinfections [48]. In a cohort of high-risk women in Kenya, 7% of the cases were found to be superinfected by analyzing three genomic regions using the NGS method [47]. Among them, 33.3% were intersubtype superinfections, whereas 66.7% were intrasubtype superinfections. These results demonstrated that NGS can sensitively detect both intersubtype and intrasubtype superinfections and has been increasingly used for the detection of superinfected cases.

Other than assay sensitivity, the size and number of genome regions used for analysis also have a significant impact on the detection of superinfection. The analysis of only small genome regions may lead to an underestimation of the number of superinfected cases [31,34,40]. For example, the examination of additional *gag* gene sequences identified two additional superinfected cases among 14 high-risk Kenyan women from whom only *env* gene sequences were initially analyzed [41].

Accurate determination of superinfection incidence

The frequency of superinfection varied significantly among different cohorts (from 1.5% to 19.4%), and the incidence of superinfection also varied from 1.44 to 19.6 per 100 person-years (Table 2). These observations suggest that the incidence of superinfection can be significantly affected by many factors in study cohorts. It also raises a question whether current data reflect the real superinfection frequencies. To precisely determine the frequency of superinfection, some key factors should be well controlled in the cohort design. First, longitudinal cohorts are required for the detection of superinfection. In crosssectional studies, dual infection can be detected, but the timing of infection for each strain cannot be determined. Therefore, superinfection can not be distinguished from coinfection [49,50]. Thus, superinfection can only be clearly defined through the long-term follow-up of HIV-1-infected individuals. The length of follow-up is also an important factor. Superinfection may occur soon after primary infection or during chronic infection [51]. Superinfection can be more frequently detected in a cohort with a longer follow-up period than a shorter one. Second, sampling frequency is critical in longitudinal studies [40]. If intervals are short (e.g., weeks) during follow-up, superinfection can be accurately and timely detected [21,40]. If intervals are long (e.g., months or years), superinfection may be missed due to the rapid recombination between primary and superinfected viruses [35,52–55], which can lead to the misclassification of the superinfection as monoinfection with divergent viruses. Therefore, the incidence or prevalence of superinfection can be underestimated in low-frequency sampling cohorts and might be the reason for the nondetection of superinfection in some studies [34,35,37,56,57]. Moreover, individuals in cohorts should not be on antiretroviral therapy. Ongoing antiretroviral therapy is likely to protect an individual from being infected by new viruses and results in low superinfection frequencies [58–60].

Superinfection and preexisting immune responses

One critical question is whether weak immune responses against HIV-1 during monoinfection lead to superinfection. HIV-1 superinfection provides a unique opportunity to investigate the relationship between superinfection and preexisting immune responses. nAbs have long been thought to play an important role in the protection from HIV-1 infection [61–63]. The absence of potent nAb responses, especially cross-reactive nAb responses against heterologous viruses, probably predisposes an HIV-1-infected individual to superinfection. Weaker crossprotective and autologous nAb responses were detected in three intrasubtype B superinfected cases before the occurrence of superinfection than in monoinfection controls in the Acute Infection Early Disease Research Program (AIEDRP) in San Diego and Los Angeles, USA [7]. In a subsequent study, the same group further confirmed their findings with a larger number of samples. Ten intrasubtype B superinfected cases had relatively weaker nAb responses against the autologous virus and two neutralization-sensitive heterologous viruses compared to 19 monoinfection controls [64]. An intrasubtype C superinfection study in a discordant couple cohort in Zambia showed that three subtype C intrasubtype superinfected cases had weaker nAb responses against their baseline autologous viruses prior to superinfection compared with monoinfection controls within one year of primary infection [12]. Moreover, antibody-dependent cellular cytotoxicity responses in three intrasubtype superinfected cases were low prior to superinfection [13]. These studies

suggest that a relatively weak nAb response is present in superinfected cases within the first year of HIV-1 infection.

Broadly neutralizing antibodies (bnAbs), which are believed to be key in preventing heterologous superinfection, usually take 2–4 years to develop. Because bnAbs are unlikely to develop within one year of infection [65,66,67], understanding whether superinfection is affected during chronic infection in which bnAbs are more likely to be elicited is important. In a high-risk women cohort, nAb responses were examined in six individuals who were superinfected by second strains between 1 and 5 years after the initial infection as well as 18 monoinfection women with similar risk factors [14]. The superinfected women had less neutralization breadth than matched controls at approximately 1 year post infection, but the breadth or potency of nAb responses was not significantly different from the control group just prior to superinfection. In fact, four of the six subjects had relatively broad and potent nAb responses prior to infection by additional strains. Importantly, the detection of nAbs against superinfection viruses in most superinfected individuals prior to their infection suggested that the level of nAbs elicited during primary infection was insufficient to block superinfection. These data indicate that preventing HIV-1 infection by a vaccine will likely require broader and more potent nAb responses than those found during primary HIV-1 infection.

Evidence supporting protection from superinfection by T cell responses is still scarce. In a recent rhesus monkey mucosal superinfection study, the polyfunctionality of CD4⁺ effector memory T cells, but not nAb responses, was shown to correlate with protection against superinfection [15]. Notably, virus-associated effector memory T cell responses might contribute to the suppression of superinfection. However, in another macaque model study, a series of SIVmac239 CD8⁺ T cell escape variants failed to infect long-term nonprogressors (LTNP) that had robust SIV-specific CD8⁺ and CD4⁺ T responses as well as neutralizing antibodies [68]. In another study, preexisting HIV-1-specific CD8⁺ T cell responses in one individual failed to protect the host from superinfection [11]. Similar HIV-1-specific cellular immune responses induced during chronic infection also did not appear to significantly contribute to protection from HIV-1 superinfection in a case-control study of high-risk women in Kenyan [69]. Taken together, more studies are warranted to better understand the role of cellular immune responses in preventing subsequent superinfection.

Other immunological factors have also been studied for their potential effects on superinfection. Antibody-dependent cell-mediated virus inhibition activity and frequencies of T cell activation markers were comparable between superinfection and monoinfection cases [70,71]. When immune functions in both plasma and cervical swab supernatants were examined to assess systemic and mucosal responses, no significant associations were detected between any of the immune functions and superinfection acquisition in a sex worker cohort in Kenya [72]. Another study showed that human leukocyte antigen alleles were found to be correlated with the differential superinfection risk in a recent MSM cohort in the USA [73].

Recombination between primary infection viruses and superinfection viruses can be rapidly detected, and recombinants could quickly replace the parental viruses soon after

superinfection under the selection of T cell or nAb pressure [35,52–55]. Thus, the rapid recombination between superinfection and primary infection viruses may allow recombinants to quickly escape the preexisting immune responses and be an important cause for the second virus to successfully establish superinfection. However, such recombinant events will not be detected if the genome regions not under selection pressure are used for analysis.

In summary, immune responses elicited by primary HIV-1 infection might have limited impact on superinfection. More potent and broader immune responses than those elicited during mono-infection may be needed for an effective HIV-1 vaccine. However, given the small numbers of cases studied in each of these cohorts and the use of different panels of viruses to determine neutralization activities, well-controlled cohorts with large numbers of cases should be studied to better understand the roles of immune responses in the prevention of superinfection.

Superinfection at different infection stages

The determination of the timing of superinfection is important for our understanding of the roles of immune responses induced by primary infection in preventing superinfection. A hypothesis is that superinfection occurs more easily at the early infection stage because of weak immunity. This hypothesis was initially supported by some case reports and cohort studies showing superinfection within one year after initial infection [4,7,12,22,28,39,42,64]. However, later studies showed that the number of superinfection within one year after the initial infection was similar to that after one year of infection [11,19,41,51,74]. Moreover, in studies for which superinfection was determined by NGS, no significant differences in superinfection frequencies between acute and chronic infection stages were observed [21,23,40,47,64]. Results from these studies indicate that the immune responses elicited by HIV-1 infection during early and late infection stages similarly failed to protect superinfection (Fig. 1).

Incidences of superinfection and primary infections

A comparison of incidences of superinfection and primary infections is important to understand whether immune responses elicited by primary infection can be protective from subsequent superinfection. Several studies showed that the incidence of superinfection is lower than that of primary infection. An earlier study using RFLP and subtype-specific primer assays found that the incidence of superinfection (intersubtype) was less than half of that of primary infection (2.2/100 person-years versus 5.8/100 person-years) in an IDU cohort in Thailand [6]. The result from a high-risk women cohort in Kenya also showed that the incidence of superinfection (intersubtype and intrasubtype) was lower than that of primary infection after adjusting variables (such as self-reported sexual risk behavior, place of work, hormonal contraceptive use, genital tract infections, years in sex work, age at first sex, follow-up time in the cohort, and calendar year) previously shown to influence HIV-1 exposure risk. After adjusting these variables, the hazard ratio for superinfection compared with primary infection was 0.47 [47]. Another study on a recent seroconverter cohort from a general population in Uganda also showed that the incidence of superinfection (intersubtype

and intrasubtype) was slightly lower than the primary infection incidence at baseline ($P=0.07$), but comparable with the primary HIV incidence during follow-up after controlling for differences in sociodemographic and behavioral characteristics [10]. Results from an MSM cohort in the USA showed that the intrasubtype superinfection incidence was similar to that of primary infection [22]. Although the rates of superinfection and primary infection are not consistent among different studies, after the use of a more sensitive NGS method and multiple proper adjustments, the incidence of superinfection is approximately half of the incidence of primary infection (Fig. 2), suggesting that primary infection is only partially protective of superinfection.

In summary, results on the incidences of superinfection and primary infection suggest that immune responses elicited by primary infection are only partially protective and not effective enough in fully preventing later superinfection. The comparison of incidences between superinfection and primary infections in a population is very difficult. Except for the methods used, sociodemographic and behavioral factors will also have significant impact on the frequency of superinfection. Superinfected individuals are inherently at a higher risk than people in the control group [10]. Moreover, behavior changes can also significantly affect the incidence. For example, the absence of superinfection one year after infection between 1985 and 1997 coincides with a reduction in sexual risk behavior in the seroincidence in an MSM cohort in Amsterdam [35,37]. Thus, the comparison between incidences of superinfection and primary infections should take into account all major risk factors to ensure that real differences are properly determined in future studies.

Broader immune responses after superinfection

Another important question is whether superinfection can result in broad neutralization activity because the presence of different envelope glycoproteins (Env) from multiple variants may have a better chance to trigger more B cell lineages for broad neutralization responses than a single form of Env [75 – 77]. Viral diversity has been found to be associated with the development of broad neutralization activity [16,53,55,67,78,79]. Dual infection resulted in a significant increase in the neutralization capacity when compared with monoinfection controls within a comparable study period [17]. In a seroincident cohort of high-risk women in Kenya, 12 superinfected individuals were tested for neutralization activities against a panel of eight viruses at matched time points post-superinfection (~5 years postinitial infection) [18]. Superinfected individuals demonstrated significantly broader neutralization responses postsuperinfection when compared with monoinfection individuals. This observation remained the same even after controlling for neutralization breadth developed prior to superinfection, contemporaneous CD4⁺ T cell counts, and viral loads. This finding suggests that sequential infections may lead to the augmentation of broad neutralization activities. The same group further confirmed this observation by studying nine additional superinfected individuals in the setting of controlling other factors that could potentially influence nAb responses [16]. The results also indicated that the length of time after superinfection could play an important role in development of neutralization breadth. The longer an individual is infected with multiple viruses, the broader nAb responses will develop. In addition, a case study showed that an individual who was superinfected three months post-primary infection mounted broad and potent neutralization responses during

three years of post-superinfection [66]. The successful isolation of bnAbs from this individual further demonstrated that bnAbs were indeed elicited [55]. In another study of a superinfected HIV-1 LTNP elite controller, cytotoxic T lymphocyte responses after superinfection were maintained in two samples separated by 9 years, and they were broad and potent [80]. This study demonstrated that strong and sustained cellular and humoral immune responses as well as low replicating viruses are associated with viral control in the superinfected LTNP elite controller. However, broad neutralization activities were not detected in some superinfected cases [52]. It seems that the timing of superinfection and genetic characteristics (diversity, length variation, potential glycosylation site, and others) of superinfection viruses might contribute to development of broad and potent nAb responses [52]. These observations indicate that multiple immunogen variants may be needed to induce cross-reactive immune responses to diverse heterologous viruses.

Summary

Weak immune responses before superinfection and similar incidence of superinfection during acute and chronic infections suggest that immune responses elicited by primary HIV-1 infection are not strong enough to confer protection from superinfection by subsequent heterologous viruses. This finding suggests that hosts with severely damaged CD4⁺ T cell populations during HIV-1 infection may not be able to mount protective immune responses when they encounter additional viruses, although both T and B cell immune responses are detectable when superinfection occurs. The ineffectiveness to prevent superinfection by primary infection indicates that an HIV-1 vaccine needs to induce more potent and broader immune responses than those elicited by primary HIV-1 infection. However, because healthy people when vaccinated have intact immune system, an HIV-1 vaccine that can induce similar immune responses as those detected in primary infection individuals may be effective to prevent HIV-1 infection. More potent and broader nAb responses detected in superinfection individuals than monoinfection subjects suggest that bnAbs are more likely to be induced by immunization of multiple different immunogens, sequentially or combinationally. Whether superinfection occurs after HIV-1-infected individuals have developed broad neutralization activities and how broadly neutralizing antibodies are induced after superinfection are important for future studies. Well-defined longitudinal cohorts with frequent sampling and standardized assays should be carried out to more precisely identify correlates between superinfection and status of immune responses as well as roles of innate immune responses in protecting superinfection.

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Fig. 1.

Comparison of superinfection frequencies at different infection stages. The numbers of superinfected cases detected within one year and after one year were compared when all methods (left) or only NGS method (right) was used ($P = 1.0$ for both comparisons). Each cohort is indicated by a distinct symbol.

Fig. 2.
Comparison of superinfection and primary infection incidence rates.

Table 1

Methods for the identification of superinfection

Method	Advantage	Limitation
RFLP	Easy	Low sensitivity
Subtype-specific PCR	Low cost	Low throughput
MHA	Large-scale screening	Low sensitivity, detect intersubtype SI only
	Multiple regions	Cloning confirmation
HMA	1.5% differences in pairwise distance	False positives
	Identify intrasubtype SI	Cloning confirmation
Population-based sequencing	Prediction by degenerate base codes	Less sensitive to minority viral population
	Initial screening method	
Bulk PCR + selective cloning	Improved sensitivity	Expensive
	Sequences for analysis	Labor intensive
		Amount of sequences
		Genomic regions
		Cannot unequivocally confirm
SGS	More sensitive and accurate than other methods	Expensive
	No PCR error, bias, and recombination	Labor intensive
NGS	Highly sensitive, 0.25%	Short read length
	High throughput	Low accuracy in fragment assembly
	Multiple regions	Sophisticated data analysis procedure

Table 2
Identification of intrasubtype and intersubtype superinfection among different cohorts

Cohort	Year	Population	Method	No. of intersubtype/intrasubtype	Frequency	Incidence (100 person-years)	Reference
Primary infection cohort, Shenyang, China	2008–2013	MSM	NGS	6/4	15.6%	19.6	Luan <i>et al.</i> [48]
AIEDRP, San Diego and Los Angeles, USA	1997–2003	MSM	Population-based sequencing, cloning	0/3	3.8%	5	Smith <i>et al.</i> [22]
Primary infection cohort, San Diego, USA	1998–2007	MSM	NGS	10/0	8.4%	4.96	Wagner <i>et al.</i> [21]
Mombasa, Kenya	1993–2000	HST	HMA cloning	3/0	14.3%	4.3	Chohan <i>et al.</i> [31]
Mombasa, Kenya	1993–2004	HST	ssPCR, SGS	4/3	19.4%	3.7	Piantadosi <i>et al.</i> [40]
Mombasa, Kenya	1993–2000	HST	SGS	1/1	14.3%	7.7	Piantadosi <i>et al.</i> [41]
Mombasa, Kenya	1993–2008	HST	Sanger sequencing	11/10	14.4%	2.61	Ronen <i>et al.</i> [47]
			NGS				
Rakai community cohort study	2002–2005	HST	NGS	2/0	18.2%	NA	Redd <i>et al.</i> [8]
Rakai, Uganda	1998–2009	HST	NGS	3/4	4.7%	1.44	Redd <i>et al.</i> [10]
Durban, South Africa	2004–2005	HST	SGS	0/3	9.3%	NA	Sheward <i>et al.</i> [43]
Seattle primary infection cohort and FSWs in the South African	2008–2009	FSW	HMA, cloning	0/1	1.6%	NA	Gottlieb <i>et al.</i> [33]
Bangkok, Thailand	1995–1996	IDU	RFLP, ssPCR	2/0	1.5%	2.2	Ramos <i>et al.</i> [4]
KwaZulu-Natal, South Africa	2007–2010	HST	NGS	0/2	2.6%	1.5	Redd <i>et al.</i> [28]

NA, not available.