A Vero Cell–Derived Whole-Virus H5N1 Vaccine Effectively Induces Neuraminidase-Inhibiting Antibodies

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A Vero cell–derived whole-virus H5N1 influenza vaccine has been shown to induce neutralizing antibodies directed against the hemagglutinin (HA) protein of diverse H5N1 strains in animal studies and clinical trials. However, neuraminidase-inhibiting (NAi) antibodies can reduce viral spread and may be of particular importance in the event of an H5N1 pandemic, where immunity due to HA antibodies is likely absent in the general population. Here we demonstrate the effective induction of NAi antibody titers after H5N1 vaccination in humans. In contrast to the immune response directed toward HA, a single vaccine dose induced a strong NAi response that was not significantly boosted by a second dose, most probably due to priming by previous vaccination or infection with seasonal influenza viruses. After 2 immunizations, seroconversion rates based on antibody titers against HA and NA were similar, indicating the induction of equally strong immune responses against both proteins by this H5N1 vaccine.

Avian H5N1 influenza viruses pose a pandemic threat with continuously occurring widespread infections of avian species, as well as sporadic human cases with a mortality rate of approximately 60% [1]. As vaccination is the most effective measure to combat an influenza pandemic, a Vero cell–derived H5N1 whole-virus vaccine was developed and was shown to induce high levels of virus-neutralizing antibodies and to provide highly efficacious cross-clade protection against lethal doses of H5N1 viruses in animals [2–4]. In clinical trials in humans, the vaccine’s immunogenicity was assessed by microneutralization (MN) and single radial hemolysis (SRH) assays [5, 6], both of which detect antibodies specific for the influenza hemagglutinin (HA) [7]. HA is the most abundant influenza virus surface protein and the most relevant target protein for vaccination because it can induce virus-neutralizing antibodies that act by hindering HA attachment to the sialic acid receptor on the host cell surface or by interfering with virus membrane fusion. Influenza virus envelopes, however, contain a second major surface protein, neuraminidase (NA), a tetrameric glycoprotein that cleaves sialic acid residues from oligosaccharides, including the HA receptor. This enzymatic cleavage is crucial for progeny virions to be released from the infected host cell, and thus NA-inhibiting (NAi) antibodies have the potential to prevent virus release. While HA-specific antibodies can neutralize virus infectivity, as first demonstrated by plaque inhibition assays, NAi antibodies are described as infection permissive; in other words, these antibodies do not block infection but can reduce virus budding and spread, as measured by a reduction of plaque size [8, 9], which in turn results in a reduction of the intensity of infection [10, 11]. A negative correlation between serum NAi antibody titers and severity of influenza illness has been found in challenge studies in animals [12–15] and
also humans [16–19]. During the H3N2 influenza pandemic of 1968/1969, it was observed that serum NAI antibody titers contribute to protection: persons with a history of H2N2 infection and thus having preexisting NAI antibodies against N2 were not as vulnerable to H3N2 infection as were N2-naive subjects [20]. Likewise, schoolchildren vaccinated with recombinant N2 were protected against the circulating H3N2 strain compared with placebo groups [21, 22]. Thus, a number of studies have demonstrated that humoral protective immunity against influenza virus infection is mediated by antibodies to both HA and NA. While both proteins are subject to continuous evolution, NA has been shown to evolve more slowly [23]. The immunological response to NA could be of particular importance in the event of an influenza pandemic, where the majority of the population would be naive for the HA protein but might possess immunity toward the NA through priming by earlier influenza infection or vaccination. The European licensing authority has realized the importance of investigating the capability of influenza vaccines to induce NAI antibodies and recommends such investigations, especially during the development of pandemic influenza vaccines [24]. To date, however, the extent to which NAI antibodies are elicited by pandemic H5N1 influenza vaccination has not been reported from investigations in humans. A possible explanation for this could be that the measurement of NAI antibodies has traditionally relied on NA inhibition assays based on thiobarbituric acid (TBA-based assay), which are cumbersome to perform [25–28]. More recently, however, assay formats for the evaluation of NAI antibody titers have been developed that are easier to perform and allow for higher sample throughput [29–34]. Here we report the results of a proof-of-concept study investigating the generation of NAI antibodies after vaccination of humans with the Vero cell culture–derived pandemic whole-virus H5N1 vaccine, using both the traditional TBA-based NA inhibition assay and the more recently developed enzyme-linked lectin NA inhibition assay (ELLA).

**MATERIALS AND METHODS**

**Clinical Study and Vaccine**

Human serum samples were derived from a randomized, phase I/II clinical study (EUDRACT 2006-001466-17) of an H5N1 monovalent A/Vietnam/1203/2004 whole-virus vaccine [6]. The vaccine was manufactured in Vero cell culture [3, 35] and inactivated with formalin and ultraviolet light. Different amounts of HA antigen were administered twice, 21 days apart, to healthy adult volunteers. Blood for serological analysis was drawn at baseline (day 0) and on days 21 and 42. Here, the serum samples from all subjects dosed with the nonadjuvanted H5N1 whole-virus formulation containing 7.5 μg HA (n = 42) or with 15 μg of nonadjuvanted antigen (n = 41) were analyzed.

**Recombinant NA**

Highly purified, full-length recombinant NA from the H5N1 strain A/Vietnam/1203/2004 (GenBank accession no. EF541467.1) was produced using a baculovirus expression system (Protein Sciences Corp).

**Determination of NA Activity**

**Thiobarbituric Acid–Based Assay**

A thiobarbituric acid (TBA)–based assay was established as described by the World Health Organization [36]. In sum, 10 μL of serially diluted recombinant NA protein was applied to 96-well plates, 5 μL fetuin (25 mg/mL; Sigma) was added, and plates were incubated at 37°C for 16–18 hours. After cooling to room temperature, 5 μL periodate reagent was added. Following incubation for 20 minutes in the dark, the reaction was stopped by addition of 25 μL arsenite reagent; 50 μL thiobarbituric acid (Sigma) was added, and plates were incubated at 99°C for 15 minutes in a thermostorer (Applied Biosystems). Plates were cooled in a water bath, and 75 μL Warrenoff reagent was added. Plates were vortexed and centrifuged for 5 minutes at 250g; 50 μL of the upper phase of each well was transferred to flat-bottom 96-well plates, and the optical density (OD) was measured at 550 nm in a microplate reader (Biotek). The standard NA dose to be used for NA inhibition tests was defined as the NA activity measured at OD 1.0.

**Enzyme-Linked Lectin Assay**

NA activity was also determined by ELLA according to Lambre et al [30]. In total, 100 μL of serially diluted recombinant NA protein was applied to 96-well plates precoated with fetuin (2.5 μg/well; Sigma) and incubated at 37°C for 16–18 hours. Plates were washed and incubated with peroxidase-labeled peanut agglutinin (100 μL/well; Sigma) for 2 hours in the dark at room temperature. Plates were then washed before 100 μL of o-phenylenediamine dihydrochloride (Sigma) was added as substrate. After 10 minutes incubation in the dark, the reaction was stopped by addition of 100 μL 0.5 mol/L H2SO4. The OD was measured at 550 nm. The standard NA dose to be used for NA inhibition tests was defined as the NA activity measured at OD 1.0.

**Determination of NA-Inhibiting Antibody Titers**

**TBA-Based Assay**

To determine NA-inhibiting antibody titers, serum samples were serially diluted in 2-fold steps, and 5 μL of each dilution was transferred to 96-well plates. Negative control serum was derived from naive rabbits, and positive control serum was obtained from rabbits immunized with the influenza A/Vietnam/1203/2004 NA. These controls were tested in every assay with the expected results required for validity of the assay. 5 μL of the standard NA dose was added to each well. Following incubation for 1 hour at room temperature, 5 μL fetuin (25 mg/mL, Sigma) was added and plates were incubated at 37°C for 16–18 hours. Residual NA
activity was determined as described above. The NAi antibody titer of a sample was defined as the reciprocal serum dilution resulting in 50% inhibition of the NA activity of the standard NA dose.

**ELLA**

Serum samples were heat-inactivated for 30 minutes at 56°C, subjected to 3 freeze-thaw cycles, and serially diluted in 2-fold steps. Positive and negative control serum samples, as well as validity criteria, were used as described for the TBA-based assay. In total, 50 µL of each dilution was applied to fetuin-coated 96-well plates (2.5 µg/well; Sigma). The standard NA dose (50 µL) was added to each well, and the remaining procedure was performed as described above. The NAi titer was defined as 50% inhibiting titer as calculated by nonlinear regression analysis.

**Statistical Analysis**

Samples were analyzed in 2 independent tests with means used for further evaluation. Statistical significant differences between pre- and postvaccination titers were determined by paired Student t test analysis, and the significance of the correlation of titers determined via different test methods or of pre- and postvaccination titers was determined by nonparametric Spearman correlation analysis (GraphPad Prism software). The confidence intervals of the increase of the geometric mean titers were calculated by applying t statistics on the log-transformed data, and the confidence intervals of the seroconversion rates according to Clopper and Pearson were calculated based on F distributions (Minitab software).

**RESULTS**

To determine the NAi antibody response induced in humans by vaccination with a Vero cell–derived whole-virus H5N1 vaccine, individual serum samples from both the 7.5-µg and 15-µg dose groups obtained prevaccination (day 0), 3 weeks after the primary immunization (day 21), and 3 weeks following the booster immunization (day 42) were analyzed with the TBA-based assay (Figure 1A) and the ELLA (Figure 1B). Already after the first dose (day 21), a highly significant induction of NAi antibodies by H5N1 vaccination was measured using both test systems. With the TBA assay, the geometric mean titer (GMT) increased from 2.1 (95% confidence interval [CI], 1.7–2.7) at baseline to 4.5 (95% CI, 3.4–6.1) on day 21 and 5.3 (95% CI, 3.9–7.1) on
day 42. The ELLA was found to be the much more sensitive assay: GMTs were calculated as 79.6 (95% CI, 62.4–101.5) at baseline and 390.8 (95% CI, 297.4–513.6) on day 21. After the second vaccination, the GMT assessed with the ELLA was 418.7 (95% CI, 317.5–552.2). Of note, although NAi antibody titers in both assays increased significantly after the first vaccination, the moderate increase in titers after administration of the second dose was not statistically significant (Figure 1). While the sensitivity of the TBA-based assay and the ELLA differed, Figure 2 shows that there was a correlation of NAi antibody titers derived from these 2 assays (Spearman $r = 0.83$, $P < .0001$), probably because both assays measure the same antibody function. Notably, however, using the TBA assay, NA-inhibiting antibodies were detectable in only 43 of 83 subjects prior to pandemic vaccination, whereas with the more sensitive ELLA all 83 study subjects tested were positive (Figure 1). Seroconversion was defined as ≥4-fold increase in NAi antibody titer postvaccination compared with titers at day 0. The seroconversion rates with the TBA assay were calculated as 21.7% on day 21 and as 33.7% on day 42 (Figure 1A), whereas for the ELLA these were 56.6% and 63.9% after the first and second vaccine doses, respectively (Figure 1B).

The vaccine dose used was found not to have a statistically significant effect on the NAi antibody response; this result is similar to the dose-independent H5-specific antibody response reported previously [6]. Numerically, for the TBA assay the GMT values 21 days after the primary vaccination were 4.7 (95% CI, 3.1–7.1) for the 7.5-μg dose group and 4.4 (95% CI, 2.8–6.9) for the 15-μg dose group. The GMTs on day 42 were 4.6 (95% CI, 3.0–7.1) and 6.0 (95% CI, 3.9–9.1) for the groups receiving 7.5 μg and 15 μg HA antigen, respectively. Similar dose-independent NAi responses were obtained with the more sensitive ELLA: the GMT values on day 21 were 400.5 (95% CI, 269.8–594.4) for the 7.5-μg group and 381.2 (95% CI, 257.2–564.8) for the 15-μg group. The day 42 GMTs were 401.7 (95% CI, 269.8–594.4) and 437.0 (95% CI, 300.5–635.4) for the 7.5-μg group and 15-μg group, respectively. Similarly, there were no significant differences regarding seroconversion or the increase of GMTs (GMI) between the dose groups (Table 1).

In previous vaccination studies, a strong dependence of the NAi antibody response on prior exposure to HA and NA molecules was reported [27, 37–39]. In the current study, all serum samples tested were positive at baseline in the ELLA (Figure 1), indicating that all subjects had prior exposure to N1 NA. To investigate whether the induction of NAi antibodies by the pandemic H5N1 vaccine was influenced by the level of preexisting anti-N1 NA immunity, the prevaccination NAi titers of the study subjects were compared with their respective postvaccination NAi titers. For both NAi assays, preexisting titers correlated positively with postimmunization titers (TBA assay: Spearman $r = 0.79$, $P < .0001$; ELLA: Spearman $r = 0.66$, $P < .0001$), suggesting that vaccination-induced NAi titers build upon preexisting anti-N1 immunity. The levels of preexisting serum NAi antibody titers did not differ significantly ($P = .05$) between subjects previously shown to have some level of anti-H5 virus-neutralizing antibodies at baseline (n = 33), as determined by MN assays [6] and those lacking them (n = 50). To evaluate the influence of preexisting H5 antibody titers on the induction of N1 antibodies by vaccination, NAi antibody titers were compared between subjects who had tested seropositive or seronegative at baseline in the MN or the SRH assays. No significant difference ($P = .05$) was seen in the day 42 postvaccination NAi antibody titers (TBA assay and ELLA) in subjects who were MN assay positive at day 0 and those who were MN assay negative. Similarly, the day 42 NAi titers did not differ significantly ($P = .05$) between groups that were reactive (n = 14) or nonreactive (n = 69) in the SRH assay at baseline. Thus, preexisting immunity toward H5 HA did not influence the N1 NAi antibody response in this study.

We further addressed the question of whether there is a correlation between antibody titers directed against N1 and H5 induced by H5N1 vaccination. The individual titer results derived from the sensitive ELLA correlated well with the previously determined corresponding MN assay results (Spearman $r = 0.67$, $P < .0001$) (Table 2). Because 2 vaccine doses induced higher virus-neutralizing antibody titers against H5 than 1 dose [6], whereas the NAi antibody response was not significantly boosted by a second immunization (Figure 1 and Table 1), the correlation between ELLA and MN results was even more significant when day 21 results were excluded from analysis (Spearman $r = 0.72$, $P < .0001$). Due to the lower sensitivity of the TBA assay compared with the ELLA, the Spearman coefficient of correlation for the TBA assay and the MN results was lower, with $r = 0.52$.
The current study represents to our knowledge the first report on the induction of anti-NA immunity in humans in response to pandemic H5N1 vaccination. By testing serum samples with the TBA assay and the ELLA, we have investigated the induction of anti-N1 NAi antibodies in subjects after 1 or 2 immunizations (Figure 1). The induction of substantial NAi antibody titers after a single vaccination, in contrast to the induction of an immune response toward H5 HA, which generally requires 2 immunizations for an optimal response [6, 40], is characteristic of a primed response. Indeed, the highly sensitive ELLA revealed that all study subjects tested had some level of preexisting N1-specific NAi antibody, presumably due to exposure to, or vaccination with, N1-containing seasonal influenza viruses. Given that H1N1 influenza A viruses have been circulating for decades and primary influenza infections typically occur during childhood, this finding is not surprising in that all study subjects were in the 18–45-year age group. Consistent with that observation, maximum NAi titers were already achieved after the first immunization, that is, a booster dose for this primed study population, with only insignificant increases in NAi titers after the second immunization.

NAi antibodies to N1 might be of particular importance in the event of a H5N1 pandemic, where the majority of the population would be naive for the H5 HA protein. Vaccination against a seasonal strain of influenza that shares the NA with a potential pandemic virus thus seems to provide for an additional benefit in a pandemic situation by inducing cross-reactive NA-specific immune responses to the HA and NA components of the vaccine.

DISCUSSION

The current study represents to our knowledge the first report on the induction of anti-NA immunity in humans in response to pandemic H5N1 vaccination. By testing serum samples with the TBA assay and the ELLA, we have investigated the induction of anti-N1 NAi antibodies in subjects after 1 or 2 immunizations (Figure 1). The induction of substantial NAi antibody titers after a single vaccination, in contrast to the induction of an immune response toward H5 HA, which generally requires 2 immunizations for an optimal response [6, 40], is characteristic of a primed response. Indeed, the highly sensitive ELLA revealed that all study subjects tested had some level of preexisting N1-specific NAi antibody, presumably due to exposure to, or vaccination with, N1-containing seasonal influenza viruses. Given that H1N1 influenza A viruses have been circulating for decades and primary influenza infections typically occur during childhood, this finding is not surprising in that all study subjects were in the 18–45-year age group. Consistent with that observation, maximum NAi titers were already achieved after the first immunization, that is, a booster dose for this primed study population, with only insignificant increases in NAi titers after the second immunization.

Table 1. Proportion of Subjects With Seroconversion and Geometric Mean of the Increase From Baseline Based on Antibodies Against H5 and N1

<table>
<thead>
<tr>
<th>Dose</th>
<th>Assay</th>
<th>Seroconversion</th>
<th>GMI Value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Day 21</td>
<td>Day 42</td>
</tr>
<tr>
<td>7.5 μg</td>
<td>TBA</td>
<td>23.8 (12.1–39.5)</td>
<td>28.6 (15.7–44.6)</td>
</tr>
<tr>
<td></td>
<td>ELLA</td>
<td>59.9 (43.3–74.4)</td>
<td>64.3 (48.0–78.4)</td>
</tr>
<tr>
<td></td>
<td>MNb</td>
<td>35.7 (21.6–52.0)</td>
<td>69.0 (52.9–82.4)</td>
</tr>
<tr>
<td></td>
<td>SRHb</td>
<td>61.9 (45.6–76.4)</td>
<td>73.8 (58.0–86.1)</td>
</tr>
<tr>
<td>15 μg</td>
<td>TBA</td>
<td>19.5 (8.8–34.9)</td>
<td>39.0 (24.2–55.5)</td>
</tr>
<tr>
<td></td>
<td>ELLA</td>
<td>53.7 (37.4–69.3)</td>
<td>63.4 (46.9–77.9)</td>
</tr>
<tr>
<td></td>
<td>MNb</td>
<td>34.9 (21.0–50.9)</td>
<td>68.3 (51.9–81.9)</td>
</tr>
<tr>
<td></td>
<td>SRHb</td>
<td>39.5 (25.0–55.6)</td>
<td>58.5 (42.1–73.3)</td>
</tr>
</tbody>
</table>

Data are % (95% confidence interval) unless otherwise indicated. In all assays the same samples were analyzed. The 95% confidence intervals were calculated using Minitab software.

Abbreviations: ELLA, enzyme-linked lectin assay; MN, microneutralization; SRH, single radial hemolysis; TBA, thiobarbituric acid.

Table 2. Correlation Between Antibody Titers Assessed by Different Test Methods

<table>
<thead>
<tr>
<th>TBA</th>
<th>ELLA</th>
<th>MN</th>
<th>SRH</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBA</td>
<td>$r = 0.83; P &lt; .0001$</td>
<td>$r = 0.52; P &lt; .0001$</td>
<td>$r = 0.38; P &lt; .0001$</td>
</tr>
<tr>
<td>ELLA</td>
<td>$r = 0.83; P &lt; .0001$</td>
<td>$r = 0.67; P &lt; .0001$</td>
<td>$r = 0.50; P &lt; .0001$</td>
</tr>
<tr>
<td>MN</td>
<td>$r = 0.52; P &lt; .0001$</td>
<td>$r = 0.67; P &lt; .0001$</td>
<td>$r = 0.71; P &lt; .0001$</td>
</tr>
<tr>
<td>SRH</td>
<td>$r = 0.38; P &lt; .0001$</td>
<td>$r = 0.50; P &lt; .0001$</td>
<td>$r = 0.71; P &lt; .0001$</td>
</tr>
</tbody>
</table>

Nonparametric Spearman correlations were calculated using GraphPad Prism software version 5.0.

Abbreviations: ELLA, enzyme-linked lectin assay; MN, microneutralization; SRH, single radial hemolysis; TBA, thiobarbituric acid.
antibodies. Indeed, experiments in mice have demonstrated that vaccination with DNA encoding the NA of a seasonal H1N1 virus provides partial cross-protection against H5N1 influenza virus challenge [41]. To further investigate the role of immunological priming in the NAi response to vaccination, an NA antibody–negative study population (eg, pediatric subjects) would be of interest. In young children who have not encountered influenza infections and are naive to both HA and NA, the effective induction of antibodies by a pandemic influenza vaccine would be of particular importance.

In the current study, vaccination-induced NAi titers correlated with preexisting anti-N1 NAi titers, that is, the antibody response to vaccination is increased in subjects with high pre-vaccination anti-N1 antibody titers as compared with those with lower titers, indicative of priming to the N1 antigen. In contrast, there was no influence of preexisting H5-specific antibodies on the NAi response to vaccination. Thus, a previously developed hypothesis that a certain NA exhibits maximum immunogenicity in populations primed with this NA but lacking antibodies to the HA protein of the vaccine virus strain [27, 37–39] is not supported by the results of our study. No statistically significant difference in NAi titers was seen between the 7.5- and 15-μg HA vaccine doses in this report, or between 3.75 and 30 μg of HA in the previous investigation of HA-specific immunogenicity of the vaccine [6]. In another study, however, 180 μg of HA antigen in a trivalent seasonal influenza split vaccine induced significantly higher NAi, as well as HA-specific antibody titers in elderly subjects, compared with a standard dose of 45 μg HA [33]. This divergent observation may be explained by the differences in vaccine compositions and the very large amount of antigen investigated.

It has previously been suggested that the antigen content of both surface glycoproteins, HA and NA, should be standardized in influenza vaccines [41]. The highly significant correlation of NAi antibody titers determined by the ELLA and HA-specific neutralizing antibody titers as determined by MN assays of the same serum samples, described in the current study, suggests that the NA and HA components of the H5N1 vaccine were similarly immunogenic in the study population (Table 2). This finding is further supported by the comparable GMI values calculated from results of the ELLA and the MN assay (Table 1). In studies administering equivalent amounts of purified HA and NA antigens to mice, equivalent HA- and NA-specific ELISA titers were found, confirming the generally equal immunogenic potential of both proteins [13]. However, seroconversion rates based on NAi titers have been reported to be lower than the rates based on HA-specific antibody titers even in NA-primed populations [38, 42]. This effect has either been assigned to greater molar amounts of HA than NA proteins on the viral surface and molecular antigenic competition between these molecules being in favor of HA [39, 42], or to the variable content of immunogenic NA in vaccines [37, 43], leading to poor NA-specific antibody responses. In light of the current investigation, the sensitivity of the assays used to measure antibodies specific for HA and NA should also be taken into consideration to explain the described divergence in antibody frequency. The seroconversion rates calculated in the present study from titers determined by the insensitive TBA assay (Figure 1, Table 1) were well aligned with those previously reported for seasonal influenza virus vaccines [25–27, 37, 38, 41]. In contrast, the day 42 seroconversion rates based on MN and SRH assay results were in line with the rates based on the ELLA titers (Table 1). Thus, beyond the earlier described effectiveness of the Vero cell–derived whole-virus H5N1 vaccine in inducing antibodies to the virus HA, we show here that this vaccine is equally effective in inducing antibodies to the NA. Consequently, it seems that adjusting the antigen amount of the vaccine according to the HA concentration, as is currently done, can be considered adequate.

To more conclusively investigate the breadth of antibody specificity following influenza vaccination, qualified assay systems for the evaluation of NAi antibodies need to be available. Our study provides the first head-to-head comparison of the TBA-based assay and the ELLA. While the results obtained with these assays correlated well (Figure 2), the ELLA showed far better sensitivity. Another advantage of the ELLA is that it does not require hazardous reagents, unlike the TBA assay. Thus, due to its safety and superior performance, the ELLA would be the assay of choice for the determination of NAi antibody responses during clinical influenza vaccine evaluations.

Notes

Acknowledgments. The authors are grateful to Nicole Hetzelt, Cherry Abraham, and Mandy Reinhardt for excellent performance of NA inhibition assays and Daniel Portsmouth for critically reading the manuscript.

Financial support. This work was supported by Baxter BioScience.

Potential conflicts of interest. All authors are employed by Baxter BioScience, the manufacturer of the Vero cell culture–derived H5N1 vaccine.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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