Antibody Responses to *Haemophilus influenzae* Type b Polysaccharide Vaccine in Relation to Km(1) and G2m(23) Immunoglobulin Allotypes

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The antibody responses of children immunized with *Haemophilus influenzae* type b polysaccharide vaccine were examined in relation to the absence or presence of the Km(1) or G2m(23) immunoglobulin allotype. Ninety-seven children, 12-83 months of age, were immunized. Sera were obtained before immunization and two months later. Total serum antibody to the type b capsule was detected by a radioactive antigen-binding assay. IgG and IgM antibody responses were measured by enzyme-linked immunosorbent assay. Antibody responses to the type b capsule were more than threefold higher in blacks with the Km(1) immunoglobulin allotype compared with those in blacks lacking this allotype ($P < .02$). The isotype affected was IgG ($P < .01$) and not IgM. Serum concentrations of IgG2, but not of IgGl, also were higher in blacks with Km(1) ($P < .003$). In whites there were no significant differences in the total or IgG-specific antibody responses to the type b capsule in relation to the Km(1) or G2m(23) allotype.

Recent evidence indicates that genetic factors may affect susceptibility to meningitis due to *Haemophilus influenzae* type b [1-3] as well as serum antibody responses to immunization with haemophilus vaccine [1, 3]. We previously reported that children with the Km(1) immunoglobulin allotype, a genetic marker on the constant region of the κ immunoglobulin light chain, have higher antibody responses to the type b capsule after immunization with *H. influenzae* type b polysaccharide (PS)-*Bordetella pertussis* vaccine compared with children who lack this allotype [3]. Black children, but not white children, with Km(1) also had a lower relative risk of developing meningitis due to *H. influenzae* type b [3]. Ambrosino et al. [1] reported that white adults with the G2m(n) allotype (designated in our study as G2m[23]) have higher IgG antibody responses to *H. influenzae* type b and pneumococcal PS vaccines than do white adults who lack this allotype. The G2m(23) allotype is of special interest because it is located on IgG2 molecules, a subclass preferentially elicited during the human immune response to PSs [4, 5]. Also, the data of Ambrosino et al. [1] indicated not only that whites lacking G2m(23) had poorer antibody responses to vaccine than did those with this marker, but also that children lacking this allotype were at increased relative risk of developing disease due to *H. influenzae* type b.

A plain *H. influenzae* type b PS vaccine was recently licensed for use in the United States [6]. Most healthy human infants <18 months of age developed serum antibody responses of <1 μg/ml to this vaccine, whereas most older children and adults showed higher responses [7]. In an efficacy trial performed in Finland, this vaccine was clearly effective in preventing disease due to *H. influenzae* type b only in children ≥24 months of age [7], whereas it was ineffective in children <18 months of age and was of uncertain efficacy in the age-group 18–23 months [6, 7]. The Finnish vaccine trial was conducted in a largely white population. Virtually no data exist on the efficacy of the *H. influenzae* type b PS vac-
cine in black children, an important target population for immunization because black children in the United States have a two- to fourfold higher incidence of meningitis due to *H. influenzae* type b than do white children [8, 9].

Our previous data indicated that the risk of developing haemophilus meningitis was higher among the 40% of black children who lack the Km(1) allotype [3]; this group is the same one that showed poor responses to immunization with *H. influenzae* type b PS—*B. pertussis* vaccine. The data of Ambrosino et al. [1] indicated that the risk of developing disease due to *H. influenzae* type b was higher among the 30% of white children who lack the G2m(23) allotype; as adults, this group was the same one that showed poor responses to immunization. One implication of these findings is that immunization of certain groups may be associated with limited success in preventing disease due to *H. influenzae* type b because of failure to confer protection on the subpopulations that are at highest risk of disease because of genetic factors. However, our previous study was conducted with a vaccine containing *H. influenzae* type b PS and inactivated *B. pertussis* cells. The latter is a strong adjuvant, and it was unknown whether children who lacked Km(1) have impaired antibody responses to the plain *H. influenzae* type b PS vaccine because altering the way an antigen is presented may influence the magnitude of an immune response in inbred strains of animals [10, 11]. Also, the vaccine study of Ambrosino et al. [1] was conducted in white adults, and it was unknown whether white children who lack G2m(23) have impaired antibody responses to *H. influenzae* type b PS vaccine.

The purposes of the present study were (1) to determine whether genes associated with the Km(1) allotype affect antibody responses to the *H. influenzae* type b PS when it is administered without *B. pertussis* vaccine and (2) to determine whether genes associated with the G2m(23) allotype, an allotype found predominantly in whites, affect antibody responses of white children to this vaccine. We also compared the respective serum concentrations of IgG1 and IgG2 in black subjects without and with Km(1). This part of the study was prompted by our recent observation that black children, but not white children, lacking Km(1) have lower serum concentrations of IgG2 than those positive for this allotype [12]. Relative to serum, the IgG2 subclass is potentially involved in the IgG antibody response to many PSs [4], including *H. influenzae* type b PS [5].

**Materials and Methods**

Subject selection and response to immunization. Ninety-seven children (51 whites and 46 blacks) from St. Louis were immunized. The children were recruited from private practices. The only criteria for inclusion in the study were that the children had no family or individual history of disease due to *H. influenzae* type b and that they were in good health.

The subjects ranged in age from 12 to 83 months (mean ± SD age, 35.7 ± 19.1 months). The subjects represent an independent sample except for six black children negative for the Km(1) allotype who served as normal subjects in a previous study of serum IgG subclasses [12].

A 0.1-ml dose containing 5 μg of the type b capsular PS vaccine was administered im. The vaccine (lot 19 [13]), produced by Dr. Porter Anderson (University of Rochester School of Medicine, Rochester, NY) under contract to the National Institutes of Health (Bethesda, Md), was stored frozen at −70 C. Before use it was thawed at room temperature (~23 C) and stored at 4 C for periods of up to two weeks. Blood samples were obtained by venipuncture before immunization and about two months later.

Serology. Concentrations of serum antibody to the type b capsular antigen were measured by a radioactive antigen-binding assay, performed as described by Kuo et al. [14] except that 125I-labeled PS was used as the test antigen instead of 3H-labeled PS. In brief, the *H. influenzae* type b PS was purified from the supernatant of stationary-phase broth cultures by sequential precipitation with ethanol, cetrimonium bromide, and ethanol, as described by Kuo [15]. The final precipitate was dissolved in 20 mM NaH2PO4 (pH 6.9), and residual contaminants were removed by adsorption with hydroxylapatite and extraction with cold phenol [16]. Protein and nucleic acid contents (<1.0% and 0.7%, respectively) of the final product were determined by UV absorption [17]. Lipopolysaccharide content (<0.06%) was determined by limulus lysate gelation [18]. The PS was coupled to tyramine by a modification of the method originally proposed by Gotschlich et al. [19] and modified by Robbins et al. [20]. The final product was stored frozen in small portions at −70 C. Before our assay an aliquot was thawed, and the PS-tyramine
was radiolabeled with $^{125}$I by the chloramine T method [21]. The average specific activity of the iodinated derivatives was 1.5 \times 10^6 \, \text{cpm/ug of PS}.

A reference serum pool from the U.S. Bureau of Biologics (Rockville, Md), containing \sim 80 \mu g of antibody to the type b capsule/ml, was used to standardize the radioactive antigen-binding assay. The smallest amount of antibody detectable was 0.025 \mu g of antibody protein/ml of serum, as determined by dilutions of this reference pool. The correlation coefficient between antibody values obtained on test serum samples assayed on different days was >.90.

IgM and IgG antibody to the type b PS were detected by an ELISA. We used a modification of the technique described by Anthony et al. [22] to measure levels of antibodies to PS of group B streptococci. The major differences between our technique and theirs was our use of \textit{H. influenzae} capsule b PS coupled to poly-l-lysine [23] as the antigen and the use of biotinylated goat antibodies to human immunoglobulins and avidin--alkaline phosphatase [24] instead of alkaline phosphatase--conjugated antiserum.

The PS was coupled to poly-l-lysine as follows. In brief, 0.75 ml (1 mg/ml) of a solution of \textit{H. influenzae} type b PS in water was added to an equal volume of 0.02 N NaOH and 30 \mu l of CNBr (25 mg/ml). The solution was maintained at 2–4 C in an ice bath, and the pH was monitored continuously and maintained at 10.9 by addition of NaOH. After 10 min, 0.75 ml of poly-l-lysine hydrobromide (0.5 mg/ml; Sigma Chemical Co., St. Louis) in 0.5 M NaHCO$_3$ (pH 8.8) and 0.5 M NaCl was added. The pH was immediately lowered to 8.5 by addition of 0.1 N HCl. The solution was then dialyzed extensively at 4 C against PBS, aliquoted, and stored frozen at -70 C.

Plates with 96 wells (model 3590; Costar, Cambridge, Mass) were incubated at room temperature for 2 hr with \textit{H. influenzae} type b PS--poly-l-lysine, prepared as described above. Two hundred microliters of antigen at a concentration of \sim 0.5–1 \mu g/ml in PBS containing 0.02% sodium azide (pH 7.4) was added to each well (the optimal antigen concentration was determined in a preliminary assay with each new lot of antigen). The plates were then washed three times with PBS containing 0.05% Tween 20 and 0.01% gelatin (washing buffer) and incubated at room temperature (\sim 23 C) for 1 hr with 200 \mu l of PBS containing 0.5% gelatin (blocking step). The plates were then washed three times as above, and 200 \mu l of the human test serum, diluted in PBS containing 0.05% Tween 20 and 0.1% gelatin (diluting buffer), was added to each well. The plates were incubated for 1 hr at 37 C and then overnight at 4 C. The next day the plates were washed three times with washing buffer and incubated at 37 C for 2 hr with biotinylated, heavy chain--specific goat antibody to human IgG or IgM (Tago, Burlingame, Calif), diluted to the working dilution recommended by the manufacturer. After three washes, 200 \mu l of avidin-coupled alkaline phosphatase, diluted in PBS containing 0.05% Tween 20 and 0.1% gelatin, was added and incubated at 37 C for 2 hr. The wells were then washed three times, and 200 \mu l of a solution of p-nitrophenyl phosphate (1 mg/ml) in 10% diethanolamine buffer (pH 9.8) was added and incubated at room temperature. The value of $A_{405}$ was monitored with a Titertek Multiscan spectrophotometer (Flow Laboratories, McLean, Va). Titers were determined graphically as the reciprocal of the serum dilution producing an $A$ value of 0.3 as described previously [18]. The IgG assay was considered complete when wells containing a 1:6,000 dilution of a local serum pool from immunized adults, which contained 52 \mu g of antibody to the type b capsule/ml as measured by the radioactive antigen–binding assay, reached an $A$ value of 0.3. The IgM assay was considered complete when wells containing a 1:3,200 dilution of the Bureau of Biologics’ serum pool reached an $A$ value of 0.3. The 0.3 value was selected because it is in the linear range of the assay and sufficiently above background values (\sim 0.15) to assure the reproducibility of our results. With this assay the Bureau of Biologics’ serum pool had an average titer of IgG antibody to \textit{H. influenzae} type b PS of 1:12,000. Paired pre- and postimmunization serum samples from each test subject were assayed in parallel on the same plate. The results of assays performed on different days agreed within twofold dilutions for >90% of serum samples. The coefficient of correlation, comparing the ELISA titers in replicate samples measured on different days, was >.90.

Serum concentrations of IgG1 and IgG2 were measured by a solid-phase inhibition RIA with subclass-specific reagents as described previously [25]. The sensitivity for the IgG1 assay was 50–128 ng/ml and that for the IgG2 assay was 70–200 ng/ml.

Determination of the Km(1) and G2m(23) allotypes was performed on coded serum samples with
an HAI assay using reagents previously described [26].

Statistical analyses. Statistical analysis was performed by using SAS and RSI software on a VAX® 11/780 computer (Digital Equipment Co., Marlboro, Mass) and SPSS software on a Harris (Hialeah, Fla) model 500 computer. Covariance analysis was used to compare the geometric mean concentrations of antibody in vaccinees grouped according to immunoglobulin allotype status. In this analysis, subjects with different allotypes were not individually matched for age. Initial analysis indicated a significant regression of the log of the postimmunization antibody concentration on age in months ($r = .64$, $P < .001$). Further analysis indicated that addition of the term age squared to the regression, while increasing the $r^2$ value only from .41 to .45, nevertheless made a significant contribution ($P < .05$). Accordingly, both age and age squared were used as covariates in analysis of the group antibody data.

In addition, our previous studies showed differences between white and black children with regard to presence of the Km(1) allotype, maturation of serum IgG2 [12], and risk of developing meningitis due to $H. influenzae$ type b [3]. Therefore, in the present study the data were analyzed separately for whites and blacks.

To compare the respective serum concentrations of IgG1 and IgG2 in black children without and with Km(1), we matched each of the 12 Km(1)-negative children for age with two Km(1)-positive children; most matches were within one month of age (mean ± SD difference, 0.5 ± 2.1 months). The value for the Km(1)-negative subject was paired with the respective mean value of the two Km(1)-positive subjects. A paired $t$ test was used to compare the respective mean concentrations of IgG1 and IgG2 in subjects without and with Km(1).

### Results

**Antibody responses in relation to Km(1) immunoglobulin allotype.** In black subjects, preimmunization serum concentrations of antibody to $H. influenzae$ type b PS were not significantly different between children who were Km(1) positive and those who were Km(1) negative (table 1). Two months after immunization both groups showed significant increases in serum concentrations of antibody to the type b capsule ($P < .02$ by paired $t$ test). However, the concentrations of antibody to the type b capsule were more than threefold higher in blacks with the Km(1) allotype ($P < .02$). Only three of 12 black children negative for Km(1) showed fourfold or greater increases in serum antibody levels compared with 20 of 34 black children positive for Km(1) ($\chi^2$

### Table 1. Antibody responses to $H. influenzae$ type b capsule in relation to presence of the Km(1) and G2m(23) immunoglobulin allotypes.

<table>
<thead>
<tr>
<th>Race, allotype (no. tested)</th>
<th>Mean age (months) at injection</th>
<th>Concentration of serum antibody to capsule (µg/ml)</th>
<th>Reciprocal postimmunization IgG titer by ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Preimunination</td>
<td>Postimmunination</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean log$_{10}$ ± SD</td>
<td>GMT log$_{10}$ ± SD</td>
</tr>
<tr>
<td>Black children</td>
<td></td>
<td>GM</td>
<td>GM</td>
</tr>
<tr>
<td>Km(1) positive (34)</td>
<td>31</td>
<td>0.42 -0.38 ± 0.61</td>
<td>3.03* -0.48 ± 0.81</td>
</tr>
<tr>
<td>Km(1) negative (12)</td>
<td>32</td>
<td>0.30 -0.53 ± 0.48</td>
<td>0.91* -0.04 ± 0.71</td>
</tr>
<tr>
<td>White children</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Km(1) positive (7)</td>
<td>35</td>
<td>0.54 -0.27 ± 0.70</td>
<td>3.89 0.59 ± 0.77</td>
</tr>
<tr>
<td>Km(1) negative (44)</td>
<td>42</td>
<td>0.66 -0.18 ± 0.71</td>
<td>4.90 0.69 ± 0.96</td>
</tr>
<tr>
<td>G2m(23) positive (39)</td>
<td>42</td>
<td>0.76 -0.12 ± 0.68</td>
<td>4.50 0.66 ± 0.90</td>
</tr>
<tr>
<td>G2m(23) negative (12)</td>
<td>39</td>
<td>0.36 -0.44 ± 0.74</td>
<td>5.60 0.75 ± 1.06</td>
</tr>
</tbody>
</table>

NOTE. Children were immunized with a single injection of $H. influenzae$ type b PS vaccine. The subjects in each group showed significant ($P < .01$) increases in antibody concentration after immunization. Blacks with Km(1) had significantly higher total antibody responses and higher IgG responses than did blacks lacking Km(1) by analysis of covariance with age and age square as covariates. In white subjects the respective antibody concentrations before or after immunization were not significantly different ($P < .05$) in a comparison of children with Km(1) with those who lacked Km(1) or children with G2m(23) with those who lacked this allotype. Postimmunization data were obtained two months after immunization. GMT = geometric mean titer. GM = geometric mean.

* $P < .02$.
† $P < .01$. 

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The mean ± SD age of the nine Km(1)-negative nonresponders (28.9 ± 17 months) was not significantly different from that of 20 Km(1)-positive responders (30.9 ± 15.4 months; P > .05).

Blacks with Km(1) also had higher IgG responses than those who lacked Km(1), with reciprocal GMTs of 141 and 34, respectively (P < .01; table 1). In contrast, there were no significant differences in the IgM antibody responses to the type b capsule of black subjects without and with Km(1) (postimmunization reciprocal GMTs, 281 and 347, respectively; t = .7, P = .5).

Seven (14%) of the 51 white subjects were positive for Km(1), a percentage not significantly different from the 18%–20% found in white subjects in our previous study [3]. The percentage of whites with Km(1) was significantly lower than that of the black subjects (74%; P < .0001 by χ² test) and reflected the recognized differences in racial frequencies of certain allotypes [27]. In white subjects, antibody responses to the H. influenzae type b PS vaccine were not significantly different between children who were Km(1) positive and those who were Km(1) negative (table 1). The postimmunization IgG titers of the two groups measured by ELISA were also not significantly different.

Antibody responses in relation to G2m(23) immunoglobulin allotype. The G2m(23) allotype is present in ~70% of white subjects [1, 3] but is infrequent in North American blacks (<15% [3]). When present in blacks this allotype may reflect racial admixture [27]. Therefore, in the present study the G2m(23) allotype was determined only in white subjects. Table 1 summarizes the antibody responses to immunization of the white subjects in relation to this allotype. Preimmunization antibody levels in the two groups were not significantly different. After immunization both groups showed significant (P < .002) increases in serum antibody levels, but the postimmunization levels of total or IgG antibody to the type b capsule were not significantly different between the two groups.

Serum concentrations of IgG1 and IgG2 in black children in relation to Km(1) allotype. We previously found that black children, but not white children, with Km(1) had higher serum concentrations of total IgG2 than did those who lacked this allotype [12]. In contrast, the IgG1 concentrations of subjects without and with Km(1) were not significantly different. In the present study we have increased the sample size and have compared the respective serum concentrations of IgG1 and IgG2 in black children without and with Km(1). The mean serum concentrations of IgG1 in the two groups were not significantly different (table 2). However, the mean serum concentration of IgG2 was significantly higher in black children with Km(1) than in those without Km(1) (t = 5.79, P = .0025). This difference remained significant (P = .0075) even after omission of the data from the six Km(1)-negative children included in our previous report [12] together with the data from their respective 12 new Km(1)-positive matches.

Discussion

We previously reported that subjects with the Km(1) immunoglobulin allotype had enhanced IgG antibody responses to the H. influenzae type b PS capsule compared with those lacking Km(1). In our earlier study we used the H. influenzae type b PS-B. pertussis vaccine, which contained B. pertussis cells as adjuvant. Therefore it was unclear whether the higher responses of subjects with Km(1) were specific for the capsular antigen or resulted from an adjuvant effect. In the present study we used the plain H. influenzae type b PS vaccine, a preparation [13] similar to that recently licensed in the United States for use in children two years of age or older [4]. The subjects studied had not participated in our previous vaccine trial and represent an independent sample. Our findings of higher IgG antibody responses to the type b capsule in black subjects with Km(1)
compared with those in blacks lacking Km(1) indicate that the differences found in our previous study were not dependent on administration of an adjuvant with the PS.

The mechanism by which black children with Km(1) have higher IgG antibody responses to the *H. influenzae* type b PS antigen is unknown. Allotypes are hereditary antigenic variants on immunoglobulin molecules that are inherited according to Mendelian laws. One explanation of the present findings may be that in blacks there are immune response genes in linkage disequilibrium with the Km(1) allele that regulate IgG antibody responses to the *H. influenzae* type b PS antigen. In contrast, these genes do not appear to regulate IgM antibody responses to the type b capsule because no significant differences were observed in these responses of black children without and with Km(1) either in the present study or in our previous study.

IgG antibody responses to many bacterial PSs, including the capsule of *H. influenzae* type b, preferentially involve the IgG2 subclass [4, 5], based on a comparison of the relative concentrations of IgG1 and IgG2 in serum [12, 28]. The present data on IgG2 (table 2) support our previous findings [12] that black children with Km(1) have higher serum concentrations of IgG2 than do those lacking the Km(1) allotype. This finding may reflect higher IgG responses to many antigens, in addition to *H. influenzae* type b PS, that preferentially elicit IgG2 antibody responses. However, the enhanced IgG antibody responses to the type b capsule of blacks with Km(1) also appears to involve IgG1 antibody. We recently measured specific IgG1 antibody responses to *H. influenzae* type b PS by ELISA, with a biotinylated murine monoclonal antibody to human IgG1 (HGI1) and an avidin–alkaline phosphatase detection system. By this technique the immunized black children with Km(1) had higher IgG1 responses than did those lacking Km(1), even though the total serum concentrations of IgG1 were not significantly different in the two groups (table 2). Thus it is likely that some underlying mechanism that affects both IgG1 and IgG2 antibody responses to PS produces the higher IgG responses in black children with Km(1). The observation that total serum concentrations of IgG1 are not influenced by genes associated with the Km(1) allotype (table 2) may reflect normal acquisition of IgG1 antibodies to protein antigens in black subjects lacking Km(1).

In contrast to black children, in white children there does not appear to be any consistent association between the Km(1) allotype and the relative risk of developing meningitis due to *H. influenzae* type b [3], relative serum concentrations of IgG2 [12], or magnitude of the antibody responses to *H. influenzae* type b PS vaccine [1] (table 1). Our preliminary report [3] of higher antibody responses in white children with Km(1) who were immunized with the *H. influenzae* type b PS–*B. pertussis* vaccine may have been spurious, a reflection of the small sample size of whites with Km(1) in that study and the borderline statistical significance (*P* = .06). Because the Km(1) locus is not thought to be involved directly in regulation of immune responses or in susceptibility to disease caused by *H. influenzae* type b, the most likely explanation for the racial differences in antibody response in relation to Km(1) is that the genes involved are in linkage disequilibrium with the Km(1) locus in blacks but not in whites. There are several other examples of the association of a particular genetic marker with either immune response or a disease state in one population but not in others [29–34], a finding suggesting that the linkage disequilibrium between the marker loci and those controlling immune responses or disease susceptibility is different in different populations.

We also found that antibody responses of white children with the G2m(23) allotype and those of white children lacking this allotype were not significantly different (table 1). This result is consistent with our previous findings of no difference in the antibody responses of white children without and with G2m(23) who were immunized with *H. influenzae* type b PS–*B. pertussis* vaccine [3]. However, the results from both of our studies differ from those reported by Ambrosino et al. [1]. In their study, white adults with G2m(23) immunized with *H. influenzae* type b and pneumococcal PS vaccines had higher IgG antibody responses to PS than did those who lacked this allotype. Ambrosino et al. [1] also reported a lower frequency of the G2m(23) allotype in white children with disease due to *H. influenzae* type b than in controls. In contrast, we reported no difference in the frequency of this allotype [3]. Our laboratories have exchanged sera for typing of G2m(23) and have found consistent results. Therefore laboratory differences do not appear to be responsible for our different findings. Further work is needed to clarify the apparently discrepant results.
In summary, the present data relating the magnitude of the antibody responses of black children immunized with *H. influenzae* type b PS vaccine to the presence of the Km(1) allotype provide further support for the existence of genetic regulation of certain immune responses of humans. Black subjects with Km(1) had higher IgG antibody responses to *H. influenzae* type b PS vaccine and higher serum concentrations of IgG2 than did those lacking this allotype. These results, together with our previous findings of lower relative risk of developing meningitis due to *H. influenzae* type b but may benefit least from immunization. Finally, it will be important to determine whether blacks lacking Km(1) also have impaired antibody responses to the new *H. influenzae* type b PS-protein conjugate vaccines [35-38]. These conjugate vaccines offer the prospect of preventing disease due to *H. influenzae* type b in children two years of age and younger, the age-group at greatest risk. However, it is not known whether the antibody responses to the type b capsule induced by the conjugate vaccines will be subject to genetic regulation similar to that observed in blacks given either the *H. influenzae* type b PS vaccine alone (table 2) or *H. influenzae* type b PS combined with *B. pertussis* vaccine [3].

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