Human Immune Proteome in Experimental Colonization with *Staphylococcus aureus*[^1][^2]

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Received 10 June 2009/Returned for modification 28 July 2009/Accepted 9 September 2009

More than 20% of adults are persistently colonized with *Staphylococcus aureus*. When hospitalized, these carriers have increased risks of infection with their own strains. However, a recent study demonstrated a lower incidence of bacteremia-related death among carriers than among noncarriers, raising the question whether the adaptive immune system protects against superantigens of their colonizing strains. We now used 2-dimensional immunoblotting to investigate the profiles of antibodies from healthy individuals against *S. aureus* extracellular proteins. Moreover, we tested whether symptom-free experimental colonization of these individuals with an *S. aureus* strain of low virulence, 8325-4, is sufficient to induce an antibody response. Sera obtained before and 4 weeks after colonization were screened for immunoglobulin G (IgG) antibody binding to extracellular staphylococcal proteins. At baseline, most volunteers harbored IgG directed against conserved virulence factors, including alpha-hemolysin (Hla), beta-hemolysin (Hlb), phospholipase C (Plc), staphylococcal serine protease (SspA), and cysteine protease (SspB). However, the variability of spot patterns and intensities was striking and could be important in case of infection. Experimental nasal colonization with *S. aureus* 8325-4 did not elicit new antibodies or boost the humoral response. Thus, the high antibody prevalence in humans is likely not induced by short-term nasal colonization, and presumably minor infections are required to trigger anti-*S. aureus* antibody responses.

*Staphylococcus aureus* is one of the most common causes of nosocomial infection, and the species is becoming increasingly resistant to antibiotics (2). Apart from being a major human pathogen, *S. aureus* is also a frequent colonizer of human skin and mucosa (34). The bacteria find their primary ecological niche in the human nose but are also able to colonize the throat, the intestines, and the perineal region, sometimes exclusively (1, 17). Approximately 20% of the adult population carry *S. aureus* in the nose persistently, and another 30% carry it intermittently, frequently only for a few days, whereas 50% are noncarriers (NC) (29, 30, 34). Nasal carriers stand an increased risk of developing severe *S. aureus* infections caused by their autologous strains, especially upon hospitalization or immune suppression (32, 35). This underlines the fact that host and environmental factors play a decisive role in determining the outcome of *S. aureus* host interactions.

In a recent large prospective study, carriers acquired *S. aureus* bacteremia more frequently than NC but, surprisingly, had a better survival rate than NC (35). This observation raises the question whether the adaptive immune system establishes immunity to the colonizing *S. aureus* strain, which could be of advantage in autologous infections. In support of this hypothesis, our group recently showed that *S. aureus* carriers raise a strong and strain-specific antibody response against the superantigen cocktail produced by their colonizing strain (12). However, *S. aureus* produces a broad repertoire of virulence factors, and the antibody response against superantigens is likely only the tip of an iceberg (8). In fact, anti-*S. aureus* antibodies against staphylococcal toxins, immune evasion molecules, and adhesins have been detected in healthy individuals as well as in patients (6, 7, 11, 31).

Virulence factor expression is strictly regulated in *S. aureus*. While adhesins are expressed by bacterial cells in logarithmic growth, the majority of known virulence factors, including most superantigens but also cytolytic toxins, proteases, lipases, and several immune evasion molecules, are secreted in the post-exponential-growth phase (23, 38). In contrast to intracellular and cell wall-associated proteins, secreted virulence factors can act systemically while bacteria remain localized. Consequently, these factors are the most likely stimuli of the adaptive immune system during epithelial colonization with *S. aureus* (28).

To date, a comprehensive investigation of anti-*S. aureus* antibody profiles from healthy individuals and their variability is still lacking. Moreover, it remains unknown which conditions (e.g., nasal colonization, minor or major infections) are required to trigger an antibody response against *S. aureus*. Therefore, we experimentally colonized the nares of 16 healthy human volunteers with *S. aureus* (36) and compared the anti-*S.
TABLE 1. Characteristics of volunteers

| Volunteer no. | Carriage status | Day from which culture was negative |
|---------------|-----------------|------------------------------------|
| 12            | PC              | 4                                  |
| 141           | IC              | Remained a carrier                 |
| 165           | NC              | 4                                  |
| 211           | NC              | 8                                  |
| 216           | PC              | 8                                  |
| 600           | NC              | 29                                 |
| 601           | PC              | Remained a carrier                 |
| 602           | IC              | Remained a carrier                 |
| 603           | PC              | 29                                 |
| 604           | NC              | 3                                  |
| 605           | NC              | 3                                  |
| 606           | PC              | 3                                  |
| 607           | PC              | 22                                 |
| 608           | NC              | 15                                 |
| 609           | NC              | 15                                 |
| 610           | NC              | 28                                 |

**aureus** antibody profiles before and 28 days after colonization. Our aims were to analyze the variability of the anti-*S. aureus* antibody profiles and to test whether experimental nasal colonization elicits or boosts an antibody response.

**MATERIALS AND METHODS**

*S. aureus*. The superantigen-negative *S. aureus* strain 8325-4 was used for experimental nasal colonization of human volunteers as described previously (36). The genome sequence of the original 8325 strain is available at http://www.genome.ou.edu (NCBI database, accession no. NC_007795). Strain 8325-4 differs from strain 8325 by the absence of three prophages (20). DU5997 is a *clfB*-deficient mutant of 8325-4, and 8325-4 Δ*clpP* is a protein A-deficient mutant of 8325-4 (21, 36).

Study design and human experimental colonization. Wertheim et al. conducted a human experimental colonization study of 16 healthy volunteers (36). Among them were six persistent carriers (PC), two intermittent carriers (IC), and eight NC (Table 1). In short, all enrolled volunteers underwent a decolonization treatment with mupirocin 5 weeks before experimental colonization. Volunteers were inoculated with a mixture of *S. aureus* strains 8325-4 and DU5997 (Δ*clfP*) in one nostril and with either 8325-4 or DU5997 in the other nostril (1 × 10⁸ CFU of each strain). Follow-up cultures were performed on days 1, 2, 3, 4, 8, 15, 22, 28, and 29 after inoculation.

Sera. Serum samples were obtained from all 16 volunteers directly before experimental inoculation and 28 days afterwards. Sera were aliquoted and stored at −80°C.

Growth conditions of bacteria and preparation of protein extracts. In this study, *S. aureus* strain NCTC8325-4 and its isogenic spa mutant DU5875 (8325-4 Δ*spa*) were used. For the preparation of protein extracts, *S. aureus* 8325-4 and its isogenic spa mutant were inoculated into tryptic soy broth to an optical density at 540 nm (OD₅₄₀) of 0.05 and were cultivated in 1.5 liters of tryptic soy broth at 37°C and 110 rpm until the bacterial culture entered the stationary phase at an OD₅₄₀ of 8 to 10.

For preparation of the extracellular protein fraction, *S. aureus* cells were removed by centrifugation (9,164 × g for 10 min at 4°C), and extracellular proteins from three 1.5-liter culture supernatants were precipitated by addition of trichloroacetic acid to 10% (wt/vol) and centrifugation at 9,164 × g for 1 h at 4°C. Protein-containing pellets were then washed six times with 70% ethanol. After the final washing step, 100% ethanol was added and then removed by centrifugation (5 min, 16,060 × g, 4°C), and the protein pellet was air dried and finally resolved in rehydration buffer containing 8 M urea, 2 M thiourea, and 2% CHAPS (3-[3-cholamidopropyl]-dimethylammonio)-1-propanesulfonate). The protein concentration was determined according to Bradford (3) with a commercially available kit (Pierce, Thermo Scientific, Bonn, Germany). Sample aliquots were stored at −80°C.

Two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) with 2D minigels. Isoelectric focusing was done with 7-cm Immobiline dry gels (GE Healthcare, Munich, Germany) with the pH ranges 4 to 7 and 6 to 11. For separations in the pI range from 4 to 7, strips were rehydrated overnight in 150 µl rehydration solution (8 M urea, 2 M thiourea, 33 mM CHAPS, 28 mM dithiothreitol [DTT], 2% Pharmalyte 3–10 carrier ampholytes, and a trace of bromophenol blue) containing 30 µg of protein extract. Strips were placed in a Multiphor II horizontal electrophoresis system (GE Healthcare), and proteins were separated at 20°C at a maximum of 2 mA and 5 W per strip by applying the following voltage profile: 200 V constant for 1 min, a gradient from 200 V to 3,500 V for 1.5 h, and 3,500 V constant for 1.5 h (total, 8,000 V). For separations in the pH range from 6 to 11, strips were rehydrated overnight in 150 µl rehydration solution (8 M urea, 2 M thiourea, 10% [vol/vol] isopropanol, 5% [vol/vol] glycerol, 162 mM DTT, 1% [vol/vol] Pharmalyte 3–10, 1% [vol/vol] Pharmalyte 8.5–10.5, and a trace of bromophenol blue) without proteins, and 30 µg of crude protein extract was loaded with the aid of sample cups at the anode. The isoelectric focusing was done in a Multiphor II device at 20°C, 2 mA, and 5 W with the following voltage profile: 200 V constant for 1 min, a gradient from 200 V to 3,500 V for 1.5 h, and 3,500 V constant for 1 h (total, 6,500 V). After isoelectric focusing, the strips were either frozen for storage or immediately equilibrated with solution A (20% [vol/vol] glycerol, 0.375 M Tris-HCl [pH 8.8], 6 M urea, 4% [wt/vol] sodium dodecyl sulfate [SDS], and 65 mM DTT) and solution B (20% [vol/vol] glycerol, 0.375 M Tris-HCl [pH 8.8], 6 M urea, 4% [wt/vol] SDS, 135 mM iodoacetamide, and a trace of bromophenol blue) for 15 min each. Separation according to apparent molecular weight by SDS-PAGE in the second dimension was performed for 12 samples in parallel on 12.5% PAGE gels on a vertical Mini Protein II Multi cell (Bio-Rad, Munich, Germany) at a constant power of 10 mA per gel for 30 min and then at 15 mA for 90 min.

2D Western blot analysis. The pH range 6 to 11 was probed with extracellular proteins of the *S. aureus* strain 8325-4. 2D immunoblotting (2D-IB) for the pH range 4 to 7 was performed with extracellular proteins from 8325-4 Δ*clpP* in order to avoid a high background due to immunoglobulin G (IgG) binding by protein A. The two strains produced very similar patterns of extracellular proteins, except for the difference in protein A (data not shown). Thirty micrograms of extracellular proteins was separated on 12.5% 2D mini-gels as described above and was transferred to a polyvinylidene difluoride membrane for Western blotting using a semidry blotting device (Milliblot graphic electrobottler II; Millipore, Billerica, MA) according to the manufacturer’s instructions. The quality of transfer was controlled by reversible staining with Pelikan Ink solution (10). After destaining, membranes were blocked. After destaining, membranes were blocked by incubating the human sera with the SuperSignal West Pico maximum-sensitivity substrate (Pierce, Roche, Mannheim, Germany) at a 1:1,000 dilution for 1 h at room temperature. After washes with TBS-T buffer, the membranes were incubated with the human sera at 4°C (diluted 1:10,000 in 5% [wt/vol] bovine serum albumin in TBS-T). Bound human IgG was detected by incubation with peroxidase-conjugated goat anti-human IgG (H+L), diluted 1:50,000 in blocking buffer for 1 h at room temperature. After a wash, the membranes were incubated with a luminol (Luminol, GE Healthcare, Munich, Germany) substrate solution (10) and exposed to light for 1 h. Image analysis was performed with AlphaEase FC software (Alpha Innotech, San Leandro, CA) and Photoshop (Adobe Systems, San Jose, CA).
autolysin (Atl), autolysin precursor (Aaa), immunodominant antigen B (IsaB), and iron-regulated surface determinant protein A (IsaA).

To reveal the proteins behind the IB spots, a reference map on which protein spots were identified was matched with the master IB containing the spot map and corresponding spot labels in Delta-2D. By these means, protein names could be assigned to 44% (52/117) and 36% (43/119) of the IB spots for pH 6 to 11 and pH 4 to 7, respectively (see Fig. S2 in the supplemental material).

**Statistics.** Statistical analyses were performed with GeneSpring, version 3.7.1. Graphs were created with GraphPad Prism, version 5. Medians are given with 90% confidence intervals. Differences in baseline IgG binding between carriers and NC were assessed with the Mann-Whitney U test. Immunodominant antigens were identified by assigning the highest ratio between median spot intensities and spot patterns. IgG binding to the conserved virulence factors SspA, SspB, and IsaA, which belong to the core immune proteome, was assessed with a Student's t test.

**RESULTS**

Our investigations were performed in the context of an experimental colonization study that compared the adherence and colonization properties of *S. aureus* 8325-4 and its isogenic clumping factor B mutant (36). 8325-4 is a laboratory strain with low virulence and without superantigen genes; it was chosen for safety reasons. As expected, all volunteers remained completely free from clinical symptoms (36). Sera obtained from these volunteers before and 4 weeks after the colonization were used to investigate the anti-*S. aureus* IgG profiles in healthy adults.

**High variability of baseline IgG binding to *S. aureus* antigens.** Because *S. aureus* releases many virulence factors, including hemolysins, proteases, and superantigens, into the extracellular space, especially during post-exponential growth (37), we focused our investigation on serum IgG binding to these antigens. We used 2D-IB to obtain a comprehensive picture of the anti-*S. aureus* antibody profiles against the post-exponential-phase extracellular proteome of *S. aureus* strain 8325-4. Before experimental colonization, we found marked interindividual variation in baseline IgG binding when we probed 2D blots with the precolonization sera from the 16 volunteers. Six representative cases are depicted in Fig. 1, which shows serum IgG binding to bacterial proteins resolved in the pH ranges 6 to 11 (A) and 4 to 7 (B) (all results are given in Fig. S1 in the supplemental material). The strong heterogeneity in IB patterns comprised total IgG binding, spot patterns, and spot intensity.

The total IgG binding, i.e., the sum of spot intensities, differed between individuals up to a factor of 20 in the pH range from 4 to 7 and a factor of 3.6 in the pH range from 6 to 11, where more immune-reactive proteins were present (Fig. 2). *S. aureus* carriers and NC did not differ significantly in total IgG binding, but the numbers are small. Moreover, total IgG binding did not correlate with persistence of strain 8325-4 in the nose following experimental colonization (data not shown).

Individuals differed strongly in their spot patterns and spot intensities (Fig. 1). For example, serum 605 reacted only with a few staphylococcal proteins, especially in the pH range from 4 to 7, while serum 211 contained high titers of IgG against a range of staphylococcal exoproteins, including the immune-dominant staphylococcal protein A (IsaA).

A measure for immune dominance, we identified the 10% of spots that were strongest on all 2D-IBs (cutoff, 5.6 arbitrary units [AU]). In Table 2 we provide an overview of their distribution. The spots (rows) were ranked by their frequencies among the 16 volunteers. Similarly, volunteers were ranked by their numbers of strong spots (columns). At the top of Table 2 are proteins with strong IgG binding in many individuals. These spots represent the pattern of immune dominance or the core immune proteome of *S. aureus* 8325-4. Most immune-reactive proteins could be identified by matching the IBs with the protein reference maps of strain 8325-4 (for annotated reference blots, see Fig. S2 in the supplemental material).

Among the 10% strongest spots that were present in at least 75% (12/16) of all individuals, we found the virulence factors alpha-hemolysin (Hla), cysteine protease (SspB), phospholipase C (Plc), serine protease-like proteins B and E (SplB, SplE), and staphylococcal serine protease (V8 protease; SspA). These virulence factors are highly conserved in the species *S. aureus*.

However, there was also much interindividual variation in spot intensities as well as in spot patterns. IgG binding to the immune-dominant antigens differed between individuals by factors of 2.3 (6-11_68; Plc) to 18.9 (4-7_59; SspA). The spot with the highest intensity in every individual represents Hla (6-11_59), which was also the most abundant spot in the protein 2D-PAGE analysis. In this single case, spot quantification was out of the linear range. Finally, the lower part of Table 2 shows that many immune-reactive spots were present in only a few individuals. Twenty-four of the 52 strongest spots appeared in fewer than half of the volunteers, underlining the impressive interindividual diversity in the adaptive immune response against *S. aureus* in the healthy population.

**Differences in baseline IgG binding between carriers and NC.** The broad interindividual variability in baseline IgG binding is one of the reasons why antibody binding differed between *S. aureus* carriers and NC. In fact, five spots were stronger in carriers (*P* = 0.05 by the Mann-Whitney U test), but none were stronger in NC (Table 3). These spots represent IgG binding to the conserved virulence factors SspA, SspB, and IsaA, which belong to the core immune proteome, as well as to two unidentified proteins. SspA, SspB, and IsaA are represented on 2D gels by several isoforms, all of which show the same tendency, i.e., stronger serum IgG binding in carriers than in NC.

**Impact of experimental colonization on the serum IgG response.** To test whether the observed highly variable anti-*S. aureus* antibody profiles are induced by bacterial colonization, we compared the sera obtained before and 4 weeks after colonization with *S. aureus* 8325-4.

In contrast with the pronounced interindividual differences in baseline antibody patterns, experimental colonization induced only moderate changes (see Fig. S1 in the supplemental material). In total, 19 spots gained intensity by a factor of at least 5 (Table 4). These moderate increases in IgG binding were observed predominantly for weak spots (exceptions are 4-7_8 and 4-7_77), which also hampered spot identification.
FIG. 1. High variability in serum IgG binding to *S. aureus* extracellular proteins in the pH ranges 6 to 11 (A) and 4 to 7 (B). IB for pH 4 to 7 was performed with extracellular proteins from 8325-4 Δspa in order to avoid background due to IgG binding to protein A. *S. aureus* extracellular proteins from cells in the stationary-growth phase were resolved by 2D-PAGE and were probed with precolonization sera from 16 volunteers. Data for six representative individuals are shown.
We observed no commonality in spots with increased IgG binding, i.e., the observed serum IgG responses to experimental colonization were very individual. In line with these findings, we observed no major differences in the serum IgA response, which was analyzed in four representative probands (see Fig. S2 in the supplemental material). In conclusion, human experimental colonization with S. aureus 8325-4 did not elicit a strong humoral immune response.

**DISCUSSION**

When we probed extracellular S. aureus proteins with serum samples from 16 volunteers, we observed a broad spectrum of anti-S. aureus antibodies in every individual. There was pronounced interindividual variability in total IgG binding, spot patterns, and spot intensities. Compared to this strong IgG response, experimental nasal colonization with S. aureus 8325-4, a laboratory strain of low virulence, induced only minor changes in the IgG binding patterns. Similar findings were obtained for IgA.

The study was focused on extracellular proteins, because virulence factors are enriched in this subproteome, and because of our finding of S. aureus strain-specific antibody responses to superantigens (12). The fluorescence-based 2D-IB method chosen for investigation is relatively simple, is highly reproducible, and allows signal quantification. In contrast to (multiplexed) enzyme-linked immunosorbent assay and genomic expression approaches, it permits the simultaneous analysis of the proteome of an individual S. aureus strain and the serum antibody response against it. However, there are limitations: Conformational epitopes may be denatured; non-protein antigens are not resolved; and the assay measures antibody binding but not function. Further studies are needed to assess the antibody response against cell wall-associated antigens and to correlate the observed antibody binding with neutralizing or opsonizing properties. Moreover, due to our study design, we may have missed exclusive throat and intestinal carriers (1, 17).

The high prevalence of a broad spectrum of antistaphylococcal antibodies in healthy adults can be explained by the ubiquity of S. aureus. Carriers are exposed to the same S. aureus strain for a long time and will experience multiple minor invasive episodes from it. Even though NC by definition are not constantly colonized on nasal epithelia, they will transiently contact different S. aureus strains, and most individuals experience minor staphylococcal skin infections. To what degree cross-reactive antibodies elicited by antigens from other bacterial species shaped the observed IgG binding patterns will need to be addressed in the future.

The core immune proteome of S. aureus 8325-4, defined as strong spots that were present in at least 75% of the individuals, consists of the well-known virulence factors Hla, SspB, Plc, StpB, StpE, and SspA. These virulence factors are conserved within the species S. aureus, and they appear to be immune dominant. IgG binding to IsaA, SspA, SspB, and two unidentified proteins was stronger in carriers than in NC. This confirms and extends our earlier observation that S. aureus carriers raise a strong antibody response against the superantigen cocktail produced by their colonizing strain (12). Similarly, Verkaik et al. observed higher IgG titers against TSST-1 and staphylococcal enterotoxin A in persistent carriers than in NC (31). We observed no spots that were more prominent in NC than in carriers.

The most striking finding with our unbiased approach, however, was the high degree of variability in IgG binding to S. aureus exoproteins in healthy adults. This may have been underrated in studies with selected antigens or serum pools. The data clearly show that our previous observation of a highly heterogeneous spectrum of superantigen-neutralizing antibodies in healthy adults was just the tip of the iceberg (11, 12; also unpublished data). Similarly, a broad range of anti-S. aureus antibody titers was reported for some toxins and surface-associated antigens (6, 31).

The high variability in antibody profiles likely reflects the individual history of encounters with S. aureus as well as the genetic diversity of the species. The latter is due to mobile genetic elements, which carry many virulence and resistance genes. Two strains can differ in as much as 20% of their genomes (15). Thus, S. aureus strains are expected to leave different imprints on the adaptive immune system. It is also possible that some antibody specificities were cross-reactive, i.e., induced by non-S. aureus antigens.

The broad and very personal antibody repertoires against
As a measure for immune dominance, we identified the 10% of spots that were strongest (gray squares) on all 2D-IBs (cutoff, 5.6 Au). This table combines the results for the pH ranges of 6 to 11 and 4 to 7. An annotated reference blot with the spot numbers is provided in Fig. S2 in the supplemental material.

IB spots were ranked by their frequencies among the 16 volunteers.

The carrier status is given for each of the 16 volunteers. Volunteers were ranked by the number of strong spots for each.

The number of sera in which the respective IB spot was defined as strong is given, along with the median spot volume (arbitrary units) and interquartile range. n.d., not determined.

n.i., not identified.

A strong 2D-IB spot covered two (or more) closely located protein spots.

| Spot No. | Protein name | Seras with strong spots | No. | Median | Interquartile range |
|----------|--------------|-------------------------|-----|--------|---------------------|
| 6-11_59  | Hla          |                         | 16  | 40.9   | 37.3 - 43.9         |
| 6-11_78  | SplB         |                         | 15  | 31.1   | 27.2 - 33.7         |
| 4-7_43   | SspB         |                         | 15  | 28.0   | 18.7 - 33.5         |
| 6-11_60  | Hla          |                         | 15  | 20.7   | 15.4 - 29.0         |
| 6-11_68  | Ptc          |                         | 15  | 18.9   | 17.1 - 26.1         |
| 6-11_55  | Hla          |                         | 14  | 15.1   | 12.9 - 22.1         |
| 6-11_87  | n.i.*        |                         | 14  | 13.4   | 11.7 - 18.2         |
| 4-7_40   | SspB         |                         | 13  | 18.8   | 14.7 - 22.1         |
| 6-11_85  | SplE         |                         | 13  | 10.5   | 7.1 - 13.4          |
| 6-11_59  | SspA         |                         | 12  | 30.6   | 17.9 - 37.5         |
| 4-7_67   | SspA         |                         | 12  | 26.5   | 19.6 - 37.8         |
| 6-11_30  | Geh          |                         | 11  | 14.2   | 11.6 - 19.3         |
| 4-7_37   | SspB, Pkg, ArgF |                   | 11  | 9.0    | 6.7 - 10.7          |
| 4-7_49   | SspB         |                         | 10  | 8.6    | 6.7 - 11.8          |
| 6-1-31   | Geh          |                         | 9   | 9.6    | 6.9 - 12.4          |
| 6-11_57  | Hlg-C        |                         | 9   | 7.3    | 6.7 - 9.9           |
| 6-11_72  | SsaA-a       |                         | 8   | 12.8   | 7.0 - 15.6          |
| 4-7_69   | SspA         |                         | 8   | 11.5   | 8.3 - 13.2          |
| 4-7_38   | n.i.         |                         | 8   | 9.6    | 8.6 - 10.4          |
| 6-11_49  | Hlb          |                         | 8   | 9.1    | 7.4 - 9.6           |
| 4-7_71   | SspA, DnaK   |                         | 7   | 13.8   | 9.1 - 15.1          |
| 6-11_48  | Hlg-B        |                         | 7   | 13.3   | 9.8 - 15.4          |
| 4-7_8    | 88196599     |                         | 7   | 7.2    | 6.5 - 9.3           |
| 6-11_18  | 8819493      |                         | 7   | 13.8   | 8.4 - 18.6          |
| 6-11_8   | n.i.         |                         | 6   | 10.2   | 8.1 - 11.6          |
| 6-11_51  | Hlb          |                         | 6   | 9.6    | 5.9 - 13.7          |
| 6-11_25  | n.i.         |                         | 6   | 8.0    | 6.4 - 9.4           |
| 4-7_89   | 88194436     |                         | 6   | 7.9    | 6.6 - 9.3           |
| 6-11_46  | 88195914     |                         | 4   | 8.3    | 7.1 - 9.5           |
| 4-7_1    | 88196599     |                         | 4   | 7.9    | 6.6 - 10.5          |
| 6-11_98  | 88193909     |                         | 4   | 7.3    | 6.6 - 10.4          |
| 4-7_86   | IsaA         |                         | 3   | 11.0   | 9.0 - 29.6          |
| 6-11_20  | 88194493     |                         | 3   | 8.0    | 7.2 - 9.9           |
| 6-11_82  | SplA         |                         | 2   | 12.0   | 10.9 - 13.1         |
| 4-7_9    | 88196599     |                         | 2   | 8.4    | 7.3 - 9.5           |
| 6-11_69  | n.i.         |                         | 2   | 7.2    | 7.1 - 7.3           |
| 4-7_58   | 88196599     |                         | 2   | 6.6    | 6.2 - 7.0           |
| 6-11_119 | IsaA         |                         | 1   | 39.5   | n.d.                |
| 4-7_117 | n.i.         |                         | 1   | 21.6   | n.d.                |
| 4-7_110 | SsaA         |                         | 1   | 10.7   | n.d.                |
| 6-11_64 | Hla          |                         | 1   | 9.2    | n.d.                |
| 6-11_107 | 88193909     |                         | 1   | 9.0    | n.d.                |
| 4-7_107 | IsaA         |                         | 1   | 8.7    | n.d.                |
| 4-7_91  | IsaA         |                         | 1   | 7.7    | n.d.                |
| 6-11_2  | Geh          |                         | 1   | 7.6    | n.d.                |
| 6-11_58 | 88194062     |                         | 1   | 7.2    | n.d.                |
| 6-11_81 | Hla          |                         | 1   | 7.1    | n.d.                |
| 6-11_61 | Hla          |                         | 1   | 6.9    | n.d.                |
| 6-11_12 | n.i.         |                         | 1   | 6.7    | n.d.                |
| 4-7_118 | IsaA         |                         | 1   | 6.1    | n.d.                |
| 6-11_22 | Lip          |                         | 1   | 6.1    | n.d.                |
| 6-11_110 | 88193909     |                         | 1   | 5.9    | n.d.                |

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A strong 2D-IB spot covered two (or more) closely located protein spots.
TABLE 3. Differences in baseline IgG binding between carriers and NC

\[
\begin{array}{|c|c|c|c|c|}
\hline
\text{Spot label} & \text{Protein}^a & \text{Carriers (PC + IC)} & \text{NC} & \text{P}^b \\
\hline
4-7_101 & NI & 0.7 (0.4–1.3) & 0.3 (0.2–0.4) & 0.027 \\
4-7_119 & IsaA_6, IsaA_7, IsaA_8 & 1.4 (1.1–1.9) & 0.5 (0.4–0.7) & 0.027 \\
4-7_49 & SspB_2 & 8.6 (6.2–12.9) & 4.3 (2.6–6.6) & 0.036 \\
4-7_69 & SspA_1, SspA_2 & 9.6 (5.6–12.6) & 2.7 (0.8–5.5) & 0.046 \\
4-7_70 & NI & 0.1 (0.1–0.2) & 0.1 (0.1–0.1) & 0.046 \\
\hline
\end{array}
\]

\(a\) Only proteins that differed significantly in IgG binding between carriers and NC are listed. NI, not identified.

\(b\) By the Mann-Whitney U test.

TABLE 4. Spots with more than fivefold increases after experimental colonization

| Spot label\(^a\) | Protein | Volunteer no. | Carriage status | Median fold increase in spot intensity (minimum–maximum)\(^b\) | Spot intensity\(^c\) |
|-----------------|---------|---------------|----------------|------------------------------------------------|------------------|
| 4-7_34          | NI\(^d\) | 165           | NC             | 11.5 (4.2–18.7)                                      | 0.1              |
| 4-7_31          | NI      | 165           | NC             | 7.7 (4.7–10.7)                                      | 0.1              |
| 4-7_5           | 88196599_1 | 165   | NC             | 6.49 (3.4–9.3)                                      | 0.1              |
| 6-11_71         | NI      | 165           | NC             | 5.9 (2.1–11.0)                                      | 0.3              |
| 6-11_15         | NI      | 604           | NC             | 8.4 (7.2–9.6)                                      | 0.1              |
| 6-11_42         | NI      | 604           | NC             | 7.4 (3.2–11.6)                                      | 0.3              |
| 4-7_8           | 88196599_2 | 608   | NC             | 12.0 (8.1–15.8)                                     | 4.0              |
| 4-7_9           | 88196599_10 | 608  | NC             | 8.6 (3.3–13.8)                                      | 2.3              |
| 4-7_61          | NI      | 608           | NC             | 6.6 (2.6–10.5)                                      | 0.1              |
| 6-11_21         | NI      | 601           | PC             | 7.1 (3.7–11.6)                                      | 0.9              |
| 6-11_113        | NI      | 601           | PC             | 2.7 (2.6–17.9)                                      | 0.3              |
| 4-7_77          | NI      | 603           | PC             | 5.6 (5.6–5.7)                                      | 2.6              |
| 4-7_115         | SsaA_b_1 | 606           | PC             | 24.8 (19.4–30.2)                                     | 0.3              |
| 4-7_3           | 88196599_8 | 606   | PC             | 9.7 (2.5–16.9)                                      | 0.7              |
| 4-7_23          | PdhD_1, PdhD_2, PdhD_3 | 606 | PC             | 7.4 (5.0–9.9)                                      | 0.1              |
| 4-7_5           | 88196599_1 | 606   | PC             | 5.9 (4.3–7.4)                                      | 0.2              |
| 4-7_88          | NI      | 606           | PC             | 5.3 (4.8–5.7)                                      | 0.1              |
| 6-11_6          | Pbp3    | 607           | PC             | 18.6 (13.6–23.6)                                    | 0.2              |
| 6-11_7          | Geh_13  | 607           | PC             | 15.4 (14.9–16.0)                                    | 0.2              |

\(a\) An annotated reference blot with the spot numbers is provided in Fig. S2 in the supplemental material.

\(b\) The ratio of the spot intensity in the postcolonization serum to that in the precolonization serum was determined for each volunteer and for each technical replicate separately. A spot increase was defined as (i) a \(\geq\)3-fold increase in the mean ratio, (ii) a \(\geq\)2.5-fold increase in at least two technical replicates, and (iii) a difference in spot intensity between the postcolonization and precolonization sera exceeding twofold the standard deviation of the technical replicates. The median, minimum, and maximum of the ratios from two to four technical replicates are given.

\(c\) Median spot intensity (in arbitrary units) of technical replicates of IBs developed with postcolonization sera.

\(d\) NI, not identified.
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