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Rapid Generation of Rotavirus-Specific Human Monoclonal Antibodies from Small-Intestinal Mucosa

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The gut mucosal surface is efficiently protected by Abs, and this site represents one of the richest compartments of Ab-secreting cells in the body. A simple and effective method to generate Ag-specific human monoclonal Abs (hmAbs) from such cells is lacking. In this paper, we describe a method to generate hmAbs from single Ag-specific IgA- or IgM-secreting cells of the intestinal mucosa. We found that CD138-positive plasma cells from the duodenum expressed surface IgA or IgM. Using eGFP-labeled virus-like particles, we harnessed the surface Ig expression to detect rotavirus-specific plasma cells at low frequency (0.03–0.35%) in 9 of 10 adult subjects. Single cells were isolated by FACS, and as they were viable, further testing of secreted Abs by ELISPOT and ELISA indicated a highly specific selection procedure. Ab genes from single cells of three donors were cloned, sequenced, and expressed as recombinant hmAbs. Of 26 cloned H chain Ab genes, 22 were IgA and 4 were IgM. The genes were highly mutated, and there was an overrepresentation of the VH4 family. Of 10 expressed hmAbs, 8 were rotavirus-reactive (6 with Kd < 1 × 10−9M). Importantly, our method allows generation of hmAbs from cells implicated in the protection of mucosal surfaces, and it can potentially be used in passive vaccination efforts and for discovery of epitopes directly relevant to human immunity. The Journal of Immunology, 2010, 185: 5377–5383.

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uman mAbs (hmAbs) are versatile reagents used for many purposes, including studies of immunological diseases and disease therapies. Use of hmAbs in passive vaccination to fight infectious disease is becoming increasingly important, especially for conditions in which vaccines are not available (1). Efforts have therefore been undertaken to establish techniques for the production of Ag-specific fully human mAbs. The most used methods established so far include the generation of EBV-immortalized B cell clones (2), single-cell PCR of Ab genes from B cells (3, 4), phage display libraries (5), and recently the genetic reprogramming of memory B cells to become Ab-secreting cells (ASCs) (6). Single-cell PCR followed by cloning of variable region genes and expression in eukaryotic cells represents a powerful tool to produce hmAbs. The advantage of this method is that Abs can be retrieved from any type of B cell, including naïve cells, memory cells (7), and ASCs (8). For passive immunization the best candidates for mAb production should be ASCs found at mucosal sites where microorganisms are encountered and fought. Not surprisingly, these sites represent the richest compartments in the body in terms of the number of ASCs (9). The predominant mucosal Abs are secretary (s) IgA and slgM (10); production of Abs reactive to commensal intestinal microbes (11), food proteins (11, 12), and self proteins (13) have been reported at this site. Notably, Ab responses generated at the mucosal or at the systemic level may differ, whereas in some cases protective effects are displayed exclusively by the former (14). Moreover, certain B cells can be found only in the effector organ and not in the circulation. This is the case for some T cell-independent responses (15) and some early immune responses from short-lived plasma cells resulting from extrafollicular development (16), which may not generate a detectable memory compartment. Isolation of ASCs with desired reactivity from the intestinal mucosa is, therefore, important both to isolate unique Ab reagents and therapeutics and to further our burgeoning understanding of mucosal immunity. The exploitation of the gut mucosal tissues as a source of Abs, however, has been hampered by the lack of a method that allows coupling the dissection of the specificity of the large diversity of ASCs with the isolation of those having desired reactivity. In this study, we present and validate a simple but powerful and accurate method to clone hmAbs with desired reactivity from plasma cells of the small intestine. The method builds on the unexpected observation of surface expression of Igs, which we harnessed to identify single Ag-specific plasma cells by flow cytometry. Importantly, the approach described in this paper overcomes the need for high frequency of specific cells.

Materials and Methods

**Biopsy samples**

Small-intestinal biopsies were obtained by esophagogastroduodenoscopy and forceps sampling from the duodenum of adult donors (six women, four...
ments; mean age 48 y, range 20–68 y) undergoing routine examination for different reasons not related to rotavirus infection. Of these, five had normal duodenum (reasons for biopsy sampling were as follows: one patient with chronic gastritis, two with reflux esophagitis, one with possible gluten intolerance later not confirmed, and one treated celiac disease patient with total recovery upon institution of a gluten-free diet), and five had some degree of villous atrophy (four of them as a consequence of partially treated or untreated celiac disease). All patients gave written informed consent before the gastroendoscopy. Ethical approval was obtained from the Regional Ethics Committee in South-Eastern Norway (Application S-97/20).

Preparation of single-cell suspensions

For preparation of cell suspensions from biopsies, we adapted protocols previously published (17, 18). Briefly, two to four biopsies were sampled in RPMI 1640 on ice and immediately rinsed in PBS. Epithelial cells and intraepithelial lymphocytes were separated by two incubations at 37°C, for 30 min, under continuous rotation in PBS added with 2 mM EDTA and 2% FCS, and discarded. The remaining material containing lamina propria lymphocytes was then treated with 1 mg/ml Blend Collagenase (Sigma-Aldrich, St. Louis, MO) in 3% FCS in PBS, at 37°C for 30 min. The cell suspension thus obtained was filtered through a 40-μm cell strainer and washed with PBS. A single-process biopsy yielded between 0.2 and 0.5 million viable cells. Between 0.4 and 2 million cells were therefore obtained from each donor.

Source and preparation of viral particles

Virus-like particles (VLPs) were produced by coinfection of Spodoptera frugiperda 9 insect cells at a multiplicity of infection of 5 PFUs/cell, with a recombinant baculovirus expressing the VP6 and another expressing eGFP–VP2 fusion protein. VLPs were purified as described previously, by double CsCl density gradient centrifugation (19). Rotavirus double-layered particles (DLPs) were purified by CsCl density gradient centrifugation from the cell culture supernatant of MA104 cells infected with the bovine rotavirus strain RF as described (19). As a control protein expressed in a baculovirus system, human recombinant transglutaminase 2 (TG2) was obtained from Phadia (Freiburg, Germany).

Flow cytometry

The following Abs and concentrations were used: anti–CD19-FITC 1:20 (BD Biosciences, San Jose, CA), anti–CD54-PacificBlue 1:25 (BD Biosciences, San Diego, CA), anti–CD138-PE 1:20 (eBioscience), anti–CD27–APC 1:20 (BD Biosciences), anti–CD41–PerCP 1:40 (BD Biosciences) (used as exclusion channel), anti–CD19–PE-Cy7 1:25 (eBioscience), anti–HLA DR–PeCy7 (eBioscience), anti–Ki–67–PE 1:20 (BD Biosciences), anti–IgA, -IgG, or -IgM FITC 1:1500, 1:2000, 1:1500, respectively (Southern Biotechnology Associates, Birmingham, AL); isotype controls from BD Biosciences. Purified eGFP (BD Clontech, Palo Alto, CA) and eGFP–VLPs were used at 0.05 μg/ml, in a final volume of 250 μl. In short, cells were resuspended in a solution of labeled Abs diluted in PBS added with 3% FCS, and incubated for 45 min in ice. After incubation, cells were briefly washed, resuspended in PBS–3% FCS added with 0.5 μg/ml proteidium iodide, and immediately analyzed on either a FACScalibur, FACSAria, or LSRII (BD Biosciences) instrument. Intracellular staining was performed as follows: Cells were stained for surface markers, washed twice, fixed for 30 min in 1% PFA, permeabilized with 0.2% saponin in PBS, stained with anti–Ki–67, washed, and analyzed.

Sorting and in vitro culture

A total of 100–250 biopsies were bulk sorted on a FACSaria instrument into 4–ml sterile tubes containing 3 ml RPMI (Life Technologies, Rockville, MD) added with 10% FCS. Tubes were centrifuged to remove sheath fluid; then cells were resuspended in fresh medium, split in the desired number of wells, and immediately after the limiting dilution of cells, one aliquot for each Ag for ELISPOT experiments and one aliquot for culturing were prepared, and finally added to either ELISPOT plates or to 96-well plates, where feeder cells (50,000 PBMCs isolated from blood of healthy donors according to standard procedures, and irradiated at 25 Gy) were preseeded. Irradiated feeder cells did not secrete detectable amounts of Abs and increased the production by plasma cells, but were not strictly necessary. Cells were cultured at 37°C, in 5% CO2, for 1 wk before being screened in ELISA. Single small biopsies were kept in culture under the same conditions (without addition of feeder cells), and the supernatant was screened after 1 wk.

ELISA

ELISA plates (96-well; Nunc, Naperville, IL) were coated overnight with either unconjugated anti-IgA (goat polyclonal; Sigma-Aldrich), TG2, rotavirus DLPs, rotavirus eGFP–VLPs, or reGFP at 5 μg/ml in PBS, overnight at 4°C. Wells were washed and blocked with 2% skimmed milk in PBS (MPBS), for 1 h at room temperature. Supernatants from cell cultures or purified hmAbs were diluted in MPBS, added to the wells, and incubated for 2 h at 37°C. Wells were extensively washed, a secondary alkaline phosphatase (AP)-conjugated anti-IgA or anti-IgG Ab (goat polyclonal; Sigma-Aldrich) diluted 1:2000 in MPBS was added, and the plate was incubated for 1 h at 37°C. After washing, immune complexes were revealed with phosphatase substrate (Sigma-Aldrich) and the plates read at 405 nm. Ab avidities were calculated by nonlinear regression (GraphPad, San Diego, CA) of ELISA curves plotted from four 3-fold dilutions of Ab starting from 3.3 × 10−8 M. For hmAbs with low avidity, experiments were repeated with dilutions starting from 10−5 M (not shown).

ELISPOT

ELISPOT was performed on MultiScreen HPS membrane plates (Millipore, Billerica, MA). The membrane was activated by adding 15 μl 35% ethanol in each well, followed by 1-min incubation. Ethanol was discarded and the plate rinsed with water. Ads (anti-IgA, anti-IgM, VLPs, or DLPs) were added at 2–10 μl/well RPMI-10%FCS, 2 h at 37°C. Cells were added to the plate and incubated for 16 h at 37°C, 5% CO2. Cells were then discarded and the plate washed six times with PBS-0.01% Tween 20 and three times with PBS. AP-conjugated secondary Ab (anti-IgA or anti-IgM, 1:2500; Sigma-Aldrich) and substrate (NBT/5-brom o-4-chloro-3-indolyl phosphate substrate and incubating for 10 min at room temperature). After extensive washing with PBS Tween 20 and PBS, detection of spots was performed by adding 100 μl/well NBT-5-bromo-4-chloro-3-indolyl phosphate substrate and incubating for 1 h at room temperature. The plate was then rinsed and dried overnight and read by a Carl Zeiss (Oberkochen, Germany) Axiosplan 2 microscope and Vision ELISPOT software.

Single-cell PCR and expression of recombinant hmAbs

PCR amplification of paired H chain variable (VH) and L chain variable (VL) Ab genes from single cells, cloning into expression vector, and production of IgG recombinant hmAbs in 293 cells was performed as described before (4). Briefly, each single cell was sorted in a well of 96-well plate containing 10 μl RNase-inhibiting cocktail buffer (10 mM Tris pH 8 and 0.25 μl RNasin (Promega, Madison, WI)). For the RT and nested PCR reactions, IgA–RT–PCR (5′-CCTGCTCAGGGTAGTTT-3′) and IgM–nested (5′-CAGAGGGTGGCAGACC-3′) primers and IgG1 (GAGAATC TTTTACGGGCTGG-3′) primers and IgG1, and IgG, and κ antisense primers previously published (4). Sense primers, annealing to the Ab leader sequences in the RT–PCR and to the first framework of the V region in the nested PCR, allow amplification of the great majority of V region genes and were previously published (4). The Ab V region genes were cloned in the AbVec-ilgG1 and AbVec-ilgKappa vectors previously described (4) and deposited in GenBank under the accession numbers FJ475055 and FJ475056, respectively (www.ncbi.nlm.nih.gov/genbank/).

SDS-PAGE and Western blot

SDS-PAGE and Western blotting were performed according to standard procedures. Briefly, VLPs were resuspended in Læmmli loading buffer with 2-mercaptoethanol as reducing agent. Samples were then either boiled for 10 min or heated at 65°C prior to separation on a 12% acrylamide gel. Next, 1 μg (for Western blotting) or 2 μg (for Coomassie blue staining) VLPs were loaded in each well. The gel was either stained with Coomassie blue or blotted on nitrocellulose membrane. For detection of antigenic proteins, the membrane was blocked with MPBS for 1 h at room temperature, incubated with each mAb at 0.5 μg/ml overnight at 4°C, then washed and incubated with AP-conjugated anti-human IgG (goat polyclonal; Sigma-Aldrich) diluted 1:2000 for 1 h at room temperature. Immunocomplexes were detected by adding NBT/5-bromo-4-chloro-3-indolyl phosphate solution.

Results

Phenotypic characterization of ASCs in the gut

Single-cell suspensions from duodenal biopsies of adult donors were prepared using a collagenase-based protocol. Flow cytometry
analysis of FSC and SSC parameters showed a heterogeneous population according to size (Fig. 1A). Typical B cell (CD19) and plasma cell (CD138, syndecan-1) markers revealed the presence of three distinct viable B cell populations (upper right panel). The cell suspensions were abundant in CD19<sup>+</sup> CD138<sup>-</sup> cells and CD19low CD138<sup>-</sup> cells, with large size (lower panels) suggesting an ASC phenotype; a CD19<sup>+</sup> CD138<sup>-</sup> population with smaller size (lower right panel), likely representing memory cells, was also identified. Most of the large CD138<sup>-</sup> cells (95%) expressed CD27, whereas a significant percentage of CD19low CD138<sup>-</sup> cells (26%) were CD45<sup>-</sup> CD27<sup>+</sup>, possibly representing the final stage of ASC differentiation (Fig. 1B). A small percentage of CD138<sup>-</sup> cells showed a CD138<sup>-</sup>, CD19<sup>-</sup>, CD45<sup>-</sup>, CD27<sup>-</sup> phenotype; these cells likely represented contaminating epithelial cells, most of which were efficiently removed in the first step of the single-cell suspension preparation procedure (see Materials and Methods). The proportion of ASCs in 10 analyzed samples averaged 3.1% (range 0.7–8.3%). Absent or low expression of HLA-DR and the proliferation marker Ki-67 (Fig. 1C) indicated that these cells belong to the plasma cell rather than to the plasmablast compartment (20, 21).

**FIGURE 1.** A. Forward and side scatter plot of single-cell suspensions from duodenal biopsy (upper left panel); expression of the B cell markers CD19 and CD138 (upper right panel; dead cells excluded by FSC/SSC gating and propidium iodide staining); forward and side scatter plots are reported in the lower panels for each identified B cell population. B and C, Phenotypic analysis based on expression of CD45 and CD27 (in B), HLA-DR and intracellular Ki-67 (in C) on the CD138<sup>-</sup> populations described in A.

**FIGURE 2.** A, Expression of surface Igs of the IgA, IgM, IgG isotypes (left, center, and right panels, respectively) on gut plasma cells (gated on large, viable, CD138<sup>+</sup>, CD27<sup>+</sup> cells; dead cells were excluded by FSC/SSC gating and propidium iodide staining). B, ELISPOT detection of Abs produced by IgA<sup>+</sup>, IgA<sup>-</sup>, IgM<sup>+</sup> and IgM<sup>-</sup> populations (viable, large, CD138<sup>+</sup>, CD27<sup>+</sup>) sorted in equal numbers according to surface Ig staining.

**FIGURE 3.** A, ELISPOT detection of IgA and IgM plasma cells producing anti-VLP Abs. Plasma cells were sorted according to large size and CD138 expression prior to seeding into wells. B, Representative plots showing detection by flow cytometry of VLP-specific plasma cells using eGFP-VLPs (upper plots) in comparison with eGFP only (lower plot). C, Frequency of VLP-reactive plasma cells as detected in samples from 10 different adult donors. Gray dots indicate three donors from which hmAbs were cloned (see below).
amounts of surface Igs, mostly IgA (left panel, 85%) and IgM (center panel, 13%). Only a few scattered IgG-positive events were found. IgA⁺, IgA⁻, IgM⁺, and IgM⁻ cells were sorted in equal numbers, and the production of Abs was assayed by ELISPOT (Fig. 2B). The ELISPOT readings indicated production of IgA in the IgA⁺ or IgM⁻ sorted populations, and similarly IgM production in the IgA⁻ and IgM⁺ sorted populations. Only a few spots were detected in the IgA⁻ and IgM⁻ sorted population, confirming that plasma cells not displaying surface Ig were present, but not frequent.

Selection of Ag-specific plasma cells

The presence of surface Igs on plasma cells—combined with the ability of sorted cells to produce Abs in vitro, hence allowing confirmation of the specificity of the staining—could provide a powerful system for isolating B cells with reactivity against an Ag of interest. To explore this, we generated fluorescent VLPs expressing the rotavirus VP2 (in fusion with eGFP) and VP6 proteins (19). Rotavirus VP2 and VP6 are highly immunogenic, and Abs, in particular, sIgA, toward these proteins are present in infected children (23–25). By ELISPOT we identified a small population (0.27% in the example reported in Fig. 3A) of intestinal plasma cells producing anti-VLP Abs. The fusion of eGFP with VP2 allowed us to identify these cells by flow cytometry. Whereas a small, but clear, VLP-reactive population was consistently detected, staining with eGFP alone did not detect any events (Fig. 3B), suggesting that the reactivity is specific toward the VP2 or VP6 proteins. In 9 of 10 donors, we detected VP2/VP6-specific plasma cells (0.18% on average; range 0.03–0.35%; Fig. 3C), whereas in one donor we did not detect any cells. The specificity of the staining was further assessed by analysis of Abs secreted by sorted plasma cells. Cells stained and not stained by the VLPs (VLP⁺ and VLP⁻, respectively) were sorted and seeded into wells. Cells were cultured for 1 wk, and both populations produced detectable amounts of IgA Abs (Fig. 4A). VLP-specific Abs were

Expression of surface Igs on gut plasma cells

Plasma cells are generally considered not to express surface Igs (22). However, we observed that this is not the case for most plasma cells of the small intestine, as depicted in Fig. 2A. Independently of CD19 expression, these cells expressed detectable amounts of surface Igs, mostly IgA (left panel, 85%) and IgM (center panel, 13%). Only a few scattered IgG-positive events were found. IgA⁺, IgA⁻, IgM⁺, and IgM⁻ cells were sorted in equal numbers, and the production of Abs was assayed by ELISPOT (Fig. 2B). The ELISPOT readings indicated production of IgA in the IgA⁺ or IgM⁻ sorted populations, and similarly IgM production in the IgA⁻ and IgM⁺ sorted populations. Only a few spots were detected in the IgA⁻ and IgM⁻ sorted population, confirming that plasma cells not displaying surface Ig were present, but not frequent.

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detected for the VLP\(^+\) sorted cells, but not for the VLP\(^-\) sorted cells. The enrichment for VLP-specific Abs was evident by their comparison with total IgA produced by the sorted VLP\(^+\) cells versus a whole cultured biopsy. Secreted IgA did not recognize eGFP, indicating recognition of VP2 or VP6 and speaking against polyreactivity. The efficacy of the sorting was further validated by ELISPOUT with VLPs and rotavirus-like DLPs as a source of native rotavirus Ag (26). The rationale for choosing these particles was to demonstrate specificity by analyzing binding to a different source of Ag. Rotavirus DLPs are mainly made of VP2 and VP6 and are purified from mammalian cells; hence they do not present as potential contaminants as VLPs purified from insect cells. A comparable number of spots were detected for total IgA, the similar potential contaminants as VLPs purified from insect cells. A comparable number of spots were detected for total IgA, the same potential contaminants as VLPs purified from insect cells; hence they do not present as a source of Ag. Rotavirus DLPs are mainly made of VP2 and VP6.

Cloning and expression of anti-rotavirus hmAbs

We next sorted single VLP-reactive cells from three donors and amplified the genes encoding for the variable regions of the Abs. The single-cell PCR technique maintains the correct pairing between H and L chains, thus resulting in the production of authentic, fully human mAbs. We retrieved and sequenced the VH region gene from 26 cells (Table I). Of these, 22 were from IgA and 4 from IgM. In the H chain repertoire, the VH4 family, and in particular the VH4-39 gene, was overrepresented (18/26 VH4; of these, 12/18 VH4-39). A \(\kappa\) VL region gene was obtained from 10 of these cells. As a proof of principle, we produced and purified the 10 hmAbs for which we had both the VH and the VL genes. These were cloned into expression vectors, and hmAbs were produced as human IgG1 in 293 cells. We assayed the hmAbs in ELISA for reactivity to the rotavirus VLPs and DLPs and, as a negative control, to the enzyme TG2. An anti-TG2 hmAb from a different study (R. Di Niro, L. Mesin, N.-Y. Zheng, J. Stamnaes, M. Morrissey, J.-H. Lee, M. Huang, K.E.A. Lundin, P.C. Wilson, and L.M. Solidor, submitted for publication) produced in the same system was included as a control. As shown in Fig. 5A, 8 of 10 hmAbs recognized the DLPs, whereas none was reactive to the control protein. Avidity, as estimated by ELISA, was very high for six Abs (\(\sim 10^{-16}\)), intermediate for one (\(\sim 10^{-9}\)), and low for the eighth (\(\sim 10^{-7}\)) (Table I). Interestingly, in the only two hmAbs found to be clonally related (2B04 and 2G01, with some CDRs and some shared somatic mutations), the avidity increased by two logs in 2G01 as a consequence of accumulation of somatic mutations in 2G01. Preliminary competitive ELISA experiments suggested recognition of at least two epitopes, of which one was recognized by the majority of hmAbs (data not shown). Given the skewing for VH4-39 gene usage, we tested in ELISA two control Abs carrying a VH4-39 H chain, obtained in previous studies, and did not detect any reactivity to VLP and DLP (data not shown).

Thus, although there is clear selection for cells using VH4-39, use of this gene alone does not account for the specific binding. To rule out the possibility that the procedure used influenced the observed VH usage by preferential amplification of VH4 genes, we amplified Ab variable regions from single intestinal plasma cells sorted without Ag selection from two different donors. Of 64 VH sequences obtained, only 7 belonged to the VH4 gene family (11%), 2 of those being VH4-39 (3%), demonstrating that the VH4 overrepresentation observed in the rotavirus-specific plasma cells (18/26, 69%) was not influenced by the procedure used. An identical pattern of recognition was observed in Western blot experiments by all the reactive Abs (with the exception of 2F02, likely under the limit of detection due to low affinity; results from a representative Ab, 2G01, are reported in Fig. 5B). The rotavirus proteins were not recognized when the VLPs were boiled and reduced, whereas reactivity was seen with particles that were not boiled prior to SDS-PAGE separation. In this latter case, detection of a band of 120 kDa strongly indicated recognition of VP6 trimers, as previously shown in other studies (27, 28).

Discussion

Mucosal tissues in general and the gut in particular represent vulnerable sites for entry of microbes. The gut surface is covered by a single layer of epithelium, and immune protection is essential. Abs, through a variety of mechanisms, both within the tissue and in the gut lumen, are players in the defense against pathogens. This is underscored by the fact that the gut is the richest compartment of ASCs in the human body. These cells represent an abundant source of Abs with therapeutic potential. How, then, to isolate the Abs of these cells? Cloning hmAbs from ASCs has so far mostly been hampered by difficulties in coupling analysis of the Ab reactivity to the ability to track the viable producing cells. This has been shown to be feasible using only technically challenging methodologies (27–29). Two of the most efficient ways to produce hmAbs, via EBV transformation (2) and genetic reprogramming (6), are specific for memory cells and not for ASCs.

Recombinant expression of hmAbs after recovery of Ab V region genes by single-cell PCR has instead been successful for both memory B cells (7) and ASCs (8). A relevant difference is that memory B cells carry surface Igs. This allows selection for specificity, using the Ag of interest in soluble and labeled form in flow cytometry. This method has been successful in the dissection of
memory responses to, among others, HIV gp140 (29), tetanus toxoid (30), and rotavirus (31, 32). Most ASCs appear to down-regulate their expression of surface IgGs (22). This downregulation hinders selection of specific cells, and the cloning by single-cell PCR of Ab genes from ASCs requires the ability to identify the peak of the immune response, so that the frequency of specific cells is high enough to give a good chance of isolating clones with the desired reactivity. Even if this inconvenience can be bypassed by intracellular staining with the Ag to detect cells producing specific Abs, there is a major drawback: Functional confirmation of the specificity of the staining (i.e., by ELISA or ELISPOT) cannot be performed, as it requires viable cells. We observed that in single-cell suspension of duodenal biopsies most large CD138+ CD27+ cells with a plasma cell-like phenotype (20, 21) had surface IgA or IgM. This notion is supported by Farstad and coworkers (17), who reported that many, but not all, large CD38+ CD20− cells of jejunal resection specimens expressed surface Igs. We harnessed the surface Ig expression to rapidly produce hmAbs reactive to rotavirus from adult individuals, giving a proof of principle that this method can be used to derive hmAbs directed against infectious agents.

Rotavirus infection is a major cause of child mortality in developing countries and hospitalization following severe diarrhea worldwide. Abs, in particular sIgA of the gut, are important for human immunity to rotavirus (33, 34). In mice in which both systemic and intestinal IgA responses were generated, only the latter provided protection (14). Further, passive administration of Abs prevents rotavirus infection in both calves and humans (35, 36). Rotavirus is made of six structural proteins; of these, VP6 forms the intermediate layer and represents 51% of the virion by mass. Interestingly VP6 is antigenically conserved among most circulating group A rotavirus strains and could, therefore, provide heterotypic protection (37). Anti-VP6 IgA Abs are generally not neutralizing in vitro but provide protection through an expulsion mechanism thought to take place inside infected epithelial cells (38, 39). VP6-specific neutralizing Abs have been generated in llama (28).

We used the broadly characterized rotavirus-like particles made of VP2 and VP6 and engineered with eGFP (19) as bait to detect specific plasma cells. These cells were detected at low frequency by ELISPOT. eGFP fluorescence was exploited by setting up a flow cytometry assay to identify cells expressing surface Igs reactive to the viral particles. We detected them in 9 of 10 donors at a low frequency (0.03–0.35%), consistent with the ELISPOT data, and, importantly, did not observe reactivity to eGFP alone, suggesting specificity to rotavirus VP6 or, less likely, VP2. The membrane expression of IgA and IgM allowed us to isolate viable Ag-specific cells. Because the cells were viable, we could undertake simple preliminary experiments to assess the specificity of the isolation. This is highly desirable before embarking on the setting up of techniques such as single-cell PCR and rAb production. ELISA and ELISPOT are suitable for confirming the specificity of the staining, and we performed both. The advantage of the ELISA method is that Abs accumulate in the medium over several days, and the supernatant can be used in a number of preliminary experiments. ELISPOT is more suitable for confirmation of the specificity when the number of cells is low, and to estimate the frequency of cells of interest among the isolated ones. Further analysis, such as assessment of neutralizing capacity, would be feasible at this stage of the procedure. It is conceivable that, by providing the proper environment, even single plasma cells may be kept in culture for some time, leading to production of Abs in amounts sufficient for simple assays. Experiments along these lines indicated to us that the likelihood of cloning specific hmAbs following this approach was high. By means of single-cell PCR and expression of recombinant hmAbs in 293 cells, we generated eight Abs specific for rotavirus from three donors, with a selection efficiency of 80%. This finding demonstrated that the majority of sorted cells was specific, supporting the notion that the frequency measured by flow cytometry reflects the true proportion of VLP-specific cells in the plasma cell compartment. Six Abs recognized their target with very high avidity (Kd < 10−10). It would be interesting to analyze whether a direct correlation exists between Ab avidity and effectiveness in functional assays, as in neutralization ones; this, however, goes beyond the scope of the present work. Although limited in terms of numbers, the repertoire interestingly showed signs of skewing toward the VH4 H chain gene family, thus not reflecting the prevalence observed in memory cells isolated by the same VLPs in the circulation (40, 41). This finding underscores that the circulating compartment and the effector mucosal site may have different characteristics, with the latter possibly being more appropriate for the isolation of relevant hmAbs.

Our protocol is currently optimized for rapid production of rIgG. This is useful for high-throughput screening of several clones, as well as characterization of the fine specificity and properties of selected ones. It is noteworthy that upon identification of Abs with desired properties, the Ab molecule can be engineered so that it is produced in alternative desired formats, for instance, sIgA (42). sIgA is chiefly responsible for protective immunity at mucosal surfaces and is likely to be optimal for passive vaccination by nasal or oral administration (43).

Essentially, we describe a procedure to isolate Ag-specific hmAbs against potentially any pathogens recognized by Abs at mucosal sites, opening new, exciting possibilities for the production of drugs for passive vaccination, for epitope discovery, and for characterizing mucosal immune responses. When compared with other approaches, this method is simpler, as it does not require challenging technical procedures (27–29); rapid, as it can be carried out in as little as 3 wk (4); and efficient, as the majority of isolated hmAbs were specific, whereas cloning from randomly sorted plasma cells would have required the production and screening of thousands of Abs. Abs taken from the mucosal compartment of subjects who have signs of protective immunity, particularly individuals who are recently infected and asymptomatic or noninfected but seropositive, would bear particular promise as a source of therapeutic agents.

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Disclosures
A patent application covering the methodology described in the article has been submitted by R.D., P.C.W., and L.M.S. All other authors have no financial conflicts of interest.

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