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Human Male Genital Tract Secretions: Both Mucosal and Systemic Immune Compartments Contribute to the Humoral Immunity[1]

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In contrast to numerous studies of female genital tract secretions, the molecular properties of Abs and the magnitude of humoral responses in human male genital tract secretions to naturally occurring Ags and to mucosal and systemic immunizations have not been extensively investigated. Therefore, seminal plasma (SP) collected from healthy individuals was analyzed with respect to Ig levels, their isotypes, molecular forms of IgA, and for the presence of Abs to naturally occurring Ags, or induced by systemic or mucosal immunizations with viral and bacterial vaccines. The results indicated that in SP, IgG and not IgA, is the dominant Ig isotype, and that IgM is present at low levels. IgA is represented by secretory IgA, polymeric IgA, and monomeric IgA. In contrast to the female genital tract secretions in which IgA2 occurs in slight excess, the distribution of IgA subclasses in SP resembles that in plasma with a pronounced preponderance of IgA1. The IgG subclass profiles in SP are also similar to those in serum. Thus, SP is an external secretion that shares common features with both typical external secretions and plasma. Specifically, SP contains naturally occurring secretory IgA Abs to environmental Ags of microbial origin and to an orally administered bacterial vaccine, and plasma-derived IgG Abs to systemically injected vaccines. Therefore, both mucosal and systemic immunization with various types of Ags can induce humoral responses in SP. These findings should be considered in immunization strategies to induce humoral responses against sexually transmitted infections, including HIV-1. ***The Journal of Immunology,*** **2005, 175: 4127–4136.**

Mucosal surfaces of the female and male genital tracts are the entry sites of sexually transmitted diseases of viral, bacterial, fungal, and parasitic origin with over 300 million cases estimated to occur annually (1). HIV-1 is no exception: worldwide, over 80% of infections are contracted through heterosexual intercourse (2). Studies performed in various species have convincingly demonstrated that the mucosal and systemic compartments of the immune system display a significant degree of independence (3). In typical external secretions such as milk, saliva, or intestinal fluid, the locally produced and selectively transported polymeric (pIgA)[3] secretory IgA (S-IgA) represents by far the dominant Ig isotype (4, 5). In contrast, immunochemical analyses of human urine and female and male genital tract secretions have demonstrated that these fluids display several features that are distinct from other secretions (6). Urine and genital tract secretions contain equal or higher levels of IgG than IgA. Furthermore, in female genital tract secretions, IgA is represented by S-IgA, pIgA, and monomeric IgA (mIgA), with a slight excess of IgA2 (7, 8). These marked differences in Ig isotype distribution in female genital tract secretions are partly due to the pronounced influence of sex hormones on the regulation of the distribution and selectivity of Ig transport during the menstrual cycle (9).

In contrast to the abundant studies of the secretions of the female genital tract, analyses of the male genital tract are limited (for review see Refs. 10 and 11). Although IgG, IgA, and IgM have been reported in both pre-ejaculate and seminal plasma (SP), their relative levels and molecular properties have varied remarkably in several studies (12–23). The discrepancies are partly due to the differences in collection procedures, methods, and standards used in Ig measurements, and the presence of abundant seminal proteolytic enzymes that are essential in the liquefaction of semen but that also degrade Ig, especially IgM and mIgA (24, 25). Even more controversial are reported differences in the level of IgG and IgA, including the proportion of S-IgA (13, 14, 18, 20). Therefore, in this study we collected semen from a large group of volunteers and analyzed molecular properties such as the proportion and levels of mIgA, pIgA, S-IgA, and IgA1 and IgA2 subclasses. The SP was collected and processed according to the protocol developed by a National Institutes of Health-sponsored study group (6), and evaluated by methods tested and verified in several multicenter studies (26).

Studies of the molecular properties of mucosal Abs are highly relevant to the determination of the origin (local vs systemic) and to the effective routes of induction of humoral immune responses (27–29). Because the immune system of the urogenital tract displays features of both the common mucosal immune system and systemic immune compartment (30, 31), in this study we evaluated the levels of naturally occurring Abs to microbial Ags, and compared the mucosal and parenteral immunization routes using various types of Ags that resulted in the induction of humoral responses in semen.

Materials and Methods

Study volunteers

Healthy, adult male volunteers 20–45 years old were recruited from donors registered at the Sperm Bank and from laboratory and office personnel

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[3] Abbreviations used in this paper: pIgA, polymeric IgA; S-IgA, secretory IgA; mIgA, monomeric IgA; SP, seminal plasma; SC, secretory component; TT, tetanus toxoid; DT, diphtheria toxoid; PnPs, pneumococcal polysaccharide; ASC, Ab-secreting cell; RSD, relative SD.

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Table I. *Ig isotypes and levels*[a]

	SP[b]			SP[c]		Pre-Ejaculate[c]	
	IgA	IgG	IgM	IgA	IgG	IgA	IgG
n	82	82	40	14	14	14	14
mean	13.865	29.379	0.464	19.790	26.994	2.681	1.252
SD	15.794	24.366	0.473	11.912	34.120	4.308	1.806
min	0.025	4.734	0.000	7.136	8.178	0.230	0.013
max	95.886	142.337	2.275	43.190	142.337	17.275	6.383
median	8.685	23.500	0.464	13.183	19.575	1.701	0.602

[a] Data shown as micrograms per milliliter.
[b] Ig isotypes and levels in human SP collected from healthy human male volunteers.
[c] Ig isotypes and levels in 14 paired samples of SP and pre-ejaculate collected from healthy human male volunteers.

and students within the University of Alabama at Birmingham (UAB) campus. The criteria for volunteer exclusion were the following: uretheral discharge; genitourinary infections; known allergies (e.g., egg proteins or lactose); infections with hepatitis B or C virus, HIV-1, or other immunodeficiency conditions. Volunteers agreed to abstain from sexual activity for 48 h before the designated day of collection. The UAB Human Use Committee approved this study.

Vaccines

The 35 enrolled volunteers were injected i.m. with one of the following vaccines purchased from the UAB pharmacy: 1) inactivated trivalent influenza virus types A and B purified subvirion Ags (Flushield/Wyeth), containing 15 μg of hemagglutinin of each A/Beijing/262/95(H1N1), A/Sydney/5/97(H3N2), and B/Harbin/7/94 influenza viruses (11 volunteers); 2) pneumococcal vaccine (Pnu-Imune 23/Lederle), consisting of 25 μg of each of the purified capsular polysaccharides from 23 types of *Streptococcus pneumoniae* (10 individuals); or 3) alum-adsorbed tetanus and diphtheria toxoids (Aventis Pasteur), 5Lf and 2Lf per dose, respectively (14 volunteers). Another group of 14 individuals was orally immunized with the live attenuated *Salmonella typhi* Ty21a vaccine (Vivotif Berna) consisting of four doses of enteric-coated gelatin capsules containing a mixture of viable (10^9–10^{10} CFU) and nonviable (5×10^9-6×10^{10} CFU) *S. typhi* Ty21a, as we previously described (32, 33).

Biological samples

The collection and processing of biological fluids were performed as described (6). Peripheral blood was obtained by venipuncture into heparinized (for cell isolation) or nonheparinized (for serum) syringes. Mononuclear cells were isolated from the heparinized blood by Ficoll-Hypaque gradient centrifugation. SP was prepared from semen obtained by masturbation. Samples of semen, placed on ice for 1 h to agglutinate and then liquefy, were diluted with an equal volume of PBS and centrifuged at $1200 \times g$ for 10 min to obtain the seminal fluid supernatants. Pre-ejaculatory fluid was obtained from donors that agreed to abstain from sexual activity for 48 h before collection. Pre-ejaculatory fluid, which appears as small droplets at the tip of the penis following sexual arousal, was collected before ejaculation by pressing the tip of the penis on the edge of a cryovial. Parotid saliva was collected into Schaefer cups placed over the parotid duct, and nasal lavages were obtained by instillation of 5 ml of prewarmed saline into the nostrils, and expelling it after ~30 s into a specimen cup. Rectal secretions were obtained by instillation of saline into the rectum with a 50-ml plastic syringe. After ~5 min, the discharged rectal content was collected into a plastic device, then the solid material was retained on cheesecloth. Approximately 20 ml of the filtered fluid was transferred into a tube containing 1% v/v protease inhibitors (Sigma-Aldrich) and further centrifuged for 10 min at $1600 \times g$. After the addition of protease inhibitors (6), all secretions were aliquoted and stored at −70°C until assayed.

Immunoglobulins

Human myeloma pIgA1, mIgA1, pIgA2 proteins, S-IgA, and free secretory component (SC) from colostrum were isolated by precipitation, electrophoretic, and chromatographic methods from existing supplies of IgA myeloma plasmas or colostrum, as described in detail (34). The purity of isolated proteins was tested by SDS-PAGE (under reducing or nonreducing conditions) and by immunoelectrophoresis using specific Abs. IgG subclass myeloma protein standards (two of each subclass with κ or λ-chains) were given to us by Dr. F. Skvaril (Cancer Institute, Tiefenau Hospital, Bern, Switzerland). The specificity of Abs to IgG and IgA subclasses was tested in our previous studies (35–37).

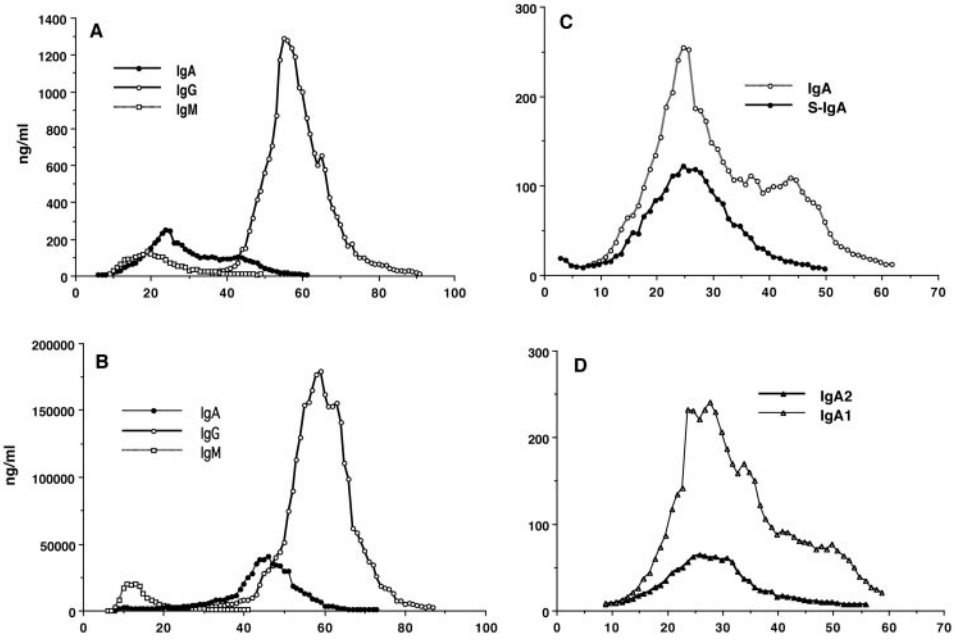

FIGURE 1. Elution profiles of pooled of SP and serum samples ($n = 5$) on a Sephacryl S-300 gel-filtration column (1.6 × 60 cm). Distribution of molecular forms of IgA, IgG, and IgM in SP (*A*) and in paired sera (*B*). Molecular forms of IgA and S-IgA (*C*) and of IgA subclasses (*D*) in SP.

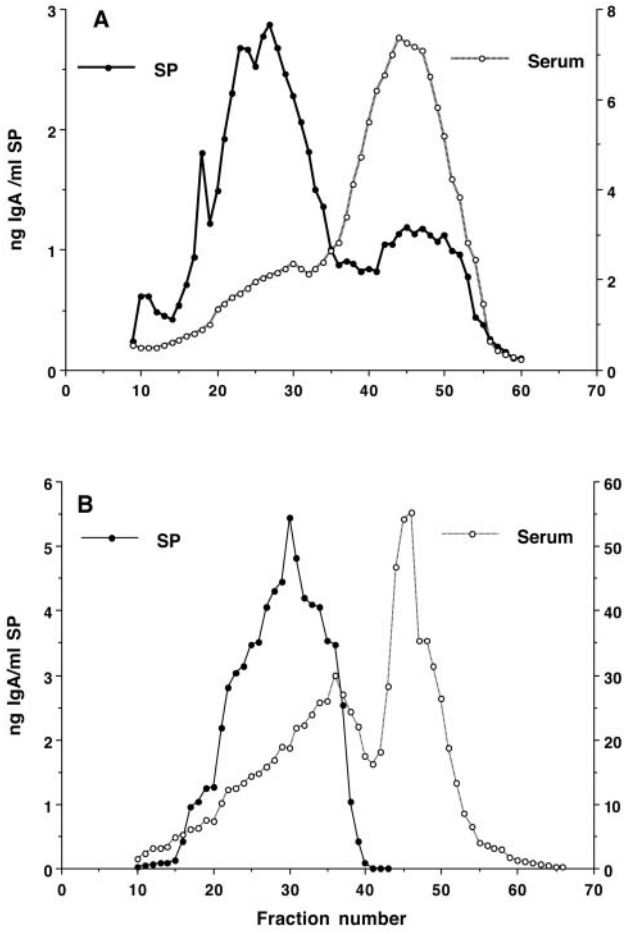

FIGURE 2. Molecular forms of naturally occurring Abs of the IgA isotype specific for Ag I/II of *S. mutans* (*A*) and influenza virus (*B*) in paired SP and serum samples.

ELISA

In all fluids, the levels of IgA, S-IgA, IgA1, IgA2, IgG, and IgM, as well as their Ab specificity, were measured by ELISA. Polystyrene 96 microwell plates (Nalge Nunc International) were coated overnight with 1 μg/ml $F(ab')_2$ of goat IgG anti-human IgA, IgG, or IgM (Jackson ImmunoResearch Laboratories), or with mouse monoclonal IgG1 anti-human IgA1 or IgA2 (Nordic Immunological Laboratories) (35–37). The following concentrations of Ags were used as capture for measurement of vaccine-specific Abs: 1/100 dilution of influenza virus vaccine, 1/500 dilution of Pnuimmune (pneumococcal polysaccharide (PnPs)), 1/50 dilution of tetanus toxoid purified vaccine (TT) (Wyeth), 0.5 μg/ml diphtheria toxoid (DT) (List Biological Laboratories), 10^7 CFU/ml *S. typhi* Ty21a, 5 μg/ml *Salmonella typhosa* LPS, 5 μg/ml OVA, 5 μg/ml bovine γ-globulin (Sigma-Aldrich), or 5 μg/ml Ag I/II of *Streptococcus mutans* (a gift from Dr. M. Russell, State University of New York (SUNY), Buffalo, NY). Coated plates were blocked with 5% FBS in PBS containing 0.05% Tween 20, and serial 2-fold dilutions of samples and standards were incubated overnight. Standard curves were generated for each plate by using serial dilutions of a calibrated pool of normal human serum (Binding Site) with known Ig isotype concentrations previously calibrated against World Health Organization standards or by using a purified human colostral S-IgA. Human sera with known Ab titers against the influenza virus, PnPs, TT, DT, or salmonella Ty21a and LPS, were included on each plate as positive control. Bound Abs were detected with biotin-labeled goat $F(ab')_2$ of IgG anti-human IgA, IgG, or IgM (BioSource International), or anti-human SC (34), followed by ExtrAvidin peroxidase conjugate (Sigma-Aldrich). The wells were developed with *o*-phenylenediamine-H_2O_2 substrate (Sigma-Aldrich). The color reaction was stopped with 1 M sulfuric acid and the absorbance at 490 nm was read in a EL312 Bio-Kinetics microplate reader (Bio-Tek Instruments). The level of Ag-specific and total Igs was calculated by interpolating the ODs on calibration curves, as previously described (32, 33) using the DeltaSoft II program (BioMettalics).

ELISPOT assay

Numbers of vaccine-specific, as well as the total IgA-, IgG-, and IgM-secreting cells in peripheral blood, were determined by the ELISPOT assay (38). MultiScreen-HA 96-well filtration plates (Millipore) were coated with the same concentration of Ags or anti-Ig reagents as the ELISA plates. The plates, blocked with complete tissue culture medium, were incubated for 3 h at 37°C and 5% CO_2 with 4–16 replicates of different concentrations of freshly isolated mononuclear cells. The IgA-, IgG-, and IgM-secreting cells were revealed after incubation with biotinylated, affinity-purified $F(ab')_2$ goat anti-human IgA, IgG, or IgM (BioSource International), followed by alkaline phosphatase-labeled ExtrAvidin (Sigma-Aldrich) and the enzyme substrate 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and NBT (Bio-Rad). The blue spots obtained were counted by means of a stereomicroscope (Wild Heerbrugg M3) and the results were expressed per 10^6 mononuclear cells.

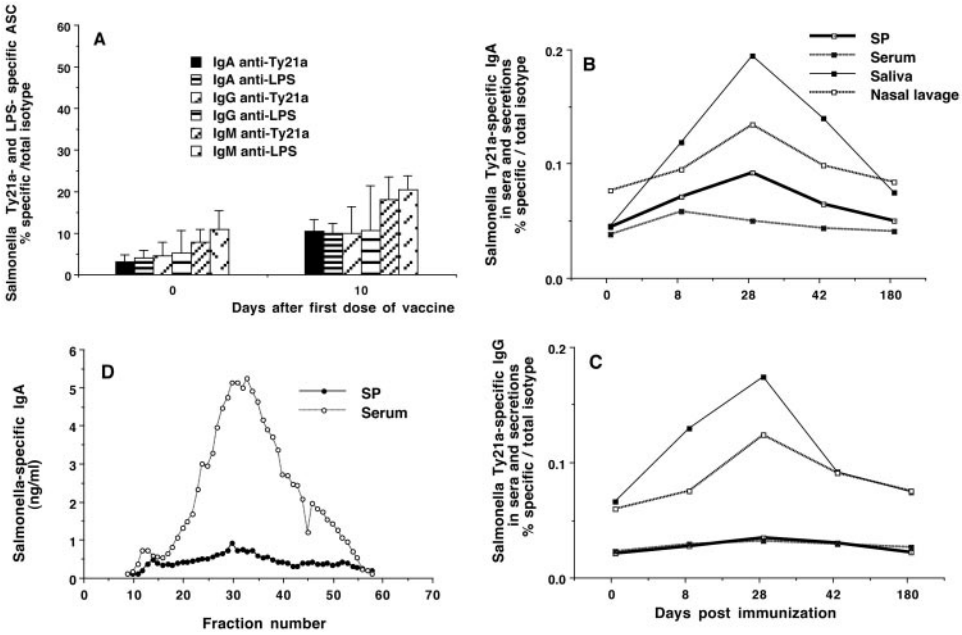

FIGURE 3. Abs induced in 14 human male volunteers orally immunized with live-attenuated Ty21a typhoid vaccine. *Salmonella* Ty21a and LPS-specific ASC in the peripheral blood of male volunteers before, and 2 days after last dose of vaccine (*A*); kinetics of *Salmonella* Ty21a-specific *IgA* (*B*) and *IgG* (*C*) Abs in SP, sera, saliva, nasal lavages; molecular mass distribution of salmonella-specific IgA in SP and in serum, 28 days after administration of or oral typhoid vaccine (*D*).

Molecular-sieve chromatography

Samples of sera (150 μl diluted with equal volume of PBS) or 500 μl of seminal fluids were fractionated on a Sephacryl S-300 HR column (Amersham Biosciences) column (1.6 × 60 cm) attached to a BioLogic DuoFlow chromatography system (Bio-Rad). The column was calibrated with Bio-Rad m.w. markers as well as with purified human mIgA, pIgA, S-IgA, IgG, and IgM. The protein profiles of the eluted samples were provided by means of a UV detector with a continuous reading at 280 nm.

SDS-PAGE and Western blot analysis

Sera and corresponding SP were separated by SDS-PAGE on an 8% gel (ISC BioExpress) and transferred onto Immulon P membranes (Millipore) by electroblotting. The membranes, blocked with superblock (Pierce), were incubated with anti-human IgG F(ab′)$_2$ of goat IgG (Southern Biotechnology Associates) or with mouse mAbs to human IgG subclasses (The Binding Site), followed by neutravidin-peroxidase (Pierce). The formed immune complexes were visualized by the ECL technique (ECL) on Kodak Scientific Imaging film after the addition of SuperSignal chemiluminescent substrate (Pierce), as described (39).

Statistical analysis

Descriptive statistics such as mean, median, and SD were used to summarize the Ig levels in SP and the immune responses induced by the various vaccines. Data were calculated on an Apple Macintosh computer with Microsoft Excel and StatView (Brain Power) programs.

Results

Ig levels in SP and in corresponding sera

The levels of IgG, IgA, and IgM were measured in 82 samples of SP and in 14 paired samples of SP and pre-ejaculate. In SP, unlike in other external secretions, including the pre-ejaculate (Table I, SP), IgG and not IgA was the dominant isotype (Table I, SP, and SP and Pre-ejaculate). Furthermore, in SP samples high interindividual variations marked by large SDs were observed (Table I). However, the variability in Ig levels of SP obtained from the same individual at various time points, up to 6 mo apart, was less pronounced. For example, the mean relative SD% (RSD) between SP collected from 11 individuals at five time points was 188.72 for IgA and 52.17 for IgG, whereas the mean RSD% within five successively collected SP samples from each of the 11 volunteers was 20.46 for IgA and 19.34 for IgG.

Molecular characteristics of Ig in SP

To determine the molecular properties of Ig in SP, we fractionated SP on a standardized molecular-sieve chromatography column (Sephacryl S-300). The protein profiles indicated at least six fractions with molecular masses of proteins ranging from >650 to 17 kDa and approximately three fractions corresponding to lower molecular mass polypeptides (dominant 1.4 kDa). When the Ig levels were measured in successive fractions obtained after Sephacryl S-300 gel-filtration of SP or the corresponding serum, IgG had an identical elution profile in both fluids; IgM in SP appeared to have a slightly lower molecular mass and a large proportion of IgA was eluted in fractions corresponding to pIgA (Fig. 1, *A* and *B*). In SP, the ratio IgG:IgA was similar to that measured in paired sera (3.46 vs 3.75). As shown in Fig. 1*C*, a relatively high proportion of IgA (~41%) was associated with SC and was therefore considered to represent S-IgA. IgA1 and IgA2 in SP displayed a similar distribution to that in serum with a predominance of the IgA1 subclass (~83% or total IgA). The proportion of IgA2 was slightly higher in fractions corresponding to S-IgA and pIgA; IgA1 predominated in the mIgA fraction (Fig. 1*D*).

The distribution of IgG subclasses was evaluated with respect to their migration pattern and the intensity of bands obtained after electrophoretic separation, Western blot, and ECL visualization of equal amounts of total IgG from samples of SP and serum, as well as those derived from known quantities of human IgG1-IgG4

Table II. *Kinetics of* Salmonella*-specific Abs in serum and secretions*[a]

Day	IgA					IgG					IgM				
	SP	Serum	Saliva	Nasal lavage	Rectal lavage	SP	Serum	Saliva	Nasal lavage	Rectal lavage	SP	Serum	Saliva	Nasal lavage	Rectal lavage
0	0.041 ± 0.016	0.035 ± 0.022	0.089 ± 0.042	0.073 ± 0.034	0.057 ± 0.035	0.017 ± 0.015	0.019 ± 0.010	0.062 ± 0.028	0.056 ± 0.045	0.090 ± 0.073	1.473 ± 1.301	0.382 ± 0.096	0.654 ± 0.174	0.682 ± 0.464	0.27 ± 0.23
8	0.068 ± 0.045	0.055 ± 0.027	0.296 ± 0.115	0.091 ± 0.032		0.024 ± 0.017	0.026 ± 0.014	0.126 ± 0.098	0.072 ± 0.046		1.662 ± 1.494	0.534 ± 0.185	0.959 ± 0.325	1.007 ± 0.549	
28	0.088 ± 0.052	0.047 ± 0.026	0.547 ± 0.191	0.131 ± 0.040	0.108 ± 0.061	0.031 ± 0.025	0.028 ± 0.016	0.171 ± 0.194	0.120 ± 0.085	0.144 ± 0.161	2.672 ± 2.359	0.506 ± 0.174	1.126 ± 0.313	1.487 ± 0.752	0.50 ± 0.49
42	0.061 ± 0.031	0.040 ± 0.021	0.388 ± 0.136	0.095 ± 0.039		0.027 ± 0.024	0.026 ± 0.014	0.088 ± 0.040	0.087 ± 0.057		1.955 ± 1.717	0.481 ± 0.159	0.983 ± 0.368	1.272 ± 0.693	
180	0.047 ± 0.022	0.037 ± 0.019	0.173 ± 0.071	0.080 ± 0.033		0.018 ± 0.012	0.023 ± 0.014	0.071 ± 0.042	0.072 ± 0.047		1.336 ± 1.161	0.418 ± 0.178	0.671 ± 0.357	1.051 ± 0.591	

[a] Data represent the mean (n = 14) percentage of specific Abs relative to total isotype ± SD.

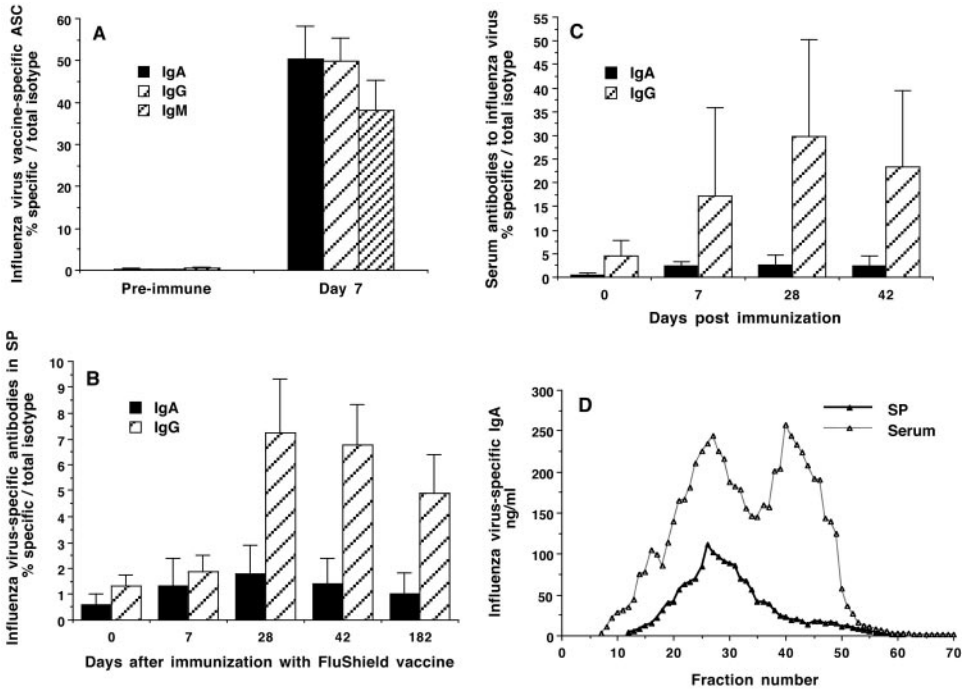

FIGURE 4. Vaccine-specific responses induced in 11 volunteers by i.m. injection with influenza virus FluShield vaccine. ASC to influenza virus vaccine before and at 1 wk after immunization (*A*); IgA and IgG Abs to influenza virus in SP (*B*) and in sera (*C*) at selected time points. Molecular form distribution of influenza virus-specific IgA in SP and in serum 28 days after immunization (*D*).

myeloma proteins. All four subclasses were present in SP samples, and their proportions among total IgG as well as their molecular mass resembled those in corresponding sera for IgG1 and IgG2. However, the percentage of IgG3 of total IgG was lower in SP compared with serum (7.28 vs 13.09) and the percentage of IgG4 was slightly higher in SP than in paired serum (20.17 vs 16.14).

Levels and properties of pre-existing Abs

Because of the relative similarity between the total Ig profiles in SP and matching serum samples, we investigated whether the levels of Ag-specific Abs in SP also paralleled the levels of such Abs in corresponding sera. For this purpose, we examined SP and corresponding sera for the presence of naturally occurring Abs to dietary or microbial Ags, such as *S. mutans* AgI/II, influenza virus and TT vaccines, OVA and bovine γ-globulin. In 12 samples analyzed we found that both serum and SP contained IgA and IgG Abs against *S. mutans*, IgG against TT and influenza virus, and very low levels of IgA against influenza virus, OVA, and bovine γ-globulin.

The molecular properties of specific Abs in SP and serum were evaluated by quantitating these Abs in successive fractions from molecular sieve separation of a pool of five samples. Interestingly, differences in the molecular forms of IgA Abs to *S. mutans* were observed: almost all *S. mutans* Ag I/II-specific IgA in SP was present as S-IgA and pIgA, while in serum, mIgA predominated (Fig. 2*A*). Moreover, Abs induced by previous influenza virus infection or immunization were represented by very low levels of IgA in SP; again, anti-influenza virus-specific pIgA predominated,

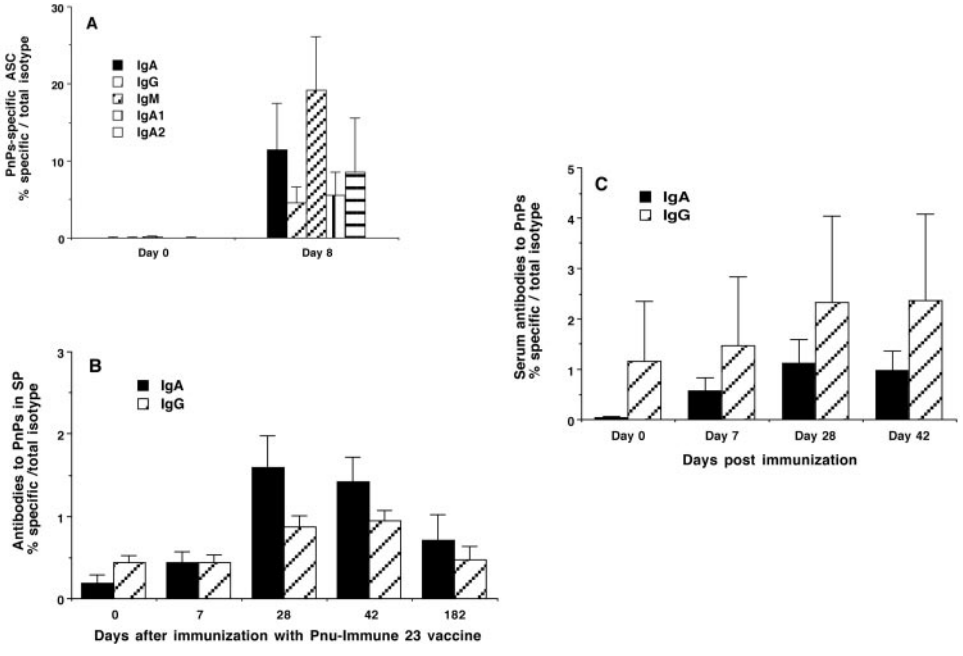

FIGURE 5. Abs induced in 10 human male volunteers by i.m. immunization with Pnu-Imune 23 vaccine. PnPs-specific ASC in peripheral blood before, and at 1 wk after immunization (*A*); kinetics of Abs induced in SP (*B*) and in sera (*C*) by PnPs vaccination.

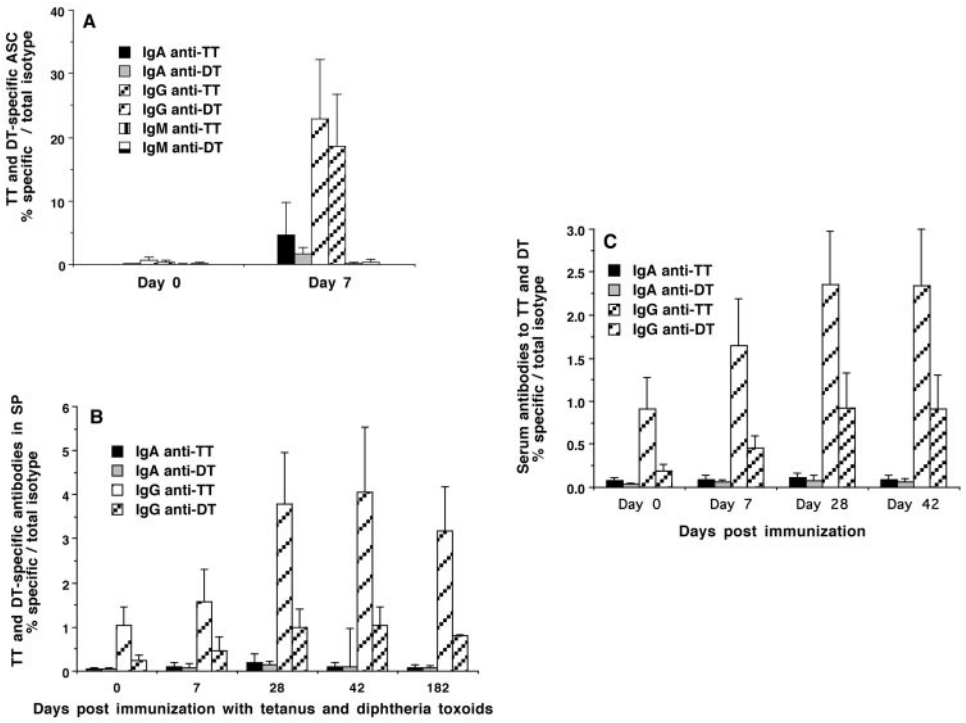

FIGURE 6. Abs induced by systemic immunization of 14 human male volunteers with alum-adsorbed tetanus and diphtheria toxoid vaccine. TT- and DT-specific ASC determined before and 7 days after vaccination (*A*); vaccine-specific IgA and IgG measured in SP (*B*) and in sera (*C*) of the immunized individuals.

whereas in serum the IgA Abs were present in both mIgA and pIgA forms (Fig. 2*B*). Influenza virus-specific IgG levels were higher than those of IgA in both serum and SP. Abs specific for TT were represented exclusively by IgG in both SP and serum. For all Ags analyzed, in SP the specific IgG Abs represented a higher percentage of total IgG than in paired sera.

Abs induced by immunization

Because the levels of pre-existing Abs to the above-selected microbial Ags were rather low, and no specific information about the time of previous infection or immunization was available, we proceeded with four immunization protocols: mucosal (oral) administration of live attenuated salmonella vaccine, and three systemic vaccinations using either glycoproteins (influenza virus), polysaccharides (PnPs), or two bacterial proteins (TT and DT) as Ags. The humoral responses induced by vaccines were measured in sera and external secretions–including SP, saliva, nasal lavage, and intestinal fluid–and Ab-secreting cells (ASC) were enumerated in peripheral blood. We selected the commercially available influenza virus, PnPs, TT, and DT as vaccines of choice to determine whether systemic or mucosal Ag exposure is able to induce Abs in the male genital tract and whether the type of Ag (proteins vs polysaccharide) affects the quality and magnitude of humoral responses in SP. The typhoid Ty21a vaccine was the only commercially available mucosal vaccine.

Mucosal immunization with Salmonella typhi *Ty21a vaccine*

The immmunogenicity of *S. typhi* vaccine was first manifested by the appearance of specific ASC in peripheral blood 2 days after the last vaccine dose (10 days after the first dose). Because of the high degree of variability among the volunteers, we expressed the vaccine-specific ASC as percentage of total ASC of the IgG, IgA and IgM isotypes. After oral immunization, *S. typhi*-specific IgA ASC increased five times, IgM four times, and IgG three times from the preimmunization levels (Fig. 3*A*). Therefore, the results generated in immunized males were comparable to those induced by the same vaccine in women (32, 33). The level of specific Abs in SP and sera increased, on day 28 after immunization, approximately two times, although in SP the levels of specific IgA, IgG, and IgM Abs were rather low (Fig. 3, *B* and *C*). When expressed as a percentage of total IgA, IgG, or IgM, in SP the levels of anti-*Salmonella* Abs were slightly higher than in matching serum samples. Higher levels of *Salmonella*-specific Abs were measured in saliva and in the nasal lavage fluid. Interestingly, when compared with the levels of specific Abs in rectal lavage fluids, SP displayed a similar profile at the peak of the humoral response (28 days after

Table III. *Levels of PnPs-specific IgA, IgG, and IgM Abs*[a]

	Serum[b]						Seminal plasma[c]					
	IgA		IgG		IgM		IgA		IgG		IgM	
Day	μg/ml	specific/total	μg/ml	specific/total	μg/ml	specific/total	ng/ml	specific/total	ng/ml	specific/total	ng/ml	specific/total
0	1.17 ± 1.04	0.04 ± 0.03	0.06 ± 0.04	1.16 ± 2.37	20.71 ± 8.76	1.16 ± 3.61	31.58 ± 43.56	0.19 ± 0.18	140.05 ± 90.30	0.44 ± 0.16	12.53 ± 13.45	2.70 ± 3.61
7	14.35 ± 12.17	0.57 ± 0.55	90.94 ± 45.68	1.46 ± 2.75	70.59 ± 43.26	3.56 ± 2.29	61.48 ± 70.05	0.44 ± 0.27	144.34 ± 84.04	0.44 ± 0.18	22.18 ± 16.72	3.81 ± 5.09
28	30.00 ± 22.67	1.13 ± 0.91	178.07 ± 60.19	2.32 ± 3.45	115.52 ± 86.68	5.38 ± 3.71	251.48 ± 242.83	1.59 ± 0.78	272.28 ± 110.57	0.87 ± 0.26	51.89 ± 49.37	5.77 ± 5.62
42	25.42 ± 20.06	0.98 ± 0.79	175.01 ± 58.64	2.36 ± 3.46	106.42 ± 83.04	5.25 ± 3.70	263.92 ± 241.72	1.42 ± 0.58	320.95 ± 128.81	0.95 ± 0.22	61.05 ± 52.70	5.04 ± 4.62
142	15.62 ± 12.97	0.57 ± 0.42	137.99 ± 43.76	2.06 ± 3.16	64.72 ± 52.73	3.47 ± 2.39	140.04 ± 174.12	0.71 ± 0.63	249.27 ± 90.74	0.47 ± 0.34	10.15 ± 10.67	1.88 ± 2.74

[a] The values represent mean ± SD, (n = 10). Levels of PnPs-specific IgA, IgG, and IgM Abs in sera[b] and external secretions[c] induced by systemic immunization with PnPs vaccine, expressed as micrograms or nanograms per milliliter and as percentage of total isotype.

vaccination) (Table II). Similar results were obtained when we analyzed ASC specific for *Salmonella* LPS. When analyzed by molecular-sieve chromatography, specific Abs were predominantly associated with pIgA forms in both sera and SP (Fig. 3*D*); specific IgG Abs in both fluids were present in the same proportion and displayed identical elution profiles. Six months after vaccination, the level of specific Abs decreased to preimmune levels (Fig. 3, *B* and *C*). Although specific Abs of the IgM isotype were also induced, their absolute levels were very low due to the low levels of total IgM in SP.

Systemic immunization induces humoral responses in SP

Influenza virus. Systemic immunization with influenza virus vaccine induced, in agreement with previous studies (40), high numbers of influenza virus-specific ASC of IgG, IgA, and IgM isotypes in peripheral blood 1 wk after immunization (Fig. 4*A*). At the peak of the serum Ab response (28 days after immunization), IgG influenza virus-specific Abs were dominant while IgA and IgM Abs represented a smaller fraction (Fig. 4*C*).

A similar increase in influenza virus-specific IgG and IgA Abs was also induced in SP, when expressed as a percentage of specific vs total isotype (Fig. 4*B*). A slightly higher proportion of specific Abs was measured in sera than in corresponding SP, saliva, or nasal lavage fluids. Six months after immunization, influenza-virus specific Abs in SP decreased from the peak levels (1 mo after immunization) by 30% for IgG and 50% for IgA. Analyses of molecular properties of total and influenza virus-specific IgA Abs revealed an interesting pattern: in SP all specific Abs were associated with pIgA, while in sera both pIgA and mIgA contained specific Abs (Fig. 4*D*). S-IgA, which represented a relatively small fraction of total IgA, displayed the highest levels of specific Ab activity. Furthermore, in SP the level of anti-influenza virus IgA was approximately half of that present in serum, although serum contained ~10 times more of total IgA than SP. This may be due to the fact that pIgA displays a higher binding of Ags than mIgA because of the bonus effect of multivalency (four to eight Ag-binding sites for IgA dimers and tetramers, respectively) (41), and more effectively neutralizes the influenza virus (42).

Pneumococcal polysaccharide vaccine. Systemic immunization with PnPs induced, in peripheral blood, ASC of the IgM (~20% of total IgM ASC were PnPs-specific), IgA (~12%), and IgG (~4%) isotypes. When examined for IgA subclasses, IgA2 PnPs-specific ASC were dominant as reported in our earlier studies (Ref. 43; Fig. 5*A*). Thus, in contrast to influenza virus ASC (Fig. 4*A*) as well as to TT and DT ASC (Fig. 6*A*), PnPs responses in peripheral blood occurred mainly in the IgM > IgA > IgG isotypes. However, 1 mo after immunization, PnPs-specific Abs (peak response in serum) were present mainly in the IgG and IgM isotypes with a low level of IgA Abs (Fig. 5*B*). A similar Ig isotype distribution of PnPs-specific Abs was observed in SP (Fig. 5*C*), saliva, and nasal secretions (Table III). Interestingly, in SP the highest levels of PnPs-specific Abs were present in the IgG and IgA isotypes. However, when expressed as percentage of total isotype, IgM was dominant, followed by IgA and IgG (Table III). Nevertheless, very low levels of total IgM were present in SP, perhaps due to the proteolytic digestion of IgM.

TT and DT vaccines. Following TT or DT vaccination, most of the vaccine-specific ASC in peripheral blood were of the IgG isotype (~20%), with <5% of IgA and very few (~0.2%) of IgM isotypes (Fig. 6*A*). In general, responses to TT were of significantly greater magnitude than to DT, perhaps reflecting more frequent TT than DT immunization in adult volunteers. TT- and DT-specific Abs of predominantly IgG isotype were detected in both sera and SP, with peak levels at 6 wk; only low levels of specific IgA and IgM Abs were induced (Fig. 6*B*). Six months after immunization, levels of specific IgG and IgA Abs decreased to approximately half of those measured at 6 wk, whereas the vaccine-specific IgM reached the preimmune levels.

Compilation of results from immunization studies clearly indicates that humoral immune responses are inducible in SP by mucosal as well as systemic immunization routes and with different types of vaccines (Table IV). The dominant isotypes of specific Abs in SP in systemically immunized individuals reflects Ab responses induced in serum, although the molecular properties of IgA Abs to influenza virus differ with respect to the representation of molecular forms.

Discussion

The comparative analyses of distribution, and molecular properties of Abs in human serum and male genital tract secretions, revealed that the Ig profiles in male genital tract secretions resembled those present in serum rather than those characteristic of typical external secretions. In contrast to saliva, milk, and intestinal fluid, in which S-IgA is by far the dominant isotype (and pIgA devoid of SC and mIgA are present in trace amounts; Refs. 4 and 6), SP contains IgG as the dominant isotype and all three molecular forms of IgA (i.e., S-IgA, pIgA and mIgA) are present in comparable quantities. We found that, in SP, only ~50% of pIgA occurs as S-IgA. This may be due to the digestion of SC by proteases found in abundance in SP (24, 25), or alternatively, pIgR-independent transport pathways may function in the genital tract (5). S-IgA is probably produced locally by the glands of Littré distributed along the penile urethra (44). Because all components necessary for selective transport are found–that is, plasma cells producing pIgA with J chain and polymeric Ig receptor on epithelial cells–these glands are analogous to other typical mucosal tissues. Although the pre-ejaculate may resemble, with respect to properties and Ig isotype distribution, other typical external secretions (e.g., saliva) (10), we were unable, due

Table III. *(Continued)*

Saliva[c]						Nasal Lavages[c]					
IgA		IgG		IgM		IgA		IgG		IgM	
ng/ml	specific/total	ng/ml	specific/total	ng/ml	specific/total	ng/ml	specific/total	ng/ml	specific/total	ng/ml	specific/total
137.61 ± 67.26	0.05 ± 0.04	10.39 ± 8.90	0.32 ± 0.35	79.51 ± 54.57	2.70 ± 3.61	21.22 ± 9.02	0.05 ± 0.03	25.32 ± 30.76	0.25 ± 0.28	8.81 ± 5.55	2.70 ± 3.61
195.25 ± 121.06	0.08 ± 0.06	13.88 ± 18.05	0.28 ± 0.29	134.84 ± 106.98	4.93 ± 3.30	43.39 ± 32.40	0.08 ± 0.05	38.26 ± 28.87	0.69 ± 0.71	35.54 ± 24.57	4.83 ± 2.97
222.33 ± 87.10	0.12 ± 0.09	38.42 ± 40.85	1.36 ± 1.01	150.41 ± 169.86	6.79 ± 4.88	84.84 ± 80.89	0.31 ± 0.45	85.02 ± 65.18	1.18 ± 0.72	46.61 ± 41.41	7.82 ± 4.26
239.12 ± 137.49	0.09 ± 0.05	58.18 ± 34.63	1.00 ± 0.80	181.20 ± 211.99	5.35 ± 4.15	49.81 ± 42.51	0.15 ± 0.13	71.87 ± 89.20	0.78 ± 0.64	47.73 ± 49.61	5.68 ± 2.54

Table IV. *Ig isotype distribution of Abs*[a]

Immunization Route	Vaccine and Test Ags	ASC		Serum		SP		Saliva		Nasal Lavage	
		Number/10^6 MNC[b]	% of total Ig isotype[c]	ng/ml[b]	% of total Ig isotype[c]	ng/ml[b]	% of total Ig isotype[c]	ng/ml[b]	% of total Ig isotype[c]	ng/ml[b]	% of total Ig isotype[c]
Mucosal (Oral)	Salmonella Ty21a or LPS	IgG > IgA	IgA = IgG	IgG > IgA	IgA > IgG	IgG > IgA	IgA > IgG	IgA ≫ IgG	IgA = IgG	IgA > IgG	IgA = IgG
	Influenza virus	IgG ≫ IgA	IgA > IgG	IgG ≫ IgA	IgG ≫ IgA	IgG ≫ IgA	IgG ≫ IgA	IgG > IgA	IgG ≫ IgA	IgG > IgA	IgG ≫ IgA
Systemic (i.m.)	PnPs	IgA ≫ IgG	IgA ≫ IgG	IgG > IgA	IgG > IgA	IgA = IgG	IgA > IgG	IgA > IgG	IgG > IgA	IgG > IgA	IgG > IgA
	TT	IgG ≫ IgA	IgG ≫ IgA	IgG ≫ IgA	IgG ≫ IgA	IgG ≫ IgA	IgG > IgA	IgG > IgA	IgG ≫ IgA	IgG ≫ IgA	IgG ≫ IgA
	DT	IgG ≫ IgA	IgG ≫ IgA	IgG ≫ IgA	IgG ≫ IgA	IgG ≫ IgA	IgG > IgA	IgG = IgA	IgG ≫ IgA	IgG ≫ IgA	IgG > IgA

[a] Ig isotype distribution of Abs induced by oral or systemic immunization with proteins, glycoproteins, or polysaccharides in peripheral blood MNC, sera, SP, saliva, and nasal secretions. Data were analyzed as [b] absolute values and as [c] percentage of total isotype.

to the low volumes available, to examine the spectrum of naturally occurring or immunization induced Abs to food and microbial Ags in this fluid. Although the origin of mIgA and pIgA is not clear, the comparable distribution of specific IgA Abs and the similar ratio of IgA1 and IgA2 subclasses suggest a plasma origin. In humans, IgA subclasses are not equally distributed in systemic and in mucosal tissues; the majority of IgA2-producing cells is found in mucosal tissues (6, 29, 45–47). Furthermore, the distribution of IgA1- or IgA2-producing cells in mucosal tissues parallels the distribution of these subclasses in the corresponding fluid (4, 45–47). Thus, SP, due to the predominance of IgA1, more resembles serum than secretions of the gastrointestinal or female genital tracts (4, 8, 29). In contrast, when IgA subclasses were quantitated in each column fraction, a higher proportion of IgA2 was detected in pIgA-containing fractions, which contrasts with serum where the ratio of the two subclasses remains constant for all molecular forms, as previously reported (48). In this respect, SP differs from the female genital tract secretions: IgA1 and IgA2 levels in the cervical fluid are similar or IgA2 is present in slight excess (8). These levels closely parallel the distribution of IgA1- or IgA2-secreting cells in the endocervix (49). In comparison, IgA1 in SP is dominant (84% of mIgA); this value corresponds to the level of IgA1 in plasma (29, 45, 50). The above-described molecular characteristics of IgA in SP, namely an increased proportion of pIgA forms and particularly the presence of S-IgA as compared with serum, indicate that at least a fraction of IgA is locally produced. This assumption was further substantiated by the prevalence of pIgA and S-IgA Abs against influenza virus, *Salmonella*, and *S. mutans* in SP, while in corresponding sera a lower proportion of pIgA and no specific S-IgA were detected.

IgG in SP displayed properties similar to its plasma counterpart: the same molecular mass and similar distribution of IgG subclasses (51). Interestingly, earlier reports suggested that IgG present in SP exhibits a higher molecular mass (by ~70 kDa) than its serum counterpart, due to the association with soluble FcγRs (52–58). However, in our studies, serum and SP IgG displayed identical molecular profiles as evaluated by molecular-sieve chromatography. Surprisingly, IgM present in SP exhibited a slightly lower molecular mass than in serum IgM. It is possible that IgM may have been partially digested by highly active proteolytic enzymes abundant in SP (24, 25).

The analyses of the molecular properties of naturally occurring and vaccine-induced Abs in external secretions, and their comparison with the properties of such Abs in serum, have provided extremely useful markers to determine with considerable precision the relative contribution of systemic or local (mucosal) sources to the total Ig pool in external secretions (28, 29). In turn, this information is important in the design of immunization strategies that are effective for the induction of humoral immune responses in various secretions (28, 56). Thus, in addition to the studies of immunochemical properties of Igs in human SP, we evaluated the antigenic specificity and properties of naturally occurring and immunization-induced Abs. The presence of S-IgA Abs in external secretions of salivary, lacrimal, and mammary glands to Ags which are encountered at anatomically remote inductive mucosal sites (e.g., intestinal or respiratory tract mucosae), has provided compelling evidence for the concept of the common mucosal immune system (56). For example, human tears, parotid saliva, and milk contain relatively high levels of naturally occurring and ingestion-induced Abs to the strictly oral bacterium, *S. mutans*, that exclusively colonizes the tooth surfaces (57–59). The presence in human SP of these S-IgA-associated Abs to *S. mutans* and the influenza virus, as well as *Salmonella*-specific Abs induced by oral

immunization, suggests that the male genital tract is also a component of the common mucosal immune system.

The distribution of specific Abs of the *IgA* isotype in external secretions parallels the distribution of IgA-producing cells in mucosal tissues (56, 60). Consequently, the degree of protection against infectious diseases of, for example, the intestinal tract, correlates better with the level of specific S-IgA Abs in the intestinal fluid than in serum (41). Other external secretions display a variable degree of contribution of Abs from the circulation. Urine, female genital tract secretions (particularly during menses Ref. 61), and lower and upper respiratory tract secretions contain significant quantities of plasma-derived IgG and mIgA (6) that may confer Ab-dependent protection from microbial infections at the corresponding mucosal surface (62). Based on the levels and molecular properties of Igs, SP obviously belongs to the same category of external secretions that have a pronounced contribution of Ig from the circulation. Consequently, in our studies, systemic immunization with DT, TT, or PnPs vaccines induced predominant IgG responses in sera as well as in SP; corresponding IgG responses in saliva and nasal washes were unimpressive, mainly due to the low levels of total IgG in these fluids. Interestingly, systemic immunization with influenza virus vaccine stimulated IgA responses in SP. This finding is in agreement with previous studies (63) in which systemic immunization (with a *Vibrio cholerae* vaccine) of women from regions with endemic cholera induced corresponding S-IgA Abs in milk; this was not the case when previously mucosally unexposed women were immunized. Presumably, previous mucosal exposure to a given Ag primes the mucosal immune system for subsequent systemic immunization.

Because local application of various Ags in the vagina, or genital tract infection (27) with gonococcus (64), *Chlamydia trachomatis* (65), or with HIV-1 (66) stimulates only modest local responses, immunization at remote inductive sites such as intestinal (including rectal), and especially nasal mucosae, appears to be more effective in the induction of humoral responses in the female genital tract secretions (27, 32, 33, 67–72).

However, in addition to the site of stimulation, the type of the Ag profoundly influences the magnitude and quality of the immune response. This is particularly evident in the case of sexually acquired HIV-1 infection. Despite the overwhelming dominance of total IgA in saliva, tears, milk, and intestinal secretions, HIV-1-specific Abs are present mainly in IgG isotype in sera and in all secretions examined while IgA Abs specific for HIV-1 are either absent or present in low levels (for experimental data and extensive review of the pertinent literature see Refs. 26 and 66). Results presented in this report demonstrate that oral or systemic immunization results in the appearance of Abs in the male genital secretions. The problems inherent to mucosal immunization (presence or absence of inductive sites, potential induction of mucosal tolerance, high doses of Ags required, unavailability of potent mucosal adjuvants suitable for use in humans, and dominant responses to live vaccine vectors) have hampered the development of mucosal vaccines (60). However, in contrast to other external secretions in which systemic immunization is usually ineffective as a means of inducing vigorous IgG or IgA responses (56, 60, 62), fluids of the genital tract contain IgG as the dominant isotype and a significant part of this IgG is derived from plasma. Therefore, systemic immunization is likely to be effective for the stimulation of humoral immune responses in the male genital tract secretions.

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Disclosures

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