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Salivary, Nasal, Genital, and Systemic Antibody Responses in Monkeys Immunized Intranasally with a Bacterial Protein Antigen and the Cholera Toxin B Subunit

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Previous attempts to induce mucosal antibodies in rhesus monkeys by enteric immunization have resulted in only modest and short-lived responses, dominated by immunoglobulin M (IgM) antibodies in the plasma. In this study, two groups of rhesus monkeys were immunized intranasally three times at 2-week intervals with a bacterial protein antigen (AgI/II) either chemically coupled to or mixed with the B subunit of cholera toxin (CT), a known potent mucosal immunogen and carrier for other immunogens. Cells secreting antibodies, predominantly of the IgA isotype, to AgI/II and to CT were detected in the peripheral blood 1 week after each immunization, indicating the dissemination of IgA-secreting precursor cells through the mucosal immune system. IgG and, to a lesser extent, IgA antibodies to both proteins were induced in the plasma commencing after the second immunization. Plasma IgE concentrations and IgE antibody levels were not consistently raised during the immunization period. IgA antibodies against AgI/II and CT were found in saliva and also in fecal extracts, and both IgA and IgG antibodies were found in nasal and vaginal washes. Nasal IgG but not IgA antibodies showed a significant positive correlation with plasma IgG antibody levels, suggesting that they were largely derived by transudation from the circulation. Analysis of the molecular form of vaginal IgA indicated that both secretory and monomeric forms of IgA were present in various proportions. Furthermore, neither IgG nor IgA antibodies in vaginal washes were correlated with plasma antibody responses, suggesting the contribution of locally synthesized antibodies of both isotypes. Comparison of the responses between the two groups of animals showed only sporadic significant differences, indicating that intranasal immunization with AgI/II either coupled to or mixed with the B subunit of CT was equally effective at inducing generalized IgA antibody responses in the mucosal immune system and predominantly IgG antibodies in the plasma.

The realization that most infectious diseases, including human immunodeficiency virus infection, arise at or are acquired through mucosal surfaces has focused attention on immunological protection of the mucosae, which in humans comprise some 400 m^2, a considerably greater expanse than the skin surface (36). These surfaces are protected by numerous innate as well as inducible (specific) factors of immunity, the latter being largely represented by secretory immunoglobulin A (S-IgA) antibodies. Conventional parenteral immunization does not effectively induce S-IgA antibodies, which are the product initially of polymeric IgA-secreting plasma cells residing beneath the epithelium of mucosal surfaces and exocrine glands and are then transported through epithelial cells by means of polymeric Ig receptor, which becomes secretory component (SC) in the complete S-IgA molecule (36). The polymeric IgA-secreting cells are terminally differentiated B cells of the mucosal immune system that originate from various mucosal inductive sites, such as Peyer's patches of the small intestine and analogous lymphoepithelial structures in other mucosal tracts (35). Much recent attention has been given to the development of peroral or enteric immunization procedures designed to induce mucosal immune responses through the gut-associated lymphoid tissues (61).

Recent studies have demonstrated that the female genital tract contains elements of the mucosal immune system, as polymeric IgA-secreting cells occur in the subepithelial stroma (25), and the epithelial cells in the human fallopian tube and endocervix and in the rat uterus express SC (25, 47). Furthermore, although no anatomical structures equivalent to Peyer's patches have been identified in the genital tract, antibody responses can be induced by instilling antigens into the vagina or uterus (34, 38, 44, 52, 58, 64), by a combination of oral and vaginal or rectal routes, or by the targeted injection of antigens in the vicinity of local lymph nodes (27, 28). The magnitude and duration of these responses, however, are usually modest, although extensive studies in rats have revealed the importance of hormonal influences in both the inductive and effector phases of the genital mucosal immune response (63). It has become clear that the mucosal immune system, although integrated, is not uniform but rather is compartmentalized, so that application of immunogens to one inductive site preferentially elicits antibodies in adjacent or physiologically related effector sites (15, 40). In particular, enteric immunization, which stimulates the gut-associated lymphoid tissue, results in IgA antibody responses preferentially in the gut and proportionately less in the respiratory and genital tracts (15, 30, 50), whereas intranasal (i.n.) immunization, which stimulates the nasal lymphoid tissue (NALT) of rodents, is more effective in generating antibody responses in the respiratory tract and salivary glands (54, 65).

A major problem with enteric immunization is that most nonviable immunogens are readily digested within the gastrointestinal tract and have no tropism for the specialized absorp-

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tive M cells that overlie the Peyer's patches. Consequently, oral administration of most protein immunogens, even in large and repeated doses, usually elicits only modest mucosal immune responses that do not persist. Several strategies have been proposed to overcome these difficulties, including harnessing the exceptional properties of cholera toxin (CT) and its nontoxic B subunit (CTB), which have been shown to induce strong and long-lasting mucosal immune responses when administered orally in small doses to experimental animals and human subjects (8, 19, 30, 50). CT acts as an adjuvant that enhances responses to other immunogens administered with it in relatively large doses (11). CTB itself can function as a carrier for other antigens conjugated to it chemically or fused to it by genetic engineering (8, 10, 13, 20, 33). We and others have shown that intragastric (i.g.) administration of small doses of bacterial protein antigens conjugated to CTB induces strong S-IgA antibody responses to the coupled antigen in most secretions, including those of the gastrointestinal, respiratory, and genital tracts (50). However, most reports show that a small adjuvant dose of intact CT must be administered with the CTB-conjugated immunogen by the i.g. route to generate responses in rodents.

To develop this approach to mucosal immunization further, we have sought to use rhesus monkeys as a model that more closely resembles humans in lymphoid anatomy and physiology. However, monkeys appear to be substantially more resistant than humans to the pharmacological and immunological properties of CT and CTB administered i.g. For example, whereas a mouse (body weight, ~25 g) can tolerate the toxic effects of 5 to 10 μg of intact CT and develop strong antibody responses to it, the diarrheagenic dose of CT in humans (body weight, ~70 kg) is approximately 1 to 5 μg, and 1 mg of CTB is immunogenic (21); yet rhesus monkeys (body weight, ~4 kg) appear to tolerate 10 μg of CT and 250 μg of CTB i.g. and display little or no immune response to either (37, 57). We have consequently experienced difficulties in inducing mucosal immune responses in rhesus monkeys by the i.g. or even intraduodenal administration of relatively large doses of various well-characterized immunogens either mixed with CT or conjugated to CTB. Antibody responses to either CT or the coadministered antigen in serum, saliva, and intestinal secretions have been weak and dominated by IgM, and the numbers of specific antibody-secreting cells in the circulation approximately 1 week after immunization have also been low and, when found, largely of the IgM class (37).

In contrast, we and other groups have reported considerable success in generating strong mucosal immune responses to immunogens coupled to CTB and administered i.n. (2, 18, 23, 42, 54, 65). In rats and mice, this is most likely because these animals possess NALT, a bilateral strip of nonencapsulated lymphoid tissue beneath the posterior nasal passage and covered by M-like epithelial cells (24, 59). After i.n. immunization of mice, specific antibody-secreting cells can be found in the NALT and its draining cervical lymph nodes (14, 66). Moreover, there is a less stringent requirement for coupling of the coadministered antigen to CTB or for adding CT as an adjuvant by the i.n. route, and indeed, smaller doses are more effective than when given by the i.g. route (65). NALT is thought to be functionally equivalent to Waldeyer's ring of tonsils and adenoids in humans (24), and although these have received little attention as potential sites for inducing mucosal immune responses in humans, it is apparent from recent as well as earlier reports that the tonsils are inductive sites of the mucosal immune system (4, 43, 45). Rhesus monkeys possess tonsils that are anatomically similar to those of humans (41), and in both macaques and humans, lymphoid tissues are associated with the nasal epithelium (3, 16, 62). We therefore decided to determine whether mucosal immune responses could be induced by i.n. immunization of these nonhuman primates. The antigen chosen was a high-molecular-weight protein surface antigen (AgI/II) of the oral bacterium *Streptococcus mutans*, which was chemically conjugated to or mixed with CTB as in our previous studies (50, 65).

MATERIALS AND METHODS

Antigens. *S. mutans* AgI/II (M_r, 167,000) was purified as described previously (48). CT and CTB (<0.1% intact CT) were purchased from List Biological Laboratories, Inc. (Campbell, Calif.). G_{M1} ganglioside was obtained from Calbiochem-Novabiochem Corp. (San Diego, Calif.). Equimolar amounts of CTB (2 mg) and AgI/II (6 mg) were conjugated by using *N*-succinimidyl-(3-[2-pyridyl]-dithio)propionate (Pharmacia Biotechnology, Inc., Piscataway, N.J.) as described previously (50); the final conjugate retained the G_{M1} ganglioside-binding property of CTB and the antigenicity of both AgI/II and CTB, as shown by G_{M1} enzyme-linked immunosorbent assay (ELISA).

Animals, immunization, and sampling. Rhesus monkeys (*Macaca mulatta*) were bred and maintained at the White Sands Research Center, Alamogordo, N.Mex., where all experimental procedures were performed. Animals were caged individually, fed a standard monkey chow diet supplemented with fruit, and provided water ad libitum. Prior to the study, the animals received a complete physical examination, including complete blood count and serum chemistry panel, by a qualified veterinarian to ensure satisfactory health and were acclimated to laboratory conditions for at least 2 weeks. Ten young adult animals, aged 5.3 to 6.6 years and weighing 6.1 to 7.6 kg (males) or 4.9 to 6.5 years and 4.5 to 6.6 kg (females) were assigned to two groups of five, A and B, balanced for age, weight, and sex, so that there were three females and two males in each group. The monkeys were sedated with ketamine for all procedures. Animal maintenance and experimental procedures were in full compliance with the Animal Welfare Act and National Institutes of Health regulations, and the experiments did not duplicate any known previous studies. To facilitate management, the procedures on the two groups were staggered by 1 week, group A first and then group B, but the results are reported in terms of experimental weeks for each group.

Preimmunization samples (see below) were collected from each animal to establish baseline antibody levels. Animals in group A were then immunized with 250 μg of AgI/II-CTB conjugate in 2 ml of sterile phosphate-buffered saline (PBS) instilled i.n. (1 ml in each nostril) on days 0, 14, and 28; group B animals were likewise immunized with 200 μg of AgI/II mixed with 100 μg of CTB. One week after each immunization and also 2 weeks after the third dose, samples were collected from each animal for the analysis of immune responses. The animals were observed twice daily for any signs of abnormality and weighed on each day of immunization or sample collection.

The following samples were obtained at each collection time and shipped to the University of Alabama at Birmingham by overnight courier. Venous blood (10 ml) was collected in a heparinized vacuum tube and shipped at ambient temperature on the same day. Saliva was collected by aspiration from the cheek pouch after placing four to five drops of 2% pilocarpine under the tongue to stimulate flow, and 1% (vol/vol) 100 mM phenylmethylsulfonyl fluoride (PMSF) in isopropanol was added as a protease inhibitor. The saliva was stored at −70°C and shipped on dry ice. Nasal wash was obtained by inserting a soft 8 French pediatric feeding tube 2 to 4 cm into the nostrils on each side, instilling 3 ml of sterile PBS into the nasal cavity, and allowing the fluid to drain into a petri dish. PMSF (as above) was added to the wash fluid, which was stored at −70°C and shipped on dry ice. Vaginal wash was collected from the three female monkeys in each group by inserting a soft 8 French pediatric feeding tube into the vaginal vault while the pelvic region was elevated and flushing several times with 3 ml of sterile PBS by means of a syringe. PMSF was added (as above), and the wash was stored at −70°C and shipped on dry ice. Approximately 5 g of fresh feces was collected and extracted with 10 ml of PBS containing soybean trypsin inhibitor (10 μg/ml) and 50 mM EDTA, as described elsewhere (13). PMSF (as above) and sodium azide (0.1%) were added to the extract, which was stored at −70°C and shipped on dry ice.

Antibody assays. Plasma (after separation of cells from the blood; see below) was analyzed for IgM, IgG, and IgA antibodies to AgI/II and to CT by ELISA, using a modification of the procedures described previously for mouse antibodies (50). Plates were coated with either AgI/II (5 μg/ml) or G_{M1} ganglioside (2.5 μg/ml) followed by CT (1 μg/ml), and duplicate serial dilutions of plasma in an appropriate range for the particular analysis were incubated on the plates overnight. Bound antibodies were developed by applying suitably diluted biotinylated goat antibodies specific for rhesus monkey IgM, IgG, or IgA (37) for 4 to 6 h followed by avidin-peroxidase (1 μg/ml; Sigma Chemical Co., St. Louis, Mo.) for 1 h. Color development with a substrate consisting of *o*-phenylenediamine and H_2O_2 in citrate-phosphate buffer (pH 4.0) was stopped with sulfuric acid after 15 min and read at 490 nm in a V_{max} microplate reader (Molecular Devices Corp., Menlo Park, Calif.) interfaced to a Macintosh computer for data retrieval. Standard curves were determined for each plate by using serial dilutions of a

TABLE 1. Numbers of circulating cells secreting IgM, IgG, and IgA before and after immunization[a]

Wk	Mean no. of spot-forming cells/10^6 mononuclear cells (SD)[b] secreting:					
	IgM		IgG		IgA	
	Group A	Group B	Group A	Group B	Group A	Group B
−1	1,190 (605)	1,055 (1,849)	3,355 (1,100)	15,540 (7,446)	182 (246)	60 (13)
1	2,630 (1,075)	191 (82)	18,700 (9,196)	9,800 (7,229)	178 (65)	50 (21)
3	339 (265)	465 (266)	3,140 (2,812)	9,570 (1,460)	60 (70)	64 (28)
5	1,168 (93)	1,048 (1,457)	12,800 (4,833)	13,300 (9,162)	96 (40)	114 (48)

[a] Immunizations were given at 0, 2, and 4 weeks.
[b] ELISPOT assay, $n = 5$.

calibrated monkey serum on wells coated with goat antibodies to monkey IgM, IgG, or IgA, as appropriate. Unknowns were interpolated on standard curves generated by a computer program with four-parameter logistic algorithms (49), and parallelism between unknown and standard dilution curves was demonstrated over the range of unknown dilutions used for calculation.

Plasma IgE concentrations were determined by ELISA on plates coated with anti-human IgE (Dako) and developed with peroxidase-conjugated anti-human IgE (Dako). To calibrate the assay, the monkey serum standard was calibrated against a human serum Ig standard (The Binding Site, Birmingham, U.K.). To estimate specific IgE antibodies, an IgE capture ELISA was modified from a method already developed for estimating mouse IgE antibodies (65a). Plates were coated with anti-human IgE, and monkey plasma serially diluted from 1:10 was applied overnight. Antibodies present in the captured IgE were revealed by applying biotinylated AgI/II (1 μg/ml) or biotinylated CT (1 μg/ml; List Biological Laboratories) followed by avidin-peroxidase (1 μg/ml) and substrate as described above. Biotinylated AgI/II was prepared from 1 mg of AgI/II dissolved in 1 ml of 0.1 M $NaHCO_3$ treated with 4 μl of biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma; 50 mg/ml in dimethyl sulfoxide) for 4 h at room temperature; the reaction was quenched with 0.1 ml of 1 M NH_4Cl, and the product was dialyzed against PBS containing 0.02% NaN_3. IgE antibody results are presented in terms of titer, defined as the highest plasma dilution that yielded an absorbance reading greater than the mean + 3 standard deviations (SD) of the plate background absorbance obtained when diluent alone was substituted for the plasma samples.

Saliva, nasal and vaginal washes, and fecal extracts were analyzed for IgA antibodies and for total IgA concentrations in parallel by similar ELISA procedures with plates coated with antigens as described above or with goat antibodies to monkey IgA, respectively. A sample of monkey colostral whey calibrated for IgA was used as a standard. Nasal and vaginal washes were also analyzed for IgG antibodies. Antibody levels in secretions were expressed relative to total corresponding Ig concentrations to compensate for the variation in flow rate of secretions (known to affect Ig concentrations inversely) and for the unknown dilution factor in the case of secretions collected by washing or extraction.

Molecular form of vaginal IgA. Vaginal washes were analyzed by size exclusion chromatography on a Perkin-Elmer series 10 high-pressure liquid chromatography (HPLC) apparatus equipped with a silica gel column (30 by 0.78 cm; Biosep SEC-S3000; Phenomenex, Torrance, Calif.) calibrated for the elution volumes of monkey S-IgA and dimeric and monomeric serum IgA (49). The eluent was 0.02 M phosphate (pH 6.8) plus 0.05 M Na_2SO_4 pumped at 1 ml/min, and fractions of three drops (~120 μl) were collected and tested for IgA by ELISA as described above. To ensure that apparently polymeric IgA represented true polymers and not immune complexes, the chromatography was repeated in a dissociating buffer (0.1 M acetate [pH 3.6] plus 0.05 M Na_2SO_4), and fractions were collected in tubes containing 0.1 ml of 1 M Tris-HCl (pH 8).

To determine whether vaginal wash IgA contained SC, 0.5-ml samples were concentrated on 10-kDa-cutoff centrifugal ultrafilter units (Ultrafree-MC; Millipore Corp., Bedford, Mass.). The concentrates were extracted from the filter in 15 μl of 5× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and electrophoresed under nonreducing conditions on 5% polyacrylamide gels. The separated bands were transferred to nitrocellulose by Western immunoblotting (17), and replicate blots were probed with biotinylated goat antibodies to monkey IgA (1:500) or to monkey SC (1:500). Bands were revealed by developing with avidin-peroxidase (1 μg/ml) and 3-amino-9-ethylcarbazole–H_2O_2 substrate. Biotinylated anti-monkey SC was prepared from the IgG fraction of goat antiserum to monkey SC (Nordic Immunological Laboratories, Capistrano Beach, Calif.), isolated on a Mono Q column (Pharmacia) equilibrated with 0.01 M Tris-HCl (pH 7.65). The IgG peak was precipitated with 50% saturated ammonium sulfate, redissolved and dialyzed against 0.1 M $NaHCO_3$, treated with biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma) dissolved in dimethyl sulfoxide (0.1 mg/mg IgG) for 4 h at room temperature, and dialyzed against PBS. Preliminary trials showed that the anti-SC reagent identified a ~80-kDa band corresponding to free SC in a reduced sample of monkey colostrum as well as high-M_r bands corresponding to S-IgA in unreduced colostrum. Anti-monkey IgA detected only the ~55-kDa IgA heavy-chain bands in reduced serum and colostrum samples, and avidin-peroxidase did not react with any bands.

Assay of antibody-secreting cells. Ig- and antibody-secreting cells were enumerated by ELISPOT assay (6) with mononuclear cells separated from heparinized blood by centrifugation on Ficoll-Hypaque; remaining erythrocytes were lysed in buffered ammonium chloride, and the cells were washed and resuspended in RPMI 1640 medium containing 10% fetal calf serum. Membrane-based microtiter plates (Multiscreen-HA; Millipore Corp.) were coated with goat antibodies to monkey IgM, IgG, or IgA, with AgI/II, or with G_{M1} and CT as described for ELISA and blocked with 10% fetal calf serum–RPMI 1640. Aliquots (0.1 ml) of mononuclear cell suspensions (up to 5×10^5 cells per well) were incubated at 37°C under 5% CO_2 in air for 3 h. After the cells were washed off, biotinylated goat antibodies to monkey IgM, IgG, or IgA were added, followed by avidin-alkaline phosphatase and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt–nitroblue tetrazolium chloride substrate. Spots were enumerated under a dissecting microscope and related to the number of cells plated.

Statistical analysis. The significance of the difference of the means between groups A and B was evaluated by Student's *t* test for unpaired samples performed on logarithmically transformed data. Group data are therefore presented as geometric means ×/÷ SD after back-transformation of the logarithmic means ± SD.

RESULTS

None of the monkeys displayed any adverse reactions or abnormalities as a result of the immunization and sampling regimen, and there was no loss of weight or appetite in any animal. The animals were returned to the colony in good health after the close of the study.

Circulating antibody-secreting cells. Prior to immunization and 1 week after each dose, peripheral blood mononuclear cells were assayed by ELISPOT to enumerate cells secreting IgM, IgG, or IgA irrespective of specificity or specific for either AgI/II or CT. The total number of cells secreting Igs fluctuated but not consistently with the immunizations (Table 1). The numbers of antibody-secreting cells were therefore expressed relative to total corresponding Ig isotype-secreting cells. There were no cells secreting antibodies specific for AgI/II or CT in the preimmune samples, but commencing with the week after the first immunization dose, cells secreting IgA antibodies to AgI/II and to CT became easily detectable (Fig. 1). In group A animals, the numbers of these cells relative to all IgA-secreting cells rose to mean peaks of ~13% (anti-AgI/II) and ~7% (anti-CT) after the third dose, whereas in group B animals, the peak responses appeared to occur after the second immunization, ~25% (anti-AgI/II) and ~14% (anti-CT). The numbers of circulating antibody-secreting cells of IgM and IgG isotypes remained low throughout the immunization period.

Plasma antibody responses. Plasma IgM, IgG, and IgA antibodies to AgI/II and to CT are shown in Fig. 2. In both groups, IgG was the predominant plasma antibody response, commencing after the second immunization. IgA antibodies showed parallel increases but at a lower level, whereas IgM antibodies changed little during the experimental period. Student's *t* tests revealed significant differences ($P < 0.05$) be-

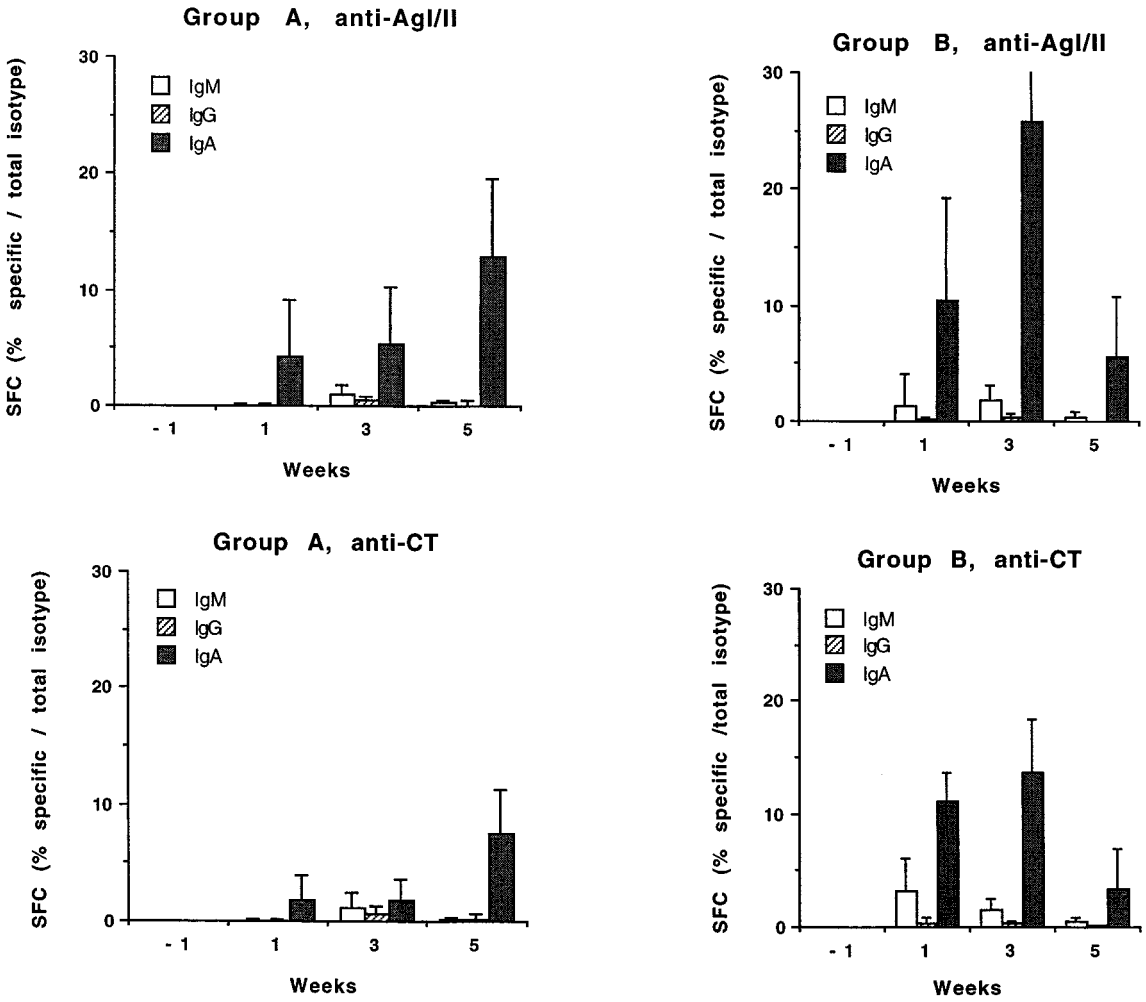

FIG. 1. Peripheral blood cells secreting IgM, IgG, and IgA antibodies to AgI/II and CT before (−1 week) and after (1, 3, and 5 weeks) immunization at 0, 2, and 4 weeks with AgI/II-CTB conjugate (group A) or AgI/II mixed with CTB (group B). Results are the numbers of antigen-specific spot-forming cells (SFC) expressed as a percentage of total Ig isotype SFC in an ELISPOT assay (mean ± SD, $n = 5$ animals).

tween the groups only for IgM anti-AgI/II at weeks 1 and 5 after the commencement of immunization. Statistically significant differences in some preimmune antibody levels were also recorded: IgM anti-AgI/II and anti-CT and IgA anti-CT. Overall, therefore, we interpret these results as showing no essential difference between the responses in the two groups.

Because of concerns that the use of CT as a mucosal adjuvant may promote IgE antibodies, which would be undesirable (31, 51, 56), we also evaluated IgE responses. Plasma IgE concentrations did not change consistently over the course of the immunization period, although some individual animals showed fluctuations in this parameter (Table 2). Likewise, titers of IgE antibodies to AgI/II did not change significantly over the immunization period (Table 2). In group A, IgE antibodies to CT appeared to rise after the second immunization but then declined by the later sampling times. Overall, we interpret these results as showing no IgE antibody response to the immunizations.

Salivary IgA antibody responses. The concentrations of total salivary IgA remained fairly constant over the course of the immunization period (Table 3), although there were differences between individual animals, ranging from 45 to 545 μg/ml. Salivary IgA antibodies to AgI/II and to CT, expressed relative to the total IgA concentration for each sample, showed increases after the second immunization (Fig. 3) in both groups of monkeys. Responses to AgI/II were equal to or greater than responses to CT. Total IgA concentrations and specific antibody levels tended to be less variable in group B than in group A animals, and preimmune mean levels of both parameters also appeared to be lower in group B, but because of the interanimal variation, these were not statistically significant (t test). Salivary IgA-specific antibody levels at the end of the experiment were similar in both groups.

Nasal secretion antibodies. Both IgA and IgG antibodies in nasal washes were assessed. Total IgA concentrations remained fairly constant over the study period (Table 4), ranging from 5.53 to 75.8 μg/ml in different individual samples. Both groups showed elevations in specific IgA antibodies to both AgI/II and CT after the second immunization, but antibodies to CT were generally at higher levels than those to AgI/II at all time points, including the preimmune samples (Fig. 4). As with salivary antibodies, nasal wash IgA antibodies to AgI/II in preimmune samples appeared to be significantly higher in group A than in group B ($P < 0.01$), but there were no significant differences in antibody levels at the end of the study period.

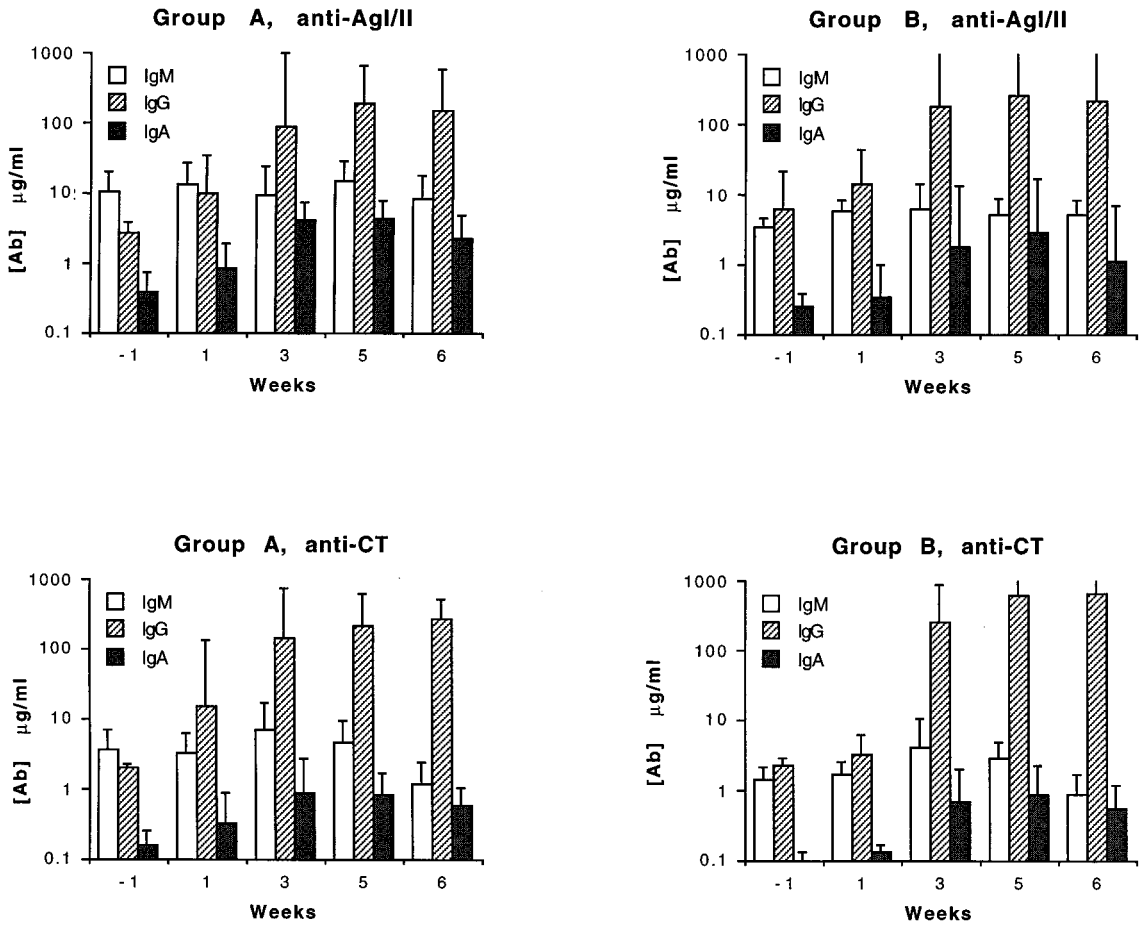

FIG. 2. Serum IgM, IgG, and IgA antibody (Ab) responses (geometric mean ×/÷ SD, $n = 5$ animals) to AgI/II and CT before (−1 week) and after (1, 3, 5, and 6 weeks) immunization at 0, 2, and 4 weeks with AgI/II-CTB conjugate (group A) or AgI/II mixed with CTB (group B).

Because human nasal washes contain IgG which is probably derived from the circulation by transudation (60), we also evaluated nasal IgG antibodies in the monkeys. Nasal washes contained only low concentrations of total IgG (0.07 to 10.2 µg/ml; overall geometric mean, 0.76 ×/÷ 2.56 µg/ml), and specific antibodies were undetectable in the preimmune samples. However, steady rises in IgG antibodies to both AgI/II and CT were recorded in both groups during the immunization period (Fig. 5). Overall, no consistent statistically significant differences were found between the two groups. Nasal wash IgG antibody responses were significantly correlated with serum antibody responses ($P < 0.005$ for anti-AgI/II and $P < 0.05$ for anti-CT, Spearman's rank correlations of nasal wash IgG antibodies with serum IgG antibodies at 5 and 6 weeks, taking groups A and B together), as expected if nasal antibodies are derived by transudation from the plasma. In contrast, nasal IgA antibodies showed no correlation with serum IgA antibodies, indicating that these are derived from independent sources.

Vaginal secretion antibodies. Total IgA concentrations in vaginal washes varied considerably, even in the same animal, during the course of the study, ranging from undetectable

TABLE 2. Total serum IgE and IgE antibody responses[a]

Wk	Total IgE[b], ng/ml (×/÷ SD)		Geometric mean titer[c] (×/÷ SD)			
			Anti-AgI/II		Anti-CT	
	Group A	Group B	Group A	Group B	Group A	Group B
−1	185 (1.60)	58.7 (1.24)	26.4 (1.5)	26.4 (1.5)	3.3 (5.2)	9.6 (4.0)
1	160 (2.29)	165 (2.29)	9.6 (3.7)	26.4 (1.5)	12.2 (3.4)	4.6 (4.1)
3	130 (2.07)	52.9 (1.44)	23.0 (2.1)	13.2 (1.9)	26.7 (1.9)	17.4 (1.4)
5	145 (2.0)	59.3 (1.33)	7.2 (3.1)	11.0 (4.9)	4.9 (8.8)	2.5 (3.5)
6	167 (2.29)	80.1 (2.56)	15.2 (1.5)	12.6 (4.3)	7.2 (3.1)	6.3 (2.8)

[a] Immunizations were given at weeks 0, 2, and 4.
[b] Geometric means, $n = 5$.
[c] Geometric mean titers, $n = 5$.

TABLE 3. Salivary IgA concentrations[a]

Wk	Geometric mean concn[b], μg/ml (×/÷ SD)	
	Group A	Group B
−1	196 (2.95)	126 (1.74)
1	165 (2.10)	169 (1.69)
3	192 (1.88)	110 (1.43)
5	135 (1.73)	87.0 (2.30)
6	108 (2.26)	109 (1.70)

[a] Immunizations were given at weeks 0, 2, and 4.
[b] Geometric means, $n = 5$.

TABLE 4. Nasal wash IgA concentrations[a]

Wk	Geometric mean concn[b], μg/ml (×/÷ SD)	
	Group A	Group B
−1	19.0 (2.06)	37.6 (1.96)
1	19.8 (2.42)	26.2 (1.45)
3	21.7 (1.56)	22.3 (1.34)
5	22.3 (1.88)	43.0 (1.33)
6	36.4 (2.48)	28.1 (1.68)

[a] Immunizations were given at weeks 0, 2, and 4.
[b] Geometric means, $n = 5$.

(<0.1 μg/ml) to 14.7 μg/ml and from 1.48 to 40.1 μg/ml, for example, in two different animals. Because of these fluctuations, which make vaginal IgA antibody responses more difficult to interpret than in the case of other secretions, these data are presented as antibody levels together with total IgA concentrations. Increased levels of specific IgA antibodies to both AgI/II and CT were found in both groups A and B after the second immunization dose (Fig. 6). There were no consistent significant differences in antibody responses between the two groups.

Vaginal wash samples were also analyzed for IgG antibodies. Concentrations of total IgG varied considerably, as expected, from undetectable in many samples (<1 μg/ml) to 86.4 μg/ml. IgG antibodies to both AgI/II and CT were detectable in those samples having readily detectable IgG levels after the second immunization (Table 5), but there were no significant differences in antibody levels between the two groups when there were sufficient numbers of IgG-positive samples to analyze.

Because of the possible contribution of significant quantities of plasma-derived Igs to the genital tract (26), attempts were made to correlate IgA and IgG antibody responses in vaginal wash samples taken 3, 5, and 6 weeks after the commencement

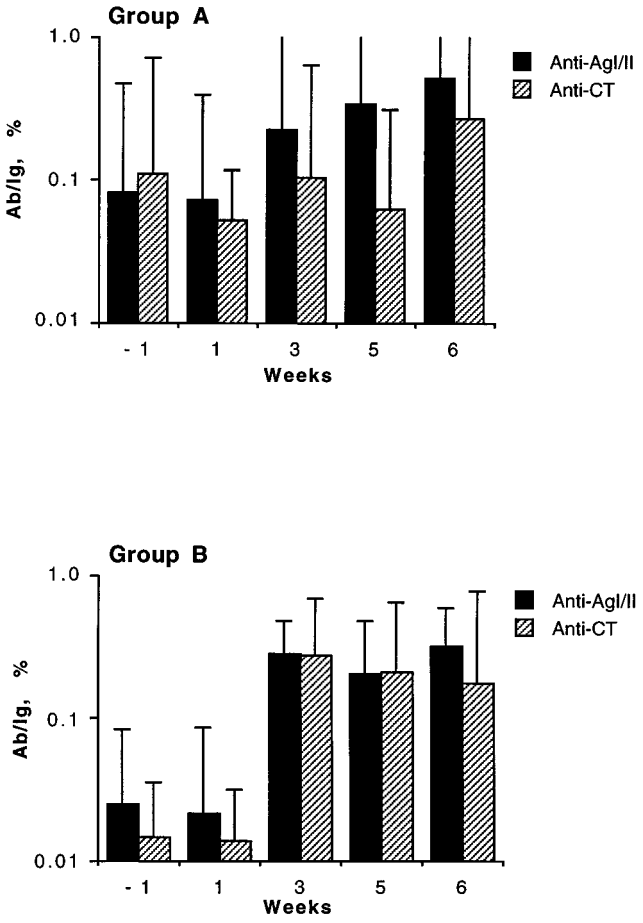

FIG. 3. Salivary IgA antibody responses to AgI/II and CT before (−1 week) and after (1, 3, 5, and 6 weeks) immunization at 0, 2, and 4 weeks with AgI/II-CTB conjugate (group A) or AgI/II mixed with CTB (group B), expressed as a percentage of total salivary IgA (geometric mean ×/÷ SD, $n = 5$ animals).

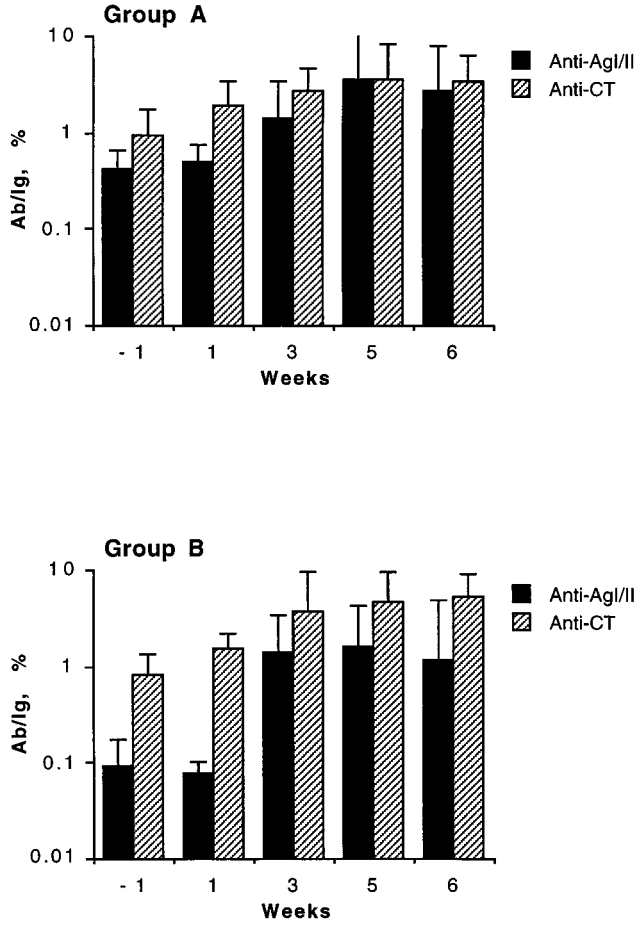

FIG. 4. Nasal IgA antibody responses to AgI/II and CT before (−1 week) and after (1, 3, 5, and 6 weeks) immunization at 0, 2, and 4 weeks with AgI/II-CTB conjugate (group A) or AgI/II mixed with CTB (group B), expressed as a percentage of total nasal IgA (geometric mean ×/÷ SD, $n = 5$ animals).

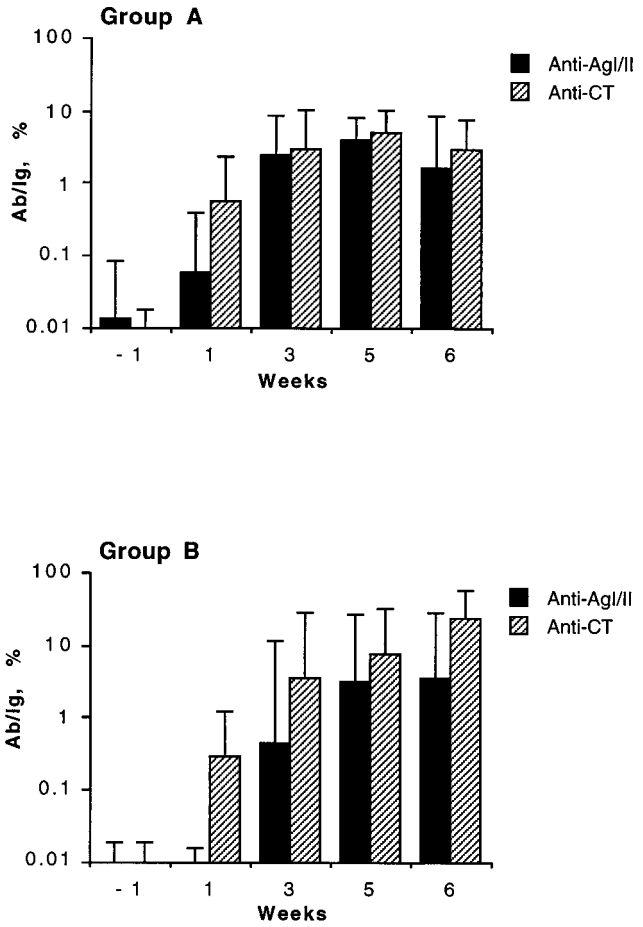

FIG. 5. Nasal IgG antibody responses to AgI/II and CT before (−1 week) and after (1, 3, 5, and 6 weeks) immunization at 0, 2, and 4 weeks with AgI/II-CTB conjugate (group A) or AgI/II mixed with CTB (group B), expressed as a percentage of total nasal IgG (geometric mean ×/÷ SD, $n = 5$ animals).

of immunization with IgA and IgG antibody responses in the plasma of the same animals, taking groups A and B together. However, no significant correlations were found even if vaginal IgA and IgG antibodies were expressed as a proportion of total IgA and IgG concentrations, whether parametric or nonparametric statistics were used.

Molecular form of vaginal IgA. It is likely that marked fluctuations in vaginal Ig levels occur during the menstrual cycle, as a result of variations in secretion of S-IgA in cervical mucus (26) as well as admixture of plasma Igs during menses, but information on hormonal cycles was not available for this study. However, none of the vaginal wash samples in this study was visibly stained with blood. In an attempt to evaluate the contribution of plasma IgA to vaginal IgA, selected vaginal wash samples (chosen on the basis of availability of sufficient material and including samples having high and low IgA concentrations as well as from different experimental time points) were analyzed for the molecular form of IgA by size exclusion HPLC. Most samples displayed two peaks coinciding with the elution positions of colostral polymeric IgA (S-IgA) and monomeric serum IgA, and although the proportions of these varied in different samples, the polymer peak was always present (Fig. 7). The polymer peak was accentuated relative to the monomer peak when the samples were rechromatographed under dissociating conditions (Fig. 7), implying that it represented true polymeric IgA rather than immune complexes or aggregates.

To confirm whether the polymeric IgA in vaginal washes represented true S-IgA, selected samples were analyzed for the presence of SC complexed with polymeric IgA by Western blotting. Some samples contained high-molecular-weight IgA (M_r, ~400,000) revealed by a band reacting with anti-IgA of the same mobility as colostral S-IgA, and a similar band was detected in these samples by antibody to SC (Fig. 8). Free SC (M_r, ~80,000) was detected in the colostral standard and in some vaginal wash samples (Fig. 8). Monomeric IgA migrating at M_r ~150,000 together with lower-M_r IgA-positive bands, presumably half-molecules or breakdown products of IgA, were also present in a few vaginal samples (Fig. 8). In many samples, however, the concentration of IgA was too low for bands to be detectable by Western blotting, even though the washes were concentrated by ultrafiltration and extraction of the filters.

Intestinal IgA antibody responses. Fecal extracts contained highly variable concentrations of total IgA, as estimated by ELISA, ranging from 0.25 to 235 μg/ml in samples from different animals and varying considerably also in sequential samples from the same animal. Group A animals displayed only a small response in IgA antibodies to AgI/II at week 1, and group B animals displayed it only at week 3 (data not shown).

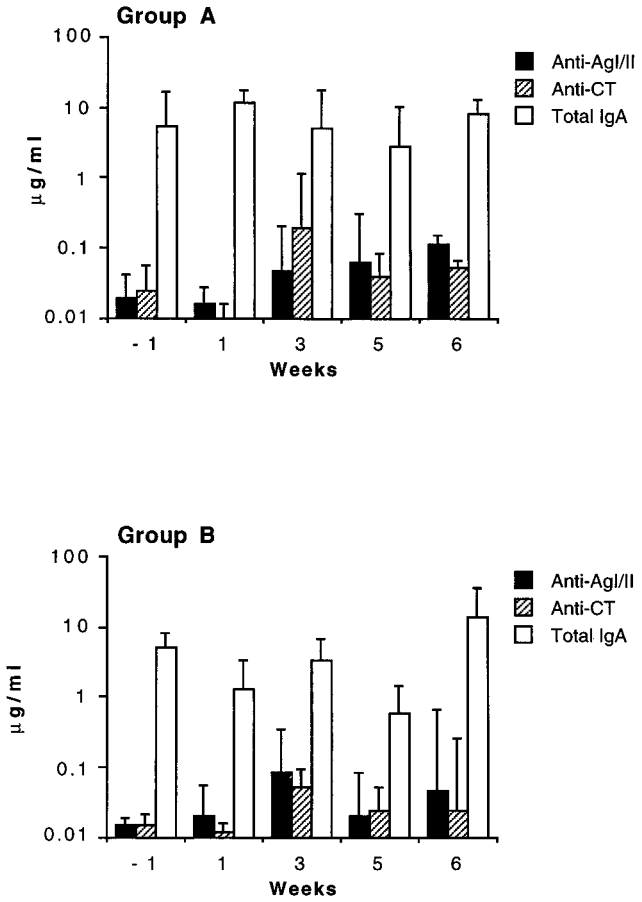

FIG. 6. Vaginal IgA antibody responses to AgI/II and CT before (−1 week) and after (1, 3, 5, and 6 weeks) immunization at 0, 2, and 4 weeks with AgI/II-CTB conjugate (group A) or AgI/II mixed with CTB (group B). Specific antibody levels are shown in comparison with total vaginal IgA concentrations (geometric means ×/÷ SD, $n = 5$ animals).

TABLE 5. Total IgG and IgG antibodies in vaginal washings[a]

Wk	Geometric mean concn[b], μg/ml (×/÷ SD)					
	Anti-AgI/II		Anti-CT		Total IgG	
	Group A	Group B	Group A	Group B	Group A	Group B
−1	0.006 (1.40)	0.005[c] (5.90)	0.001 (25.1)	0[c]	21.5 (2.94)	5.06[c] (5.74)
1	0.001 (1.87)	0.001[d]	0	0[d]	6.47 (3.84)	1.17[d]
3	0.154[c] (9.69)	0.690[d]	0.046[c] (6.79)	0.053[d]	15.2[c] (11.7)	15.8[d]
5	0.078 (9.34)	0.170[d]	0.023 (61.0)	0.025[d]	4.71 (2.55)	8.73[d]
6	0.172[c] (5.58)	0.029 (16.9)	0.090[c] (5.77)	0.013 (38.6)	15.7[c] (3.26)	3.71 (2.70)

[a] Immunizations were given at weeks 0, 2, and 4.
[b] Geometric means, $n = 3$ except as noted.
[c] $n = 2$ (IgG undetectable in one sample).
[d] $n = 1$ (IgG undetectable in two samples).

Group A animals also responded weakly with IgA antibodies to CT at weeks 1 and 3, but group B fecal extracts displayed higher preimmune anti-CT IgA antibody levels and did not show elevations during the immunization period. The highly variable concentrations of IgA and levels of IgA antibodies in fecal extracts probably reflect degradation in vivo by intestinal and microbial proteases (12).

DISCUSSION

In marked contrast to the previously reported modest responses in monkeys immunized i.g. or intraduodenally (37), i.n. immunization of rhesus monkeys with *S. mutans* AgI/II, either chemically conjugated to or mixed with CTB, resulted in readily detectable antibody responses to both components of the vaccine in plasma, saliva, and nasal and vaginal secretions. Plasma antibody responses were predominantly IgG, as expected, together with an IgA response which may be underestimated because of its shorter circulating half-life (5), and IgA antibodies were found in saliva and in nasal and vaginal washes. In general, antibody responses to AgI/II were comparable to those to CTB (assayed on CT for reasons of economy). If CTB can be regarded as a potent mucosal immunogen in monkeys, at least by the i.n. route, as it is in rodents and humans, then these findings imply that the present strategies of immunization result in similarly potent responses against the coadministered antigen. The volume of buffer in which the immunogen was administered was chosen to be sufficient to wet the nasal passages and reach the tonsils in the pharynx but not so large as to flood into the trachea or esophagus. Because of previous failures to generate strong immune responses by i.g. administration of similar vaccine materials in monkeys, we may reasonably infer that stimulation of gut-associated lymphoid tissue by swallowed immunogen cannot account for the responses obtained. However, the possible contribution of responses generated in the tracheobronchial tree, a route of immunization which has been found to induce vaginal antibodies in monkeys (32), cannot be eliminated. The present experimental design did not include monkeys immunized i.n. with AgI/II alone because of the failure of i.g. immunization to induce responses to AgI/II regardless of whether CT was given as an adjuvant or if AgI/II was coupled to CTB. The original intention of this study was to determine if, as in rodents, i.n. immunization with AgI/II coupled to or mixed with CTB would generate mucosal immune responses in monkeys and, if so, how long these would persist, whether they could be recalled by booster immunizations given later, and whether i.n. immunization would afford protection against oral colonization with *S. mutans* and the consequent development of dental caries. For reasons beyond our control, these further aims could not be pursued.

These results demonstrate the dissemination of IgA antibody responses induced i.n. to several distinct effector sites of the mucosal immune system, including the orogastrointestinal and genital tracts. Although the quantification of antibodies in fecal extracts was unsatisfactory, probably because of degradation of IgA in the feces (12), this method of assessment was the only practicable means available to us, and only weak and intermittent IgA antibody responses could be inferred. Salivary, nasal, and genital IgA antibody responses were much clearer and consistent with each other. Moreover, the finding of distinct IgA antibody-secreting cell responses in the peripheral blood after each immunization clearly demonstrated the trafficking of these cells from inductive to effector sites of the common mucosal immune system, as established in humans and other experimental animals (7–9, 22, 29, 35, 39, 40, 46, 50, 65).

Previous extensive studies in mice had shown that i.n. immunization with AgI/II coupled to CTB was highly effective at inducing IgA antibodies in saliva and other secretions as well as predominantly IgG antibodies in the circulation (65). I.n. immunization with AgI/II mixed with CTB greatly enhanced the response to AgI/II alone, but this was consistently lower than the response to AgI/II-CTB conjugate, and in neither case did the addition of CT as an adjuvant further elevate the antibody levels. We therefore anticipated that comparable differential responses would be elicited by i.n. immunization of monkeys. However, we cannot infer significant differences between the responses in monkeys immunized i.n. with AgI/II conjugated to CTB and those immunized with a mixture of the two components. A further divergence of results obtained in these two species is that monkeys responded after the second immunization dose, whereas mice required three immunizations (65) before substantial elevations of antibody levels occurred.

Both IgA and IgG were present in vaginal washings at broadly comparable concentrations, although both varied markedly between animals and over time, and both IgA- and IgG-specific antibodies to the component antigens of the immunogen were detected. Sources of variation in Ig concentrations must include the efficiency of washing in collecting the samples as well as changes in secretion or transport of Igs into the genital tract during the course of hormonal cycles. It has been shown that secretion of IgA into cervical mucus in humans is maximal shortly before ovulation (26), and estrogens and progesterone influence the expression of SC in uterine cells and the recruitment of IgA-secreting cells in the genital tract of rats (63). Furthermore, the presence of plasma-derived

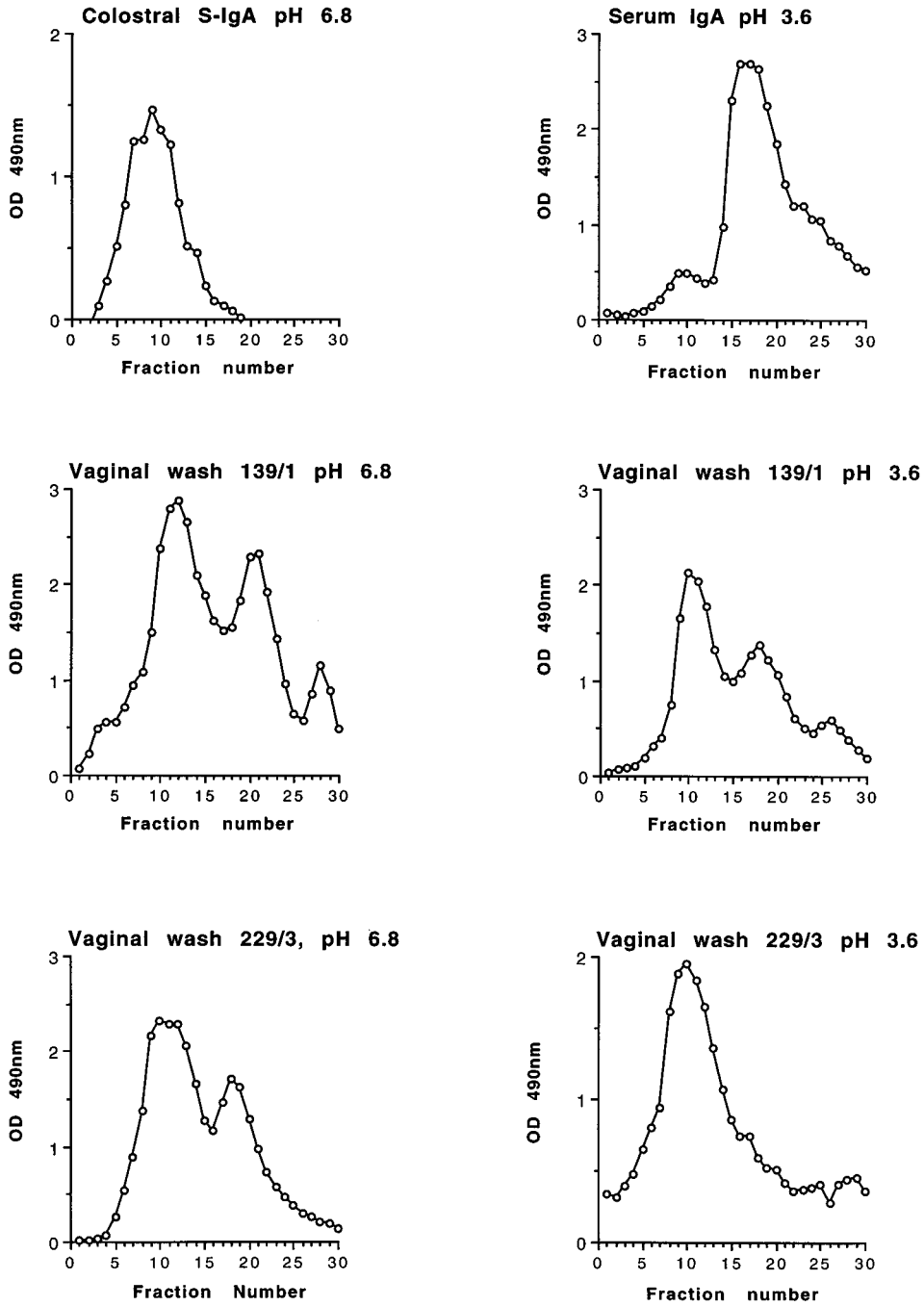

FIG. 7. Polymeric and monomeric forms of vaginal IgA shown by size exclusion HPLC. Panels show the elution profiles (OD_{490} in ELISA) of monkey colostral S-IgA and serum IgA as markers and two representative vaginal wash samples (from animals 139 and 229, taken at 1 and 3 weeks, respectively) chromatographed at neutral and acidic pHs.

Igs in the genital tract varies considerably with the menstrual cycle. However, none of the female monkeys was menstruating at the time of sample collection. The finding that IgA concentrations and the IgA antibody response were comparable to those of IgG suggests that at least some of the IgA must be locally synthesized rather than derived by transport or transudation from the plasma. Plasma antibodies induced by i.n. immunization were overwhelmingly of the IgG isotype, and in monkeys, the concentration of IgG in plasma is sixfold higher than that of IgA (37). Furthermore, analysis of the molecular form of IgA in the vaginal washes showed that substantial proportions of polymeric IgA were present, whereas monomeric IgA predominates in the circulation of monkeys, as in humans (25). Some samples of vaginal wash revealed the presence of high-M_r forms of IgA associated with SC. Thus, at least part of the genital tract IgA included S-IgA, most likely originating in the mucosal immune system. However, despite the lack of correlation, our data do not eliminate the possibility that plasma-derived IgA and particularly IgG antibodies may also have contributed to those found in the vaginal wash. On

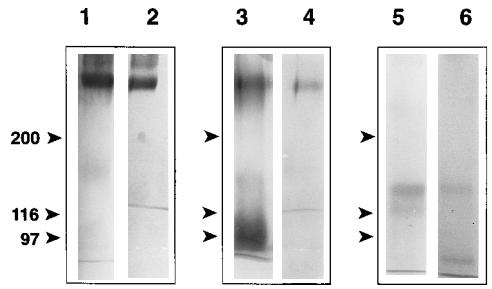

FIG. 8. Western blots showing polymeric IgA associated with SC, monomeric IgA, and free SC in vaginal wash samples. Lanes 1 and 2, monkey colostral S-IgA (standard); lanes 3 and 4, vaginal wash sample from animal 229, taken at 3 weeks; lanes 5 and 6, vaginal wash sample from animal 265, taken at 5 weeks. Lanes 1, 3, and 5, anti-monkey IgA; lanes 2, 4, and 6, anti-monkey SC. The migration positions of molecular mass standards (200, 116, and 97 kDa) are indicated by arrowheads on the left.

the other hand, careful estimates of specific antibody activity relative to total corresponding isotype in pre-AIDS patients suggest that even IgG antibodies may also be locally synthesized in the human female (and male) genital tract (1).

There has been controversy over the contribution of and requirement for intact CT in preparations of CTB used as a mucosal adjuvant. Commercially available CTB usually contains small amounts of intact toxin, and recent reports have indicated that recombinant CTB (or the homologous B subunit of *Escherichia coli* heat-labile enterotoxin), devoid of toxicity, not only lacks adjuvant activity (55) but actually induces oral tolerance (53). As the CTB preparations used in this study contained <0.1% CT, which is ample to act as an adjuvant by the i.n. route in mice (65), we cannot eliminate this effect in our conclusions. However, this level of contamination is inadequate to permit CTB to serve as an adjuvant by the i.g. route in mice or monkeys. Furthermore, recent experiments in our laboratory with recombinant CTB either mixed with or conjugated to AgI/II have shown that i.n. immunization of mice with this antigen does not depend upon trace amounts of intact CT (65a). Moreover, a genetically engineered chimeric protein containing a 42-kDa fragment of AgI/II linked to CTB is immunogenic alone by the i.g. or i.n. route, although the addition of CT as an adjuvant enhances the mucosal IgA response (13). Further work will be necessary to resolve these uncertainties in primates. However, it should be noted that highly purified or recombinant CTB is immunogenic by the i.g. route in humans in the absence of CT (9, 21), which is far too toxic for human use.

One concern expressed about the use of CT as a mucosal adjuvant is that it has been shown to induce IgE antibody responses when administered i.g. together with some but not all protein antigens in mice (31, 51, 56). Although this effect seems to vary with different mouse strains and is not induced by CTB, it would compromise the use of CT or CTB as immunoenhancing agents, especially by the i.n. route. However, we did not detect any IgE antibody response to either AgI/II or CT itself as a result of i.n. immunization in monkeys, and no adverse reactions to repeated i.n. immunization were reported.

The results of the present study demonstrate the dissemination of IgA antibody responses to the salivary glands as well as to the nasal and genital secretions after i.n. immunization. Our previous studies with rodents and humans have shown that the i.n. route is particularly effective for generating IgA antibodies in the saliva and indeed in the genital tract (23, 39, 65). Moreover, the finding of distinct IgA antibody-secreting cell responses in the peripheral blood after each immunization demonstrated the circulation of these cells from inductive to effector sites of the common mucosal immune system (7, 9, 22, 29, 35, 39). Thus, it is clear that i.n. immunization is an effective route for generating mucosal immune responses in a primate, at least when the vaccine includes CTB as an enhancing agent. The mechanisms underlying such a differential distribution of IgA antibody-secreting cells have not been clarified, but recent studies with human subjects have shown that the circulating IgA antibody-secreting cells induced by intratonsillar injection frequently display L-selectin and less often the gastrointestinal homing receptor $\alpha_4\beta_7$ integrin, whereas the cells induced by oral administration of CTB show more $\alpha_4\beta_7$ integrin and less L-selectin (46). Thus, IgA-secreting cells induced in the upper respiratory tract may have a greater propensity to relocate in compartments other than the gastrointestinal tract, such as the genital tract and rectum as well as the respiratory tract and salivary glands. The exact site and cellular mechanisms involved in the induction of these responses, and why the i.n. route is more effective than i.g. immunization, remain to be clarified.

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