Impairment by Mucosal Adjuvants and Cross-Reactivity with Variant Peptides of the Mucosal Immunity Induced by Injection of the Fusion Peptide PADRE-ELDKWA

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Secretory immunity protects against mucosal transmission of viruses, as demonstrated with the oral poliovirus vaccine. In a previous study we showed that this immunity could be induced in mice by injection of a fusion peptide consisting of an unnatural peptide-like sequence (PADRE) and a viral epitope (ELDKWASLW). PADRE is a T-helper-cell epitope able to bind most major histocompatibility complex class II molecules of different haplotypes in mice and humans and to increase antibody responses. ELDKWA is a well-known consensual sequence of gp41 involved in a key structure of human immunodeficiency virus (HIV) type 1. Here, the antibody response to the native form of ELDKWA was mainly of the immunoglobulin A isotype and selectively occurred in mucosa. Adjuvants, such as cholera toxin and cytosome polyguanine, were useless and even competed with PADRE for the response. Interestingly, these antibodies were cross-reactive with the three major variants of the epitope, as shown both by direct enzyme-linked immunosorbent assay and by inhibition. This unconventional route of mucosal immunization allows control of the administered dose. The lack of adjuvant and the cross-reactivity of the antibodies increase the safety and the spectrum of the candidate vaccine, respectively. The drug-like nature of the construct suggests further improvements by synthesis of more antigenic sequences. The reasonable cost of short peptides at the industrial level and their purity make this approach of interest for future vaccines against mucosal transmission of HIV or other pathogens.

Vaccination is a method of choice for eradication of infectious diseases, including infection by HIV-1. Unfortunately, despite tremendous efforts using sophisticated methods of molecular biology, no clear protection against this disease has emerged from traditional approaches of vaccination of the systemic immune system in naive subjects. Additional methods of immunization against the virus thus are of interest to complement, potentiate, or replace the usual strategies. This is the case for vaccines inducing local immunity, which may protect against mucosa-transmitted viral diseases, as demonstrated by the efficacy of the oral polio vaccine in humans (30). Nevertheless, vaccination of the mucosal immune system rarely has been successful (6), leading to investigations of unconventional methods of inducing mucosal immunity by i.m. administration of a soluble Ag. This selected Ag was a fusion peptide consisting of a protective epitope of HIV-1 gp 41 (ELDKWA) with an unnatural major histocompatibility complex class II-binding peptide (PADRE).

In contrast with the systemic immune system, which is involved in protection against pathogens within the body, mucosal immunity forms a barrier preventing penetration of microorganisms through the epithelial border. Except for reducing the viral load in the gut reservoir (4), this immunity is rarely involved in recovery from disease. Conversely, it can decrease the inoculum to a harmless level (17). pIgA is the major isotype produced by mucosal plasma cells (8). It is actively transported across the epithelium with the help of the pIg receptor (22).

The complex of pIgA with its receptor is cleaved from the apical membrane to form slgA, which is then released in the mucosal lumen. The major functions of slgA include immune exclusion and immune elimination. Immune exclusion (31) maintains the pathogen in the mucosal lumen by various mechanisms, such as agglutination, neutralization, and inhibition of interactions with the epithelium. Immune elimination also involves various mechanisms, such as clearance of intracellular or subepithelial pathogens by their transport to the lumen during transcytosis of the corresponding Abs (20). In the secretory lumen, the immune complexes formed with slgA are eventually cleared by conveyance with the mucus stream. These mechanisms explain why a neutralizing activity of Abs in the mucosa is of interest but not an absolute requirement for protection against pathogens (9). However, since the secretory immune system acts only during the first step of infection, a preliminary contact with the Ag is a prerequisite for its role of immune barrier against transmission when the activity of natural Abs is overwhelmed (20, 27).

The mechanisms of induction and development of a mucosal immune response differ from those of systemic immunity. The first step consists of a translocation of the luminal pathogen to Ag-presenting cells via epithelial M cells (18). The Ag-presenting cells are located in the mucosa-associated lymphoid tissues, where specific T and B lymphocytes are selected. Following a maturation cycle in the circulation, the B cells gain access to effector areas where Abs are produced by plasma cells dispersed throughout the subepithelial stroma. Induction of this humoral immunity is delayed, and the response is compartmentalized (5, 7, 21). Moreover, the persistence of the secretory immune response usually depends on that of the corresponding Ag. Therefore, most research programs deal with
In the present study, our aim was to improve this mucosal immune response to the i.m.-administered fusion peptide. We investigate two mucosal adjuvants, since we have already observed that Alum, the most commonly used systemic adjuvant, exerts an inhibitory effect on this response (12). We show that these adjuvants, CT and CpG, also suppress the response and are therefore not suitable for this immunization. In addition, we analyze the reactivity of the mucosal IgA Abs to ELDKWA with the three variants of this epitope and find them to be cross-reactive.

**MATERIALS AND METHODS**

**Abbreviations.** i.m., intramuscular; Ag, antigen; Ab, antibody; Ig, immunoglobulin; IgA, immunoglobulin A; pIg and pIgA, polymeric Ig and IgA; sIgA, secretory IgA; PADRE, pan DR epitope; CT, cholera toxin; CpG, cytosine-guanosine; OVA, ovalbumin; HIV-1, human immunodeficiency virus type 1; aa, amino acid; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunoassorbent assay; OD, optical density; i.m., intramuscular; MAb, monoclonal antibody.

**Immunization and collection of specimens.** Groups of five 2-month-old female BALB/c mice were i.m. injected in the thigh, four times at monthly intervals, with 0.2 ml of the PADRE-ELDKWA peptide in PBS. In some experiments, different concentrations of CT (Sigma Chemical, St. Louis, Mo.) or CpG ODN1826 (5′-TCC-ATG-ACG-TCG-ACG-CTG-3′; MWG-Biotech, Courtabeuf, France) were mixed with the peptide in the same final volume of 0.2 ml. The specimens were collected and treated by using the perfusion extraction method, PERFEXT, slightly modified (33). This method allowed measurement of the Ab response in local plasma cells, irrespective of the number of specific B cells. Briefly, the mice were exsanguinated 2 weeks following the last booster injection. The liver was fully bled, and the organs (spleen, jejunum, ileum, cecum, and colon) were freed from the peritoneum and the intestinal content. They were homogenized in the presence of protease inhibitors and extensively washed to remove the extracellular fluid. The pellet was suspended in a final dilution of about 1:10 and frozen at −20°C. Disruption of the cell membranes, to release intracellular IgG, was achieved by thawing overnight at 4°C in the presence of 2% (vol/vol) saponin (final dilution) and then cleared by centrifugation. Aliquots of the supernatants were stored at −20°C until use. Vaginal fluids were collected by washing with 0.1 ml of PBS and pooled. Mucus and cells from each pool were removed by centrifugation at 10,000 × g, and the pellets were controlled for the absence of red blood cells. The supernatants were stored at −80°C.

**Antigens.** The peptides (Table 1) were synthesized by NeoSystem (Strasbourg, France). Their purity and molecular mass were controlled by liquid chromatography and mass spectroscopy. The consensual immunizing Ag consisted of PADRE with the ELDKWA peptide (aa 659 to 664) extended with its adjacent C terminus sequence in gp41 SLW (aa 665 to 667). The Ags used for detection, DRE with the ELDKWA peptide (aa 659 to 664) extended with its adjacent C terminus sequence in gp41 SLW (aa 665 to 667), were saturated with 100 μl of ELDKWA-OVA (4 μg/ml) per well (Table 1).

**Antibody assays.** Microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 μl of ELDKWA-OVA (4 μg/ml) per well (Table 1). Washes were carried out with 0.1% Tween 20 in PBS. The free reactive groups were saturated with 100 μl of 0.5% gelatin in PBS per well. The wells were washed with PBS-Tween and incubated with 50 μl of diluted samples for 1 h at 37°C. Following additional washes and incubation with a peroxidase-labeled sheep Ab to the murine α or γ chain (Sigma), the enzyme was revealed with o-phenylene diamine. The reproducibility of the OD values was ±10%. The level of IgA Abs was expressed in micrograms per milliliter by comparison with the curve of a calibrated reference pool present in the same plate. The calibration (in micrograms per milliliter of IgA Abs) of this pool of jejunum and ileum extracts, selected for a high level of IgA Abs to ELDKWA, was carried out by comparison with the IgA concentration curve of the MAb TEP C15 (Sigma). OD values lower than the exponential segment of the reference curve were considered not significant. The results of the pools of vaginal fluids were expressed as relative Ab levels both because of the variable dilution of this fluid and the variable efficiency...
of the washing. The relative level (in arbitrary units) was defined as the ratio of Ab activity to the concentration of total Ig (12). Because of their low levels, the IgG Abs were expressed only by OD values.

**Statistical analysis.** The IgA Ab levels in the tissue extracts from mice immunized in the presence, or absence, of adjuvant were compared by using the two-tailed \textit{U} test of Mann and Whitney. The comparison was carried out between the corresponding organs of the adjuvant-free group and of the adjuvant-treated groups. In the case of colon extracts, the sensitivity of the comparison was increased by joining the results for all CT-treated animals (\( n = 15 \)) in one group, which was compared with the adjuvant-free group (\( n = 5 \)).

**Cross-reactivity and inhibition experiments.** Cross-reactivity to variant peptides was investigated with ELISA plates coated with OVA-linked peptides. Positive and negative controls were coated with ELDKWA-OVA or p145-OVA, respectively. Binding to these molecules was assayed with dilutions of the reference pool of IgA Abs. For inhibition experiments, dilutions of this pool were incubated with various concentrations of ELDKWA-OVA, or of its variants, for 1 h at 37°C. The remaining Ab activity in response to ELDKWA-OVA was then investigated with the ELISA. The percentage of inhibition was calculated by comparing the ODs at 490 nm of the Abs incubated with peptides or with PBS.

**RESULTS**

Decrease of locally synthesized IgA Abs in the presence of CT or CpG. Uncleaved CT being nontoxic in adult mice, these animals remained healthy during the course of experiments. Although CT is a mucosal adjuvant that enhances the Ab response during transcutaneous immunization (3), it was found here to impair the IgA response to 1 \( \mu \)g of PADRE-ELDKWA/injection (\( P \leq 0.01 \)). This result was observed in all mucosal organs (Fig. 1) as well as in the pooled vaginal washes (Fig. 2). IgA Abs were not detected in serum nor in the spleen.

Interestingly, the level of IgA Abs in the pooled vaginal washes was higher at the dose of 1 \( \mu \)g of CT/injection than at 0.1 and 0.01 \( \mu \)g. Six groups of mice immunized with doses of 0.1 or 0.01 \( \mu \)g of PADRE-ELDKWA together with CT (1, 0.1, or 0.01 \( \mu \)g) exhibited lower responses than those with 1 \( \mu \)g of PADRE-ELDKWA plus 1 \( \mu \)g of CT (data not shown). This confirms that 1 \( \mu \)g of PADRE-ELDKWA is the optimal dose, whatever the concentration of CT. No Ab response was observed in a control group of mice injected with 1 \( \mu \)g of CT alone. CpG also
decreased the IgA Ab response to PADRE-ELDKWA (Fig. 3), but more severely, and no IgA Ab was detected in the vaginal fluid. Investigation of IgG Abs (Table 2) showed low-level and variable responses with no evidence of an inhibitory effect of the adjuvants.

**Reactivity of Abs to ELDKWA with the variant peptides.** A reactivity was observed when the pool of mucosal IgA Abs to ELDKWA was assayed with ELEKWA-OVA, ELNKWA-OVA, and ELDEWA-OVA (Fig. 4). The curves of activity were similar to that of the positive control ELDKWA-OVA. As expected, no reactivity was observed with the negative control p145-OVA, containing the ASREAK peptide from the streptococcal protein M.

**Inhibition of the Ag-Ab reaction.** Incubation of the IgA pool of Abs to ELDKWA with the four OVA-coupled HIV peptides dose dependently inhibited the reactivity of this pool with ELDKWA-OVA (Fig. 5). The inhibition curves were similar.

**DISCUSSION**

We investigate here an alternative method of induction of IgA Abs to a protective epitope of HIV. We show that addition of mucosal adjuvants impairs the response and that the induced Abs to ELDKWA cross-react with the variants of this consensual epitope. We have already shown that the response

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**TABLE 2.** Low values of IgG antibodies to ELDKWA in the organs and serum of mice immunized with PADRE-ELDKWA in the presence or absence of CT or CPG

| Adjuvant (concn [µg]) | OD_{490} of IgG in organs and serum* |
|-----------------------|-------------------------------------|
|                       | Spleen | Jejunum | Ileum | Cecum | Colon | Serum |
| None                  | 0.06 (0.0–0.26) | 0.11 (0.07–0.14) | 0.10 (0.04–0.14) | 0.18 (0.15–0.45) | 0.12 (0.09–0.20) | 0.13 (0.01–0.19) |
| CT (1)                | 0.15 (0.04–0.23) | 0.07 (0.06–0.07) | 0.21 (0.17–0.23) | 0.13 (0.11–0.24) | 0.08 (0.06–0.10) | 0.14 (0.06–0.18) |
| CT (0.1)              | 0.20 (0.05–0.38) | 0.11 (0.06–0.12) | 0.20 (0.08–0.28) | 0.09 (0.04–0.17) | 0.14 (0.08–0.15) | 0.09 (0.04–0.12) |
| CT (0.01)             | 0.03 (0.01–0.13) | 0.17 (0.10–0.25) | 0.15 (0.08–0.27) | 0.11 (0.05–0.14) | 0.19 (0.13–0.24) | 0.05 (0.04–0.06) |
| CPG (1)               | 0.01 (0.00–0.07) | 0.15 (0.06–0.20) | 0.26 (0.05–0.39) | 0.07 (0.03–0.08) | 0.15 (0.09–0.28) | 0.03 (0.02–0.04) |
| CPG (0.1)             | 0.00 (0.00–0.06) | 0.12 (0.08–0.26) | 0.14 (0.10–0.22) | 0.11 (0.02–0.14) | 0.15 (0.10–0.16) | 0.02 (0.02–0.09) |
| CPG (0.01)            | 0.04 (0.00–0.05) | 0.16 (0.09–0.28) | 0.16 (0.11–0.32) | 0.02 (0.00–0.14) | 0.10 (0.10–0.19) | 0.04 (0.02–0.07) |

* Median values and ranges.

**OD** at 490 nm.
is long lasting and indicated that the method may be suitable for mass vaccinations in humans.

To avoid anatomic and physiologic differences between humans and mice, the Ags were administered at a distance from the pharyngeal mucosa (8). Similarly, the transcytosis of serum-derived plgA through murine hepatocytes (14) led us to investigate plgA Abs in tissue extracts and not in the content of the gut lumen. We tried to improve the immune response by analyzing the effects of two different adjuvants: CT and CpG. CT is a major adjuvant of the mucosal IgA response, either under its complete form or under its B subunit (28). In humans, CT (but not its B subunit) is highly toxic and must be used as a genetically modified nontoxic derivative (25). This is the case for a recent oral anticholera vaccine (24). CpG is also a mucosal adjuvant (13). Impairment of the anti-ELDKWA responses (Fig. 1 to 3) is unusual and at variance with the increased serum IgG response to a fucopentose-PADRE conjugate in the presence of different adjuvants (1). We notice that the three adjuvants shown to suppress the mucosal IgA response anti-ELDKWA usually display different mechanisms that improve the immune response. Alum acts by a depot effect, leading to a long-lasting release of the Ag. CT enhances the penetration of the Ag through the gut epithelium and the skin and acts as both an immunogen and an adjuvant (28). CPG induces both specific and nonspecific immunostimulations via a Toll-like receptor (16).

The linear conjugation of PADRE with a B-cell epitope allows activation and Ag presentation of adjacent cells. PADRE may also be involved in the rejection of tumors when associated with a CD8-reactive foreign peptide (34). An interesting problem is the contrast between the selective induction of a mucosal response and the low level of serum Abs. This contrast may be due both to PADRE and to ELDKWA, since injection of soluble Ags containing various haptens and carriers leads to different responses in serum as well as in mucosa (11).

The cross-reactivity between the consensual epitope ELDKWA and variant peptides is of major interest for using a single Ag to protect against most HIV-1 clades. In contrast with the gp41 mutations induced by the synthetic peptide enfuvirtide (T-20), which render the virus resistant to this treatment (15, 29), the variations in the ELDKWA sequence may not impair the protection by sIgA Abs. Only three variants, corresponding to a change of aa 670 or 671, were described in primary isolates or in strains obtained by immune selection (26, 32). They were shown to be cross-reactive (35), as dem-

FIG. 4. Cross-reactivity of IgA Abs to ELDKWA with the variant peptides. Abs were from the pool of jejunum and ileum extracts of mice immunized with PADRE-ELDKWA. The ELISA plates were coated with ELDKWA-OVA, ELEKWA-OVA, ELNKWA-OVA, or ELDEWA-OVA. The negative control ASREAK peptide from the streptococcal protein M was assayed with p145-OVA-coated wells. The dilution curves of the consensual sequence and those of the variants were similar.

FIG. 5. Inhibition of IgA Abs to ELDKWA following incubation with the OVA-linked peptides. The reference pool of IgA Abs was incubated with dilutions of ELDKWA-OVA or with the OVA-linked variants and assayed against ELDKWA-OVA. The concentration of inhibitor corresponds to that of the whole OVA peptide. The concentration of the ELDKWA epitope alone would be ~15-fold lower.
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