INTRODUCTION

The mucous membrane is the main interface between the human body and the surrounding environment. With its permeability for metabolic exchange and an overall surface area of around 400 m², it is the main entry port for microorganisms. To protect the host from pathogenic invaders at such sites, mammals have developed a mucosal immune system with the key features of local antigen-specific lymphocyte expansion and homing of effector and memory cells. Upon pathogen transcytosis, subsequent uptake by mucosa-associated antigen-presenting cells and transport to local lymph nodes, antigen-specific lymphocytes get activated and expand in numbers. This activation process leads to the differentiation of naïve cells into effector and memory cells with appropriate homing receptors on their surface. The interaction of these receptors with their ligands then controls the subsequent tissue-specific migration of the antigen–primed leukocytes (Butcher & Picker, 1996). For example, leukocytes that were antigen-triggered in the gut by dendritic cells from Peyer’s patches express the integrin α₄β₇, which binds to the mucosal vascular addressin MAdCAM-1 (Johansson-Lindbom et al., 2003; Mora et al., 2003). This interaction mediates homing of the respective lymphocytes back to the Peyer’s patches and the lamina propria (Berlin et al., 1993).

The expression of tissue-specific homing receptors on the surface of effector/memory lymphocytes and their temporal recirculation in blood after antigen encounter allows a simple quality control of immunization routes. By intracellular cytokine staining of antigen-triggered leukocytes directly from blood, it is possible to quantify antigen-specific CD4⁺ and CD8⁺ T cells and to characterize their differentiation phenotype and their homing commitments. A 6 h duration of antigenic ex vivo activation ensures that only specific effector and memory CD4⁺ and CD8⁺ T cells are detected (Heintel et al., 2002). Naïve T cells, in contrast, need longer times of activation to differentiate and produce cytokines. The additional analysis of CD45RA/RO and CD27 expression allows us to typify the differentiation status, while via CCR7, CD62L and α₄β₇, their homing to lymph nodes and the gut can be identified, respectively (Mackay, 1999; Sallusto et al., 1999).

Poliovirus is a prototype for gastrointestinal viral infections and is close to worldwide eradication due to highly effective vaccines. Poliovirus is a single-stranded RNA virus of the family Picornaviridae and may cause an acute paralytic disease in non-immune humans (Bodian & Horstmann, 1965). It is transmitted via the faecal–oral route. It initially penetrates through the M cells of the intestinal epithelium (Sicinski et al., 1990) and replicates in the Peyer’s patches. The virus then circulates through the blood and may enter the central nervous system where it causes paralysis (Bodian & Horstmann, 1965). Mucosal immunity is of particular importance in protection against poliovirus infection (Faden et al., 1990; Ogra, 1968). Protection is mediated through neutralizing antibodies and at least four neutralizing antibody epitopes have been identified (Minor, 1990; Minor et al., 1990). However, poliovirus-specific T cells are an important component in poliovirus defence. This was demonstrated by adoptive transfer experiments in poliovirus-receptor transgenic mice, which showed

Gut-homing (α₄β₇⁺) Th1 memory responses after inactivated poliovirus immunization in poliovirus orally pre-immunized donors

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Mucosal infections are prevented by a specialized local immune system. Immune cells of this compartment can also be found in the blood and are characterized by the expression of mucosa-specific homing molecules. Here, the cellular immune responses after inactivated poliovirus immunization (IPV) in poliovirus orally pre-immunized donors were investigated. Subcutaneous IPV induced a transient increase in the proliferative response against poliovirus antigen and in the number of poliovirus-specific CD4⁺ T cells in the blood of the vaccinees. These cells were characterized to be of the effector memory type (CD45RA⁻/CD45RO⁺/CD27⁻/CD27⁺) and expressed the homing molecule α₄β₇, indicating their origin from the gut. Together these data show the recurrence of gut-derived poliovirus-specific cells upon IPV and evaluate the whole-blood assay as a powerful tool for monitoring the success of a vaccination.
that only primed B cells together with polyclonal poliovirus-specific T cells protected from a lethal intravenous wildtype poliovirus challenge (Mahon et al., 1995).

For several prevalent and threatening infections, i.e. human immunodeficiency virus (HIV), a mucosal antiviral cellular immune response is considered to be an integral part of any preventative vaccine candidate. From the vast number of vaccine studies it seems apparent that mucosal homing could only be achieved with a mucosal antigenic trigger. However, it seems possible that subsequent non-mucosal booster immunizations may break mucosal commitment as has been suggested (Kantele et al., 1999). To examine whether virus-specific T cells after mucosal vaccination may be boosted via a non-mucosal immunization and whether the mucosal commitment would be broken or not, a group of nine volunteers was followed after poliovirus vaccination. All volunteers have been vaccinated more than 10 years ago with an oral live-attenuated vaccine (OPV). Their cellular immune response to a booster immunization with an inactivated poliovirus vaccine (IPV) was followed with respect to poliovirus-specific T cell frequencies, proliferation, differentiation phenotype and homing commitment. Consistently, an expansion of gut-homing Th1 effector memory responses was observed suggesting that the location of the initial immune trigger determined the subsequent homing properties of the effector T cells.

METHODS

Subjects. Nine healthy volunteers were immunized with a poliovirus vaccine. Their mean age was 38.2 ± 8.7 years, with their last oral poliovirus immunization more than 10 years ago. Initially, three were immunized orally with the live attenuated vaccine OPV-Virelon (Chiron Behring) and tested for neutralizing antibody titres three were immunized orally with the live attenuated vaccine OPV-OPV (Chiron Behring) and produced poliovirus vaccine. Their mean age was 38.2 ± 8.7 years, with their last oral poliovirus immunization more than 10 years ago. Initially, three were immunized orally with the live attenuated vaccine OPV-Virelon (Chiron Behring) and tested for neutralizing antibody titres three were immunized orally with the live attenuated vaccine OPV-OPV (Chiron Behring) and produced poliovirus vaccine. Their mean age was 38.2 ± 8.7 years, with their last oral poliovirus immunization more than 10 years ago with an oral live-attenuated vaccine (OPV). Their cellular immune response to a booster immunization with an inactivated poliovirus vaccine (IPV) was followed with respect to poliovirus-specific T cell frequencies, proliferation, differentiation phenotype and homing commitment. Consistently, an expansion of gut-homing Th1 effector memory responses was observed suggesting that the location of the initial immune trigger determined the subsequent homing properties of the effector T cells.

Poliovirus neutralization assay. Serial twofold diluted serum samples (dilution from 1:4 to 1:4096) were examined in 96-well flat-bottomed culture plates (Nunc). Diluted sera were mixed with 100 TCID50 of poliovirus types 1–3 each (in-house serotyped patient isolates) and incubated for 1 h at 37 °C with 5 % CO2 in a standard cell incubator. Green monkey kidney (GMK) cells (2.5 × 10^5) were added per well and cultured for 4 days. The cells were fixed with 1 % glutaraldehyde and stained with 1 % crystal violet in PBS. The poliovirus-induced cytopathic effect was analysed by light microscopy. The neutralizing antibody titre was considered to be the highest dilution of serum that protected the duplicate cultures from the cytopathic effect. The variance between repeated titre determinations was within one dilution step.

Poliovirus-specific proliferation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMC) were isolated from 10 ml heparinized venous blood by Ficoll-Hypaque density centrifugation (PAA). Cells were adjusted to 1 × 10^8 cells ml^-1 in RPMI 1640 supplemented with 10 % human AB serum (Sigma). Alternatively, serum-free medium (AIM-V; Gibco) was used for cultivation. Triplicate cultures of 1 × 10^5 PBMC in 100 μl culture medium were seeded in 96-well round-bottomed microtitre plates (Greiner). PBMC were stimulated from day 5 to day 6 in the presence of 10 μl poliovirus antigen ml^-1 (equal volumes of complement fixation reagents for poliovirus types 1–3; BioWhittaker). Stimulation with 50 ng 2FE tetanus toxoid (Chiron Behring) ml^-1 was used as a positive control. Twelve hours before harvesting, 1 μCi [3H]thymidine (Amersham) per well was added to the PBMC. Cells were harvested on glass fibre filters (Wallac), fused with scintillator wax (Wallac) and [3H]thymidine uptake was measured in a scintillation counter (Wallac).

Stimulation of poliovirus-specific CD4+ and CD8+ T cells within whole blood. Human venous heparinized blood was collected at days 0, 7, 14 and 21 after immunization. Whole blood was stimulated with titrated amounts of poliovirus antigen (equal volumes of complement fixation reagents for poliovirus types 1–3, BioWhittaker) in the presence of 1 μg αCD28 and αCD49d ml^-1 (clones CD28.2 and 9F10, respectively; Becton Dickinson) as previously described (Heintel et al., 2002). As a negative control, blood cells were incubated with the complement fixation reagent control, which did not contain any poliovirus protein (BioWhittaker). Initially we also used IPV (Chiron Behring) and produced poliovirus ourselves from the supernatants of poliovirus-infected GMK cells for stimulation, which gave similar results. As a positive control, cells were stimulated with SEB at 2.5 μg ml^-1 (Toxin Technologies). Cells were incubated in polypropylene tubes at 37 °C at 6 % CO2 for a total of 6 h. During the last 4 h, 10 μg Brefeldin A (Sigma) ml^-1 was added to block extracellular transport of cytokines. Thereafter, the blood was treated with 2 ml EDTA for 15 min, the erythrocytes subsequently lysed and leukocytes fixed for 10 min using a Becton Dickinson lysing solution following the manufacturer’s instruction. Cells were washed once with FACS buffer (PBS plus 5 % filtered FCS, 0.05 % sodium azide) and either immediately processed for FACS analysis or left overnight at 4 °C.

Determination and characterization of poliovirus-specific T cells. Fixed leukocytes were permeabilized with 2 ml FACS buffer containing 0.1 % saponin (Sigma) for 10 min at room temperature. Thereafter, they were immunostained for 45 min at room temperature in the dark using saturating conditions for the following phycoerythrin (PE)- or FITC-labelleld monoclonal antibodies: αCD4 (clone RPA-T4), αCD8 (clone RPA-T8), αIFN-γ, αZL-4, αZL-2, αCD45RA, αCD45RO, αCD27 (all purchased from Becton Dickinson). The PC5-labelled αCD4 (clone 13B8.2), αCD8 (clone B9.11) and the PE-labelled αCD69 (clone TP1.55.3) antibodies were purchased from Coulter-Immunotech. Cells were washed once with 3 ml FACS buffer and fixed in 1 % paraformaldehyde. At least 20,000 CD4+ or CD8+ lymphocytes were analysed on a FACSscan (Becton Dickinson) using the CellQuest Software version 3.1. The percentage of poliovirus-specific T cells was calculated by subtracting the frequency of IFN-γ-positive cells in the control stimulation from the frequency in the poliovirus antigen stimulation.

The quantification of homing-receptor-positive poliovirus-specific T cells was performed by indirect staining since directly fluorescent-labelled antibodies were not available. For the detection of αCD28 and CD62L, mouse monoclonal antibodies were used as the primary antibodies and a fluorescent-labelled rabbit anti-mouse as secondary antibody. To ensure homing-receptor specificity, poliovirus-specific T cell stimulation in these cases was performed in the absence of the mouse-derived co-stimulatory antibodies αCD28 and αCD49d, and cell staining for homing-receptor detection was performed before staining for CD4 and IFN-γ.

Stimulated cells were incubated for 45 min at room temperature with the mouse monoclonal antibodies specific for the gut-homing integrin α4β7 (clone ACT-1, kindly provided by Millennium, Cambridge, MA, USA, and Alf Hamann, Berlin) or the lymph node-homing selectin CD62L (clone Dreg-56; Becton Dickinson). After washing the cells with FACS buffer, a PE-cyanin-5 (PC5)-labelled rabbit F(ab anti-mouse Ig
(DAKO-Cytomation) was used as secondary antibody. To quantify the cytokine receptor CCR7 surface expression, cells were incubated with the rat aCCR-7 antibody (clone 3D12, kindly provided by Martin Lipp, Berlin) and subsequently with a donkey anti-rat FITC-labelled secondary antibody (Coulter-Immunotech). Incubation conditions to detect the other antigens were as described above.

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**Fig. 1.** Increase in neutralizing poliovirus antibody titres of nine poliovirus booster-immunized volunteers. The neutralizing antibody titres of three orally (A) and nine intramuscularly (B) booster-immunized volunteers before (day 0) and after vaccination (day 21) are shown. The oral- and intramuscular-applied vaccines were OPV-Virelon and IPV-Virelon, respectively. Titres were determined against poliovirus types 1–3 by serial twofold serum dilutions.
RESULTS

Increase in poliovirus-specific humoral immune responses after IPV booster immunization

To demonstrate an immunological response after poliovirus booster immunization with an oral, live-attenuated vaccine (OPV-Virelon) and/or an intramuscular, inactivated vaccine (IPV-Virelon), neutralizing antibody titres were determined from the nine volunteers. All of the volunteers had been immunized more than 10 years ago with the live attenuated poliovirus vaccine according to the approved recommendations at that time. Three of them were given OPV-Virelon and the poliovirus neutralizing antibody titres against all three poliovirus serotypes before (day 0) and 21 days after oral booster immunization were determined. Only a marginal increase in neutralizing antibody titres was observed (Fig. 1A), which is compatible with previous studies (Ogra, 1968). One month later, these three and another six volunteers were given a single dose of IPV-Virelon. High neutralizing antibody titres were detected in eight of the nine subjects at day 21 after IPV immunization (Fig. 1B). In particular, subjects 1, 2 and 4 showed an increase beyond 1:1024 at day 21 compared with antibody titres below 1:128 before IPV immunization. Only donor 3, who already had an overall high antibody titre of 1:128 before immunization, showed a marginal increase in neutralizing antibody titre after immunization. Thus, the poliovirus booster immunization was successful in all cases with a striking increase in neutralizing antibody titres, especially with the IPV regimen, as previously shown.

IPV booster immunization induces a poliovirus-specific proliferative response in PBMC

To test whether the booster immunization led to an expansion of poliovirus-specific leukocytes, the poliovirus-specific proliferative response was determined ex vivo with PBMC. These were isolated from all volunteers at days 0, 7, 14 and 21 after OPV and IPV immunization. The proliferative response was measured in the presence of 10 μl poliovirus antigen ml⁻¹ in a standard proliferation assay. No proliferative response before booster immunization (lymphocyte proliferation index (LPI) < 10; Fig. 2) could be detected. In the OPV-immunized group, only one donor showed a slight proliferative response at day 14 after immunization (LPI = 10). In the IPV-immunized group, eight of the nine individuals showed a proliferative response (LPI > 10) at

![Fig. 2. Increase in the poliovirus-specific proliferative response after booster immunization. The proliferative response of poliovirus antigen-stimulated PBMC of three orally (A) and nine intramuscularly (B) booster-immunized volunteers was determined by [³H]thymidine uptake before (day 0) and 7, 14 and 21 days after immunization. Numbers indicate the lymphocyte proliferation index (LPI), which is the quotient of antigen-specific proliferation to non-specific proliferation. Mean ± SD of triplicate measurements are shown. Each bar represents an individual vaccinee.](image-url)
day 14 after immunization, with six of the donors showing a 20-fold increase compared with the LPI before immunization (Fig. 2). The proliferative response had dropped back to baseline levels by day 21. This demonstrated that the IPV booster immunization successfully boosted a cellular poliovirus-specific immune response.

**Quantification of poliovirus-specific Th1 lymphocytes after IPV booster immunization**

The quantification of poliovirus-specific CD4⁺ and CD8⁺ lymphocytes was performed with intracellular cytokine staining directly from blood, as previously described for HIV and cytomegalovirus (CMV) (Sester et al., 2000, 2001). Briefly, heparinized whole blood was incubated for 6 h with poliovirus antigen \textit{ex vivo}. During this time, effector and memory CD4⁺ and CD8⁺ T cells are specifically stimulated, resulting in the upregulation of CD69 and the production of cytokines. The presence of Brefeldin A inhibits cytokine export from the cell and therefore allows intracellular staining, which can subsequently be analysed by flow cytometry. The frequency of poliovirus-specific CD8⁺ and CD4⁺ T cells was given as the percentage of CD8⁺ or CD4⁺ T cells positive for both CD69 and IFN-γ.

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**Fig. 3.** Detection and kinetics of poliovirus-specific T cells after oral (OPV) and intramuscular (IPV) booster immunization. (A) Detection of poliovirus-specific CD4⁺ and CD8⁺ T cells of a representative vaccinee before (day 0) and 14 days after IPV immunization. (B) Kinetics of poliovirus-specific CD4⁺ T cells at days 0, 7, 14 and 21 after OPV and IPV booster immunization. Whole blood cells were stimulated with poliovirus antigen or an equivalent amount of control antigen. The frequency of antigen-specific T cells at days 0 (before booster immunization) to day 21 after immunization is given as the relative number of CD4⁺ or CD8⁺ T cells positive for both CD69 and IFN-γ.
Poliovirus antigen specifically stimulated CD4⁺ T cells from IPV-immunized individuals. The control antigen failed to induce any relevant cytokine release. No IFN-γ production before or after IPV immunization was detected in the CD8⁺ T cells. Induction of IFN-γ in CD4⁺ T cells indicates a Th1 phenotype. Th2 cytokines, like IL-4, were below the detection limit (data not shown). A dot plot of a flow cytometric analysis for a representative volunteer is shown in Fig. 3(A). Fourteen days post-immunization, 0·37% of CD4⁺ T cells produced IFN-γ. Only 0·09% of CD4⁺ T cells produced IFN-γ before immunization at day 0.

Only IPV immunization led to the expansion of poliovirus-specific CD4⁺ T cells, which peaked at days 7 and 14 post-immunization (Fig. 3B). For eight vaccinees, a significant increase of IFN-γ-secreting CD4⁺ T cells was observed. Comparison of the frequencies of poliovirus-specific CD4⁺ T cells amongst the reacting volunteers showed considerable variation, which ranged from 0·09 to 0·51%.

**IPV booster immunization induces poliovirus-specific gut-homing effector memory lymphocytes**

To characterize the differentiation phenotype of the observed poliovirus-specific Th1 lymphocytes, the reciprocal expression of the memory markers CD45RA and CD45RO was analysed. The majority of poliovirus-specific T cells were of a memory phenotype, as shown by expression of CD45RO and lack of CD45RA (e.g. 86·41 ± 7·89% RO⁺ versus 22·82 ± 8·90% RA⁺, Table 1). CD27 was analysed as a maturation marker of T cells (Hamann et al., 1997; Hintzen et al., 1993). Most of the poliovirus-specific Th1 cells were CD27⁺ (87·40 ± 11·85%, Table 1), demonstrating that these were not fully mature effector T cells. Finally, the chemokine receptor CCR7 was used to divide the observed poliovirus-specific lymphocytes into central memory T cells or CCR7⁻ circulating effector memory lymphocytes (Sallusto et al., 1999). Of the poliovirus-specific Th1 cells, 91·77 ± 7·17% were CCR7⁻ (Table 1), showing that they were homing to peripheral tissues and not to lymph nodes.

To examine further the homing capacities of the poliovirus-specific T cells, we examined the expression of the gut-homing integrin α4β7 and the lymph node-homing selectin CD62L. The percentage of cells positive for α4β7 was 72·23 ± 13·00%, with the remaining 27·41 ± 9·55% being positive for the lymph node-homing selectin CD62L (Table 1). A representative example of the characterization of the poliovirus-specific T cell response before and at day 14 after IPV immunization is shown in Fig. 4. Taken together, the intramuscular poliovirus booster immunization in orally poliovirus pre-immunized volunteers induced high poliovirus neutralizing antibody titres and Th1 cells with an effector memory phenotype. These poliovirus-specific Th1 cells had gut-homing (α4β7⁻) capacities.

**DISCUSSION**

A single intramuscular immunization of mucosally virus-primed humans can efficiently booster a cellular gut-homing immune response. The poliovirus-specific T cell immune response after an IPV booster immunization of previously OPV-immunized volunteers was characterized in detail by intracellular cytokine staining of ex vivo antigen-triggered lymphocytes. Of particular interest was the expansion of virus-specific CD4⁺ T cells that expressed IFN-γ and thus had a Th1 phenotype. The majority of these antigen-specific T cells in addition expressed CD45RO, CD27 and α4β7, but lacked CCR7 and CD45RA. Therefore, they were effector memory cells with gut-homing capacities. Since the expression of CD27 usually correlates with a proliferation potential, it seems likely that the poliovirus-specific T cells would expand in numbers after a subsequent poliovirus challenge (Hamann et al., 1997). Our data are compatible with recent findings in rhesus macaques, where

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**Table 1.** Poliovirus-specific CD4⁺ T cells in peripheral blood are gut-homing effector memory cells

Numbers indicate the percentage of cells positive (+) (CD45RA, CD45RO, CD27, α4β7, CD62L) or negative (−) (CCR7) for the respective cell surface antigens among IFN-γ-positive CD4⁺ T cells stimulated with poliovirus antigen. ND, Not determined.
Fig. 4. Characterization of poliovirus-specific CD4⁺ T cells of one representative volunteer at days 0 and 14 after intramuscular booster immunization. The surface marker expression on poliovirus-specific CD4⁺ T cells was determined by staining with fluorescence-labelled CD69-, CD45RA-, CD45RO-, CCR7- and CD27-specific antibodies. Gut (α4β7⁺-) and lymph node (CD62L⁺-) homing receptor expression was analysed by indirect staining with a PC5-conjugated secondary antibody. The numbers indicate CD4⁺ and IFN-γ⁺-positive cells expressing the respective surface markers.
a peripheral booster immunization with vaccinia recombinants augmented the gut-homing cellular immune response after mucosal priming with recombinant *Salmonella* (Evans et al., 2003). Together this would suggest a flexibility in choosing peripheral boosting strategies for gut-homing T cell responses. It also shows that certain schemes of mucosal priming and peripheral boosting do not break the mucosal homing commitment, a possibility that has been suggested previously (Kantele et al., 1999).

To our knowledge, this is the first description of a gut-homing (αββ*)+* poliovirus-specific T cell effector memory response in humans. It is well documented that the neutralization of poliovirus infection is mainly due to antibodies. In particular, mucosal IgA responses from gut-homing B cells seem to be required for efficient protection, their induction being dependent on mucosal priming (Herremans et al., 1999). However, transfer experiments in poliovirus-receptor transgenic mice have demonstrated an important role for virus-specific T cells. Mice were only protected from infection when primed B cells were transferred together with polyclonal poliovirus-specific T cells. The transfer of each leukocyte population alone was not protective. Subsequent experiments with T cell clones showed further that Th1 cells can mediate the protection *in vivo* through their helper activity for humoral immunity (Mahon et al., 1995).

The detection, quantification and characterization of virus-specific T cells directly from blood via intracellular cytokine staining is a rapid and reliable means to follow not only virus infections but also vaccinations. While the induction of a cellular immune response may easily be seen and a quantitative comparison of different vaccination schedules performed, a direct correlation with protection is at present not possible. This would require the establishment of correlates between the levels of response and protection from infection or disease development, as has been done for antibody titres. To date, such a correlate has been defined only for CMV-induced disease in immunosuppressed patients after renal transplantation. When the frequency of CMV-specific CD4*+* T cells dropped below 0-25 % of the CD4*+* T cell population, the patients lost immune control over CMV and had to be treated with antiviral agents (Sester et al., 2001).

It has become increasingly apparent that an efficient antiviral immune response requires the combination of various effector and helper functions. Similar elegant experiments, as mentioned above for poliovirus infections in transgenic mice, have been performed in other mouse virus systems. For example, cooperativity between neutralizing antibodies and T cells is necessary to prevent persistent lymphocytic choriomeningitis virus infections (Baldridge et al., 1997). Likewise, CD4*+* T cells, CD8*+* T cells and B cells are involved in protection against Friend retrovirus infection (Dittmer et al., 1999). In consequence, this means that the improvement of new vaccination strategies always should include the testing of the comprehensive adaptive immune response.

In conclusion, peripheral booster immunization with an inactivated vaccine can expand gut-homing memory CD4*+* T cell responses in mucosally vaccine-primed humans. Thus the mucosal commitment induced by an attenuated oral poliovirus vaccine is not broken, even after an extended time period of 10 years. The ease by which such T cell responses can now be measured directly from the blood of vaccinees should encourage investigators to incorporate the respective assays into their vaccine trials.

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