

Biosensor-based characterization of serum antibodies during development of an anti-IgE immunotherapeutic against allergy and asthma

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Antibody responses, induced in *Cynomolgus* monkey by recombinant IgE-derived immunotherapeutic protein against atopic allergies and asthma, were characterized using label-free, real-time protein interaction analysis. The effects of two different immunotherapeutic proteins were compared. Active concentrations of specific anti-IgE antibodies formed were determined in sera sampled at multiple time points, using conditions of total mass transport limitation that were proved to exist on the sensor surface. These concentrations varied from about 0.4 to 35 µg/ml among the monkeys and throughout the immunization period. Based on these concentrations, the rate and affinity constants for the binding of antibody populations to the antigen could be determined. The apparent equilibrium dissociation constant decreased during the immunization period, for all the monkeys, by a factor between 6 and 50, ending at values from $\sim 2 \times 10^{-9}$ to $\sim 2 \times 10^{-11}$ M among the animals. This affinity maturation was attributable to the changes in both rate constants, although the magnitude of the contribution of each constant depended partly on specimen, but primarily on the immunotherapeutic used. The immunotherapeutic proteins examined showed excellent immunogenic properties, providing the basis for a new and effective treatment for allergy and asthma. Copyright © 2006 John Wiley & Sons, Ltd.

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INTRODUCTION

In the presence of allergen, cross-linking of IgE bound to the high-affinity IgE-receptors, FcεRI, on the mast cells surface causes de-granulation of mast cells and release of multiple mediators (histamine, prostaglandins, leukotrienes, cytokines) that are responsible for the common symptoms of the allergic reaction, such as inflammation, itching, coughing, lacrimation, bronchoconstriction, mucus secretion, vomiting, and diarrhea. A new strategy to treat allergy and asthma is to prevent IgE from binding to FcεRI by inducing self anti-IgE antibodies (Vernersson *et al.*, 2002). A specially designed, recombinant IgE-derived immunotherapeutic

protein induces and stimulates the production of anti-IgE antibodies that neutralize the circulating IgE molecules (Vernersson *et al.*, 2002). Thereby, the IgE/FcεRI cross-linking is blocked and the allergic reaction is avoided. This immunotherapeutic approach is allergen-independent. Detailed information about the level and character of antibody responses is extremely helpful for an accurate assessment of the immunotherapeutic protein, the efficacy of the immunotherapy, and for an optimal design of treatment regimes.

In this work, we used label-free protein interaction analysis based on surface plasmon resonance (SPR) detection (Jonsson *et al.*, 1991; Karlsson *et al.*, 1991) to characterize serum antibody responses in two groups of *Cynomolgus* monkeys. Each group consisted of five individuals and was treated with either of two variants of anti-IgE immunotherapeutic protein candidates, during time periods of different lengths. SPR response depends on a change in refractive index in the close vicinity of the sensor surface, and is proportional to the mass of the molecules bound on the surface (Stenberg *et al.*, 1991). In the assay set-up used here, a recombinant antigen protein was immobilized on a sensor surface, and diluted sera from the animals tested were injected into a constant buffer flow in contact with the surface. The amount and character of serum antibodies, selectively bound to the antigen, were assessed. The nature of antibody–antigen interaction enabled us to

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Abbreviations used: app, subscript denoting an apparent rate or affinity constant; k_a , the association rate constant; k_d , the dissociation rate constant; K_D , dissociation equilibrium constant; k_t , mass transfer coefficient; k_m , mass transfer constant; CCC, recombinant protein comprising C2, C3, and C4 regions of the *Cynomolgus* IgE ε-heavy chain; OCO, recombinant protein comprising C3 region of the *Cynomolgus* IgE ε-heavy chain flanked by the C2 and C4 domains of the opossum IgE ε-heavy chain; OCOCO, recombinant protein comprising C2, C3, C2, C3, and C4 regions of the opossum (O) and *Cynomolgus* (C) IgE ε-heavy chains, respectively; RI, refractive index contribution; R_{max} , observed theoretical maximal response that corresponds to the total concentration of ligand immobilized on the sensor surface; RU, resonance unit; SPR, surface plasmon resonance.

determine the active concentration (i.e., the concentration of biologically active molecules with respect to the interaction partner) under conditions of total mass transport limitation (Karlsson *et al.*, 1993, 1994). Once the concentrations were established, it was possible to characterize the interaction between specific serum antibodies and the antigen. The observed rate and affinity constants allowed an adequate assessment and comparison of immunotherapeutics. Our approach to characterization of antibody responses provides high information content and allows fast monitoring of the changing qualities of specific serum antibodies over time. This method should therefore be of considerable value in clinical trials, and consequently, we aim to employ the same approach in phase I and II studies.

Theoretical considerations for biosensor analyses

In the Biacore instrument, the binding of a molecule, delivered in a constant flow (referred to as the analyte) to an interacting partner attached to the sensor surface (referred to as the ligand), is a two-step process. In the first step, the analyte is transferred from the bulk to the sensor surface and in the second step the interaction between the two molecules takes place (Glaser, 1993). When the diffusion from the bulk to the surface is slower than the rate of the complex formation, the measurement of an active concentration of the analyte is possible under conditions of complete (Karlsson *et al.*, 1993, 1994) or partial (Christensen, 1997; Sigmundsson *et al.*, 2002) mass transport limitation. The method does not require a standard calibration curve and therefore enables us to measure concentrations directly in complex samples (such as sera and crude cell extracts), provided that unspecific binding can be disregarded. The conditions of complete mass transport limitation occur when the binding of a high molecular weight analyte to its binding partner, immobilized to the high-density sensor surface, is characterized by the k_a value of $\geq 1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Karlsson *et al.*, 1994). The initial slope of the binding curve is then given by the following expression (Karlsson *et al.*, 1994):

$$\frac{dR}{dt} = M_w \times k_m \times G \times [A_{\text{bulk}}] \quad (1)$$

where M_w is the molecular weight of the analyte, k_m is the mass transfer coefficient, and G is the conversion factor between the response and surface concentration values, calculated assuming that a mass concentration increase on the sensor surface of 1 pg/mm^2 gives a response increase of 1 RU/s (Stenberg *et al.*, 1991). The k_m value, for the case of complete mass transport limitation, is given by the following equation (Sjölander and Urbaniczky, 1991; Karlsson *et al.*, 1994):

$$k_m = 0.98 \times \sqrt[3]{\frac{D^2 \times f}{0.3 \times h^2 \times w \times l}} \quad (2)$$

where D is the diffusion coefficient, f is the volumetric flow rate, and h , w , and l are the height, width, and length of the flow cell, respectively. The product, $M_w \times k_m \times G$, is called mass transfer constant and is denoted as k_t . Given M_w , k_m ,

and G , the active concentration of the sample, $[A_{\text{bulk}}]$, can be determined.

To examine whether or not the interaction is mass transport limited, the binding rates of samples having the same concentration, observed at different flow rates, can be compared. If the binding slope observed is dependent on the flow rate, the interaction is limited by mass transport.

From Equations (1) and (2) we get:

$$\frac{dR}{dt} = M_w \times G \times [A_{\text{bulk}}] \times 0.98 \times \sqrt[3]{\frac{D^2 \times f}{0.3 \times h^2 \times w \times l}}$$

For the same experimental condition (i.e., the same flow cell, same sample, and same sample concentration), where only the flow rate varies, we can simplify this equation to:

$$\frac{dR}{dt} = \text{constant} \times \sqrt[3]{f}$$

Complete mass transport limitation occurs if the ratio of the initial binding rates measured at two different flow rates, f_1 and f_2 , is given by the following formula:

$$\frac{dR/dt_{(f_1)}}{dR/dt_{(f_2)}} = \sqrt[3]{\frac{f_1}{f_2}} \quad (3)$$

where $dR/dt_{(f_1)}$ and $dR/dt_{(f_2)}$ are the initial binding rates at flow rate f_1 and f_2 , respectively.

RESULTS

Purity of IgE-derived proteins

The purity of the recombinant proteins, OCO comprising the C3 domain of *Cynomolgus* (C) ϵ -heavy chain flanked by the C2 and C4 domains of the opossum (O) ϵ -heavy chain (Zetterström and Johansson, 1981) and OCOCO containing the C2, C3, C2, C3, and C4 regions of the opossum and *Cynomolgus* ϵ -heavy chains (Figure 1), have been analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Figure 2A) and identified by immunoblotting with an antibody recognizing the recombinant proteins (Figure 2B). Both OCO and OCOCO are

(A)

CH2	CH3	CH4
Cynomolgus	Cynomolgus	Cynomolgus

(B)

CH2	CH3	CH4
Opossum	Cynomolgus	Opossum

(C)

CH2	CH3	CH4	CH3	CH4
Opossum	Cynomolgus	Opossum	Cynomolgus	Opossum

Figure 1. Schematic presentation of IgE-derived recombinant protein constructs: (A) CCC, (B) OCO, and (C) OCOCO.

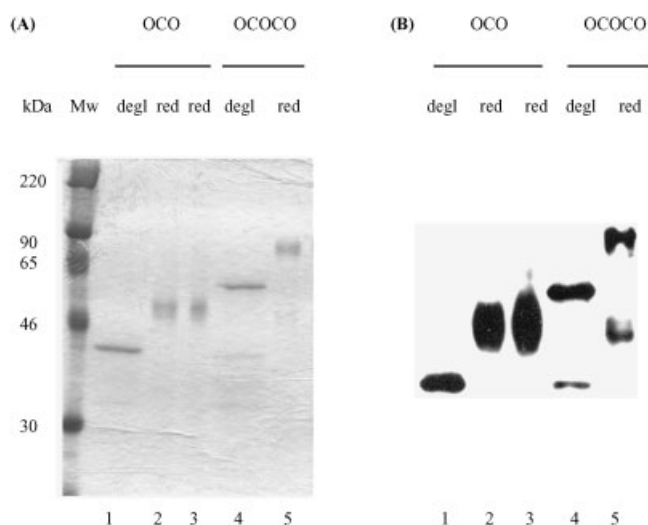


Figure 2. OCO and OCOCO proteins treated with N-Glycosidase (degl) or untreated (red), were separated by SDS-PAGE. After electrophoresis, proteins were either Coomassie-stained (A) or transferred to a membrane for detection by immunoblotting (B). Deglycosylated proteins: OCO (lane 1) and OCOCO (lane 4), reduced proteins: OCO (lanes 2 and 3) and OCOCO (lane 5). The positions of size markers (in kDa) are indicated on the left.

glycoproteins and the carbohydrate moiety can be removed by N-glycosidase (Figure 2). The additional OCOCO band seen on SDS-PAGE and Western blot is likely to represent degradation product. Densitometric analysis of the SDS-PAGE gel revealed purity greater than 90% for OCO and >80% for OCOCO (see Figure 2A).

The biosensor response reflects specific binding of anti-IgE antibodies to antigen immobilized on sensor surface

To control that only a specific antigen-antibody interaction was monitored, sera from all the animals, sampled 1 week before the first immunization, were controlled for binding to CCC. The sera were diluted 1:20 in running buffer and injected at flow rate 5 $\mu\text{L}/\text{min}$ over the antigen (CCC) surface immobilized to $\sim 12\,000$ RU. The initial portions of binding curves, comprising the region from ~ 20 to ~ 40 s from the sample injections, were fitted to a linear least-squares regression analysis. The curves obtained showed no binding, as manifested by no increase of response after sample injection, thus suggesting that no serum components, except for specific antibodies, bind to the antigen.

To examine the unspecific binding of sera to dextran surface, the sera from all 10 individuals, sampled after ninth week, were analyzed in the absence and in the presence of 1 mg/ml CM-dextran, as described above. The addition of dextran to the sera reduces unspecific binding to sensor surface, although for diluted serum samples this addition was found to be unnecessary (Karlsson *et al.*, 1994). The difference between the slopes measured in the absence and in the presence of CM-dextran was $2.2 \pm 1.2\%$ (mean $\pm$ SEM; $n = 10$). This indicates that the initial binding slopes were not affected by unspecific binding, which also

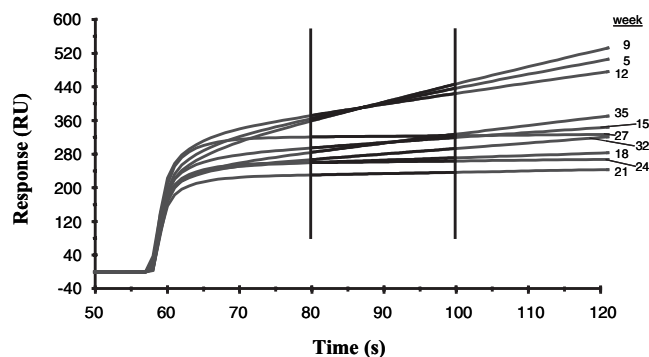


Figure 3. A representative overlay plot of the sensorgrams obtained when sera from one individual, sampled at multiple time points during the immunization study, were injected at a flow rate of 5 $\mu\text{L}/\text{min}$ over the high-density antigen surface. The analysis was conducted at 37°C. The weeks from the first immunization are indicated on the right of the corresponding sensorgrams. The vertical lines mark the initial binding slopes that were used to determine the active concentrations.

shows that the use of CM-dextran is not required. Accordingly, CM-dextran was not added to the serum samples during the concentration and kinetic measurements.

Verification of total mass transport limited conditions on sensor surface

To confirm complete mass transport limitation conditions, serum from one individual, sampled at multiple time points during the immunization period, was analyzed similarly as above, but at three flow rates: 1, 10, and 30 $\mu\text{L}/\text{min}$. The initial binding slopes were measured at all three flow rates, and the ratio between them was calculated. These were compared to the values expected, calculated as the cube roots from the ratio of the respective flow rates according to Equation (3). The ratio of the slopes (mean $\pm$ SEM; $n = 10$) and the expected values were: 2.17 ± 0.09 ($dR/dt_{10\,\mu\text{L}/\text{min}}/dR/dt_{1\,\mu\text{L}/\text{min}}$) and 2.15 , 3.33 ± 0.32 ($dR/dt_{30\,\mu\text{L}/\text{min}}/dR/dt_{1\,\mu\text{L}/\text{min}}$) and 3.11 , and 1.54 ± 0.17 ($dR/dt_{30\,\mu\text{L}/\text{min}}/dR/dt_{10\,\mu\text{L}/\text{min}}$) and 1.44 , respectively. The ratios experimentally determined approximate the values expected well enough to assume the complete mass transport limitation.

Concentration of specific antibodies

To determine the antibody concentration, the initial binding slope was measured as described above (Figure 3). The k_m value was calculated from Equation (2), for the flow rate 8.33×10^{-11} m³/s, using the flow cell dimensions, w , h , and l , of 500×10^{-6} , 40×10^{-6} , and 1.972×10^{-3} m, respectively. D for IgG (Sorber, 1973), re-calculated for the temperature of 310 K and for the relative viscosity of water at this temperature of 0.6915 (Cantor and Shimmel, 1980), was 6.12×10^{-11} m²/s. For a molecular weight of IgG of 156 000 Da, k_t was calculated to 1.33×10^9 RU/(M·s).

The concentrations of serum antibodies, calculated using Equation (1), ranged from 0.35 ± 0.01 to 35.19 ± 0.02 $\mu\text{g}/\text{mL}$ (~ 2 to ~ 200 nM). The change of the concentrations of IgG in serum over time, in all the monkeys, is shown in

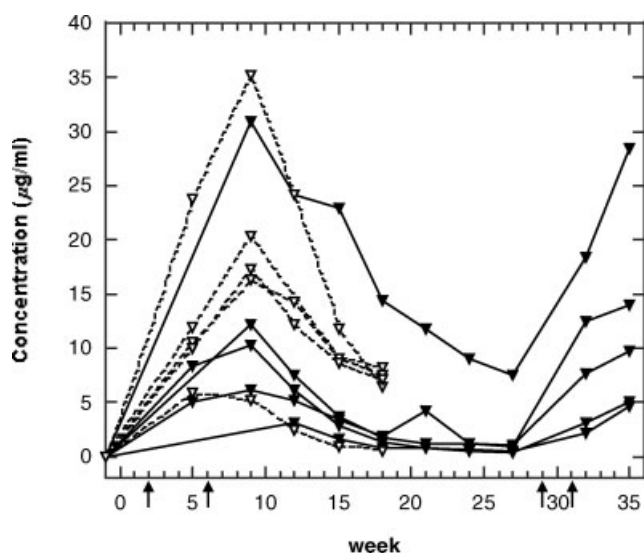


Figure 4. The concentrations of serum anti-IgE antibodies in 10 *Cynomolgus* monkeys immunized with the OCO variant, —▲—, and the OCOCO variant, —▽—, plotted over time. The absence of error bars reflects that the standard error was too low to be represented in the graph. The standard error for presented data was not greater than $\pm 6\%$. Arrows on the horizontal axis indicate booster administrations. The antibody concentrations in the IgM-containing serum samples of OCO-treated individuals (three samples at week 5 and one sample at week 9) were not determined.

Figure 4. The concentration patterns were consistent among the individual primates, although there was a considerable variation in the absolute concentration levels between the individuals. All the animals analyzed showed an antibody concentration peak after the first and second booster administration, approximately at week 9. The group immunized with the OCO construct showed a second concentration increase after the third and fourth booster administration and a concentration minimum at weeks 18–27 from the first immunization occasion.

Analysis of antibody classes

The active concentrations determined are valid, provided that the diffusion coefficient used for their calculation is appropriate. Since the value of the diffusion coefficient depends on the radius of the antibody, the possible presence of IgM in the serum samples would undermine the reliability of the active concentrations obtained. Therefore, sera of all the individuals were examined for the presence of IgM. The rabbit anti-human IgM (DakoCytomation, Solna, Sweden), controlled for specificity and cross-reactivity towards *Cynomolgus* IgM, was injected directly after the serum sample injection. IgM was detected in the serum from three individuals sampled at week 5 and in the serum from one individual sampled at week 9. All these individuals were treated with the OCO form of therapeutic protein. In the process of determination of the concentration, the diffusion coefficient of IgG was used and therefore, the concentrations, determined for the serum containing both classes of antibodies, turned out to be unreliable.

Kinetics and affinity of interaction with specific serum antibodies

The kinetic analysis was performed on the binding curves obtained when specific serum antibodies bound to the low-density antigen (CCC) surface. These conditions favored the measurement of the rate constants because mass transport limitation was expected to be less prominent. The use of low antibodies concentrations, of 2 nM, enabled us to measure the high-affinity interaction, whereby the contribution of a possible low-affinity binding was avoided. Dilution to the same concentration simplified the evaluation, although it resulted in a poor match of sample compositions, which was reflected in the varying refractive index contributions during the injections (Figure 5). The consecutive injection of sera sampled at different time points from one individual, over the same surface, gave a set of sensorgrams. Each such set was, after the reference and blank subtraction, fitted to the bimolecular binding model (Karlsson and Fält, 1997; Karlsson, 1999). The resulting fits were characterized by low residuals and low values of the goodness-of-fit χ^2 (Figure 5). The 1:1 binding model offers a simplified description of the interaction, because the bivalency and heterogeneity of the antibodies are not taken into account. The rate constants, thus determined, reflect the average properties of the polyclonal antibody pool, containing subpopulations with varying concentrations and affinities.

In this evaluation, the $R_{\max}$ and k_t parameters were fitted globally, giving the constants for the entire curve set, while the k_a , k_d , and RI parameters were fitted locally, characterizing each single curve. $R_{\max}$ depends on two factors: (i) the immobilization level and activity of the ligand on surface, and (ii) the molecular weight of the antibodies in the mobile phase. The use of the common surface and the common size of the antibodies justified a calculation of a common $R_{\max}$. The second globally fitted parameter, k_t , is the function of the flow cell dimensions and the molecular properties of antibodies, and therefore was also expected to have the same value for all the curves. Since each curve corresponded to a sample with different kinetic properties and with varying refractive index contribution, k_a , k_d , and RI were calculated separately. By a local fitting of k_a , k_d , and RI and a global fitting of $R_{\max}$ and k_t , we obtained a fast and robust estimation of the apparent association and dissociation rate constants, $k_{a,\text{app}}$ and $k_{d,\text{app}}$, and affinity constants, $K_{D,\text{app}}$.

Here, we increased the throughput of kinetic characterization by using only one sample concentration, which often provides sufficient kinetic information. However, the correctness of the kinetic constants determined had to be verified because of two major difficulties. The first of these is the choice of an appropriate single concentration that provides the true rate constants. If the concentration selected is too high, the kinetic information can be lost because of a very fast saturation of the binding sites on the surface. Conversely, too low concentration produces binding that is insufficient for a reliable determination of $R_{\max}$ and k_a , and only the product $R_{\max} \times k_a$ is meaningful. The second difficulty is concerned with mass transport limitation. To ensure the determination of reliable rate constants, the transport of antibodies to the surface has to be significantly faster than their binding to the immobilized interaction

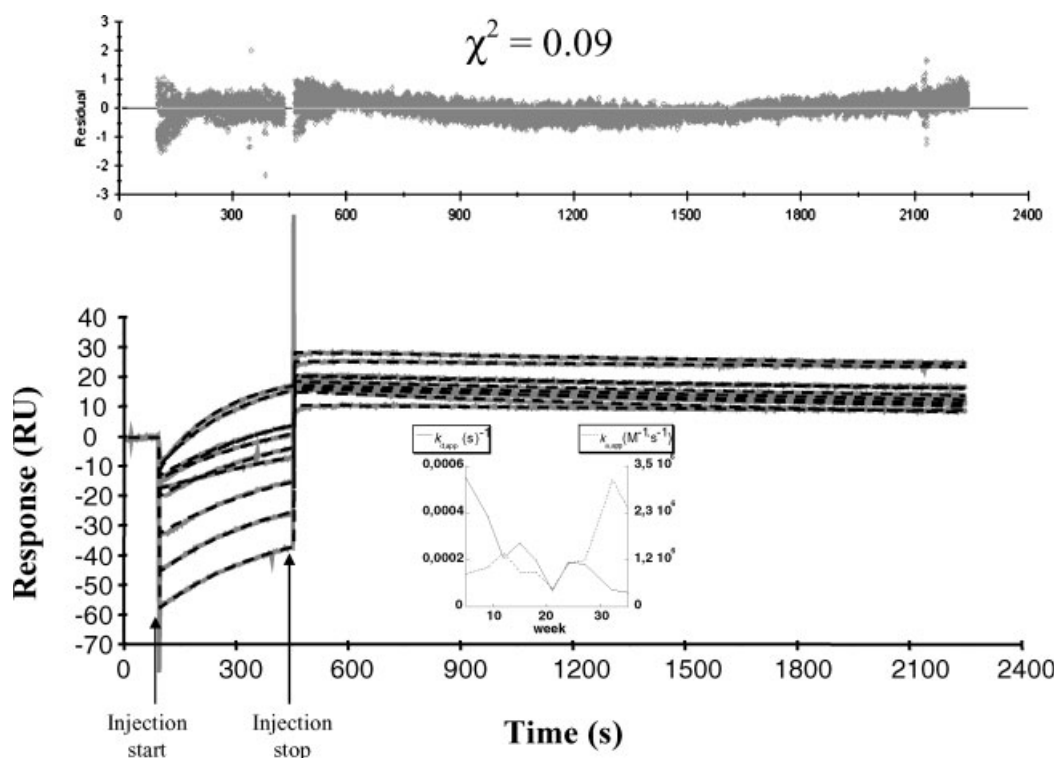


Figure 5. A representative overlay plot of sensorgrams obtained from interaction between anti-IgE antibodies from one individual and the antigen (characterized at 37°C). Serum samples from different time points were diluted to 2 nM and injected at a flow rate of 30 $\mu\text{L}/\text{min}$ over the active surface (containing ~ 50 RU of the antigen and ~ 2000 RU methoxyPEG amine) and reference surface (with ~ 2000 RU methoxyPEG amine). The curves were fitted to the bi-molecular evaluation model (Biacore T100 Evaluation Software) in which k_a , k_d , and RI were fitted locally and R_{max} and k_t were fitted globally. The overlaid dashed lines show the fitted curves. The drop in response after the injection start illustrates a shift of bulk refractive index due to a change in buffer composition. The inset represents the changes of $k_{a,\text{app}}$ and $k_{d,\text{app}}$ over time.

partner. In cases where mass transport limitation prevails, k_d and k_a are unreliable, although their ratio, defined as K_D , is correctly determined (Karlsson, 1999).

To deal with these two issues, a number of simulations on the fitted curves were performed. In the first set of simulations, R_{max} and k_a were multiplied and divided by the same factor (0.1–10), respectively, so that the $R_{\text{max}} \times k_a$ product was constant. Similarly, in the second simulation set, k_d and k_a were varied simultaneously, keeping the ratio between them unchanged. In both simulations, the remaining parameters were held unaffected. In all these cases, the shape of the simulated curves changed clearly along with the R_{max} and k_a or k_a and k_d values, respectively, showing that the fitting depended on the actual values, which, in turn, confirmed the validity of kinetic constants.

By adopting the evaluation procedure described, we were able to monitor how the polyclonal pool was enriched with higher affinity antibodies during the immunization period. In all the individuals, $k_{d,\text{app}}$ decreased and the $k_{a,\text{app}}$ increased over time, although the magnitude of these changes varied from one animal to another (Figures 6 and 7). The lowest and the highest $k_{a,\text{app}}$ values, determined in the monkeys immunized with the OCO form, were $(2.00 \pm 0.03) \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ at week 5 and $(3.67 \pm 0.03) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at week 35, respectively, whereas in the group immunized with the OCOCO variant, the

analogous values were: $(4.70 \pm 0.16) \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ at week 5 and $(1.31 \pm 0.02) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at week 18, respectively (Figure 6A). The corresponding $k_{d,\text{app}}$ values were $(5.49 \pm 0.04) \times 10^{-4} \text{ s}^{-1}$ at week 5 and $(3.18 \pm 0.09) \times 10^{-5} \text{ s}^{-1}$ at week 35, and $(4.88 \pm 0.04) \times 10^{-4} \text{ s}^{-1}$ at week 5 and $(1.82 \pm 0.09) \times 10^{-5} \text{ s}^{-1}$ at week 18 in the OCO and OCOCO group, respectively (Figure 6B). In the monkeys immunized with the OCO form, the $k_{a,\text{app}}$ and $k_{d,\text{app}}$ values changed 4- to 10-fold and 3- to 7-fold, respectively, giving the 20–50 times improvement of the antibodies affinity in individual monkeys (Figures 6 and 7). The $k_{a,\text{app}}$ values in animals immunized with the OCOCO variant were slightly lower than in the previous group, and increased from 2 to 5 times, whereas the $k_{d,\text{app}}$ values varied somewhat more dramatically and decreased from 3 to 18 times among the individuals (Figures 6 and 7). In this group of animals, the corresponding affinity improvement, expressed in terms of decrease of the apparent dissociation equilibrium constants, varied from 6 to 50 times (Figure 7C).

DISCUSSION

In this work, we have shown the excellent immunogenic properties of the two IgE-derived immunotherapeutic

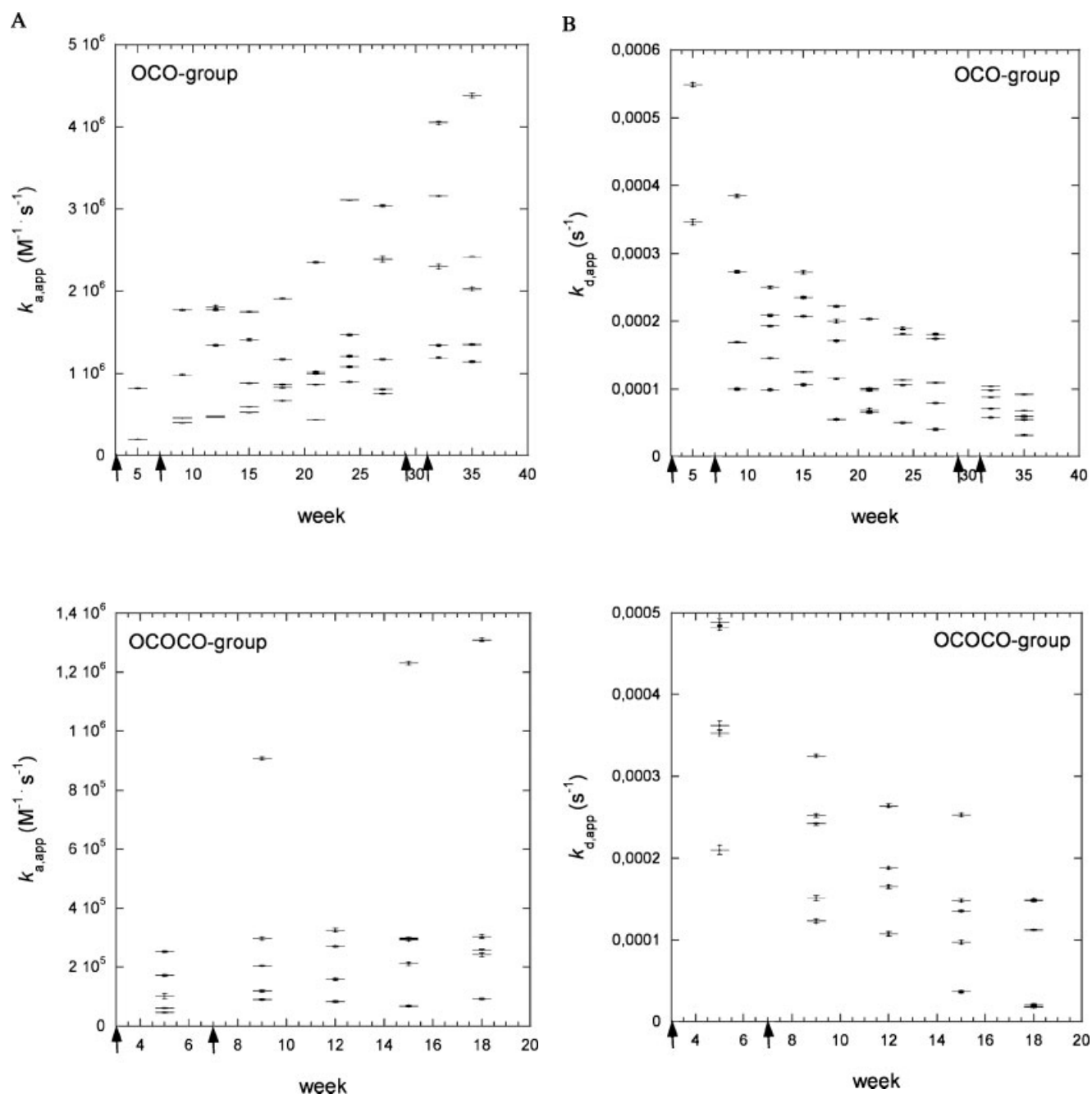


Figure 6. The $k_{a,app}$ (A) and $k_{d,app}$ (B) values for the interaction between CCC and serum antibodies from monkeys immunized with OCO and OCOCO. No values were determined for samples containing IgM. Error bars represent the standard errors. In the cases where error bars are absent, the standard error was too low ($\leq \pm 1\%$) to be represented in the graph. Arrows on the horizontal axis indicate booster administrations.

proteins intended to be used in allergy treatment. By determining the concentrations and affinities of the antibodies induced in response to these proteins, we obtained information that is important for selection of protein candidate as well as for an optimal design of dosing regimens and the choice of dose size.

The average concentrations of the antibodies populations in serum samples were determined using the fact that total mass transport limited conditions prevailed on the sensor surface. The existence of such conditions indicated that an

overwhelming majority of the antibodies bound to the antigen with the association rate constant of at least $1 \times 10^5 M^{-1} \cdot s^{-1}$ (Karlsson *et al.*, 1994). Consequently, in the present work, we characterized the populations of fast-binding, high-affinity antibodies, which are likely to be of particular interest with respect to their ability to prevent allergic reaction.

The active concentration patterns in all the monkeys showed peak values at week 9, which varied from 5.16 ± 0.01 to $35.19 \pm 0.02 \mu g/ml$ among the animals

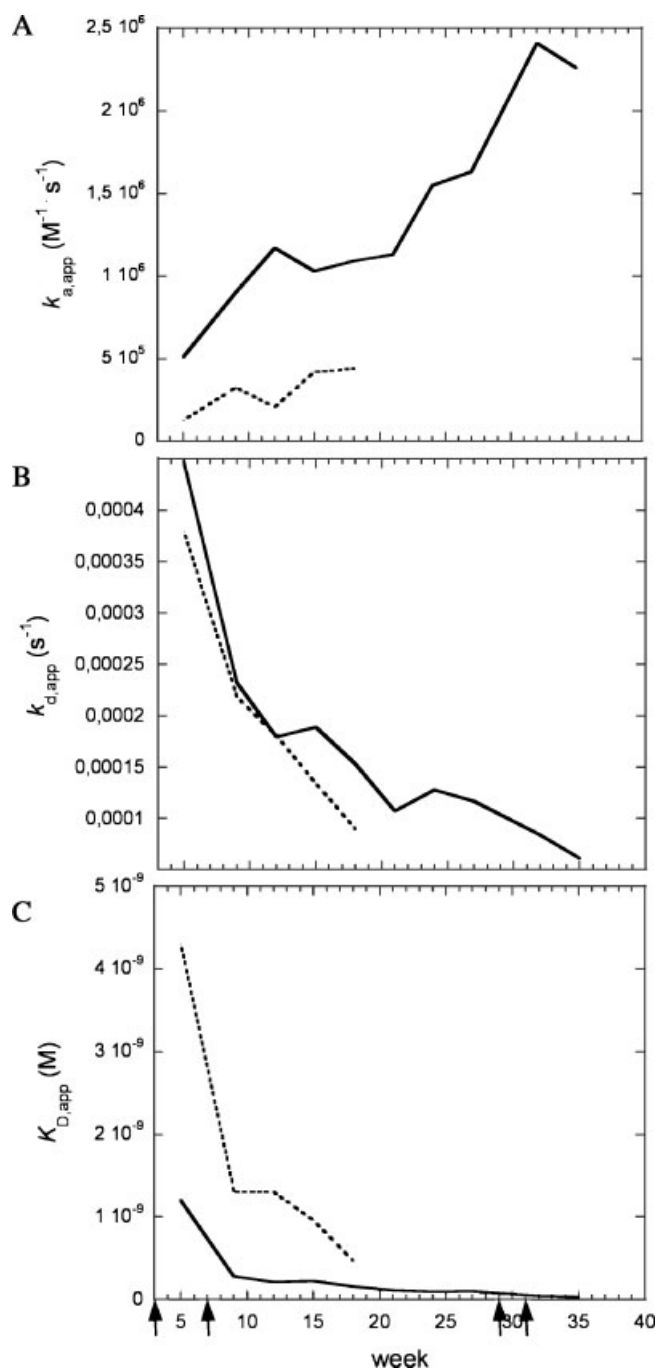


Figure 7. Comparison of the average values of (A) $k_{a,app}$, (B) $k_{d,app}$, and (C) $K_{D,app}$ for the interaction between CCC and serum antibodies from monkeys immunized with OCO, denoted as —, and OCOCO, denoted as ----. The $K_{D,app}$ values were calculated from the ratio between $k_{d,app}$ and $k_{a,app}$. Arrows on the horizontal axis indicate booster administrations.

(Figure 4). In the OCO group, studied during a 35-week period, the concentrations fell to minimum values around week 27 (0.35 ± 0.01 to $7.53 \pm 0.02 \mu\text{g/ml}$ among the animals). After booster administrations at weeks 29 and 31, antibody concentrations in this group started to rise

again, reaching at week 35, approximately the same values as at week 9 (from 4.57 ± 0.03 to $28.38 \pm 0.02 \mu\text{g/ml}$) (Figure 4). Given that blood IgE concentration in patients with moderate atopic conditions (asthma, allergic rhinitis, atopic dermatitis) ranges from 0.3 to $0.5 \mu\text{g/ml}$ (Zetterström and Johansson, 1981), and that the required anti-IgE response should be at least 10 times higher, the results presented here indicate a promising therapeutic effect in humans.

In the kinetic evaluation, the order of magnitude of the apparent association (10^5 – $10^6 M^{-1} \cdot s^{-1}$), dissociation (10^{-4} – $10^{-5} s^{-1}$), and affinity constants (10^{-9} – $10^{-11} M$) obtained (Figure 7) indicated very rapid and stable interactions with high average affinities. Already at the beginning of the immunization period, the $K_{D,app}$ values ranged from 3×10^{-8} to $1 \times 10^{-9} M$ among the monkeys and showed further gradual decrease by factors 6–50, depending on the immunotherapeutic protein form used (Figure 7C) and on the animal tested. The average changes in association and dissociation rate constants contributed almost equally to the affinity maturation in the monkey group immunized with the OCO variant. The antibodies generated in the individuals immunized with the OCOCO construct had approximately 10 times lower average affinities. This weaker binding was mainly attributable to a reduced $k_{a,app}$. The lowest apparent association rate constants observed were consistent with the requirement for the condition for the total mass transport limitation (Karlsson *et al.*, 1994). At the end of the immunization study, the affinities of monkeys treated with the OCO and OCOCO forms ranged from 7×10^{-11} to $2 \times 10^{-11} M$ and from 2×10^{-9} to $8 \times 10^{-11} M$, respectively. Considering that the affinity of the interaction between IgE and IgE-receptors is about $10^{-10} M$ (Miller *et al.*, 1989), the produced anti-IgE antibodies may not only block the binding of IgE to its receptor but also compete with the receptor for the binding to IgE. Since our results show that the conceptual immunotherapeutic protein, OCO, induces anti-IgE antibodies with approximately 10 times higher average affinity than the OCOCO variant, an effective human-specific immunotherapy may perhaps be most likely to derive from the protein construct comprising one homologous Cε3 domain flanked by the opossum Cε2 and Cε4 domains. The neutralizing activity of antibodies may not always be directly correlated with antigen-binding characteristics *per se*. Consequently, a biological cell-based assay would be helpful to determine whether or not the antibodies are indeed capable of neutralizing the biological effect of circulating IgE molecules.

In conclusion, this study demonstrates the usefulness and potential of a new approach to pre-clinical drug development of immunotherapies. Biosensor-based evaluation of the concentration and affinity maturation of anti-IgE antibodies indicate that the quantity and quality of the immune response fulfil the criteria for clinical efficacy. Discrimination between the anti-IgE responses induced by two different immunotherapeutic proteins was also readily achieved, thereby facilitating selection of candidate drug. In the pivotal early clinical development phases, a similar evaluation of IgE responses in healthy volunteers from clinical phase I studies should also help to optimize dose selection for phase II studies in patients.

MATERIALS AND METHODS

Instrumentation and software

Biacore[®] T100 (Biacore AB, Uppsala, Sweden) was used for SPR studies aiming to determine the active concentrations and qualities of the serum antibodies. Biacore T100 Control Software, Biacore T100 Evaluation Software, BIAevaluation (all from Biacore AB), and KaleidaGraph (Synergy Software, Reading, PA) were used for running, analysis, and presentation of the data obtained, respectively.

Construction of cDNA

Cynomolgus monkey (*Macaca fascicularis*) DNA constructs were made from spleen cDNA using PCR amplification and subsequent subcloning. The CCC fragment (1035 bp) encoding the entire C2, C3, and C4 regions of the *Cynomolgus* ϵ -heavy chain was amplified with the following primers: 5'-GAC CTC GAG TCA ATG GTG ATG GTG ATG GTGTCC TGA TCC TTT ACC GGG ATT TAC AGA CAC C3' and 5'-GAC GAATTC GTC AAC AAA ACC TTT GGC GTC AGC TCC AGG3'. The original cDNA clones, encoding the ϵ -heavy chain of opossum (*Monodelphis domestica*) IgE, were derived from total spleen cDNA libraries (Aveskough and Hellman, 1998) and used for construction of OCO (1077 bp) and OCOCO (1734 bp). For purification purposes, the 3' and 5' primers, directed against the opossum C2 and C4 domains, both contained an 18-nucleotide sequence encoding a histidine hexapeptide.

Cloning and expression of recombinant proteins

The PCR products were purified from agarose gels and cloned in the Bluescript vector (Stratagene, La Jolla, CA) and subsequently subcloned into the *EcoRI* and *XhoI* sites of the expression vector pCEP-Pu2. Nucleotide sequences were confirmed using the dideoxy chain-termination method (Cybergene, Huddinge, Sweden).

The pCEP-Pu2 vector is a modified version of the episomal expression pCEP4 vector of 10.4 kb (Invitrogen, Breda, The Netherlands). The major modifications, including replacing the hygromycin resistance gene with the gene for puromycin resistance to allow for puromycin selection in mammalian cells, and insertion of a 105-bp region containing some of the 5'UTR and the signal peptide sequence region of human BM40, were done by Kohfeldt *et al.* (1997, 1998) and resulted in the vector pCEP-Pu/BM40s. We further modified this vector. An *EcoRI* site, present upstream of the ampicillin-resistance gene, was removed by fill-in treatment and religation. In addition, the polylinker was extended to include an *EcoRI* and a *NotI* site between the *NheI* and *XhoI* sites. The resulting expression vector was named pCEP-Pu2.

Human embryonic kidney cells, 293-EBNA (Invitrogen), were cultured to 90% confluency in 6-well plates, washed with neutral phosphate-buffered saline (PBS) and serum-free Dulbecco's modified eagle's medium (DMEM) (Gibco BRL, Life Technologies, Cleveland, OH), respectively, and

transfected as follows. About 5 μ g DNA in sterile water and 15 μ l Lipofectamine 2000 (Gibco BRL) were each diluted in serum-free DMEM to 250 μ l. Subsequently, the DNA and Lipofectamine preparations were mixed, and incubated for 20 min at room temperature. The DNA/Lipofectamine mixtures were then added to the washed cells. After 12–16 h, the medium was supplemented with fetal bovine serum (FBS) (Gibco BRL) to a final concentration of 10%. The cultures were split 1:4 the following day and 1–2 days later, selection was initiated by the addition of 1.5 μ g/ml puromycin (P8833) (Sigma, St. Louis, MO) to the culture medium consisting of DMEM supplemented with 2 mM L-glutamine (Gibco BRL), 50 mg/ml gentamicin (Gibco BRL), 5% FBS, and 0.25 mg/ml G418 sulphate (Geneticin) (Calbiochem, San Diego, CA). Growing cells were expanded, and conditioned media were collected every 4–5 days. Cells and debris were removed by centrifugation for 10 min at $\sim$ 1200g.

Protein purification

Ni-NTA agarose slurry (0.75 ml) (Qiagen GmbH, Hilden, Germany) containing approximately 0.35 ml beads in 20% ethanol, was added per 500 ml of conditioned media. After it was incubated overnight at 4°C on a shaker, the Ni-NTA agarose was pelleted by centrifugation for 10 min at approximately 2000g and transferred to columns. The beds were washed with PBS, pH 7.0, in the presence of 1 M NaCl and 0.1% Tween-20 and eluted with 100 mM imidazole (Merck, Darmstadt, Germany) in 20 mM Tris, pH 8.0, with 0.1 M NaCl, according to the manufacturer's instructions. Protein-containing fractions were pooled and dialyzed against PBS, pH 7.0, by using a membrane with a molecular weight cut-off of 12 000–14 000 Da (Spectra/Por Membranes, Spectrum Medical Industries, San Francisco, CA). To reduce the volume required for immunization, we concentrated the samples by using Centricon PLUS-20 microconcentrators (Millipore, Bedford, MA). Final protein concentrations were estimated both by comparison with a bovine serum albumin (BSA) standard curve on SDS-PAGE and by Bradford (BIO-RAD Protein Assay, Bio-Rad Laboratories, Temecula, CA). A Rainbow protein molecular weight marker (Amersham International, Buckinghamshire, UK) was used for size estimation.

Immunization procedures

The species-specific anti-IgE immunotherapeutic proteins, OCO and OCOCO, were emulsified 1:1 with the water-in-oil adjuvant Montanide ISA 51 (Seppic, Paris, France). The study was conducted in purpose-bred male *Cynomolgus* monkeys (Sennaville, Mauritius) at Covance Laboratories Ltd, Harrogate, UK. OCO and OCOCO were administered subcutaneously on the back to five monkeys each. The primary immunization dose was 500 μ g of each anti-IgE immunotherapeutic protein. The monkeys treated with OCO received in total four booster administrations during study weeks 3, 7, 29, and 31. The booster doses were 300 μ g for both OCO and OCOCO. The group treated with OCOCO was terminated in study week 18 and, consequently, those

animals received two booster doses in weeks 3 and 7. Sera for the study were sampled in weeks 1, 5, 9, 12, 15, 18 (both groups) and 21, 24, 27, 32, and 35 (OCO-treated animals only).

Preparation of sensor surfaces

All immobilizations were performed on sensor chip CM5 series S, in 0.010 M Hepes (pH 7.4) containing 0.15 M NaCl, 0.0034 M EDTA, and 0.005% polysorbate 20, at flow rate 10 μ l/min and at temperature 25°C. Since native IgE from *Cynomolgus* monkeys is not commercially available, the recombinant *Cynomolgus* IgE heavy-chain construct (CCC) was used as a ligand in the study, aimed to examine the species-specific anti-IgE responses. Standard amine coupling procedure (Biacore Sensor Surface Handbook) was used to prepare the surface for determination of the active concentrations. The sensor surface was activated 7 min with EDC/NHS followed by 20 min injection of the CCC construct diluted to 100 μ g/ml in 5 mM Na-malate (pH 6.5) and deactivated 7 min with ethanolamine. The immobilization level obtained was approximately 12 000 RU.

For the qualitative characterization of antibodies, methoxyPEG amine (Shearwater, Dallas, TX) was immobilized to the surface prior to antigen immobilization (Biacore, 2003). In the immobilization method, the sensor surface was activated 7 min with EDC/NHS, followed by 7 min injection of methoxyPEG amine dissolved to 7 mM in 0.01 M Na-tetraborate (pH 8.5) containing 1 M NaCl. These two steps were repeated and the surface was activated once again with

EDC/NHS with subsequent 3–5 min injection of 1 μ g/ml CCC construct (in the malate buffer as above) and the final deactivation with ethanolamine (7 min). The reference surface was prepared in the same way, but the last EDC/NHS activation and the injection of the CCC construct were omitted. The level of immobilized methoxyPEG amine was about 2000 RU on both the active and the reference surfaces, and the antigen was immobilized to about 50 RU.

Biosensor assays—general conditions

Serum samples were stored in sample compartment of the instrument at approximately 8°C until injection, whereas all the analyses on the chip were performed at 37°C. The running buffer used was the same as that during the immobilization. To determine the active concentrations of antibodies, serum diluted 1:20 in running buffer was injected at flow rate 5 μ l/min. To characterize the interaction between the antigen and specific serum antibodies from all the samples, the latter were diluted to the same concentration of 2 nM and injected for 6 min, followed by 30 min dissociation at flow rate 30 μ l/min. Each cycle was finished with two regenerations pulses: 1 min injection of 0.01 M glycine-HCl (pH 2.2) and 30 s injection of 0.005 M NaOH, both at flow rate 50 μ l/min.

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