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Validation of an ELISA for determination of antibodies induced in monkeys against Epi-hNE4, a recombinant protein inhibitor of human neutrophil elastase

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Abstract

The engineered protein inhibitor of human neutrophil elastase, Epi-hNE4, is being developed for the treatment of cystic fibrosis. Like many recombinant proteins, Epi-hNE4 may induce antibodies in pre-clinical species and in humans. The aim of this report was to validate an ELISA to assess its immunogenicity in monkeys. We have designed and optimized a classical ELISA in which Epi-hNE4 was coated directly on microtitre plates and the antibodies were detected using a secondary antibody labelled with peroxidase. We report implementation of the recent recommendations proposed for the validation of immunogenicity assessment. The cut-off point was determined by means of statistical analysis of negative samples. Linearity, reproducibility, stability and specificity were estimated using quality control samples obtained from a pool of positive samples. The method was applied to monkeys given Epi-hNE4 by inhalation. A confirmation test and a neutralization assay were developed in order to further assess positive samples. In conclusion, we present here one of the first examples of validation in application of recent recommendations [A.R. Mire-Sluis, Y.C. Barrett, V. Devanarayan, E. Koren, H. Liu, M. Maia, T. Parish, G. Scott, G. Shankar, E. Shores, S.J. Swanson, G. Taniguchi, D. Wierda, L.A. Zuckerman, J. Immunol. Methods 289 (2004) 1–16].

Keywords: Immunogenicity; ELISA; Validation; Recombinant; Epi-hNE4

1. Introduction

Biopharmaceuticals represent more than 25% of new products brought to market [2,3]. Although recombinant therapeutic proteins and monoclonal antibody-based products are designed to closely resemble their endogenous form, they may be identified as foreign by the immune system thus leading to the generation of specific antibodies. The incidence and characteristics of antibodies may depend on the structure of the therapeutic protein, its mode of administration and other factors which have been reviewed elsewhere [4,5]. Apart from some rare significant adverse reactions [6], unwanted antibodies usually affect the efficacy of drugs either by modifying their pharmacokinetic properties or by neutralizing their therapeutic activity [7–10]. Since this can impair clinical responses to treatment, the assessment of immunogenicity has become a safety and regulatory concern.

Induced antibodies may be characterized through a combination of analytical methods including binding assays such as ELISA (enzyme-linked immunosorbent assay) which is the technique of choice because of its sensitivity and high throughput [11]. ELISA relies on the detection of antibody bound to solid phase antigen adsorbed by a secondary reagent (classical ELISA) or by the labelled antigen (bridging ELISA). Irrespective of the format, these assays are semi-quantitative and their validation is an issue which has been addressed very recently in a consortium paper published in order to provide scientific background to standardization of immunogenicity assays [1]. So far, few papers have proposed examples of data or discussed issues related to these recommendations [12,13].

Epi-hNE4 is a 56-amino-acid recombinant protein derived from the second Kunitz-type domain of inter-alpha-inhibitor

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protein (ITI-D2) which is able to inhibit human neutrophil elastase with a Ki of 10^{-12} M [14]. The molecule was developed by Dyax Corp. (Cambridge, MA) using its proprietary phage display technology. *In vitro* and *in vivo* pharmacological studies have demonstrated its capacity to inhibit human neutrophil elastase (hNE) and to afford protection against lesions induced by hNE of sputum from children with cystic fibrosis [15]. Therefore, Epi-hNE4 represents a new class of cystic fibrosis medication differing from mucolytics like DNAase and from antibiotics like tobramycin. In order to assess its clinical efficacy, various preliminary toxicological studies in different species are currently underway. The opportunity to participate in the toxicological evaluation of this new biopharmaceutical offered us the possibility to apply the recommendations in the development of immunogenicity testing in monkey serum samples. Here we present and discuss the results of the development and validation of assays intended to assess the presence of antibodies to Epi-hNE4.

2. Materials and methods

2.1. Materials and reagents

Epi-hNE4, a 6237 Da protein (EACNLPIVRGPCIAFF-PRWAFDAVKGKCVLFPYGGC QGNGNKFYSEKECREY-CGVP) which contains three disulphide bridges, was discovered by Dyax Corp. (Cambridge, USA) and produced in a genetically modified strain of the yeast *Pichia pastoris* GS115. The protein was provided as a 12.2 mg/ml liquid solution by Debiopharm (Lausanne, Switzerland). Unless otherwise indicated, all reagents were from Sigma (St. Louis, MO). Purified monkey IgG and goat antiserum to monkey IgG (whole molecule) were from MP Biomedical (France). The peroxidase-conjugated goat IgG fraction to monkey IgG (whole molecule) was from Organon Technika (Durham, NC) and was used for the validation at 100 ng/ml (10,000-fold dilution). Peroxidase-conjugated protein G and protein A were from Pierce (Rockford, IL). The saturation buffer contained 0.1 M phosphate pH 7.4 (monobasic and dibasic potassium phosphate), 0.15 M NaCl, 0.1% BSA, 5×10^{-3} M EDTA and 0.1% Tween-20. The washing buffer contained 0.01 M, phosphate pH 7.4 plus 0.05% Tween-20. Washing was performed using an Autowasher 96 (Labsystems, Eragny, France). The dilution buffer was the same as the saturation buffer but without Tween-20. Staining was performed with tetramethylbenzidine TMB (MP Biomedical, France) for secondary peroxidase-labelled reagent and with the Ellman reagent (Spi-Bio, France) for acetylcholinesterase-labelled reagents. Plates were read with a Multiskan RC (Labsystems, Eragny, France).

2.2. Assay design and development

Owing the availability of specific reagents, two ELISA formats (direct and bridging) were tested. The direct format (which was further selected for assay validation) was performed using microtitre plates (Nunc, Denmark) coated with Epi-hNE4 at the concentration of 5 μg/ml in 0.05 M potassium phosphate buffer, pH 7.4, in a volume of 200 μl per well. After overnight incubation at room temperature and one wash cycle, 0.3 ml of the saturation buffer was added to all wells. The plates were then stored at about 4 °C for up to 2 months with good stability. Before use, the plates were washed and 50 μl of diluted monkey serum in assay buffer were added in duplicate to the wells before overnight incubation at approximately +4 °C. Then, the plates were incubated with 100 μl of a goat anti-monkey immunoglobulin labelled with peroxidase (or other reagents in the initial steps of assay development), at the concentration of 100 ng/ml at room temperature during 4 h. After a final washing step, 200 μl of substrate (TMB) were added and the enzymatic reaction was stopped by addition of 50 μl of 1 M hydrochloric acid after 30 min of incubation. Absorbances were measured at 450 nm. All measurements were made atleast in duplicate. Assay optimization involved the study of various parameters such as sample dilution, incubation time, the choice and concentration of secondary reagent (goat anti-monkey immunoglobulin, peroxidase-labelled protein A or G). The selection of optimal conditions was based on the degree of non-specific binding and the signal obtained for a pool of positive samples.

For the bridging format, all the initial steps were identical to the previous format excepted that after sample incubation, an enzymatic tracer (acetylcholinesterase-labelled Epi-hNE4, 100 μl at 200 ng/ml, Spi-Bio, France) was added overnight at 4 °C. At the end of the reaction, the plates were washed and 200 μl of Ellman reagent (Spi-Bio, France) were added to each well and the enzymatic reaction was monitored at 414 nm.

2.3. Assay validation

Assay validation was conducted following as closely as possible suggested recommendations [1]. This included determination of the cut-off point, and study of matrix interference, specificity, precision, stability and linearity. Positive samples were obtained from an initial pre-clinical study in which animals received Epi-hNE4 by inhalation: the absorbances obtained from samples taken at different times until 28 weeks were compared to pre-administration values (negative samples). Each sample whose absorbance was at least four times the pre-dose absorbance was considered positive. Pre-dose samples ($n = 28$) were pooled in order to obtain a negative control (QC−) which was used for the validation. Positive samples ($n = 44$) were pooled in order to obtain a high QC (quality control) which was diluted 20- and 80-fold in the pool of negative sample (QC−) in order to obtain a mid QC and low QC, respectively.

The cut-off point was determined after measuring the variability of 28 individual negative samples which were assayed on three different days. The cut-off point was obtained by a normalization approach to account for inter-assay variability. An upper negative limit at 95% was calculated on each day and was obtained as the mean of the absorbances of 28 samples plus 1.645 S.D. [1]. On each day, a normalization factor was calculated as the ratio between the mean plus 1.645 S.D. and the absorbance value of the negative control (QC−). The mean value of the normalization factor obtained on the three different days was then used to determine a cut-off point value for in-study runs, *i.e.* the

absorbance above which an individual result will be considered positive. Precision was studied by calculating the intra-day and inter-day variability ($n = 4$) of the negative control and the three QCs (high, mid and low).

Matrix effects were determined by studying the variability of 10 individual negative samples spiked with the high QC at a 2- or 10-fold dilution. In the absence of purified monkey antibodies, an indirect approach was to calculate a recovery using polyclonal rabbit antiserum spiked in the pool of negative controls or in buffer. Rabbit immunoglobulins were detected with a mouse monoclonal antibody against rabbit immunoglobulin labelled with acetylcholinesterase [16]. Stability was assessed as the variability of the absorbances of the high QC which were tested after incubation for 24 h at −20 °C, three freeze-thaw cycles and after 1 month and 6 months at −20 °C. Linearity was obtained by serially diluting the high QC sample in the negative control.

In order to assess possible interference due to the endogenous protein potentially present in the samples, the high QC sample was tested in the presence of Epi-hNE4 at the concentrations of 0, 0.1, 1 and 10 and 100 μg/ml. The result of this test formed the basis for the development of a confirmation test for any positive sample obtained at the primary screen.

Samples which signal was inhibited of at least 50% when incubated with 100 μg/ml of antigen (compared to signal obtained in absence of competitor) were confirmed as positive. To validate this level of 50% we have applied the confirmation test to samples known to be negative (pre-dose samples) but that gave a signal above the cut-off in the screening assay.

2.4. Enzyme immunoassay of Epi-hNE4 in samples

Epi-hNE4 was administered (one primary injection followed by boost injections, 1 mg each in complete Freund's adjuvant) to rabbits in order to obtain antibodies. An enzymatic tracer was obtained by conjugation of Epi-hNE4 to acetylcholinesterase as indicated above. In order to remove interfering antibodies, plasma samples were assayed after acidification with 0.1% TFA and extraction with a Sep-Pak cartridge (Waters, France). After washing and elution with acetonitrile, the eluate was dried and diluted in 0.1 M phosphate buffer pH 7.4 with 0.15 M NaCl, 5 mM EDTA, 0.1% bovine serum albumin and 0.01% sodium azide. The assays were then performed in 96 well microtitre plates coated with mouse monoclonal antibodies specific for rabbit immunoglobulins (Spi-Bio, Montigny-Le-Bretonneux, France) and the enzymatic activity was recorded using Ellman reagent. Unknown concentrations were calculated from a standard curve modelled with a cubic spline transformation (Immunofit, Beckman, Gagny, France). All measurements for standards and samples were made in duplicate. The limit of quantification was 5 ng/ml.

2.5. Animal studies

The method was applied to monkey samples which were obtained from a subchronic toxicity study in which the animals (groups of 4–7 males and females for all doses) were treated at 0, 0.75, 1.8 and 3 mg/kg by daily 30-min inhalation for 25 weeks. Serum samples were obtained before administration and at weeks 4, 13 and 25 post-initial dosing and were stored at −20 °C before analysis. All samples were tested for their antibody content by ELISA and the Epi-hNE4 concentration was measured by the enzyme immunoassay described above.

3. Results

The immunogenicity of Epi-hNE4 was assessed in three steps: a binding assay as a screening assay, a confirmation test for positive samples using an indirect immunodepletion step, and finally a neutralization assay (not described in this report) using a bioassay based on its inhibition of human neutrophil elastase, the biological target of Epi-hNE4.

3.1. Screening assay

3.1.1. Optimization of direct ELISA assay

Two ELISA formats (direct and bridging) were developed for the initial screen and confirmation assay. Initial assays demonstrated that the responses of positive samples were lower in the bridging format, which led us to develop a direct ELISA format. Reagent concentrations (*i.e.* coated antigen and secondary labelled reagent) were optimized using either Epi-hNE4 or monkey immunoglobulins coated on microtitre plates and tracers consisting of either protein A, protein G or goat anti-monkey IgG conjugated to peroxidase. The criteria of optimal conditions were based on non-specific binding, the respective signals of the high QC and the QC− (negative control) and their ratio. Although the absorbances were higher with peroxidase-labelled proteins A and G, we found that these reagents gave higher non-specific binding and greater variability in the results. Fig. 1 shows the effect on assay responses of the concentration of peroxidase-labelled goat anti-monkey immunoglobulins and sample dilution. Based on the absorbances for the negative control which we chose to maintain below 0.2, a concentration of 100 ng/ml was selected for the secondary reagent (Fig. 1A). The absorbances for serial dilution of the high QC in assay buffer indicated that the maximal response was obtained for dilutions between 1/50 and 1/200 (Fig. 1B) and the 100-fold dilution was then selected for assay validation as a compromise between signal intensity and sensitivity. The effect of the number of plate washing cycles (at each step) was also studied and we found that between 2 and 20 cycles, no modification of the assay response was observed (data not shown).

3.1.2. Statistical approach

The first step of the validation was to determine the assay cut-off point, *i.e.* the level of response at which a sample is defined as positive or negative. This was obtained statistically using negative samples and a normalization approach based on an inter-day analysis of variability. Twenty-eight serum samples from untreated monkeys and a serum pool from negative animals were analyzed on three different days (Table 1). The mean and the standard deviation were calculated for each day (homogeneity of variance checked using a Bartlett's test). On

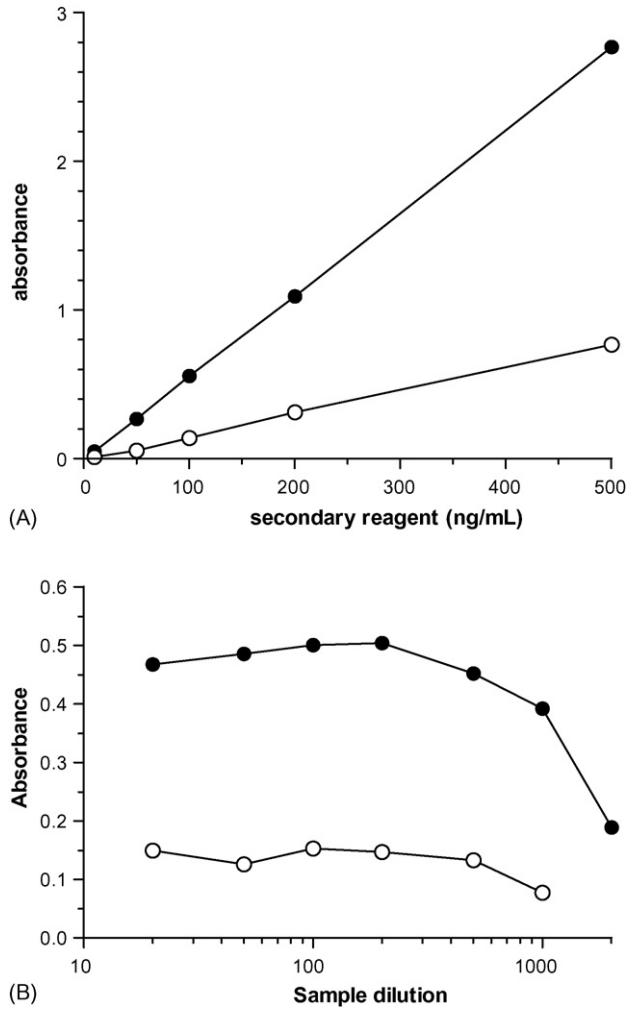

Fig. 1. Optimization of the secondary reagent concentration and sample dilution. (A) Specific signal (pool of positive samples, ●) and non-specific signal (pool of negative samples, ○) as a function of the concentration of the secondary reagent (anti-monkey immunoglobulins labelled with peroxidase). (B) Effect of sample dilution (in assay buffer) on the specific signal (pool of positive samples, ●) and non-specific signal (pool of negative samples, ○).

Table 1
Determination of the cut-off point

Sample #	Day 1	Day 2	Day 3
1	0.027	0.035	0.025
2	0.015	0.018	0.015
3	0.016	0.018	0.011
4	0.018	0.017	0.015
5	0.025	0.019	0.019
6	0.025	0.021	0.022
7	0.020	0.022	0.027
8	0.024	0.022	0.026
9	0.017	0.018	0.021
10	0.040	0.045	0.039
11	0.034	0.046	0.050
12	0.029	0.049	0.038
13	0.028	0.026	0.026
14	0.046	0.048	0.053
15	0.065	0.098	0.083
16	0.045	0.064	0.066
17	0.070	0.092	0.100
18	0.053	0.066	0.059
19	0.070	0.098	0.081
20	0.028	0.036	0.038
21	0.048	0.063	0.046
22	0.061	0.081	0.060
23	0.042	0.049	0.039
24	0.104	0.124	0.103
25	0.071	0.111	0.089
26	0.087	0.127	0.103
27	0.033	0.037	0.034
28	0.040	0.061	0.057
Mean	0.042	0.054	0.048
S.D.	0.023	0.034	0.028
Mean + 1.645 S.D.	0.080	0.110	0.095
Negative pool	0.076	0.105	0.077
Normalization factor	1.05	1.05	1.23

Values underlined are above the cut-off point.

each day, an upper negative limit (*i.e.* the cut-off point) was calculated as the mean plus 1.645 S.D. which consists in eliminating 95% of negative samples. The mean cut-off point had a reasonable variability (mean of 0.095 with a CV of 16%). In order to correct the inherent inter-day drift, the cut-off point was normalized by comparing it to that of a pool of negative samples to be used for the assay of real samples. The mean ratio (cut-off point/negative pool, Table 1) was 1.11 ± 0.10 which represents a variability significantly lower than that obtained for the inter-day cut-off points. In-study samples were assayed using this normalized factor. The precision of the assay was assessed by studying the intra-day and inter-day variability of the response of the high, mid and low QCs. The results shown in Table 2 indicate signal variabilities for either the absorbances or the absorbance ratio in the range of 4–13%. A supplementary experiment was performed using a polyclonal rabbit antiserum which was serially diluted either in buffer or in the negative control (monkey serum) and further assayed after a 100-fold

Table 2
Repeatability (intra-day) and reproducibility (inter-day)

	High QC	Mid QC	Low QC	Negative pool
Intra-day				
Mean absorbance (CV)	0.628 (4.9%)	0.287 (7.9%)	0.141 (7.0%)	0.063 (9.6%)
Ratio[a]	8.5 (10.0%)	4.6 (7.8%)	2.2 (7.1%)	
Inter-day				
Mean absorbance (CV)	0.679 (10.0%)	0.328 (7.6%)	0.172 (8.7%)	0.076 (12.2%)
Ratio[a]	9.0 (9.4%)	4.0 (7.4%)	2.3 (7.0%)	

[a] Absorbance of the QC divided by absorbance of the negative pool, $n = 4$ for all determinations.

Table 3
Determination of recovery

Dilution	Assay buffer	Monkey serum	% recovery
1/1000	0.388	0.431	112
1/5000	0.394	0.41	104
1/10000	0.375	0.344	92
1/50000	0.142	0.097	68

Table 4
Study of matrix effects

Samples #	Dilution	
	2-fold	10-fold
1	0.64	0.472
2	0.605	0.444
3	0.627	0.476
4	0.622	0.485
5	0.638	0.339
6	0.619	0.368
7	0.588	0.447
8	0.608	0.425
9	0.686	0.453
10	0.62	0.348
Mean ± S.D.	0.625 ± 0.026	0.426 ± 0.054
CV	4%	12.60%

The high QC was diluted 2- or 10-fold in 10 individual negative samples.

dilution. Although, the best test would have been the use of purified monkey antibodies, this experiment was also used as an indicator of recovery since it was considered to reflect the possible inhibition of serum components on binding of antibodies to antigen-coated solid phase. As shown in Table 3, the ratios of absorbances between monkey sample and assay buffer were above 68%, indicating that at the tested concentration there was no significant inhibition of binding. Matrix interferences were also tested by diluting the high QC in 10 different negative samples and at two dilutions (2- and 10-fold). As shown in Table 4, a CV of 12.6% was obtained with the highest dilution. Although this was higher than the intra-assay variability (Table 2), it was considered to be indicative of the absence of important sample-to-sample matrix interference. The results of the linearity study are shown in Fig. 2. Serial dilution of the high QC sample in the negative control (QC−) gave absorbances which increased with the sample concentration with a tendency to a plateau at low dilutions.

3.2. Confirmation test

Finally, the effect of the presence of the antigen itself was assessed to discard the theoretical 5% of false positive generated by the screening assay. The high QC was diluted 100-fold and then spiked with increasing concentrations of the antigen. The experiment was repeated on three different days and the results are shown in Fig. 3. The lowest concentration (0.1 μg/ml) decreased the signal obtained in the absence of Epi-hNE4 (signal of reference) by only 14%, while the highest concentration tested (100 μg/ml) gave an absorbance still above the assay cut-off point and decreased the signal of reference by 75%. These results were not changed if, in order to mimic an *in vivo* situation, the samples were pre-incubated with the antigen for 24 h. We used this information to implement an indirect immunodepletion test used to confirm the binding assay [13]. The aim was to identify false-positive results obtained with the screening assay by analyzing them in the presence of 100 μg/ml Epi-hNE4. A decrease by 50% was chosen as a limit to identify false positives from positive samples: any sample above the cut-off during the screening assay that is not inhibited by more than 50% in the presence of 100 μg/ml of Epi-hNE4 was considered as false-positive in a confirmation test.

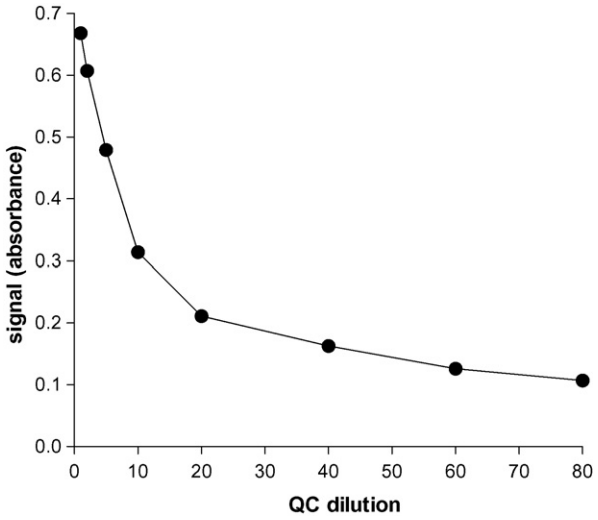

Fig. 2. Study of linearity: the high QC was tested undiluted and serially diluted in a pool of negative samples (QC−) and each new sample was assayed by ELISA (after a 100-fold dilution in assay buffer, *i.e.* the optimal dilution determined from Fig. 1).

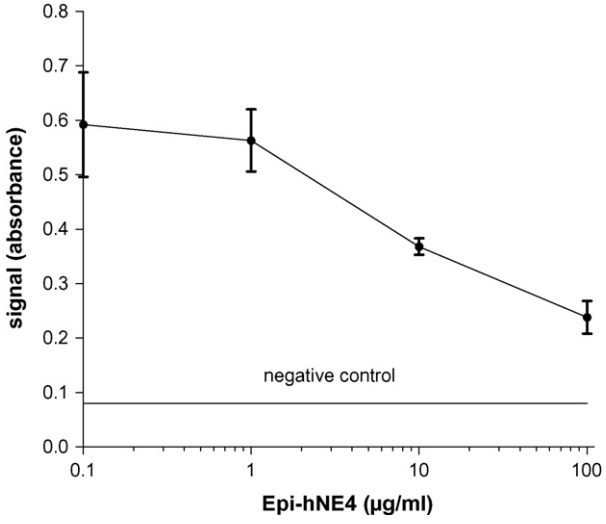

Fig. 3. Study of potential interference by the antigen: the high QC was diluted 100-fold in assay buffer and co-incubated with various concentrations of Epi-hNE4, before ELISA. The absorbance in the absence of Epi-hNE4 was 0.680. The specific signal was calculated from the difference in absorbances between the total signal and the signal of the negative pool (0.080). IC50 (8 μg/ml) was calculated as the concentration that inhibits 50% of the specific signal.

We have applied the confirmation test to samples known to be negative (pre-dose samples) but which produced a positive response in the screening test. The two samples of the study (see Table 1) spiked with 100 μg/ml of Epi-hNE4 showed a maximum decrease of their signal by 30%. Further data were obtained from another study: samples taken before the administration of the recombinant protein were screened with the first test. Seven of them were above the cut-off (*i.e.* they were false positive), but after antigen depletion, the signal reduction was $21 \pm 28\%$. Owing this variability, we choose 50% as a security level (which correspond to the mean plus one standard deviation, or two-fold the signal reduction). It should be pointed out that there is currently no accepted guideline for determining the minimum reduction signal in the confirmative assay, and this issue has been recently discussed [17].

3.3. Stability assessment

Stability was assessed by assaying the high QC and a pool of negative control samples and comparing their signal and ratio. No significant (>15%) variation from initial values was found under the tested conditions (24 h at 20 °C and 1 month and 6 months at −20 °C, three freeze-thaw cycles).

3.4. Pre-clinical results

Following this validation, the assay was applied to samples from monkeys given different doses of Epi-hNE4 by inhalation. The results presented in Fig. 4 indicate that antibodies to Epi-hNE4 were present at the first sampling time (4 weeks). Whatever the dose, sex and sampling time, more than 75% of animals were positive (not shown in the figure). There was no significant difference between males and females and the lowest dose tended to higher antibody contents. The mean concentrations of Epi-hNE4 of each group were less than 300 ng/ml, indicating the absence of potential interference by the endogenous antigen in the antibody assay. It should be pointed out that the immunoassay was free of interference by endogenous antibodies. This was done using a specific sample preparation procedure which involved the denaturation of endogenous antibodies by trifluroacetic acid and extraction with the Sep-Pak cartridge.

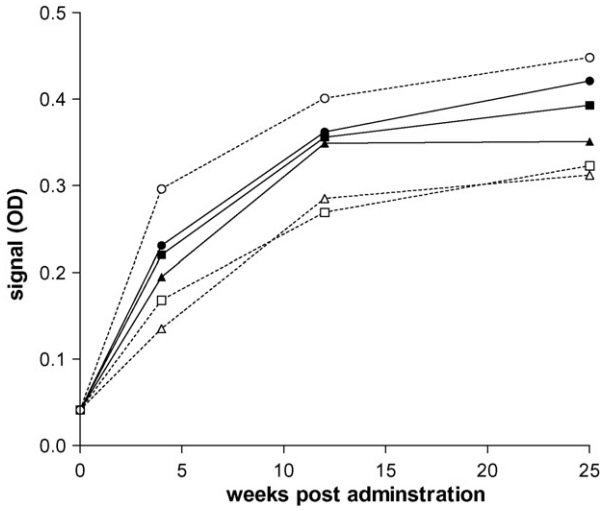

Fig. 4. Mean (from $n = 4$–6 animals per group) absorbances in the ELISA of monkey sera (open symbols: females; closed symbols: males; ○ and ●: dose 0.75 mg/kg; ■ and □: dose 1.8 mg/kg; ▲ and △: dose 3 mg/kg) after daily administration of Epi-hNE4 by inhalation.

4. Discussion

The recent exponential growth of recombinant proteins and antibodies has raised issues such as the assessment of their immunogenicity. Assay developments have already been described [12,18–20] and specific recommendations have been proposed [1]. We tried to follow these recommendations for the assessment in monkey serum of antibodies to Epi-hNE4, a recombinant protein being developed for the treatment of cystic fibrosis. The data presented in this paper illustrate how the validation may be applied in classical assay situations. Among the various techniques used to monitor antibodies to biopharmaceuticals, we chose a direct ELISA format which has the advantage of sensitivity and high throughput [11,13].

A first point to consider is the choice of a standard or a positive control in order to monitor assay performance. Chimeric antibodies or purified rabbit polyclonal antibodies used as standards allow true quantitative assay only if the affinity or isotypes are the same as those of polyclonal antibodies present in the sample [21]. A second difficulty is that these standards may be unavailable for initial pre-clinical studies. Finally, and despite the fact that they may be only used for quantification, standards may not be good candidates for validation of long-term stability and linearity, or for assay steps such as plate washing where high-affinity antibodies may provide better reproducibility than real samples.

For these reasons, a positive control obtained in animals after immunization with the protein represents a better alternative, when available. Owing to the natural maturation of antibody affinity with time or after repeated administration, it is likely that polyclonal antibodies of various affinities may be obtained. In the phases of our development, we had the opportunity to obtain samples from monkeys given Epi-hNE4 daily by inhalation. We developed an initial screening using an unvalidated assay by comparing the assay response for the same animal before and after selected times after administration.

For immunogenicity testing, the absence of a standard means that the classical estimation of a quantification limit must be replaced by the evaluation of a cut-off point. We followed the recommended strategy of establishing an upper negative limit which results in a 5% false-positive rate, and then a normalization approach which aims to study inter-assay drift in the cut-off point. The mean ratio between the cut-off point and the value for a pool of negative samples gave a mean normalization factor which was used as a criterion of positivity for the assays of toxicological samples. In our example, we demonstrated that this gave better reproducibility than that obtained using a fixed absorbance.

The positive QCs and the negative pool samples were used for tests of stability, linearity, repeatability and reproducibility. Typically, we found intra-day and inter-day variabilities below 12% which provided a basis for in-study criteria of acceptance. So far, there is no specific recommendation, but we estimate that QC values or the QC/negative ratio around ±25% of their validated value can be accepted for the validation of in-study assays.

Assay specificity, linearity and matrix interference were assessed by various methods owing to the absence of standards. Our first approach consisted of the dilution of the high QC in different negative samples in order to assess both selectivity and matrix interference. Interference by the drug itself has to be considered in the specificity study. ELISA cannot be specific in immunogenicity testing since any response can be decreased by the antigen. The amount of antigen to be added in order to inhibit the response is dependent on many parameters, such as the affinity and concentration of the antibodies present in the samples or the concentration of the antigen coated on the solid phase.

Using the high QC, we found that the specific binding was 50% inhibited with a concentration of 8 μg/ml, which is largely superior to that found in the samples (300 ng/ml). It should be pointed out that any evaluation of antigen interference requires that the method used to quantify the protein in biological fluids be free of interference from induced antibodies, a difficulty which has rarely been addressed. In contrast, antigen interference in ELISA can be circumvented by special sample preparation [22]. Finally, and in the now commonly applied strategy of immunogenicity testing which consists of stepwise screening/confirmation/neutralizing assays, the use of a competitor mixed with the sample allows an indirect immunodepletion procedure that can be used to eliminate the statistically 5% false-positive samples [13]. Furthermore, the levels of competitor used to inhibit the binding of antibodies to microtitre plates can also be used as an indicator of the mean affinity of the antibodies present in the samples [23]. However, the exact determination of affinity and concentration remains an interesting challenge since it will allow calculating the concentration of therapeutic protein bound to the induced antibodies.

In conclusion, this report describes one of the first applications of the recent recommendations. Most of the technical issues that we faced (absence of standards, determination of the cut-off point, classical ELISA format, low-affinity animal samples, etc.) are typical situations for numerous proteins undergoing pre-clinical trials. Our report should provide data for future implementation of new validations.

Acknowledgement

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