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# Low pH inactivation for xenotropic gamma retrovirus in recombinant human TNF-α receptor immunoglobulin G and mechanism of inactivation

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ABSTRACT

CHO-derived recombinant proteins for human therapeutic are used commonly. There are noninfectious endogenous retroviruses in CHO cells. Validation study for inactivation process is required. Murine xenotropic gamma retrovirus (X-MulV) is a model virus in validation study. In our previous study, optimum conditions for X-MulV inactivation were sifted. In this study, we performed a further research on low pH inactivation for evaluation of X-MulV clearance in manufacturing of recombinant human TNF-α receptor immunoglobulin G fusion proteins (rhTNF-α) for injection. Cell-based infectivity assay was used for the evaluation of X-MulV clearance. RhTNF-α were spiked with X-MulV and were inactivated at pH 3.60 ~ 3.90, 25 ± 2 °C, and 0 ~ 240 min, respectively. Samples incubated at the conditions for 15 ~ 180 min were not inactivated effectively. For 4 h incubation, $\log_{10}$ reductions were achieved 5.0 $\log_{10}$. Biological activity of rhTNF-α incubated at pH 3.60, 25 °C for 4 h, which was assayed on murine L929 fibroblasts cells, was not affected by low pH. *Env* gene of X-MulV, which was detected by conventional PCR method for the first time, was not detected after incubation at pH 3.60, and it may be the mechanism of low pH inactivation.


## 1. Introduction

Endogenous contaminants are contained in biological products of human origin, animal origin and microorganism origin. WHO, FDA, Europe Agency for evaluation of medicinal products and China Food and Drug Administration have formulated guidelines for endogenous contaminant in biological products. It is stipulated that there must be a removal or an inactivation procedure for endogenous contaminant in manufacturing, and assessment study must be conducted at a lab-scale [1–4].

Chinese hamster ovary (CHO) cells are commonly used in manufacturing of pharmaceutical proteins; however, there are noninfectious endogenous retroviruses in CHO cells [5]. For the safety of human use, inactivation process in manufacturing is compulsive, and evaluation of this process at a lab-scale is compulsive, too [6]. Murine xenotropic gamma retrovirus (X-MulV) is the surrogate for the retrovirus in CHO cells in assessment study.

In our previous study, we investigated the impacts of pH, temperature, time and protein content on X-MulV inactivation. By orthogonal analysis and regression analysis, the optimal conditions that were selected included pH 3.50, 25 °C and 4 h, which were applicable in a lab environment for inactivation of CHO-derived proteins. However, parameter values are not fixed at a certain value, especially the parameter of temperature; usually the temperature will fluctuate. Thus, the ranges of allowable variation of pH values, temperature and time were investigated in this study.

Simultaneously, the biological activity of recombinant proteins which were incubated in low pH condition was tested. In addition, the mechanism of low pH inactivation was examined: the effect of low pH on *env* gene was detected by conventional PCR method for the first time.

## 2. Materials and methods

### 2.1. Cells and viruses

PG-4, a feline brain cell line (S + L-), was purchased from ATCC (Manassas, Catalog No. CRL-2032). The cells were grown in McCoy's 5A media (Invitrogen) with 10% (V/V) fetal calf serum (FBS). Murine L929 fibroblasts cells (tumorigenic positive) were purchased from

*Abbreviations:* CHO, Chinese hamster ovary; X-MulV, Murine xenotropic gamma retrovirus; rhTNF-α, Recombinant human TNF receptor immunoglobulin G fusion proteins; PCR, Polymerase chain reaction; *Env*, Envelope.

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National Institutes for Food and Drug Control (NIFDC, Beijing), and incubated in MEM medium with 10% equine serum. All medium were added with 2 mM of L-glutamine, 100 U/ml of penicillin and 100 mg/ml of streptomycin. The cells were cultured at 37 °C in a 5% $CO_2$ environment.

Murine xenotropic gamma retrovirus, strain pNFS Th-1, was purchased from ATCC (Catalog No.VR-1447). The virus was incubated on CHO cells in DMEM/F12 (1:1) medium with 5% FBS, and harvested by ultracentrifugation on day 7 post-infection. The CHO cell line was gifted by Dr. Minggang Bi (Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing). Titer of the viral stock was 8.78 ± 0.25 $\log_{10}$ PFU/ml, which was titered on PG-4 cells [8].

### 2.2. Source of rhTNF proteins

Three batches of recombinant human TNF-α receptor immunoglobulin G fusion proteins for injection (rhTNF-α), with the protein content of 2.0 mg/ml, were provided by Baiaotai Biotechnology Co., Ltd., Guangzhou. Four batches of rhTNF-α, with the protein content of 5.0 mg/ml, were provided by Celgenpharm Co., Ltd., Shanghai.

### 2.3. Low pH incubations

Virus stocks and rhTNF-α were mixed together with the ratio of 1:9 (v/v). The mixture was split into tubes, which were incubated for 0, 15, 30, 60, 120, 180 and 240 min at 25 ± 2 °C at pH 3.60 ~ 3.90 for viral inactivation; respectively. The samples were adjusted to pH 7.00 with 1.0 mol/L Tris-based solution and were filtered by 0.22 μm filter units. One ml of the adjusted mixtures was saved as samples for cell-based infectivity assay. Samples at pH 7.00 were also prepared and incubated for 0 h. All samples were prepared in duplicate.

### 2.4. PG-4 S + L-assay

Infectivity assay was performed as our previous study [8]. Briefly, PG-4 S + L-cells were seeded into 6-well plates at a density of approximately 2 × $10^6$ cells/ml. All samples including positive and negative controls were prepared in triplicate. For 24 h of inoculation, the media was replaced with the diluted viral sample buffer (explained in 2.3.), and 8.0 μg/ml of polybrene was contained. As for the negative control, the medium was replaced with non-complete medium. Incubation lasted for seven days at 37 °C. Cytopathic effects were observed under the inverted microscope in order to record the number of plaques. Each sample was set in triplicate.

The virus titers were calculated as follows: PFU/ml = dilution folds × means of plaques observed × the reciprocal of volume of inoculation. Viral titers were expressed in a logarithmic form of PFU.

Virus titers that were decreased greater than 4.0 $\log_{10}$PFU indicated an effective inactivation. For the three generations of blind passages, if no lesions were observed, they were considered as effectively inactivated.

### 2.5. Env gene detection

PG-4 cell cultures contained non-inactivated or inactivated X-MulV were extracted using Trizol (Invitrogen), chloroform, isopropanol and 75% alcohol. Then the RNA was dissolved in 0.1% DEPC-processed water. One microgram of total RNA from each sample was used as a template for cDNA synthesis by a First Strand cDNA Synthesis Kit (Toyobo). A 305 bp fragment from the 3′ end of the *env* gene was amplified. An equal volume of cDNA product was used in the PCR performed using the Power Taq PCR Master Mix (BioTeke). Two primers were used for *env* gene sequencing: 5′ CAC TGG CGT AGT AAG GGA TAG C 3′ (forward) and 5′ GCT ATC CCT TAC TAC GCC AGT G 3′ (reverse), which were synthesized by Invitrogen. The PCR reaction conditions were set according to the protocol coming with primers. A 4-min hot-start Taq activation at 94 °C and 30 cycles of amplification are contained. Each amplification cycle was composed of 15 s denaturation at 94 °C, 30 s annealing at 55 °C, 60 s extension at 72 °C. The PCR products were examined using an agarose gel containing ethidium bromide (Sigma), and exposed on a UV transilluminator (Model: GBOX-HR, Gene Company Ltd.). To confirm the specificity of the RT-PCR, the amplified DNA fragments were sequenced by Life Technology Co.. Sensitivity assay of *env* gene amplification was tested with the thermal cycler using 2-fold serial dilutions of cDNA.

**Table 1**
$\log_{10}$ reductions of X-MulV incubated at pH 3.6 ~ 3.90, 25 °C and 4 h.

| pH | 7.00 | 3.60 | 3.70 | 3.80 | 3.90 |
|---|---|---|---|---|---|
| Lot 1 | 1.64 | 5.12 | >5.96 | 5.96 | 5.96 |
| Lot 2 | 1.67 | 5.48 | 5.96 | 5.57 | 5.57 |
| Lot 3 | 1.81 | >5.96 | 5.66 | 5.81 | 5.81 |
| Lot 4 | 1.91 | 5.46 | 5.31 | 5.81 | 5.42 |

Notes: RhTNF-α was provided by Celgenpharm Co., Ltd.. >5.96 represented no virus was detected with the limit of sensitivity of the order of 2.82 $\log_{10}$PFU. The non-inactivated samples were diluted $10^6$ folds for titer detection, and the inactivated ones were diluted $10^3$ folds. Values were mean of three wells in one copy and each sample was set in duplicate.

### 2.6. Cell growth inhibition assay

The growth inhibitory effects of rhTNF-α on L929 cells was measured by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, tetrazolium salt) staining. Briefly, the cells were dispensed in 96-well plates with 1 × $10^5$ cells/ml. After 24 h incubation, they were treated with or without rhTNF-α at given concentrations for 24 h, containing 1.0 μg/ml actinomycin D. The cells were then stained with 5.0 mg/ml MTT at 37 °C for 4 h, followed by lysis in DMSO. The absorbance was measured using a microplate reader (Model: VarioskanFlash, Thermal) with a 570 nm filter. The percentage of cell growth inhibition was calculated as follows: cell inhibition (%) = 100 − (A570, control − A570, experiment)/A570, control × 100.

### 2.7. Statistical analysis

$IC_{50}$ values were calculated via Bliss analysis by SPSS 13.0 statistical software.

## 3. Results

### 3.1. Virus titers at different pH, temperatures and time

The results are listed in Tables 1–3. Reductions of viral titers at pH 3.60 ~ 3.90 and 23 ~ 27 °C were all greater than 5.0 $\log_{10}$; respectively. Plaques in samples incubated at pH 3.60 and 25 °C

**Table 2**
$\log_{10}$ reductions of X-MulV incubated at pH 3.60, 23 ~ 27 °C and 4 h.

| (°C) | 23 | 24 | 25 | 26 | 27 |
|---|---|---|---|---|---|
| Lot 1 | 5.46 | 5.11 | 5.53 | 5.05 | 4.81 |
| Lot 2 | 5.96 | 5.81 | 5.61 | 5.57 | 5.81 |
| Lot 3 | 5.37 | 5.48 | 5.31 | 5.57 | 5.23 |
| Lot 4 | 5.96 | 5.18 | 5.37 | 5.25 | 5.66 |

Notes: RhTNF-α was provided by Celgenpharm Co., Ltd.. Values were mean of three wells in one copy and each sample was set in duplicate.