

Expression of the chimeric IgE gene in cell culture and in various mouse tissues

D.V. Rodin ^{a,*}, B.V. Rad'ko ^{b,1}, V.A. Kolesnikov ^a, O.L. Polanovsky ^a, S.M. Deyev ^b

^a Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow 117984, Russia

^b Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow 117997, Russia

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Abstract

Transient expression of recombinant gene constructs is now more widely used in gene therapy as well as in DNA vaccination. In this study, the ability of one and the same genetic construct to drive gene expression both in cell culture and in tissues of the whole organism was demonstrated. Chinese hamster ovarian cells (CHO) were transfected in vitro with plasmids bearing the genes for chimeric IgE (mouse/human) antibodies under control of the human cytomegalovirus (hCMV) promoter. Secretion of recombinant IgE antibodies by transfected cells reached 60% of the intracellular concentration of antibodies. The same gene constructs were introduced into various mouse tissues using ballistic transfection in vivo. The IgE content in blood after transfection of cartilage was found to be several times lower than after transfection of the liver, spleen, or foot pad. At the same time, the content of antibodies to the xenogenous determinants of IgE was essentially independent of the tissue type. These data can be employed in selecting conditions for genetic immunization and gene therapy.

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1. Introduction

Transient expression of recombinant genes in eukaryotic cells is of fundamental importance in gene therapy and DNA vaccination studies. It is believed that gene expression in cell culture is reliable for the preliminary testing of an engineered construct before its application in vivo. To address this issue, plasmids containing genes of chimeric (mouse/human) antibodies [1–4] under control of the hCMV promoter, were introduced into CHO-K1 cells. Chimeric antibodies are of substantial practical importance, in particular in tumor targeting and therapy [5–8], and chimeric IgE antibodies have been shown to be more effective than IgG in immunoglobulin-dependant carcinoma cell killing [9]. The IgE isotype is anticipated to be therapeutically effective, not only

for cancer treatment, but also for peripheral diseases and parasite infections [10–12]. The introduction of chimeric antibodies into a patient by transfection of recombinant DNA constructs holds promise if it can be shown that the method provides not only the stable long-term expression of the gene and secretion of the resulting protein into the bloodstream, but also that these antibodies efficiently recognize natural epitopes in vivo. Thus, studying gene expression in a eukaryotic system is of interest for the development and improvement of gene therapy approaches.

The aim of this work was to evaluate the efficacy of expression of chimeric immunoglobulin genes: expression profiles in vivo and in vitro may differ in, and since optimum functioning of immunoglobulin requires a complicated process of folding.

We designed vector plasmids for efficient expression of chimeric (mouse/human) IgE genes under control of the hCMV promoter and transfected them into CHO-K1 cells. Expression of transfected genes in vitro was estimated and the results were compared with those obtained using the same constructs in vivo [1].

Abbreviations: CHO, Chinese hamster ovarian cells; hCMV, human cytomegalovirus; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; IPTG, Isopropyl β-D-thiogalactopyranoside; TMB, tetramethylbenzidine.

* Corresponding author. Tel.: +7-095-1359958; fax: +7-095-3306392.

E-mail address: rodin@imb.ac.ru (D.V. Rodin).

¹ Authors who equally contributed to this study.

2. Materials and methods

2.1. Plasmid constructions

Gene manipulations were carried out as described [13] in *E. coli* strain JM103 using enzymes supplied by “Fermentas” (Lithuania). Final constructs were verified by standard dideoxy sequencing. To construct a plasmid carrying the hCMV regulatory sites a 293 bp fragment of plasmid pCMV containing the hCMV promoter was cloned into *EcoRI*/*SacI* digested plasmid pUC19. After transformation, colonies with the insert were selected on agar plates containing X-gal and IPTG. The presence of the insert was checked by PAGE of the resulting plasmid, pUC.CMV293, cleaved with *EcoRI* and *SacI* restriction endonucleases. Plasmid pUC.pur was designed by cloning of a *HindIII*/*EcoRI* DNA fragment from plasmid pPAC1 [14] into plasmid pUC19. This DNA fragment carries the *N*-acetyl transferase gene of *Streptomyces alboniger* and after transfection it confers puromycin resistance on mammalian cells.

A plasmid carrying the gene of the mouse immunoglobulin light chain under control of the hCMV promoter. A fragment, VkCk, was obtained by *HindIII* digestion of plasmid pIG.5k [2], and was cloned into *HindIII* digested plasmid pUC.CMV293. The resulting construct, pUC.CMV.VkCk, with the properly oriented fragment VkCk was digested with endonuclease *EcoRI* and then subjected to partial cleavage with restriction endonuclease *HindIII*. The resulting fragments were separated on an agarose gel and the excised *EcoRI*-CMV.VkCk-*HindIII* fragment was eluted from the gel slice. The isolated fragment was cloned into plasmid pUC.pur cleaved with *EcoRI* endonuclease. Ligation was performed to join the *EcoRI* ends of the plasmid and the insert. Then the *HindIII* end of the insert and the free *EcoRI* end of the plasmid were filled in using Klenow fragment and ligated at the blunt ends. Fig. 1A shows the schematic organization of the hCMV enhancer/promoter and the 5' end of the immunoglobulin light chain Vk gene of plasmid pUC.pur.CMV.VkCk.

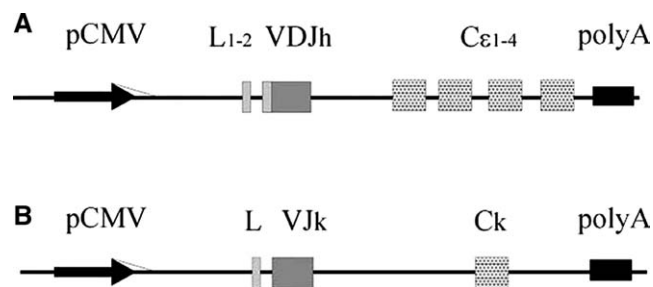


Fig. 1. Organization of chimeric mouse/human IgE genes. (A) Location of exons and promoter/enhancer region in chimeric heavy chain gene from the plasmid pUC.CMV.VhCε. The leader peptide (L) encoded by two exons (L1-2); variable gene (*Vh*) contains gene segments *Vh*, *D* and *Jh*; constant gene *Cε* consisting of four exons. PolyA is on the 3'-end of the constant gene. (B) Scheme of organization of chimeric light chain gene from the plasmid pUC.CMV.VkCk. The leader peptide (L); variable gene (*Vk*) contains gene segments *Vk* and *Jk*; constant gene *Ck*. PolyA is on the 3'-end of the constant gene.

A plasmid carrying the gene of the human immunoglobulin heavy chain under control of the hCMV promoter. Plasmid pIG.1e [2] was hydrolyzed with restriction endonucleases *XbaI* and *PstI*. The resulting VhCε fragment was eluted from low-melting agarose, made blunt using Klenow fragment, and cloned into *SmaI* digested plasmid pUC19.CMV293 to produce plasmid pUC.CMV.VhCε. The insert orientation was determined by restriction analysis of isolated plasmid DNA. Fig. 1B gives a scheme of the hCMV enhancer/promoter region and the 5' end of the heavy immunoglobulin chain *Vh* gene from plasmid pUC.CMV.VhCε.

2.2. Transfection

Plasmid DNA for transfection was isolated by alkaline lysis and purified by ultracentrifugation in a CsCl concentration gradient [13]. Co-transfection of CHO cells was performed using the calcium phosphate method. Equimolar amounts of plasmids pUC.CMV.VhCε and pUC.pur.CMV.VkCk (about 6 μg each) were added to a 50 ml flask at a cell confluence of 30–40%.

Control CHO cells were transfected with plasmid pCMV.LacZ. In this case cells were stained with X-gal through 48 h. Two days after transfection, puromycin was added to the culture to a concentration of 5 μg/ml and resistant clones were isolated. CHO cells were grown for 24 h at 37 °C in a CO₂-incubator to confluency in 50 ml flasks containing DMEM supplemented with 10% FCS (Gibco BRL). Cells were collected, counted in Goryaev's cell counter and lysed by sonication on ice or by quick freeze-drying in liquid nitrogen and warm water.

2.3. Fluorescence microscopy

CHO cells transfected with plasmids pUC.CMV.VhCε and pUC.pur.CMV.VkCk producing ε and κ chains, respectively (as shown by ELISA assay), were grown on glass slides and fixed with methanol. Slides with cells were dried and incubated in 4xSSC with mouse antibodies against constant human IgE domains. The samples were then incubated with FITC-conjugated rabbit anti-mouse antibodies. Cell nuclei were stained with propidium iodide. Kodak (Great Britain) color film was used with a Leitz Planoscope microscope for taking pictures of fluorescent preparations.

2.4. Ballistic transfection

Genetic constructions were introduced using the installation and the technique described [25]; the ratio of microparticles of gold, tungsten, platinum, and palladium in the mixture was 4:4:1:1. Solutions of plasmids pUC.CMV.VhCε and pUC.CMV.VkCk (15 μl each, DNA concentration 1 μg/μl) were added to 10–15 mg of the particles. The precipitation of DNA on the particle surface was intensified by the calcium phosphate precipitation at room temperature. The particles were accelerated on the surface of a macroaccelerator by

mercury fulminate explosion. The macroaccelerator was stopped at a distance of 12–23 cm from the object, and the particles were ejected as a stream 2.5–6 cm in diameter.

2.5. Mouse blood serum

The blood for the immunochemical testing was collected from the mouse tail vein into a polypropylene test tube (1.5 ml, Eppendorf) placed so as to prevent the drop from sliding to the bottom. After 15–45 min, the serum was accumulated in the lower part of the drop. Hemoglobin, considerably hindering the measurements, coagulated and was adsorbed on the test tube wall. To facilitate the separation of the serum, the test tube inner surface was covered with a thin layer of Repel-Silane (LKB).

2.6. ELISA

A standard test system for human IgE was used (Biotechnologiya Co., Russia). The protein concentration was calculated from the absorbancy of the solutions at 450 nm (the maximum absorbance wavelength when TMB was used as a substrate in a Roche buffer) measured on a Multiscan spectrophotometer.

3. Results

CHO-K1 cells and various mouse tissues were transfected with plasmids pUC.CMV.VhC ϵ and pUC.pur.CMV.VkC κ . In these constructs genes of mouse light and human heavy chains of recombinant immunoglobulins were under control of hCMV promoter (Fig. 1). On 5'-end this promoter was preceded by the enhancer region that was also included in the construct.

3.1. In vitro expression

It was shown by fluorescence microscopy that CHO cells efficiently express C ϵ domains of chimeric antibodies (Fig. 2). Cells, not producing C ϵ domain of heavy chain of chimeric antibodies, were considered as negative control and allowed one to estimate on the photograph the level of expression in the cells producing heavy chains of immunoglobulins. Different clones of transfected CHO cells were characterized by different levels of biosynthesis of recombinant IgE. The clones of CHO cells effectively co-transfected with plasmids pUC.CMV.VhC ϵ and pUC.pur.CMV.VkC κ , were grown up to monolayer, incubated in the medium, collected, and disintegrated and the amount of antibodies, synthesized in these cells, was determined by immunoenzyme assay. Co-transfection with plasmids pUC.CMV.VhC ϵ and pUC.pur.CMV.VkC κ resulted in different expression of chimeric IgE by different CHO clones: from 0.5 to 15 ng per 10⁶ cells.

3.2. In vivo expression

We used large amounts of DNA in order to increase the probability of the efficient occurrence of the whole IgE

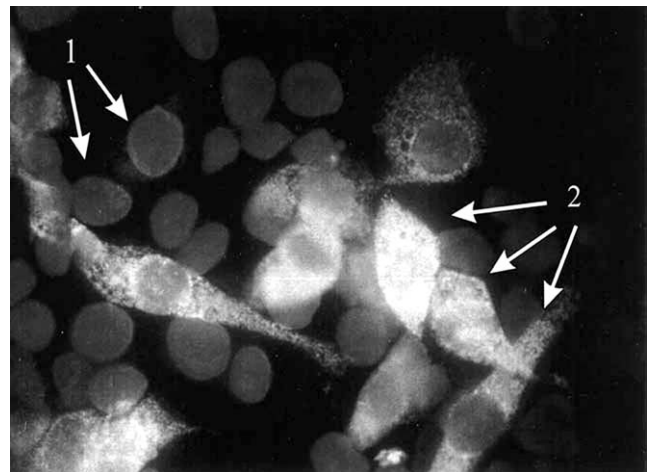


Fig. 2. Fluorescent microscopy of CHO cells producing chimeric IgE. The production of IgE was visualized by immunodetection with mice anti-human ϵ -chain antibodies and FITS-labeled rabbit anti-mice antibodies. The arrows show cell nucleus (1) and colored cytoplasm (2).

sequence. The amount of the synthesized hybrid IgE, whose constant domains were human and the variable ones were murine, was evaluated using the immunochemical test. The concentration of the transgenic IgE in blood grew over the first days after transfection and gradually diminished for 3 weeks. Upon transfection of all tissues except cartilage, the concentration of IgE over the first 3 weeks was sufficient in all the tissues except cartilage (Table 1). The concentration of antibodies to chimeric IgE synthesized by immune system in response to the appearance of xenogenous determinants was about 30 ng/ml in all groups of mice. In the case of the mice spleen, liver, and paw pads transfection in vivo the content of chimeric IgE in the blood stream was several times higher than in the case of transfection of the mice ear cartilaginous tissue.

4. Discussion and conclusion

4.1. Optimal construct of chimeric immunoglobulin

Earlier [1,2] the chimeric IgE genes were designed, in which fragments encoding variable domains of the light [15] and heavy [16] chains of mouse antibody are joined, respectively, with fragments of light (κ -type) [17] and heavy (ϵ -type) [18] constant gene of human antibodies. These genes were expressed in vitro and functional activity of recombinant IgE was demonstrated [2–4]. However, the RNA polymerase and bacteriophage T7 promoter, used in these con-

Table 1
Top-levels of the chimeric IgE expression in tissues of different mice organs

Organ	IgE (ng/ml)	Antibodies to IgE (ng/ml)
Spleen	14 $\pm$ 5	29 $\pm$ 10
Liver	10 $\pm$ 5	37 $\pm$ 15
Paw pads	29 $\pm$ 10	29 $\pm$ 10
Ear cartilaginous tissue	<1	25 $\pm$ 17

structs, are not recognized by eukaryotic RNA polymerases and thus cannot perform efficient transcription, except cases when gene of this prokaryotic polymerase is deliberately introduced into transfected cells, like it was done in the previous work [3,4]. To design universal construct, the chimeric immunoglobulin genes are put under control of the hCMV promoter that is widely used for gene expression in eukaryotic cells. TATA boxes were preserved upstream of both light and heavy immunoglobulin genes, whereas splicing sites, promoting normal RNA processing, were retained at the ends of exons.

In addition, the design of such genetic constructs in plasmids was considered, which would allow an optimal selection of the producer cell clones after transfection. Just owing to this, the puromycin resistance gene was cloned only into the plasmid containing the recombinant gene of the light immunoglobulin chain. This was done for final selection of the producer cell clones by the immunoenzyme kit for identification of heavy chain of human ϵ -type immunoglobulins: cells that survived in the presence of puromycin, carry gene of the κ -type light chain. Thus, a cumbersome procedure of screening the target transfectome producers was markedly simplified.

The enhancer/promoter of human cytomegalovirus, used in pUC.CMV.VhC ϵ and pUC.pur.CMV.VkC κ , is efficient in a broad range of hosts. Among such powerful virus enhancers like those from genomes of *Herpesvirus saimiri*, mouse cytomegalovirus, avian Rous sarcoma virus (RSV), and hepatitis B virus, enhancer of hCMV exhibits the highest activity not only in human (HeLa) and monkey (CV1) cells, but in cells of other species as well (mouse fibroblasts and cells of the frog kidney (*Xenopus laevis*)) [19].

4.2. Efficiency of chimeric IgE expression in various tissues

The fact that different levels of recombinant IgE biosynthesis were observed in different CHO cell clones fully correlates with facts described by other authors [20]. Transfected DNA is inserted into different sites of chromosomes due to random distribution of integration sites, and the so-called “position effect” is revealed. In addition, it is known that transfection by calcium phosphate method results in insertion of several copies of the same gene (6–12 copies) into the same site of the genome. The number of copies of the inserted DNA fragment undoubtedly can also influence the expression level of transfected genes.

A high efficiency of the spleen, liver, and paw pads transfection in comparison to that of the ear cartilaginous tissue can be explained as follows. First, this may be a consequence of a more efficient expression of transfected genes in the spleen, liver, and paw pads transfection in comparison to that of the ear cartilaginous tissue and, second, this may be due to a higher level of secretion of chimeric IgE by these tissues.

The level of chimeric IgE expression by CHO cells obtained in this work is comparable to expression levels of

recombinant antibodies in the mammalian cell culture obtained under control of other promoters without gene amplification. Thus, the antibody synthesis in SP2/0 cells under control of SV40 promoter made up 38 ng/ml [21], though there are examples of a markedly higher level. Thus, it reached 300 ng/ml in lymphoid cells Ag8.653 under control of immunoglobulin promoters [22] and 500 ng/ml in CHO cells under control of human β -actin promoter [23].

Previously, we described synthesis and secretion of chimeric IgE by myeloma cells SP2/0 [4]. Others [24–26] observed different levels of secretion of chimeric antibodies in CHO cells and mouse NS/0 myeloma cells. Probably the immunoglobulin structure can influence the efficiency of secretion in different cell types. It is known that sequences of FR1 region of immunoglobulin heavy chains [26] influence their secretion ability. It is possible that the structure of chimeric IgE used in this work does not stimulate such active secretion, at least from CHO cells. At the same time it should be noticed that native mouse IgG1 antibodies against porcine transferrin, which carry variable domains identical to those of chimeric IgE, are well secreted. It can be supposed in this context that the capability of secretion depends on the isotype of chimeric immunoglobulin.

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