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Preliminary report

# Anti-tumor activity of a peptide combining patterns of insect alloferons and mammalian immunoglobulins in naïve and tumor antigen vaccinated mice

Sergey Chernysh [a,*], Irina Kozuharova [b]

[a] Laboratory of Insect Biopharmacology and Immunology, Faculty of Biology and Soil Science, St. Petersburg State University, Oranienbaum str. 2, 198904 St. Petersburg, Russia
[b] Laboratory of Genetic Mechanisms of Cell Differentiation and Malignization, Institute of Cytology, Russian Academy of Science, Tichoretsky Prospect 4, St. Petersburg 194064, Russia

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ABSTRACT

Alloferons are a group of naturally occurring peptides primarily isolated from insects and capable of stimulating mouse and human NK cell cytotoxicity towards cancer cells. In this paper we examined anti-tumor activity of alloferon-1 and its novel structural analog referred to as allostatine. The activity was tested in naïve and preventively tumor antigen vaccinated DBA/2 mice subcutaneously grafted with syngenic P388D1 mouse leukemia cells. In naïve animals allostatine demonstrated tumoristatic activity prevailing over alloferon-1 effect. The preventive vaccination caused only weak tumoristatic effect in 27% of vaccinated animals. The vaccination efficacy was dramatically enhanced by allostatine but not alloferon-1 administration: 65% of allostatine treated animals benefitted from tumoristatic effect and 30% was completely cured so that total number of positive responders grew to 95%. Thus, alloferon-1 and especially allostatine are worthy of further consideration as potential anti-cancer drugs. Allostatine seems to be particularly perspective for adjuvant cancer immunotherapy. Sequence similarity search revealed evolutionary conserved allostatine-like pattern inserted to CDR3 region of human and mouse immunoglobulins. By analogy with allostatine, the pattern may execute some unknown so far function in anti-tumor immune response regulation.

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## 1. Introduction

A great number of immunologicals like vaccines, monoclonal antibodies and immune response modifiers have been developed for the purpose of active and passive cancer immunotherapy. However, poor immunogenicity, great variability and immunosuppressive activity of cancer cells considerably limit the immunotherapy efficacy [1]. Hunting for an immunological "magic bullet" assisting the immune system to recognize and attack cancerous growth remains one of the most relevant directions in the field. Following this direction, a novel family of antiviral and anti-tumor peptides named alloferons was formerly isolated from an insect species, maggots of dipteran *Calliphora vicina* [2]. Natural killer (NK) cells were identified as the peptide pharmacological target responding to alloferon-1 with immediate growth of cytotoxic activity [2–5]. Other kinds of pharmacological activity like induction of interferon synthesis [2], suppression of virus proliferation [6–8], deblocking of NF-kB mediated signaling pathway [9], modification of cytokine production [10], and adjuvant activity in combination with cancer chemotherapy [11] have been published. Alloferon-1 antiviral efficacy correlated with enhancement of NK cell cytotoxicity was clinically proven in the treatment of persistent viral infections [3,12].

Alloferons' physiological role in the host organism is not yet known however it is noticeable that cytotoxic hemocytes functionally similar to mammalian NK cells represent a significant portion of the maggot blood cells [13]. From that standpoint alloferons look like an evolutionary conservative family of NK cell regulatory peptides active both in insects and mammals.

In this paper we have used alloferon-1 primary structure as a platform for design of a novel molecule demonstrating better anti-tumor activity and potentially applicable in the field of cancer immunotherapy. In the beginning we tried to identify alloferon-1 structural analogs among mammalian immunologically relevant peptides and proteins. No close analogs were found while substitution of two amino acids in the alloferon-1 sequence revealed similar patterns incorporated into human immunoglobulins and some other immunologically relevant proteins. The "humanized" peptide referred here to as allostatine and its parental molecule, alloferon-1 was tested in a mouse tumor transplantation model in order to characterize their anti-tumor activity in the regimen of monotherapies and in combination with a tumor antigen vaccination.

* Corresponding author. Tel./fax: +7 812 4289076, +7 9219585235 (mobile).
*E-mail addresses:* sichernysh@yahoo.com (S. Chernysh), kojuxarova@mail.ru (I. Kozuharova).

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## 2. Materials and methods

### 2.1. Animals

Two-month old DBA/2 female mice with a body mass of 20–22 g were obtained from Rappolovo laboratory animal breeding nursery, St. Petersburg.

### 2.2. Tumor cells

Mouse leukemia P388D1 cell line syngenic to DBA/2 mice was obtained from the Institute of Cytology cell culture center, St. Petersburg and used for transplantation or tumor antigen preparation.

### 2.3. Vaccination

P388D1 cells were inactivated by x-ray irradiation in a 15,000 rad dose and used as corpuscular tumor antigen. The antigen in a dose of 3000 cells was inoculated twice into groin lymphatic nodes on days 1 (primary vaccination) and 13 (boosting vaccination) of the experiment.

### 2.4. Tumors cell transplantation

P388D1 tumor cells suspended in 200 μl of HEPES solution were inoculated subcutaneously in the mouse's spinal region 10 days after the boosting vaccination.

### 2.5. Antitumor activity assay

Allostatine and alloferon-1 anti-tumor activity was assayed using DBA/2 mice grafted with 3000 P388D1 tumor cells per animal according to the protocol described formerly [11]. The cells, unless rejected, form carcinoma-like solid tumor at the site of inoculation. Tumor in situ appearance and linear size measured as average of shortest and longest tumor diameter were monitored twice a week for 60 days after transplantation. Tumors in the untreated control animals became palpable within 20 days after transplantation and reached up to 3 cm in size in the next month. The 60 day checking period allows a conclusion to be drawn about transplanted tumor survival or elimination. In the latter case the anti-tumor effect was determined as tumoricidal. The effect was characterized as tumoristatic if a tumor appeared later than 20 days after transplantation when 100% of control animals developed palpable tumors. All animal experiments were approved by the author's institutional review board at the Institute of Cytology.

### 2.6. Peptides

Allostatine is a linear peptide consisting of a 13 amino acid sequence His–Gly–Val–Ser–Gly–Trp–Gly–Gln–His–Gly–Thr–His–Gly with empiric formula $C_{56}H_{77}N_{21}O_{17}$ and molecular mass 1316 Da. The peptide in the form of acetic acid salt was synthesized by Diapharm Co, St. Petersburg by solid phase synthesis using Fmoc/But strategy and purified by reverse phase HPLC. Final purity of the peptide measured by HPLC was over 98%.

Alloferon-1 in the form of acetic acid salt was synthesized by Peptide synthesis ltd, Moscow as described [14]. The peptide purity measured by HPLC was over 98%. Anti-tumor activity of the same product was previously tested using the DBA/2 mice P388D1 tumor transplantation model [11].

### 2.7. Peptides administration

Allostatine and alloferon-1 in a single 25 μg dose diluted in 200 μl of HEPES solution were injected intraperitoneally three times: days 1, 13 and 22 of the experiment. The injections were synchronized with primary vaccination, boosting vaccination and tumor transplantation, correspondingly. Control animals received an equal volume of the solvent.

### 2.8. Statistics and computation

Experimental data were summarized by descriptive statistics (mean and standard error of the mean for continued variables; frequency and percentage for categorical variables). Statistical analyses were

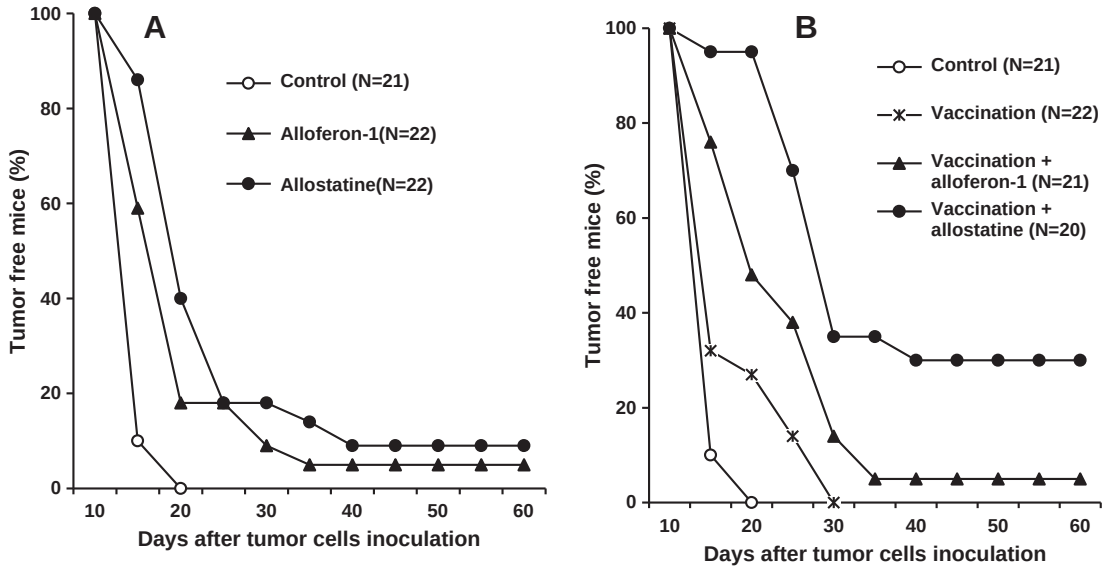


**Fig. 1.** In situ appearance of transplanted P388D1 tumor in naïve (A) and vaccinated (B) DBA2 mice treated with allostatine or alloferon-1. (A) Naïve animals. Allostatine and alloferon-1 in a single 25 μg dose diluted in 200 μl of HEPES solution were injected intraperitoneally into DBA2 mice three times: days 1, 13 and 22 of the experiment. Control animals received an equal volume of the solvent. Syngenic P388D1 murine lymphoma cells suspended in 200 μl of HEPES solution were inoculated subcutaneously in the mouse's spinal region in a dose 3000 cells per animal at day 22 of the experiment. Tumor in situ appearance and linear size measured as an average of shortest and longest tumor diameter were monitored twice a week for 60 days after transplantation. Allostatine caused significant (z test) delay of tumor appearance as compared to the control at days 15 ($P < 0.001$) and 20 ($P < 0.01$). (B) Vaccinated animals. DBA2 mice were injected with the peptides or a solvent in the same way as the naïve animals. Simultaneously they were subcutaneously vaccinated with x-ray killed P388D1 cells at days 1 and 13 of the experiment and grafted at day 22 with alive P388D1 tumor cells in the same way as naïve animals. Allostatine administration significantly (z test) suppressed tumor growth in most vaccinated animals compared to vaccination-only ($P < 0.001$ days 15–25, $< 0.01$ days 30–35, $< 0.05$ days 40–60). Alloferon-1 effect was significantly weaker compared to allostatine ($P < 0.01$ day 20, $P < 0.05$ day 35).

performed using analysis of variance (ANOVA) for continued variables and z test for categorical variables. A peptide sequence similarity study was performed using the SIB BLAST network service, NCBI BLAST software resources and UniProtKB protein knowledgebase. The peptide accession numbers are listed in the Table 2.

## 3. Results

All control animals developed detectable carcinoma-like tumors during 20 days after cancer cell transplantation (Fig. 1). Allostatine and alloferon-1 caused similar delay of tumor appearance in naïve animals as compared to the control group however most animals finally developed tumors except one in alloferon-1 and two in allostatine treated groups (Fig. 1A). In vaccinated animals (Fig. 1B) the vaccination-only had a short-term tumoristatic effect on some of treated individuals. The effect was more evident in alloferon-1 treated group, though it was significantly weaker compared to allostatine where tumor growth was suppressed in the most of vaccinated animals. One third of allostatine treated group did not develop tumor over the whole checking period ($P<0.05$ compared to control and vaccination-only groups).

Detailed characteristics of cancerous growth in tumor bearing animals are summarized in Table 1. All the treatments significantly ($P < 0.001$) extended tumor latency period (interval between tumor cell inoculation and detectable tumor appearance) and reduced size of the first detected tumor as compared to the control group. The longest latency period was found in vaccinated animals treated with allostatine versus vaccination-only ($P<0.001$), allostatine alone ($P<0.01$), alloferon-1 alone ($P < 0.001$) and vaccination coupled with alloferon-1 treatment ($P<0.05$). Allostatine treatment of naïve animals extended the latency period over alloferon-1 as well ($P = 0.015$). Tumor growth rates demonstrate similar trends at the early stages of tumor development: all the treatments caused growth suppression in comparison with untreated control group ($P<0.001$). The vaccination coupled with allostatine treatment prevailed over vaccination ($P = 0.001$), allostatine ($P < 0.05$) and alloferon-1 ($P < 0.001$) applied alone. Allostatine advantage over alloferon-1 in naïve individuals was also significant ($P < 0.01$). None of the treatments were effective at the advanced stages of cancerous growth.

Fig. 2 illustrates comparative anti-tumor efficacy of the treatments. The total number of positive responders benefitting from tumoricidal (complete tumor elimination) or tumoristatic (delay in detectable tumor appearance) effects significantly exceeded the control level under all treatments except alloferon-1. The maximum overall efficacy was established in vaccinated individuals treated with allostatine. Total anti-tumor activity here significantly exceeded vaccination-only, allostatine and alloferon-1 monotherapies as well as the vaccination combined with alloferon-1 treatment.

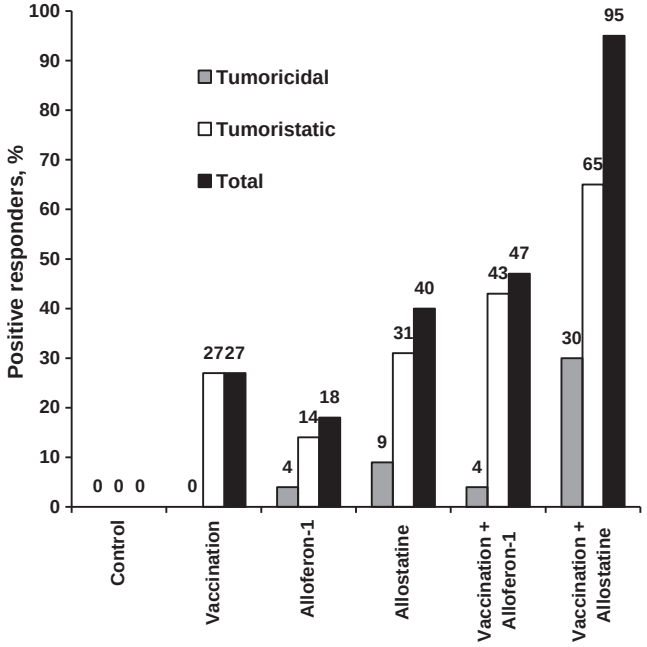


**Fig. 2.** Comparative anti-tumor activity of P388D1 tumor vaccine, allostatine, alloferon-1 and their combination. Tumoricidal activity is determined as a number of tumor-free animals two months after tumor cell transplantation; tumoristatic activity corresponds to a number of animals that developed detectable tumor later than in 20 days post transplantation. Total numbers of positive responders benefitting from tumoricidal (complete tumor elimination) or tumoristatic (delay in detectable tumor appearance) effects significantly exceeded the control level in all groups except alloferon-1 treated naïve animals. Overall anti-tumor activity in vaccinated individuals treated with allostatine significantly exceeded (z test) vaccination-only ($P < 0.001$), allostatine ($P < 0.001$) and alloferon-1 ($P < 0.001$) monotherapies as well as vaccination combined with alloferon-1 treatment ($P = 0.002$).

## 4. Discussion

Alloferon-1 anti-tumor activity in the P388D1/DBA2 mouse tumor transplantation model strongly depends on the initial dimension of tumor cell population. Transplantation of 100 cells can be cured by the peptide administration whereas the doses over 1000 cells become partially or completely resistant to the treatment [2]. In the experiments described here 3000 cell dose has been used and alloferon-1 effect in naïve animals was only tumoristatic as would be expected. The effect was essentially the same in preventively vaccinated animals: alloferon-1 administration caused a moderate delay in tumor growth at early stage of cancer development. Allostatine effect in naïve animals was also mainly tumoristatic although exceeding that of alloferon-1. The vaccinated animals, however, responded to allostatine treatment with remarkable enhancement of anti-tumor defense: one third of the recipients remained tumor-free and overall number of positive responders benefitting from tumoricidal and tumoristatic effects grew to 95% compared to 27% in the vaccination-only ($P<0.001$) and 47% in alloferon-1 ($P = 0.002$) treated groups, correspondingly.

The activity growth correlates with two amino acid substitutions in alloferon-1 primary sequence which makes allostatine structure similar to the pattern common among mammalian immunoglobulins (Table 2). The pattern belongs to the immunoglobulin heavy chain

**Table 1**
Tumor growth characteristics in DBA/2 mice inoculated with P388D1 mouse lymphoma cells.

| Treatment | $N_0$ | Tumor bearing animals | | Tumor latency period, days | Size of first detected tumor, mm | Tumor growth rate, mm/day | |
|---|---|---|---|---|---|---|---|
| | | N | % | | | Early stage[a] | Advanced stage[b] |
| Control | 21 | 21 | 100 | 15.57 ± 0.39 | 12.76 ± 0.60 | 0.83 ± 0.046 | 1.30 ± 0.104 |
| Vaccination | 22 | 22 | 100 | 18.55 ± 1.18 | 9.36 ± 0.61 | 0.54 ± 0.046 | 1.48 ± 0.088 |
| Alloferon-1 | 22 | 21 | 95 | 17.38 ± 0.73 | 9.33 ± 0.54 | 0.56 ± 0.030 | 1.35 ± 0.064 |
| Allostatine | 22 | 20 | 91 | 21.45 ± 1.44 | 8.67 ± 0.48 | 0.43 ± 0.034 | 1.34 ± 0.069 |
| Vaccination + alloferon-1 | 21 | 20 | 95 | 22.65 ± 1.44 | 8.50 ± 0.43 | 0.38 ± 0.016 | 1.50 ± 0.097 |
| Vaccination + allostatine | 20 | 14 | 70 | 27.36 ± 1.40 | 8.79 ± 0.64 | 0.33 ± 0.026 | 1.60 ± 0.193 |

[a] From tumor cell transplantation to detectable tumor appearance.
[b] From detectable tumor appearance to the endpoint.

**Table 2**
Sequence similarity of alloferon-1, allostatine and the immunoglobulin polypeptide binding site.

| Peptide reference/position | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Alloferon-1 P83412.1 | H | G | V | S | G | H | G | Q | H | G | V | H | G |
| Allostatine | H | G | V | S | G | W | G | Q | H | G | T | H | G |
| Human Ig | | | | S | G | W | G | Q | – | G | T | | |
| gbAAT02043.1, f 94–100 | | | | | | | | | | | | | |
| gbAAZ08856.1, f 107–113 | | | | | | | | | | | | | |
| gbAEX29562.1, f 109–115 | | | | | | | | | | | | | |
| dbjBAI52147.1, f 119–125 | | | | | | | | | | | | | |
| gbAAC18216.1, f 131–137 | | | | | | | | | | | | | |
| gbAAB41737.1, f 134–140 | | | | | | | | | | | | | |
| gbAAF15590.1, f 104–110 | | | | | | | | | | | | | |
| gbAAK51358.1, f 88–94 | | | | | | | | | | | | | |
| Mouse Ig | | | | | G | W | G | Q | – | G | T | | |
| gbABV01604.1, f 10–15 | | | | | | | | | | | | | |
| gbAAX90105.1, f 13–18 | | | | | | | | | | | | | |
| gbABB46044.1, f 15–20 | | | | | | | | | | | | | |
| gbAAA38086.1, f 76–81 | | | | | | | | | | | | | |
| gbAEQ62373.1, f 94–99 | | | | | | | | | | | | | |
| embCAB64123.1, f 98–103 | | | | | | | | | | | | | |

CDR3 region determining antibody complementarity to a variety of antigens. The pattern remains highly conservative within and between human and mouse genomes despite the fact that CDR3 is the most variable part of immunoglobulins. The pattern includes a tryptophan–glycine sequence (position 6–7 in Table 2) known as a polypeptide binding site of the immunoglobulins. Sequence similarity search revealed similar patterns in evolutionary conserved cystein-rich domain of macrophage scavenger receptors, in active center of transmembrane serine proteases and several other protein families performing their physiological functions by the mechanism of protein–protein interaction. Detailed analysis of these data is out of the paper scope and will be published elsewhere. Here we would like to attract attention to the mentioned in Table 2 allostatine-like pattern of the immunoglobulins that, by analogy with allostatine, may be involved in some unexplored so far mechanisms of the immune response regulation. It is noticeable that the pattern belongs to the immunoglobulin Fab fragment and has nothing similar with Fc fragment patterns performing known effector functions of the immunoglobulins.

The mechanism of allostatine action needs further clarification. Since an enhancement of mouse [2] and human [3,5] NK cell mediated cytotoxicity is known immunomodulatory effect of alloferon-1, allostatine interplay with effector and regulatory functions of NK cells should be considered in the first place although other mechanisms cannot be excluded from consideration at present. Structural likenesses of alloferon-1 and allostatine as well as near resemblance of their tumoristatic efficacy in naïve animals support this assumption. The fact that vaccinated mice more actively responded to allostatine treatment is also consistent with the assumption inasmuch as the acquired immune response should increase NK cells' anti-tumor efficacy through the mechanism of antibody dependent cellular cytotoxicity. The question why the vaccination boosted allostatine but not alloferon-1 efficacy remains unanswered.

Allostatine anti-tumor activity has been studied solely on the P388D1/DBA2 syngenic mouse tumor transplantation model. Data characterizing allostatine properties in other tumor models are not available for the time being. The model peculiarity is the very low immunogenicity of P388D1 cells transplanted to DBA2 mice. From that standpoint it adequately simulates critically important feature of genuine cancer. It makes this model especially relevant for the evaluation of potential anti-cancer drugs with immunomodulating properties [15].

Results described in this preliminary report point out two possible directions for allostatine application in the field of cancer immunotherapy. Particularly, it may be useful as an adjuvant boosting cancer vaccines efficacy. Currently available adjuvants were mainly developed for the purpose of anti-infective vaccination and are often not sufficiently effective as additives to cancer vaccines [16]. Significant tumoristatic effect achieved in nonvaccinated animals shows allostatine prospects independent of the vaccination approach as well.

## Conflict of interest

The authors declare that they have no conflict of interest.

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