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# *Infliximab effect on the levels of TNF-α and TGF-β in blood of irradiated patients*

Wpływ Infliximabu na poziom TNF-α and TGF-β we krwi napromienianych pacjentów

## INTRODUCTION

Ionizing radiation is commonly used in the treatment of neoplasms. It induces a cascade of events at all levels of organization units of the organism (cell, tissue, organ etc), which results in an early post-radiation inflammatory reaction and late post-radiation fibrosis. Ionizing radiation induces, among other things, a gene encoding multifunctional cytokine TNF - tumor necrosis factor, and other cytokines and growth factors exerting an autocrine and paracrine influence on both tumor and normal tissues. Other authors [9, 20] suggest that the reactions of tumors and normal tissues exposed to the ionizing radiation are likely to be affected not only by the direct radiation results on DNA but also by the activity of cytokines and growth factors. Rubin et al. [19] maintain that late post-radiation damage is due, among other reasons, to cytokines, which launch a cascade of events leading up to post-radiation fibrosis. Early molecular cell response, which develops within several seconds to several minutes, involves an increased gene expression followed by the activation of a pathological path of changes inducing inflammatory condition in the early post-radiation reaction and fibrosis in the late reaction. This response is connected with an increased expression of different pro-inflammatory cytokines including interleukin IL-1-α, IL-1-β, TNF-α, IL-6, IL-8, and TGF-β. TNF-α is a 17-kD polypeptide of pleiotropic properties. The compound modifies the immune system and inflammatory reactions [20]. TNF-α is a key inflammatory factor, a mediator of a wide variety of functional activities. Its biological effect can be either beneficial or harmful. It plays a mediating role in tissue remodeling, protects against infections and induces an acute phase of inflammatory state. It bears cytotoxic abilities and may cause cachexy, tissue damage, irreversible shock effects and even

death. TNF-α inhibits lipoprotein lipase activity, induces the secretion of other cytokines e.g. IL-6, activates endothelium cell adhesion and stimulates fibroblast proliferation [1, 3,].

TGF-β is supposed to be the major cytokine responsible for post-radiation fibrosis of healthy tissues [1, 13] and actively modifies post-radiation changes [6]. The growth of TGF-β level induces the expression of collagen synthesis gene which triggers off the production of fibrosis of hyaline membranes [12, 7]. This observation is of great clinical importance as it can orient towards further research on radioprotection [8]. The cytokine has an autoinductive and chemotactic effect on monocytes and macrophages and may thus further raise the growth factor level at the site of damage. TGF –β is a strong chemotactic factor for fibroblasts and stimulates the production of collagen. It is also believed to increase the accumulation of extracellular matrix through the inhibition of its degradation, and to induce the differentiation of immature terminal progenitory fibroblasts into post-mitotic fibrocytes whereby an increased collagen synthesis occurs, which leads to post-irradiation fibrosis [5, 14-18]. In certain diseases, a connection between TGF-β and fibrosis intensity has been reported [4, 23]. TGF-β administration proved to have a stimulating effect on fibrosis or the production of the connective tissue components *in vitro* and *in vivo* [2, 21]. Biologically, TGF-β assumes a latent form of L-TGF-β [10]. Thorough investigation of Barcellos-Hoff et al. [10] revealed that irradiation with 50-200 Gy of 60 Co gamma of recombinant native human L-TGF-β (rL-TGF-β) in iron containing saline resulted in generating active TGF-β. TGF-β can be produced by monocytes and macrophages activated by radiation. Cytokine response to post-radiation damage may last for weeks and even months [7] during ionizing radiation an inducing effect of TGF-α on TGF-β expression has been observed [13]. Thus the radiation causes an increase in the level of both cytokines. Our main concern was how to reduce the negative effects of the cytokines on human organism. One of the compound reducing the effect of TNF-α, and consequently the secretion of TGF-β and intensity of collagen synthesis, is Infliximab. Infliximab (also reffered to as cA2 and Remicade) is a chimeric human-murine IgG1K monoclonal antibody that binds to TNF-α in vivo and in vitro, thus inhibiting the harmful activity of this inflammatory cytokine. Infliximab administration caused a considerable fall in the level of inflammatory markers, IL-6 and CRP, which are often significantly elevated in rheumatoid arthritis patients. The results confirm a remarkable pharmacological effect of Infliximab and the role it plays in blocking TNF-α and reducing inflammatory conditions in rheumatoid arthritis.

The main purpose of this study was to discover the way and methods of reducing post-radiation damage of normal tissues and provide an adequate scientific justification for using Infliximab as an effective radio protector in the neoplasm radiotherapy.

## MATERIAL AND METHODS

A group of 98 patients of the Lublin Regional Oncology Center were subjected to the experiment. Randomly selected patients were assigned to 3 groups according to the radiation exposure. The randomization was done according to the assumption that post-radiation reactions are of general and local nature [12]. All randomized patients, whose whole blood was tested, had not been subjected to any therapeutic procedures like surgery, chemotherapy or irradiation for at least 4 weeks before the first blood tests. Patients were irradiated with the Total dose of 54Gy ÷ 66Gy, fractions 2Gy/daily, exposed 5 days a week.

Blood samples for morphologic tests were collected just after the irradiation course into 4% tripotassium versenate solution and then tested by means of CELL DYN 3700 ABBOT haematological analyser a few hours after being taken. The samples for whole blood cell culture were collected on heparin. The samples were suspended in RPMI 1640 growth medium standardized according to the number of leukocytes ($10^6$ leukocytes /ml). Two milliliters of whole blood was taken from each patient immediately before irradiation and 100µl sample of the blood was placed in wells with 0.8 mg/ml of Infliximab (Centocor Inc. USA) or without the preparation. The same procedure was applied to blood samples taken immediately after the irradiation.

Each blood sample culture was incubated for 6, 12, 24, 48, 72 or 96 h. The culture was carried out under standard conditions (37ºC and 5% $CO_2$ concentration). The SIGMA antimycotic solution (10µl/ml) was added to protect the cultures from microorganism growth. The supernatant of the cultures was collected after the lapse of the mentioned incubation time and the material was frozen for further analysis. The concentrations of TNF-α and TGF-β cytokines were examined in the supernatant with ELISA, using Bender MedSystems-Austria commercial kits. Test sensitivity for the TNF-α - was 5.8 pg/ml and for TGF-β - 1.9 pg/ml.

The data were expressed as median, interquartile range. ANOVA on the van der Waerden normal scores was used for comparison of changes from baseline at each post irradiation point. Significant differences were further tested by Dunnett's comparison to the control group. The Mann-Whitney L'test was used to compare the TNF-α and TGF-β data for the irradiated and unexposed group. Comparison between the reductions of TGF-β in the cA2 treated group was made using the Kruskal-Wallis test. Associations between parameters were defined using Spearman's rank correlation coefficient (p). No adjustment was made for multiplicity of time point or laboratory parameters. The analyses were performed using Statistica 8.1 StatSoft Inc. Software.

## RESULTS

The results of morphological parameters of peripheral blood are presented in Table 1.

Table 1. Selected parameters of peripheral blood morphology in the pre-irradiated and post-irradiated patients

| | Patients before irradiation | | Patients after irradiation | | p |
|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | |
| HB g/dl | 13.1 | ±1.65 | 12.56 | ±1.25 | 0.2259 |
| RBC 106/µl | 4.61 | ±0.874 | 4.07 | ±0.535 | 0.0359 |
| Ht % | 39.07 | ±4.76 | 37.66 | ±3.92 | 0.1274 |
| WBC 103/µl | 5.68 | ±1.47 | 5.65 | ±1.91 | 0.846 |
| PLT 103/µl | 188.85 | ±43.19 | 227 | ±58.78 | 0.0225 |
| Lymphocytes % | 25 | ±2.57 | 29.71 | ±4.76 | 0.0014 |
| Monocytes % | 6.52 | ±1.06 | 9.2 | ±3.76 | 0.0357 |
| Granulocytes % | 64.53 | ±8.35 | 61.88 | ±6.47 | 0.0667 |

The determination of TNF-α and TGF-β levels in the whole blood culture indicated that in the cell-free supernatant of the culture without cA2, TNF-α levels were 12.04 pg/ml and showed no deviation from TNF-α standard values (10 pg/ml) (Fig.1). On the other hand, post-irradiated patients showed a rapid and significant increase ($P<0.01$) in TNF-α level in the 6$^{th}$ h of incubation and this level persisted till the 12$^{th}$ h. In the 24$^{th}$ h there was a drop to initial level, which remained unchanged till the 72$^{nd}$ h, whereas after 96 h the level went up to the level observed at the 12$^{th}$ h (Fig.2). TNF-α level in cultures with cA2 and before irradiation showed paradoxical growth in the 6$^{th}$ h, and the tendency persisted for 24 h, but from the 48$^{th}$ hour a slight decrease was observed. In the blood of post-irradiated patients with cA2 addition, a surprisingly high increase in TNF-α level was detected at the 6$^{th}$ (38.5pg/ml), 12$^{th}$ (44.6 pg/ml) and 24$^{th}$ hour (59.2 pg/ml), while starting from the 48$^{th}$ hour a slight decline from 45.8 to 37.1 pg/ml at the end of the experiment was recorded (Fig.2). TGF-β levels in blood culture without cA2 before irradiation showed continuous rise from 3978 to 5242 pg/ml at the 6$^{th}$ hour, to 6207 at the 12$^{th}$ hour, to 7795 pg/ml at the 24$^{th}$ hour, to 8010 pg/ml at the 48$^{th}$ hour, to 8411 pg/ml at the 72$^{nd}$ hour and to 8950 pg/ml at the 96$^{th}$ hour (Fig. 3). In the post-irradiated group without cA2, a continuous growth was recorded till the 48$^{th}$ hour (from 4758 to 6140 pg/ml at the 6$^{th}$ hour, to 7184 pg/ml at the 12$^{th}$ hour, to 13324 pg/ml at the 24$^{th}$ hour and then a slight decline to 12925 pg/ml at the 40$^{th}$ hour, to 12537 and 11950 pg/ml at the 72$^{nd}$ and 96 $^{th}$ hour, respectively (Fig.4). In the cultures with cA2, TGF-β levels before irradiation showed also the peak value at the 48$^{th}$ hour (from 4050 to 5339 pg/ml at the 6$^{th}$ hour; 5500 pg/ml at the 12$^{th}$ hour, 8940 pg/ml at the 24$^{th}$ hour and 7340 pg/ml at the 48$^{th}$ hour) and then started to go down (6500 pg/ml at the 72$^{nd}$ hour and 5720 pg/ml at the 96$^{th}$ hour) (Fig.3). In the post-irradiated group, during the first 6 hours, there was a growth from 4717 pg/ml to 7462 pg/ml, and then a paradoxical increase to 16885 pg/ml at the 12$^{th}$ hour. From the 12$^{th}$ hour the values started to decrease (13568 pg/ml at the 24$^{th}$ hour, 12183 pg/ml at the 48$^{th}$ hour, 9075 pg/ml at the 72$^{nd}$ hour and 6895 pg/ml at the 96$^{th}$ hour).

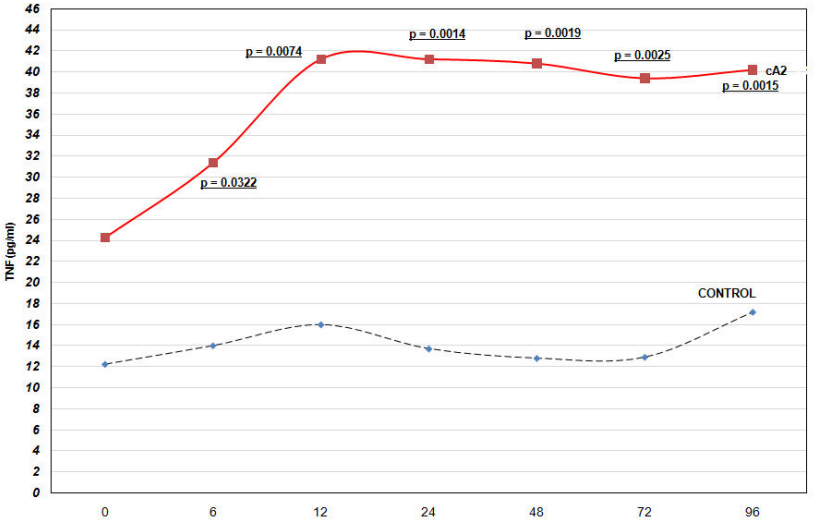


Figure 1. Effects of cA2 on TNF-α level in the whole blood of patients before irradiation in vitro

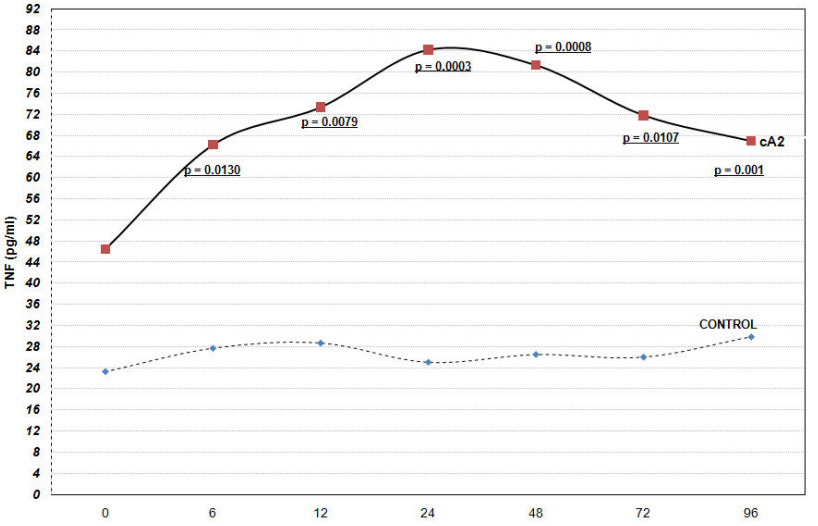


Figure 2. Effects of cA2 on TNF-α level in the whole blood of irradiated patients

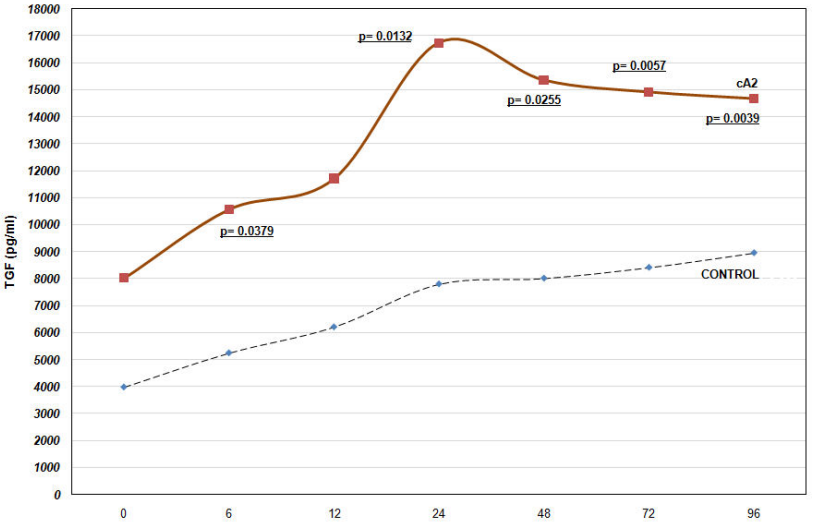


Figure 3. Effects of cA2 on TGF-β level in the whole blood of patients before irradiation

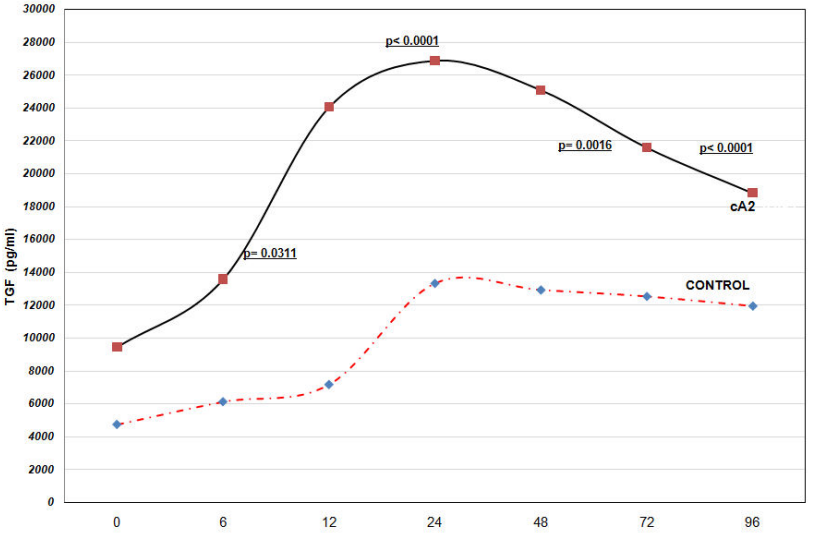


Figure 4. Effects of cA2 on TGF-β level in the whole blood of irradiated patients

## DISCUSSION

The fact that there is no effective method of the treatment in the case of post-radiation fibrosis encouraged us to do a thorough, several years' research into this problem. Positive clinical results obtained with Infliximab, which proved to have a modifying effect on TNF-α level in rheumatoid arthritis patients, inspired us to try out its properties in the prophylaxis of post-radiation fibrosis. Infliximab reduces TNF-α levels and thus regulates TGF-β levels as well. We took advantage of the fact that TNF-α is one of the factors effecting TGF-β levels by inducing TGF-β expression [13, 22], and so we assumed that lower TNF-α level would result in lower TGF-β expression. There are no reports in the literature available about radio-protective role of Infliximab in irradiated patients. Post-radiation fibrosis is a serious clinical complication influencing the quality of life of irradiated patients, leading to the deterioration of their circulatory and respiratory efficiency and even to death, depending on the extent of the fibrotic changes.

The results of our experiment show a considerable growth of TNF-α content in the whole blood culture from irradiated patients without Infliximab, persisting for 12h and then, from the 24$^{th}$ h, falling back to initial values. At the 96$^{th}$ h it went up again to reach the 12h level. When Infliximab was administered to the culture of blood taken from patients before irradiation, a paradoxically high TNF-α level was recorded 6, 12 and 24h after the administration. From the 48$^{th}$hour on we noted a slight TNF-α decline. According to the literature, a paradoxical TNF-α growth in blood circulation after Infliximab administration results from the formation of TNF-α and anti-TNF-α immune complex.

Post-irradiated Infliximab blood showed a surprisingly high growth of TNF-α level and then a slight decrease (Fig.2). However, in the blood without Infliximab, TNF-α levels were twice as high as the values recorded in Infliximab-free culture before irradiation. Similarly oscillating values were

recorded in rheumatoid arthritis, where pharmacological effect of Infliximab manifested itself in the regression of arthritis as a result of significant TNF-α level decrease [11].

The effect of Infliximab on TGF-β level seems to be interesting. A continuous increase in TGF-β levels in whole blood cultures without cA2 before irradiation was noted (Fig.3). In post-irradiated group without cA2, the level went up and then slightly declined (Fig.4). Cultures including cA2, before irradiation showed a growing tendency in TGF-β content and then a decline in its concentration (Fig.3). In post-irradiated group the levels started to grow and then to fall. According to the results, a considerable decrease in TGF-β levels was achieved, starting from the 24$^{th}$hour, which in the final time point (96$^{th}$ hour) came down to the value only slightly higher than before irradiation.

The obtained results confirmed the hypothesis of decreasing the TGF-β expression by inactivating TNF-α with a monoclonal antibody (Infliximab) in the patients' whole blood culture in vitro. These observations are a good starting point for further experiments in vitro and in vivo, whose main objective is to reduce post radiation fibrosis. Encouraging results obtained in the treatment of rheumatoid arthritis, where TNF-α plays a major role in the pathology of the disease, are another argument for further research into the problem of irradiation where TNF-α is also a key cytokine in the development radiation injury.

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## ABSTRACT

TGF-β is supposed to be the major cytokine responsible for post-radiation fibrosis of healthy tissues and actively modifies post-radiation changes. The growth of TGF-β level induces the expression of collagen synthesis gene which triggers off the production of fibrosis of hyaline membranes. The main purpose of this study was to discover the way and methods of reducing post-radiation damage of normal tissues and provide an adequate scientific justification for using Infliximab as an effective radio protector in the neoplasm radiotherapy. A group of 98 patients were subjected to the experiment. Randomly selected patients were assigned to 3 groups according to the radiation exposure. The samples of whole blood were suspended in RPMI 1640 growth medium standardized according to the number of leukocytes. Two milliliters of whole blood was taken from each patient immediately before irradiation and 100μl sample of the blood was placed in wells with 0.8mg/ml of Infliximab or without the preparation. TGF-β levels in blood culture without cA2 before irradiation showed continuous rise from 3978 to 8950 pg/ml at the 96$^{th}$hour. In the post-irradiated

group without cA2, a continuous growth was recorded till the 48$^{th}$hour (from 4758 to 13324 pg/ml at the 24$^{th}$hour) and then a slight decline to 11950 pg/ml at 96$^{th}$hour, respectively. In the cultures with cA2, TGF-β levels before irradiation showed also the peak value at the 48$^{th}$ h (from 4050 to 7340 pg/ml at the 48$^{th}$hour) and then started to go down (6500 pg/ml at the 72$^{nd}$hour and 5720 pg/ml at the 96$^{th}$hour). In the post-irradiated group, during the first 6 hours, there was a growth from 4717 pg/ml to 7462 pg/ml, and then a paradoxical increase to 16885 pg/ml at the 12$^{th}$hour. From the 12$^{th}$hour the values started to decrease to 6895 pg/ml at the 96$^{th}$ h. The obtained results confirmed the hypothesis of decreasing the TGF-β expression by inactivating TNF-α with a monoclonal antibody (Infliximab) in the patients' whole blood culture in vitro. These observations are a good starting point for further experiments in vitro and in vivo, whose main objective is to reduce post radiation fibrosis.

*Keywords:* radiation fibrosis, monoclonal antibody, TNF-α, TGF-β, cytokines

## STRESZCZENIE

TGF-β jest prawdopodobnie najważniejszą cytokiną odpowiedzialną za zwłóknienia popromienne zdrowych tkanek aktywnie modyfikującą zmiany popromienne. Wzrost poziomu TGF-β indukuje ekspresję genu odpowiedzialnego za syntezę kolagenu wywołującego kaskadę zwłóknień. Głównym celem prezentowanych badań było dążenie do uchwycenia sposobu oraz metod ograniczenia uszkodzeń popromiennych zdrowych tkanek oraz ocena możliwości zastosowania Infliksimabu jako radioprotektora w radioterapii nowotworów. Badaniami objęto grupę 98 pacjentów. Losowo wybrani pacjenci zostali podzieleni na 3 grupy w zależności od poziomu ekspozycji na promieniowanie.

Dwa mililitry pełnej krwi pobierano od każdego pacjenta bezpośrednio przed napromienieniem a następnie 100µl próbkę pełnej krwi uzupełniano dawką 0,8 mg/ml Infliksimabu lub też poddawano hodowli bez wzbogacenia.

Hodowla pełnej krwi bez cA2 przed napromienieniem wykazywała ciągły wzrost TGF-β od 3978 do 8950 pg/ml w czasie 96 godzin. W grupie poddanej napromienieniu bez dodatku cA2 obserwowano wzrost poziomu w okresie 48 godzin (odpowiednio 4758 – 13324 pg/ml w czasie pierwszych 24 godzin), a następnie łagodny spadek do poziomu 11950 pg/ml w 96 godzinie. W kulturach zawierających cA2 poziom TGF-β wykazywał także radykalny wzrost (4050 – 7340 pg/ml) a następnie spadek do poziomu 6500 pg/ml po 72 godzinach oraz 5720 pg/ml po 96 godzinach. W grupie poddanej napromienianiu w ciągu pierwszych 6 godzin obserwowano wzrost z poziomu 4717 pg/ml do poziomu 7462 pg/ml, a następnie radykalny wzrost do poziomu 16885 pg/ml w 12 godzinie. Począwszy od 12 godziny wartości zaczynają spadać do poziomu 6895 pg/ml w 96 godzinie. Uzyskane rezultaty potwierdzają tezę o redukcji ekspresji TGF-β poprzez inaktywację TNF-α za pomocą przeciwciała monoklonalnego (Infliksimabu) w pełnej krwi pacjentów in vitro oraz in vivo co może prowadzić do zmniejszenia zwłóknień popromiennych.

*Słowa kluczowe:* zwłóknienia popromienne, przeciwciało monoklonalne, TNF-α, TGF-β, cytokiny