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Individual Differentiation of Innate Antiviral Immunity in Humans; the Role of Endogenous Interferons and Tumor Necrosis Factor in the Immunity of Leukocytes

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Abstract. The natural antiviral immunity of human lymphocytes, leukocytes from peripheral blood and whole-blood cultures was studied using the method of infection with two viruses belonging to different taxonomic groups, vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV). The kinetics of virus replication in the kinds of cultures and the dependence of culture infection on pre-infection incubation time were studied. When the cultures were infected immediately after preparation, most of them were found to be resistant to the viruses. However, when they were infected after several (1–5) days of incubation, VSV and EMCV multiplied in the cultures to high titers. The time of losing resistance was individually differentiated. The results indicate the presence of a non-specific antiviral immunity characteristic for individuals. The antiviral immunity of healthy donors was compared with that of people suffering from recurrent infections of the upper respiratory tract. This latter group expressed statistically significant lower innate immunity than healthy donors. However, there were no differences in interferon (IFN) and tumor necrosis factor (TNF) production between these groups. In order to examine the contribution of the endogenous IFNs and TNF-α in maintaining innate immunity, specific antibodies against IFN-α, IFN-β, IFN-γ and TNF-α were added to VSV-infected leukocytes resistant to infection. The antibodies reduced the antiviral resistance in 9 of 16 experiments. The results suggest that both endogenous interferons and TNF-α may participate in the constitution of innate immunity, though they are not the only mediators of it.

Key words: innate immunity; human leukocytes; viral infections; interferons; tumor necrosis factor.

Introduction

In contrast to acquired antiviral immunity, which has been studied in detail, research on non-specific antiviral immunity is not advanced. Cytokines expressing antiviral activity are considered to play an important role in the innate immunity of mammals. KHAN et al.[5, 6] found in biopsy materials from 500 accident victims the presence of interferon (IFN)-α-containing spindle-shaped cells distributed among most of the organs. The majority of the people were probably healthy. Such cells were found not only in adults, but also in fetal and infant tissues. BRETT et al.[1] found IFN-γ-containing cells dispersed in normal human tissues. These cells probably participate in the innate system of immunity. Recently, precursors of dendritic cells were found to produce large amounts of IFN-α and are thought to play an important role in the innate system[4, 13]. The IFNs,

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however, are not the only cytokines with antiviral activity; interleukin (IL)-12, IL-18, tumor necrosis factor (TNF)-α, other cytokines of the TNF family, and some of the chemokines also express it. The results of experiments in which cytokine genes were inserted into a viral genome and mice were infected with the recombinant virus, after which the course of infection was studied, gave insight into a possible role for cytokines in non-specific immunity. The results are discussed in the review by RAMSHAW et al.[11] Usually, the first period after infection (1–3 days) is the best for analysis in the context of innate immunity because the effectory functions of acquired immunity are practically not effective yet. In this way, antiviral effects were shown for IFN-γ, IL-2, TNF-α, CD40 ligand, Fas ligand, IL-12 and IL-6. Which of these cytokines really plays a role in innate immunity *in vivo* is not known. The effectory function of natural killer (NK) cells and γδ T lymphocytes as well as complement-dependent killing are considered as important elements of innate antiviral immunity. Natural antibodies of the immunoglobulin M (IgM) class, with a wide antiviral spectrum and non-specific inhibitors present in human and animal sera, may also participate in this immunity[7, 12].

During our study concerning the sensitivity of human embryonic explants of placenta, amniotic membranes and endothelium from the umbilical cord vein to viral infections, we noticed that some of the organs were resistant to laboratory strains of viruses belonging to different taxonomic groups and that in some of them the viruses replicated, sometimes to high titers[2, 9, 15]. The natural antiviral immunity present in the embryonic tissues was non-specific for viruses. The immunity probably protects the organs *in vivo* against viral infection and prevents vertical transmission of viruses. These observations stimulated us to use the direct method of infection with an indicatory virus to study the innate immunity of human leukocytes.

Materials and Methods

Blood donors. Peripheral vein blood was taken from a group of healthy volunteers and from otherwise healthy persons with histories of recurrent infections of the upper respiratory tract. The latter group was sent to the Laboratory of Virology of our Institute to find the reason for their low immunity. The persons were 18–72 years old.

Whole-blood cultures. Cultures were prepared from heparinized (10 U/ml) peripheral blood. The blood was suspended in Dulbecco-modified Eagle medium (DMEM) supplemented with 2% heat-inactivated calf serum (c.s.) in a ratio of 1:9.

Cultures of lymphocytes. Lymphocytes were isolated from peripheral blood by gradient centrifugation in Lymphoprep with a density 1.077 g/ml (Nycomed Pharma, Oslo, Norway). Four ml of blood diluted in RPMI medium (1:1) was layered on 3 ml of Lymphoprep and centrifuged for 40 min at 400 × g. Next, the lymphocytes present in the interphase were collected, washed 2 times with RPMI with 2% c.s. and suspended in this medium at a density of 2×10^6 cells/ml.

Cultures of leukocytes. Leukocytes were isolated from heparinized peripheral blood by gradient centrifugation in Gradisol G with a density of 1.115 g/ml (Aqua Medica, Poznań, Poland). Five ml of blood were layered on 3 ml of Gradisol and centrifuged for 25 min at 400 × g. The leukocytes from the interphase were collected, washed 2 times with RPMI supplemented with 2% c.s. and suspended in the medium at a density of 2×10^6 cells/ml.

L_{929} cells (ATCC CCL 1). The murine fibroblast-like cell line was maintained in Eagle medium (MEM) supplemented with 10% c.s., antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and 2 mM L-glutamine.

A_{549} cells (ATCC CCL 185). The human epithelial-like cell line was maintained in DMEM supplemented with 10% c.s., antibiotics and L-glutamine.

Viruses. Vesicular stomatitis virus (VSV), Indiana strain, *Rhabdoviridae*, vesiculovirus, was multiplied and titrated in L_{929} cells, encephalomyocarditis virus (EMCV), Columbia MM strain (ColMM), *Picornaviridae*, cardiovirus, was multiplied and titrated in L_{929} cells.

Viral infection of leukocyte, lymphocyte and whole-blood cultures. The lymphocyte and leukocyte cultures (2×10^6 cells/ml) and also whole blood cultures were infected with VSV or EMCV at a concentration of 100 tissue culture infectious dose in 50% of cells ($TCID_{50}$) per 1 ml. After 40 min of adsorption, the virus was washed out 3 times with 5 ml MEM and the cells were suspended in 1 ml of MEM with 2% c.s. A sample of infected cells kept at a temperature of +4°C served as a control of the starting level of the virus. The other cell samples were incubated at 37°C and samples of supernatant were collected each day and virus was titrated in L_{929} cells. The titer of virus was expressed in $TCID_{50}$ based on the cytopathic effect caused by the virus in about 50% of the cells. Freshly isolated cells, as well as the preincubated and those for production of cytokines, were infected with viruses in the same way.

Cytokine assay. IFN was titrated using the biological method. The inhibition of the cytopathic effect caused by EMCV on the human cell line A_{549} was estimated under the microscope. The results obtained were corrected according to the internal laboratory standard of human IFN-α/β added to every experiment calibrated against the international standard of human leukocyte IFN 69/19.

TNF was assayed according to FLICK and GIFFORD[3]. The cytotoxic activity of TNF in the samples was measured in L_{929} cells in the presence of actinomycin D (Serva) at a final concentration of 2 μg/ml MEM. Recombinant human TNF-α (Genetech) was used as the standard in all assays.

Antibodies against cytokines and their titration. In the experiments we used:

- anti-IFN-α, polyclonal, sheep antiserum against human leukocyte interferon (Sigma-Aldrich, St. Louis, MO, USA),
- anti-IFN-β, polyclonal, sheep antiserum against human fibroblast interferon (National Institutes of Health, Bethesda, MD, USA),
- anti-IFN-γ, polyclonal, goat antiserum against human interferon (Sigma-Aldrich, St. Louis, MO, USA),
- anti-TNF-α, polyclonal, rabbit antiserum against human TNF-α (Sigma-Aldrich, St. Louis, MO, USA).

At the beginning of the experiments, the antibodies were titrated. Briefly, commercial preparations of human IFN-α, IFN-γ, TNF-α and IFN-β were titrated with the methods described above. Several dilutions of all antibodies were prepared on 96 well plates. Next, 32 U of the appropriate cytokine (IFNs or TNF-α) in 100 μl of culture medium was added to every well with the diluted antibodies. The dilution of TNF was prepared in the medium with 2 μg of actinomycin D per 1 ml. After 2 h of incubation of the plates at room temperature, their contents were transferred with a multicanal pipette to the previously prepared 24-hour cultures of A_{549} (in the case of IFNs) or L_{929} (in the case of TNF). After one day of incubation, the neutralization of the cytotoxic activity of TNF was observed under a microscope. Neutralization of the EMCV-inhibiting activity of IFN was observed after 2 days of incubation.

In the next experiments, anti-IFN-α antibody was used at a concentration neutralizing 500 U of IFN-α/ml, anti-IFN-β at a concentration neutralizing 1000 U of IFN-β, anti-IFN-γ at a concentration neutralizing 600 U of IFN-γ, and anti-TNF-α at a concentration neutralizing 600 U of TNF-α.

Virucidal activity of human sera. Sera of blood donors were diluted (1/8– 1/2560) on 96-well plates. To every well a dose of 30 $TCID_{50}$ VSV or EMCV was added and incubated for 2 h at room temperature. The contents of the plates were transferred to prepared, 24-hour cultures of L_{929} cells on 96-well plates. The virus-neutralizing titer was estimated under the microscope.

Statistics. Statistics was performed with the non-parametric Mann-Whitney and Wilcoxon tests for pairs. The results were considered as significant when $p<0.05$.

Results

In the study, two kinds of cultures were used: whole-blood cultures prepared from the peripheral blood of volunteers and cultures of lymphocytes isolated from the blood. Both cultures were infected with VSV immediately after preparation (time=0) and the kinetics of virus replication was measured. The results obtained from 29 independent experiments are presented in Fig. 1. Both cultures (whole-blood cultures and cultures of lymphocytes isolated from the blood) obtained from about 80% of the blood donors were found to be resistant to VSV infection.

Then the sensitivities of the cultures to viruses belonging to different taxonomic groups were compared. The effect of incubation time before infection on culture sensitivity to the viruses was also estimated. Both kinds of cultures were infected with VSV (n= 29) or with EMCV (n=11). The kinetics of virus replication in the cultures infected at time t=0 or after one (t=24), two (t=48), three (t=72) or more days were compared. The results obtained showed that 80% of the cultures infected with VSV at t=0 were resistant to the virus. Incubation of the cultures before infection resulted in a reduction of resistance and, consequently, in VSV replication. The effect of incubation time on the sensitivity of lymphocytes from different persons to VSV is presented in Fig. 2. The kinetics of VSV replication and the time of acquiring sensitivity to infection were individually differentiated. Sometimes a one-day incubation (t=24) of leukocytes before infection was enough to obtain full permissivity of leukocytes to VSV (Fig. 2A, B) and sometimes a longer time was necessary (Fig. 2C, D, E). Similar results were obtained when whole-blood cultures were infected with VSV (not presented).

In Fig. 3, the kinetics of EMCV replication in lymphocytes is presented. The cells were generally less sensitive to EMCV than to VSV infection, but individ-

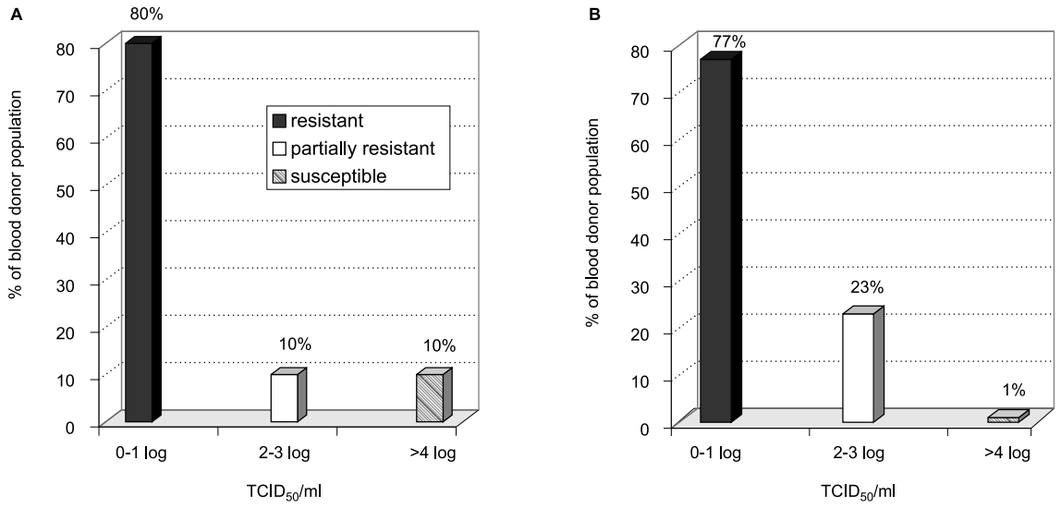

Fig. 1. Sensitivity of freshly isolated lymphocytes (**A**) and full blood cultures (**B**) to VSV infection (t= 0, n= 29)

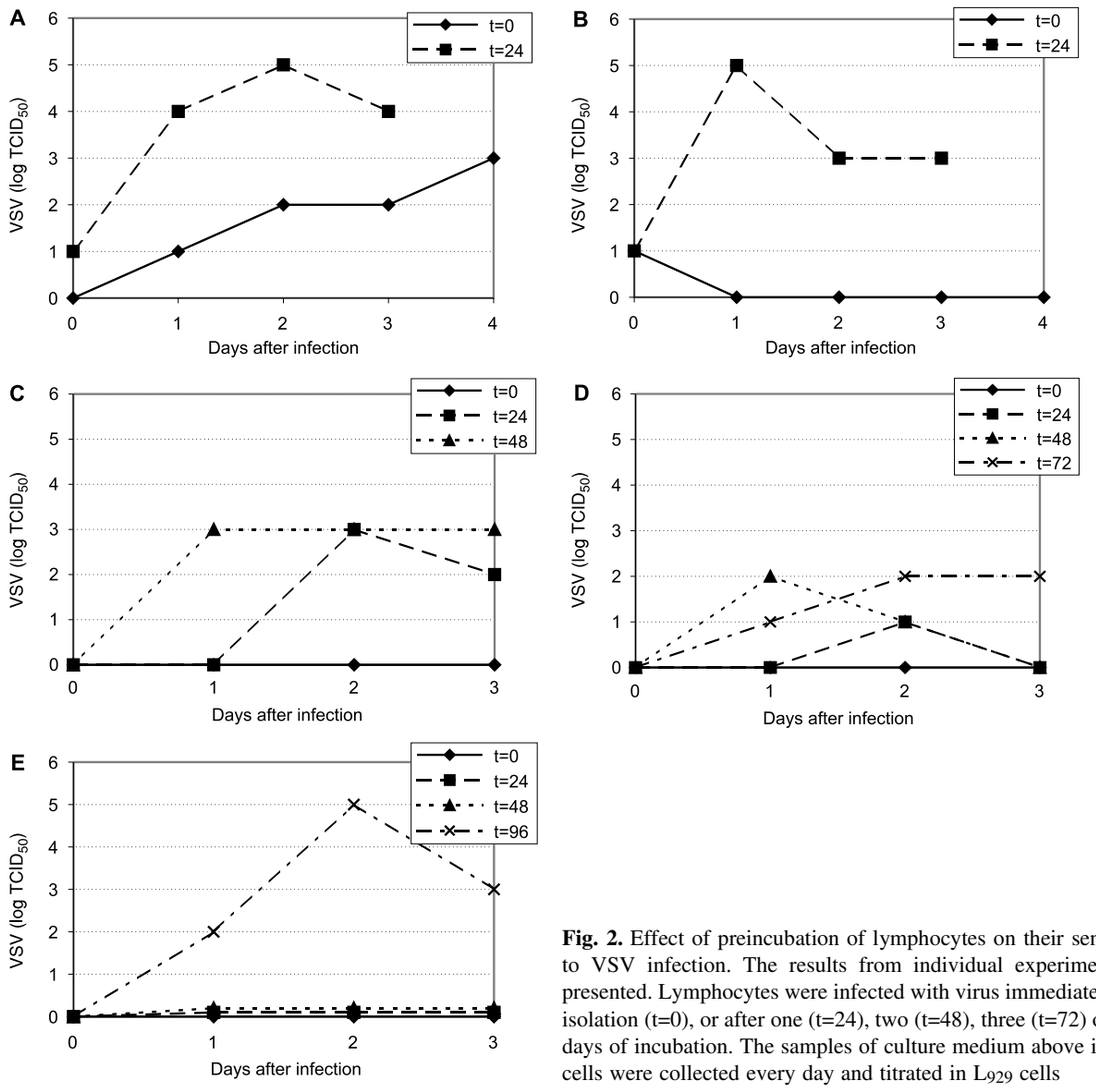

Fig. 2. Effect of preincubation of lymphocytes on their sensitivity to VSV infection. The results from individual experiments are presented. Lymphocytes were infected with virus immediately after isolation (t=0), or after one (t=24), two (t=48), three (t=72) or more days of incubation. The samples of culture medium above infected cells were collected every day and titrated in L_{929} cells

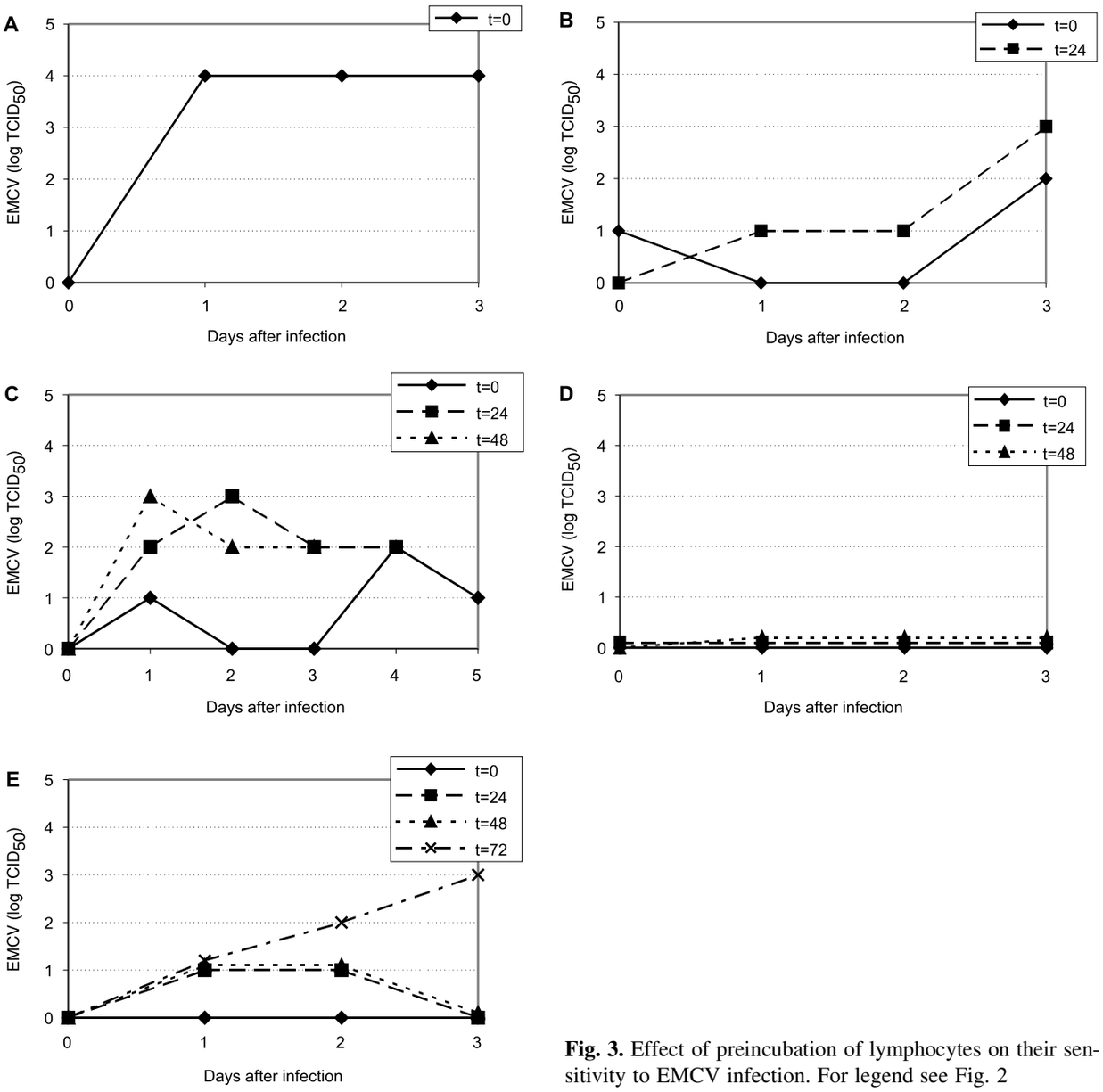

Fig. 3. Effect of preincubation of lymphocytes on their sensitivity to EMCV infection. For legend see Fig. 2

ual differentiation in the sensitivity of lymphocytes to EMCV was also observed. Among the 11 experiments, 5 lymphocyte cultures were found to be completely resistant to viral infection (titer of EMCV was 1–2 log $TCID_{50}$), 5 partially resistant (2–3 log $TCID_{50}$) and one sensitive (4 log $TCID_{50}$). The kinetics of EMCV replication in whole-blood cultures was also studied. The results (not presented here) were similar to those shown for lymphocytes. To exclude the participation of acquired immunity in the observed resistance, sera from the blood of donors were searched for the presence of a virucidal activity. The obtained results revealed that there was no activity against VSV and EMCV in 12 serum samples from blood donors (data not shown).

The blood of some persons with recurrent infections of the upper respiratory tract (though otherwise healthy) was sent to our laboratory in order to explain the reason for frequent (5–12 times per year) microbial (usually viral) infections. A defect of the interferon system was suggested. We checked the ability of their lymphocytes to produce IFN and TNF and, additionally, the level of non-specific immunity as measured by

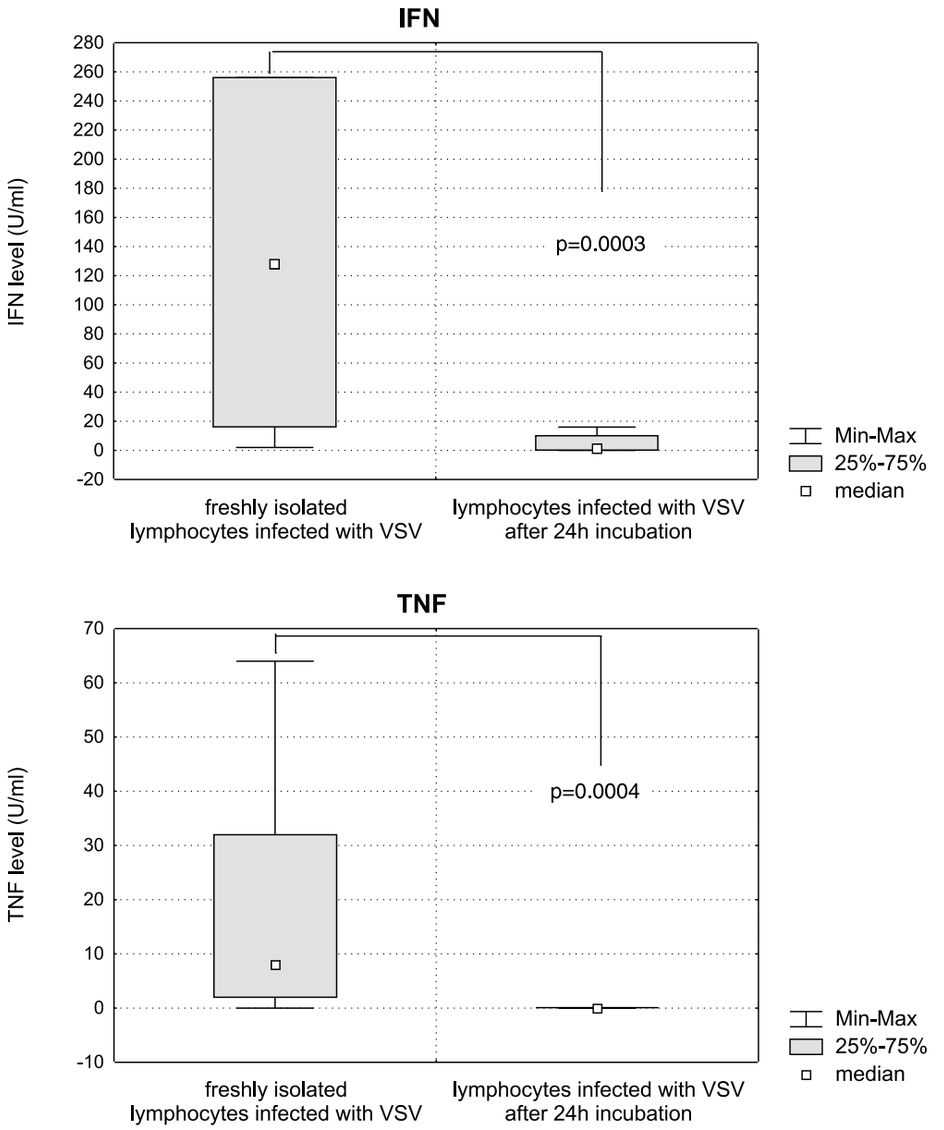

Fig. 4. Production of IFN and TNF by VSV-infected lymphocytes; the effect of preincubation time on cytokine production. The samples (n=10) were collected 24 h after infection. In order to inactivate VSV, the samples were UV irradiated (on ice) before cytokine titration. Median values and quartiles of cytokine levels are presented. The statistics were performed using the Wilcoxon test

the sensitivity of the cultures to VSV infection. First, the production of IFN and TNF by freshly isolated and one-day-cultured lymphocytes was compared. The results, presented in Fig. 4, showed that only freshly isolated cells produce cytokines after VSV infection. A comparison of IFN and TNF production by the lymphocytes of healthy donors and the group of people with frequent infections of the upper respiratory tract revealed no statistically significant differences (Fig. 5). The spontaneous production of both cytokines occurred more often in the group of persons with recurrent infections. In contrast to these results, statistically significant difference in VSV titers between these two groups was seen. The results are presented in Fig. 6.

The kinetics of VSV replication in lymphocytes and in cultures of whole blood were usually similar in the person under study, though sometimes they differed. The results obtained in whole-blood cultures seem to express the actual state of the innate immunity of the person better than those of lymphocytes. However, hemoglobin, which is sometimes present in the medium above whole-blood cultures incubated for several days, makes microscopic observation difficult. For this reason, the next experiments were performed only on all leukocytes isolated from the peripheral blood.

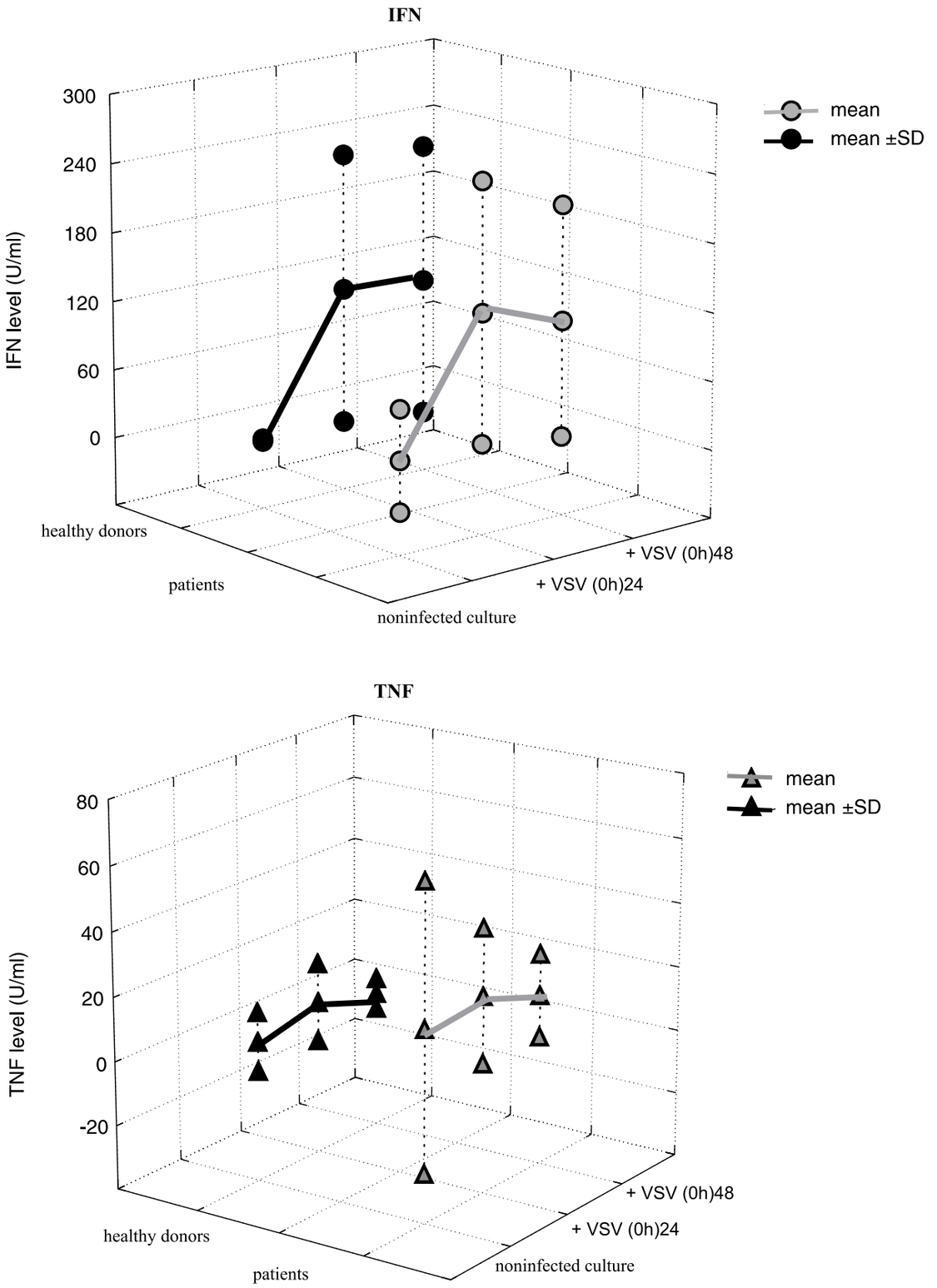

Fig. 5. Interferon and TNF production by lymphocytes of healthy donors (n=20) and patients with recurrent infections of the upper respiratory tract (n=9). The lymphocytes were infected just after cells isolation (t=0) and media were collected one (t=24) or two (t=48) days after VSV infection. The statistics were performed with the Mann-Whitney test, $p>0.05$

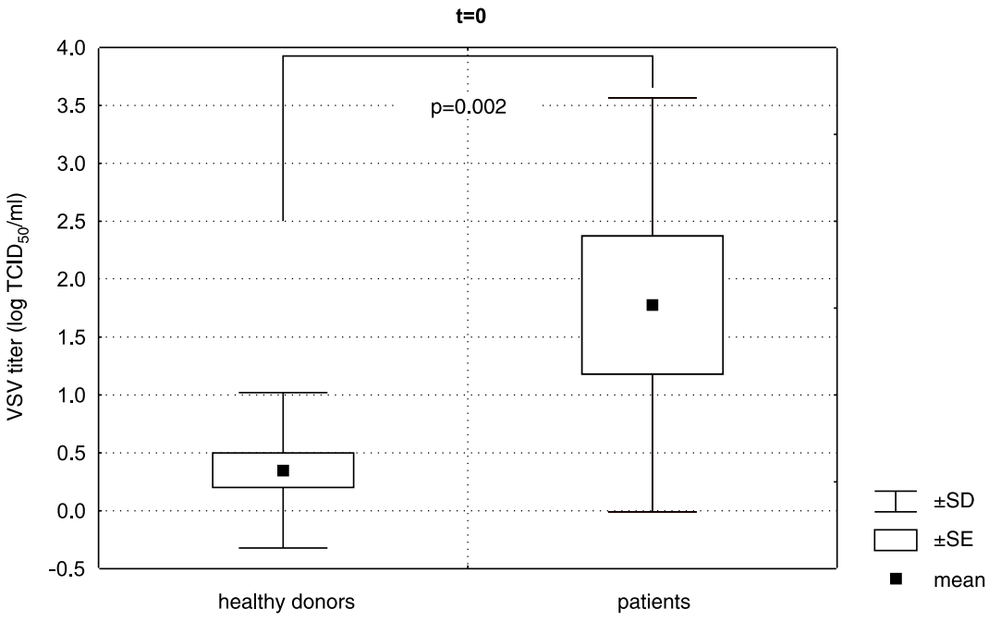

Fig. 6. Statistical significance of VSV replication in lymphocytes from healthy donors (n=20) and patients with recurrent infections (n=9). Mean values and standard deviations are presented. The statistics were performed with the Mann-Whitney test and concern the second day after viral infection

These next experiments concerned the participation of endogenous IFNs and TNF in maintaining the innate immunity of blood leukocytes. In order to eliminate cytokines released from leukocytes we used commercially available anti-TNF-α, anti-IFN-α, anti-IFN-β, and anti-IFN-γ antibodies. Their cytokine-neutralizing activity was titrated against the appropriate cytokine and used in experiments at a final concentration which neutralized cytokine concentrations above those produced by VSV-infected leukocytes. Anti-IFN-α antibody was used at a concentration neutralizing 500 U of IFN-α, anti-IFN-β: 1000 U of IFN-β, anti-IFN-γ: 600 U of IFN-γ, and anti-TNF-α: 600 U of TNF-α. The leukocytes isolated from the peripheral blood of donors were infected with VSV at a concentration of 100 $TCID_{50}$/ml immediately after preparation. After 40 min of virus adsorption and washing, the suspension of infected cells (2×10^6/ml) was distributed into 6 samples. One sample (control) was placed at +4°C, antibodies against cytokines were added to the other samples, and to one sample only culture medium was added. Antibody-treated and -untreated cells were incubated at 37°C for several days. Every day samples of supernatant were collected and the VSV was titrated in L_{929} cells. The kinetics of VSV replication in antibody--treated and untreated cells were compared. The group of 21 persons consisted of 12 healthy blood donors and 9 with frequent infections of the upper respiratory tract. In this group of 21 persons there were 7 with leukocytes completely resistant to viral infection, 9 persons with leukocytes partially resistant, and 5 with the cells very sensitive to VSV. The anti-cytokine antibodies stimulated virus replication in 4 of the 7 completely resistant leukocytes and in 5 of the 9 partially resistant. The effect of anti-cytokine antibodies on the innate immunity in 4 selected persons with leukocytes completely or partially resistant to VSV is presented in Fig. 7. The results show that the neutralization of IFN-α, IFN-β, IFN-γ or TNF-α produced by VSV-infected cultures might stimulate virus replication. The observed effect indicated a participation of endogenous IFNs and TNF-α in maintaining innate immunity. The stimulation of VSV replication in the presence of anti-cytokine antibodies was, however, not observed in every experiment. This indicates the possible contribution to innate antiviral immunity of mediators other than the IFNs and TNF-α.

Discussion

The study of the susceptibility of human whole--blood cultures and lymphocytes to viral infection revealed the presence of antiviral immunity in about 80% of the healthy persons included in the experiments. A similar frequency and degree of antiviral immunity, measured by the resistance of resident peritoneal cells

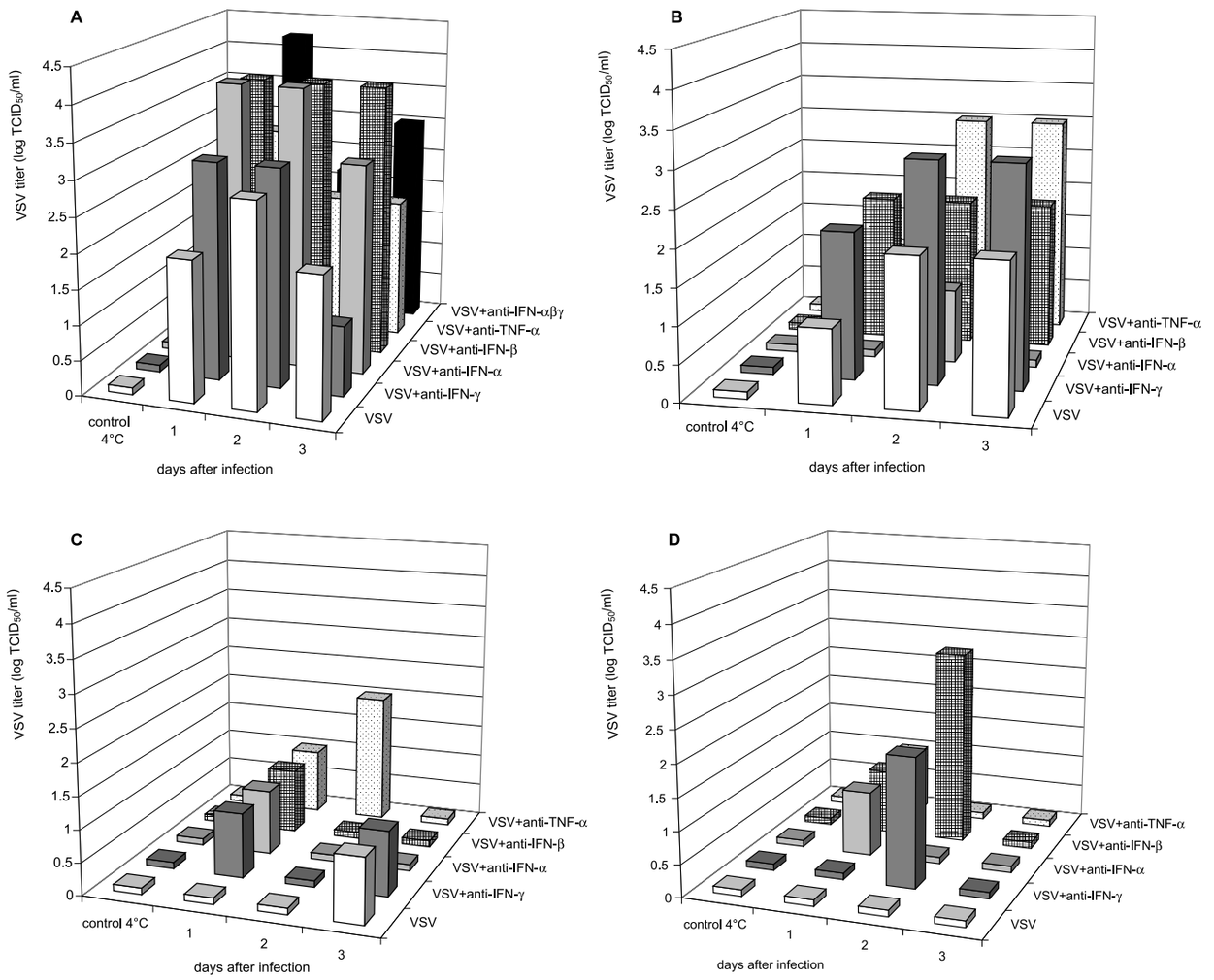

Fig. 7. Effect of antibodies against IFNs and TNF-α on VSV replication in partially (**A**, **B**) and completely virus-resistant leukocytes (**C**, **D**). The kinetics of VSV replication in antibody-treated and -untreated leukocytes of four selected persons are presented

to VSV infection, was found in a healthy BALB/c mice population[8]. The immunity is non-specific, because it prevents human and murine cells from infection with RNA- and DNA-containing viruses with and without envelopes. This was shown in this study and in a previous one[14]. The innate immunity is also present in different embryonic tissues[2, 9, 15].

The immunity was characteristic for freshly isolated cells and tissues. After several (1–5) days of incubation before viral infection, immunity was gradually reduced and the cultured cells acquired susceptibility to infection with the indicatory virus. The results of our study are consistent with those found by PLAEGER-MARSHALL et al.[10], who found higher HSV replication in preincubated macrophages than in freshly isolated ones, and with the results of our earlier studies[7, 9, 14].

The reason of the reduction of natural immunity during *in vitro* culture is not known. The cells responsible for the immunity may either die during *in vitro* incubation, or they may lose the ability to produce mediators of immunity. Survival of leukocytes changed during one day of incubation. Intravital staining with MTT revealed a 20–50% loss in leukocyte number in 3 of 6 experiments (not shown). The loss of the total number of cells was not correlated with reduction of innate antiviral immunity. Perhaps the reduction may be correlated with the death of special type of cells i.e. dendritic or NK cells. Identification of cells which participate in maintaining of innate antiviral immunity of leukocytes is now under the study. The lack of IFN and TNF production by one-day-cultured lymphocytes in most experiments may indicate a loss of ability to pro-

duce mediators of immunity during *in vitro* culture. Leukocytes with innate immunity usually produces more cytokines than the leukocytes with reduced immunity. Statistical significance, however, was found only for IFN and IL-12. The production of cytokines by leukocytes which express different level of innate immunity is the subject of another article, which will be published in future. The other possibilities are a shell of innate immunity receptors or an increase in the number of receptors for viruses during the incubation of leukocytes. The receptors engaged in maintaining of antiviral innate immunity are not completely known. Lack of differences in VSV adsorption by freshly isolated and cultured cells indicate that the number of receptors for the virus not changed during incubation. Production of inhibitors of natural immunity in the later period of incubation or proliferation of cells sensitive to virus may be considered also.

In our study, reduced non-specific antiviral immunity and normal IFN and TNF production in people with frequent infections of the upper respiratory tract was observed. The infections were mostly of viral origin. Using the method of leukocyte infection with an indicatory virus, it was possible, however, to demonstrate the disturbed innate immunity. These results and the individual differences in the innate antiviral immunity, expressed not only by resistance to the indicatory virus or different levels of virus replication, but also by the individual times of immunity loss during culture of leukocytes *in vitro*, arouse hope for a practical use of the direct method of infection of leukocytes with indicatory virus.

The study of the possible participation of endogenous IFNs and TNF-α in the natural antiviral immunity of leukocytes revealed that neutralization of released cytokines may or may not stimulate VSV replication in resistant leukocytes. Similar individual responses to anti-IFNs and anti-TNF-α antibodies were obtained in the case of VSV infection-resistant organ cultures of amniotic membrane and placenta[10]. All the results indicate that the interferons and TNF-α participate in the constitution of the immunity, but other cytokines or natural mediators may also take part. It is possible that the cytokine network responsible for innate immunity is so strong that elimination of endogenous interferons and TNF from infected leukocytes or organ cultures does not extensively disturb the immunity.

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