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BOTH MATERNAL AND NEWBORN IGMS INHIBIT TRANSMISSIBLE GASTROENTERITIS VIRUS INTERNALIZATION IN LLC-PK1 CELLS

Immunoglobulins M (IgMs) are the evolutionally oldest class of antibodies in higher eukaryotes. This pool of antibodies is one of the first to appear in humans and begins to besynthesized at the early stages of the neonatal period. Most of the repertoire of IgMs of the newborns consists of the so-called «natural», or «naive», antibodies synthesized by the body without external antigenic stimulation. In addition to the classical functions of human immunoglobulins M (such as antigen recognition and initiation of innate immune responses), antibodies of this class exhibit a variety of non-canonical functions. The non-canonical functions are the action of antibodies as agonists/antagonists of various receptors, cleavage of antigen due to the catalytic activity of IgM, direct inactivation of pathogens in the absence of effector cells and molecules, etc. ***The aim*** *of this work was to study and compare the antiviral activity of total preparations of immunoglobulin M of newborns and adults, obtained from umbilical cord and venous blood sera, on the LLC-PK1 cell line model infected with the transmissible gastroenteritis virus (TGEV). In addition, in the course of the studies, a decision was made to investigate the effect of combined preparations of immunoglobulin M on the change in signal transduction in the epidermal growth factor receptor as one of the mechanisms of TGEV internalization during infection of target cells.* ***Methods.*** *Highly purified preparations of total IgM of adults or newborns were obtained using the methodologies of sequential salt*

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fractionation and affinity chromatography. This work used the model of the interaction of the transmissible gastroenteritis virus with cells of the LLC-PK1 line and the monitoring of changes in the phosphorylation state of the epidermal growth factor receptor of these cells during virus infection to study the effect of human IgM on the internalization of the virus and its interaction with the receptor system of the host cell. The degree of cytopathogenic effect of the virus was determined visually by changes in cell morphology. The mean infectious dose for transmissible gastroenteritis virus in the cell culture of the LLC-PK1 line was determined by the Reed-Muench method. Analysis of changes in the phosphorylation of the epidermal growth factor receptor was performed using the Western blot analysis method. ***Results.*** *The addition of a total high-purified sample of human IgM reduces the degree of efficiency of TGEV infection of the LLC-PK1 cell line and modulates the phosphorylation levels of these cells.* ***Conclusions.*** *The total preparations of IgM obtained from human venous blood of adults and from umbilical cord blood of newborns can affect the internalization of the transmissible gastroenteritis virus in the LLC-PK1 cell line. The original model of virus (TGEV) — cell line (LLC-PK1) was applied and tested to study the effect of native total preparations of immunoglobulin M on the internalization of the virus into the cell. The obtained data can be useful in further studies for a better understanding of the process of development and functioning of the immune system of newborns.*

Keywords: *Immunoglobulin M, cord blood serum, adult venous blood serum, epidermal growth factor receptor.*

The phenomenon of the innate immune system is the immediate protection of the host the attack by a certain pathogen. It consists of barriers that prevent and inhibit the spread of viruses, bacteria, parasites, and other foreign species.

Natural antibodies (nAbs) of higher eukaryotes are components of innate immunity. Even without visible antigenic stimulation, the presence of natural circulating antibodies is already detected in human cord blood [1]. Such antibodies are produced spontaneously without antigenic stimulus and they are called naive (natural) antibodies [2]. Usually, the class of immunoglobulins M (IgM) predominates in the body among the natural antibodies [3], whereas antibodies of the IgG and IgA classes are found in smaller quantities [4, 5].

It has been confirmed that a pool of antibodies that arises spontaneously without antigenic stimulation is already present in the umbilical cord blood of fetuses and newborns [6], as well as in blood of normal healthy individuals [3, 7, 8]. The repertoire of natural antibodies is quite conservative [1, 9—11]. These antibodies are components of the humoral link of mammalian immunity. During an infectious involvement within the body, natural (innate) immunity is provided, first of all, with polyreactive IgM secreted by class B-1 lymphocytes. B-1 cells are generated mainly during the fetal and neonatal development from progenitor cells located in the liver and omentum [12]. In adults, the population of B-1 cells is maintained at a constant level. This population of cells is capable of self-repair, which makes it possible to maintain a constant level of natural antibodies in the blood serum [13—15]. The main source of natural antibodies in the body are peritoneal B-1 cells [16]. Also, they are produced by B-1 cells located in the bone marrow [17] and spleen [18—21]. Bone marrow B cell progenitors produce B-2 and B-1 lymphocytes in the fetus and in adults [15, 22, 23].

There is much evidence that natural antibodies play a critical role in protecting the host from bacterial and viral infections through activating them at the earliest stages of interaction with the pathogen, similar to membrane-bound pathogens (PRRs) [24—26].

IgMs are the first to react against foreign invasion, including viral pathogens that cause global pandemics. This is the only class of antibodies to be present in all vertebrate species [27]. In the human body, the total pool of IgMs is available at a high enough concentration in the blood serum (~1.47 mg/mL) [28].

Natural IgMs are low-affinity and polyreactive compared to other classes of antibodies.

The properties mentioned above are determined by their unique structure. Secretory IgM is predominantly a pentamer and contains an additional J-chain. Even at low affinities, natural IgMs play a critical role in the primary protection of the host from dangerous foreign agents [29]. It should be noted that the high avidity may allow them to adapt more effectively and recognize viral particles, despite the high frequency of mutations in the latter. Therefore, serum IgMs may be effective in the prevention of generalized infections [30].

Natural IgMs use the following three mechanisms to protect the body from viral infections: (i) neutralization of them by direct binding to viral proteins or viral receptors that are expressed on potential viral target cells, with or without complement; (ii) capture of virions by aggregation; (iii) transport of viral antigens in the form of immune complexes (ICs) to lymphoid tissues, thereby contributing to the emergence of an adaptive immune response to the activation of the complement system or interaction with the Fc receptor [29].

Growth factor receptor (GFR) signaling is crucial for intiation of infection caused by certain types of viruses [31]. Activation of GFR leads to the modulation of a wide range of cellular processes, such as proliferation, adhesion, or differentiation [32]. Various viruses, such as Epstein-Barr virus, influenza, or hepatitis C, have been shown to use epidermal GFR (EGFR) as a cell entry receptor [33, 34]. In addition, EGFR activation may inhibit interferon signaling and thus the antiviral response occurring in respiratory viral diseases such as influenza A or rhinovirus infection [35]. There is evidence that activation of GFR signaling may also play a significant role in infection with respiratory viruses such as SARS-CoV-2 [36].

In recent years, the studies of many viruses have shown that the effect of host cell signaling can be an influential aspect for virus replication and have grounds for obtaining effective therapeutic tools for viral diseases [31, 37]. The mechanism of regulation of GFR signaling in SARS-CoV-2 infection is currently the subject of speculation. It should be noted that inhibition of GFR signaling may be a useful approach in the treatment of SARS-CoV-induced fibrosis [38] and also serves as a potential tool for the treatment of COVID-19.

One of the conditions for tissue tropism of coronaviruses is the type and distribution of relevant receptors on the cell surface [39]. The ability of coronaviruses to transfer successfully from one species to another can also be explained by the use of various penetration strategies to infect the target cell [31].

It should be emphasized that coronaviral infections of swine and other respiratory coronaviruses are used as a model for the study of severe acute respiratory syndrome (SARS), which is characterized by symptoms like the pneumonia caused with the coronavirus SARS-CoV [40]. An example that serves as a model in this case is the transmissible gastroenteritis virus (TGEV), which belongs to the coronavirus family and causes a highly contagious disease in piglets. The TGEV as a tropism both for the gastrointestinal tract and the respiratory tract's epithelial cells. It is similar by structure but very distinct in the type of target cell from the porcine respiratory coronavirus (PRCV), which affects the respiratory system. The latter has been shown to arise from TGEV by deletion of part of the viral protein S, resulting in a change in the tropism of the virus from the gastrointestinal tract to the respiratory tract [41].

The LLC-PK1 cell line (renal epithelial cell line, LLC-PK) is used to model swine intestinal coronavirus infections. It is known to be quite susceptible to porcine enteroviruses, rotaviruses, and coronaviruses. Epidermal GFRs are localized along the nephron [42] and play a key role in normal physiology and renal pathology. The proximal region of the renal tubules contains the highest concentration of EGFR in the structure

of the organelles [43]. Therefore, the combination of two important aspects of susceptibility to TGEV infection and the high concentration of EGFR in LLC-PK1 cells, prompted the use of this cell line in the study of the interaction of human ImGMs with the viruses from the Coronaviridae family.

The **aim** of this study was to investigate the susceptibility of LLC-PK1 cells to infection induced by porcine intestinal coronavirus such as TGEV and the effect of naive neonatal IgM that derives from the umbilical cord blood pool as well as venous blood pool of adult donors during infection of the above cells with TGEV. This study describes events of signal transduction in the host cells infected with TGEV coronavirus and shows the transmission of GFR signals as a probable pathway required for replication of this virus. It was found that the addition of total IgMs changes the degree of effectiveness of TGEV infection of the LLC-PK1 cell line and modulates the levels of EGFR phosphorylation of these cells.

Materials and methods. *Cells, viruses, and reagents.* LLC-PK1 cells used in this study were of ATCC; Manassas, VA.

TGEV, an etiologic agent of porcine transmissible gastroenteritis (PTG), highly virulent strain D_{52} of low passages in cell cultures, was taken rom the collections of the Gromashevsky Institute of Epidemiology and Infectious Diseases of the NAMS of Ukraine.

Virus inoculation and cytopathic effect (CPE). LLC-PK1 cells were grown in 24-well plates to the confluence and then inoculated with TGEV at a multiplicity of infection (MOI) of 0.01. The optional infection doses used for TGEV were based on our preliminary experiments. After incubation for 1 h at 37 °C to allow virus attachment, the inocula were discarded and replaced with DMEM (Sigma). CPE was observed at 6, 12, 18, 24, and 30 h of postinfection (hpi).

Median tissue culture infectious dose (TCID50) assay and proliferation curve. The LLC-PK1 cells were inoculated with TGEV at an MOI of 0.01. Samples were collected at 6, 12, 18, 24, and 30 hpi, frozen and thawed three times, and centrifuged at 4 °C with 12,000 rpm for 10 min, and the supernatants were collected. TCID50 assays were performed as described previously [14]. Briefly, cell monolayers grown in 96-well plates were washed twice with DMEM. Virus-containing supernatants were serially diluted tenfold, and appropriate dilutions of virus suspension were chosen to inoculate LLC-PK1 cells, with eight replicates at each dilution. After the cells were cultured at 37 °C with 5% CO_2 for 3—4 days, the TCID50 was calculated by the Reed-Muench method, and the virus proliferation curves were plotted from three independent experiments.

Sample and sera collection. Information on gestational term, health of mother and child was obtained through an interview with the curator and clinical data from the hospital.

10 maternal-cord serum pairs were collected from apparently normal mothers and from the umbilical cord blood of their infants at the time of delivery at Maternity Hospital 5, Kyiv, Ukraine. A written consent was obtained from all donors, and all study procedures were confirmed by the ethical committee of Research Institute «MedLife» (Protocol No 2). Umbilical cord blood was collected immediately after birth. Maternal blood was obtained by venipuncture on the day of delivery. Freshly harvested blood was allowed to clot by incubation for 30 min at 37 °C, then stored for 12 h at 4 °C, and serum was obtained after centrifugation at 1,500 g for 30 min at 4 °C. All sera were stored in a 50% ammonium sulfate solution at 4 °C and were not heated before use. The study was conducted after the approval of the institutional ethical board review and the signed informed consent of each participant.

IgM purification. To obtain total IgM from umbilical cord blood pool as well as venous blood pool of maternal adult donors, each serum was treated with saturated ammonium sulfate to 50% salt concentration in the working

solution at 4 °C. After centrifugation at 2,500 g for 1 h, the precipitate was solubilized in phosphate-buffered saline (PBS) and again treated with ammonium sulfate at 4 °C overnight (degree of saturation with ammonium sulfate was 35%). After centrifugation at 2,500 g for 1 h at 4 °C, the precipitate was solubilized in PBS. Fractions of cord blood samples were combined together. The same procedure was carried out with samples from venous maternal blood. Dialysis of the above samples was perfomed overnight at 4 °C. The dialysate was filtered through a 0.45 μm filter and then passed through a G-agarose column (ABT, Spain) for maximum depletion of IgG from the samples. Then total serum IgM was purified and concentrated using column chromatography with anti-IgM-resin (Thermo Scientific, Holland). Then the purified total IgM preparations were filtered through a 0.2 μm filter in sterile conditions and stored in a 0.1 M glycine buffer, pH 7.4 in aliquots at 4 °C.

Quantification of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and protein concentration in IgM samples. All total IgM samples purified from maternal-cord serum pairs and proteins from cell lysates of LLC-PK1 were analyzed by SDS-PAGE in 7—22% gradient of polyacrylamide gel under reducing conditions, as described by laemmli [44].

Protein concentration (IgM — heavy chain) was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. Coomassie brilliant blue R-250 (Bio-Rad, Hercules, CA) was used to stain the gel, and the molecular mass marker (ApplyChem, Germany) and bovine serum albumin sample with certain concentrations were used for standartization. Quantification of total IgM purified samples was conducted by using the Image Studio lite Software 5.2 (LI-COR Biosciences, US). The heavy chain of IgM samples was used for determination of protein concentration in the samples.

Determination of the cytotoxic concentration of samples (CC50). LLC-PK1 cell cultures were used to determine the CC50 of the samples. During the experiments, at least ten rows of wells of the immunological plates with the cell culture were used for each dilution of the sample in the culture medium (RPMI 1640, Sigma). The plates with the cellculture were incubated at 37 °C with 5% CO_2 atmosphere supply for 5 days. Observations were made daily on test and control samples of cultures in order to establish the presence or absence of cytopathogenic activity (CPA).

The degree of cytopathic effect (CPE) was determined by changes in the morphology of cells (rounding, wrinkling of cells, rejection of the well surfaces, and degeneracy of changed cells).

We used the 4+ descriptive system ranked from + to ++++.

Thus, «—» means the absence of cell degeneration; «+» — no more than 25% of the cell monolayer is affected (i.e., protection of cells of the monolayer from the antiviral drug is 75%); «++» — no more than 50% of the cell monolayer is affected; «+++» — no more than 75% of the cell monolayer is affected; «++++» — complete degeneration of the cell monolayer.

According to the CC50 of the sample, the largest amount that did not cause cell degeneration was taken.

Determination of effective concentration (EC50). EC50 is the minimum concentration of the sample that inhibits the development of a specific CPE virus by 50%. To determine the EC50, the test viruses at a dose of 100 TCD50/0.1 mL were added to the cell culture and incubated for 60 min at 37 °C. After adsorption of the virus on the cells, the residues were removed, the cells were washed with a nutrient medium, after which samples in different concentrations were added to the supporting cell growth medium (RPMI-1640 + 2% fetal bovine serum (FBS), Sigma). The absence of CPE in the treated cultures, its presence in the control, as well as the decrease in the infectious titer in the treated cultures, and the difference in the infectious titers in the experiment com-

pared to the control virus allowed us to establish the EC50 of the sample.

To study the antiviral activity of the samples, daily cultures of LLC-PK1 cells were selected. The culture growth medium was drained, and the investigated drugs at different concentrations were added to the monolayer of cells. After 1 hof contact, the virus was introduced at a dose of 100 TCD50. The cultures were incubated in a thermostat with CO_2 supply for 2 daysand monitored daily with a microscope. There was noted the reproduction of the virus by the cytopathogenic effect of TGEV on LLC-PK1 cells unlike the control cultures where the monolayer was not exposed to any influences.

Criterion for assessing the antiviral activity of substances in in vitro systems. Transplanted LLC-PK1 cell culture was used to study the anticoronavirus activity of the samples. Cells were grown in plates on RPMI-1640 medium + 10% FBS (Sigma) at a 37 °C in a thermostat with CO_2 supply.

Cytotoxic concentration CC50, that is, the concentration of substances that contribute to a decrease in the viability of cell culture by 50%, was determined during the analysis of the cytotoxic effect of the studied substances in accordance with the regulatory recommendations for studies of antiviral samples *in vitro*. To determine the antiviral activity of the tested substances, the effective concentration (EC50) was determined, that is, the concentration of the tested substance at which the level of virus replication in the infected cell culture is suppressed by 50%. After determining the indicators of cytotoxic and antiviral action, the selectivity index (SI) was calculated as the ratio of CC50 to EC50. The substances that had SI ≥ 16 in the *in vitro* system were considered more active and promising for further research on animals.

Determination of EGFR phosphorylation in LLC-PK1 cell culture using the Western blot analysis. LLC-PK1 cells were cultured in RPMI-1640 medium containing 10% FBS, 1% penicillin/streptomycin at 37°C in 5% CO_2. Cells were seeded in 24-well plates at a density of 1.5x105 cells/1 mL of RPMI-1640 medium per well and grown to 70—80% monolayer. Cells were then washed three times with RPMI-1640 medium and incubated overnight in the absence of FBS. The cells depleted in this way were exposed overnight to the samples under study. Cells were incubated with EGF from murine submaxillary gland (E4127, Sigma), TGEV and IgM virions separately or, in the case of TGEV and IgM, in a mixture for 60 min at 37 °C. Then the cells were washed twice with RPMI-1640 medium and lysed in RIPA buffer containing 1 mM EDTA. Aliquots of 25 μg of protein were separated in 7—22% SDS-PAGE and transferred to a nitrocellulose membrane.

The EGF receptor was detected in the Western blot using primary monoclonal Anti-Epidermal Growth Factor Receptor's antibody produced in mice (E2760, Sigma), and the level of autophosphorylation was identified using Anti-Phosphotyrosine Antibody, clone 4G10 (05-321, Sigma). Equal amounts of LLC-PK1 cell lysates samples were subjected to 7—22% SDS-PAGE. Next, the proteins were transferred to 0.45 μm nitrocellulose membranes (Amersham Biosciences, GE Healthcare, Germany) using a trans-blot cell (Bio-Rad, US). Then tne membranes were incubated with anti-phosphotyrosine antibodies and visualized using ECL reagent (Sigma, England).

In our previous studies, the optimal quantitative indicators of the studied agents for the phosphokinase activity of the epidermal growth receptor have been determined (data not shown).

Comparison and quantification of protein phosphorylation signals. Comparison and quantification of Protein phosphorylation signals from LLC-PK1 cell lysates were conducted using the Image Studio lite Software 5.2 (LI-COR Biosciences, US).

Statistical methods of analysis. Statistical analysis of the data obtained was carried out using the current statistical methods, Excel 2000 computer

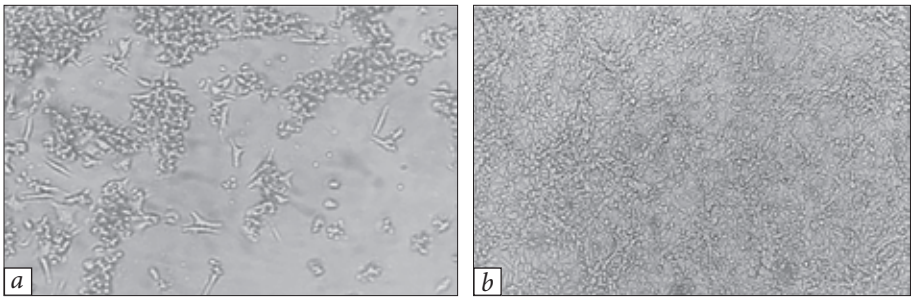

Fig. 1. Comparative characteristics of the culture of LLC-PK1 cells in a normal state (*a*) and when affected by the TGEV (*b*) (10×40 res)

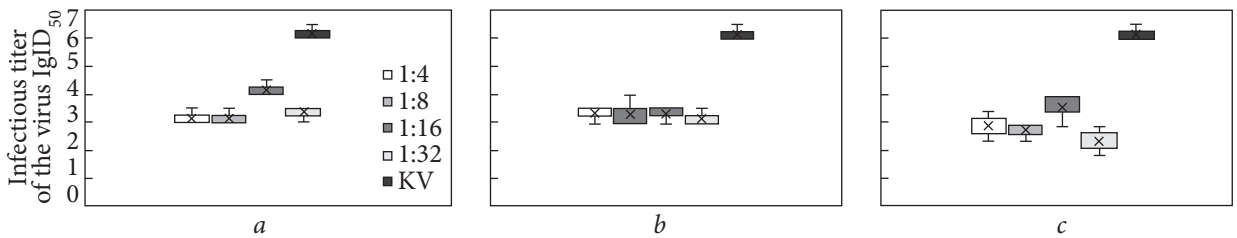

Fig. 2. Determination of anti-coronavirus activity of immunoglobulin preparations (EC50) in LLC-PK1 cell culture: *a* — total IgM obtained from serums of venous blood of parturients; *b* — total IgM obtained from umbilical cord blood sera of newborns; *c* — total IgG obtained from donor blood sera (commercial preparation)

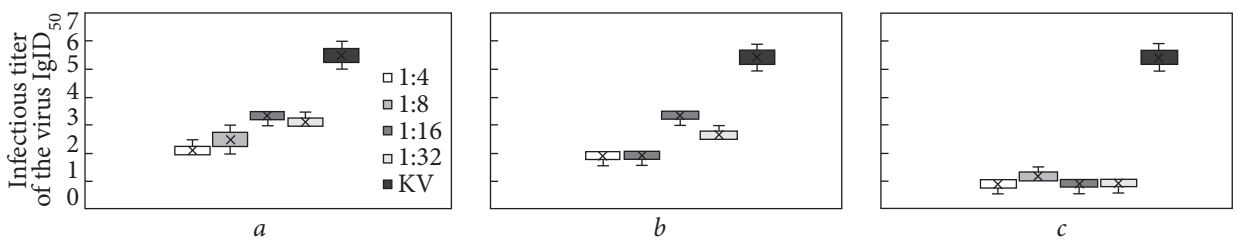

Fig. 3. The infectious titer of the TGEV in the wells with the LLC-PK1 cell line treated with various dilutions of the drugs and the TGEV 100 ID50: *a* — total IgM obtained from sera of donor venous blood of parturients; *b* — composite preparation of total IgG of donor venous blood/total IgM of donor venous blood of parturients; *c* — composite preparation of total IgG of donor venous blood/ total IgM of umbilical cord blood of newborns; KB — control of the infectious titer of a cell culture affected by the TGEV virus without addition of immunoglobulin preparations

program, and OriginPro 2018 software. One-way repeated analysis ANOVA was performed to evaluate the effects of EGF, TGEV, and human IgM obtained from the adult venous blood and from newborns umbilical cord IgM on the degree of EGFR phosphorylation in LLC-PK1 cell culture. The Bonferroni adjustment was applied to reduce the instance of a false positive.

Results. The cytopathogenic effect of the TGEV on cells is morphologically manifested in the formation of small cell degeneration (Fig.1).

After 3 days, the culture medium was collected from the tablet wells, and the infectious titer was determined.

The effect of the studied samples on the reproduction of TGEV was well-determined when

following the drug administration scheme used: simultaneous introduction of the TGEV and the studied samples, i.e. during the adsorption of TGEV onto cells.

All studied samples were non-toxic when tested on LLC-PK1 cell culture. The pig's TGEV strain was used with an infectious titer of 5.0—8.5 Lg ID50.

Total IgM samples obtained from both adult donors and umbilical cord blood serum demonstrated antiviral activity of the studied preparations, which was determined when a separate preparation was added to the TGEV-infected cell culture of the LLC-PK1 line. The determination of the antiviral activity EC50 of the total IgM samples in LLC-PK1 cell culture is presented in Fig. 2.

In order to create a composition of the total human IgG/IgM preparation, where M immunoglobulins would act as a total preparation obtained from the umbilical cord blood of newborns, or a total IgM preparation obtained from the venous blood of mothers in labor, the anti-coronavirus activity of such composite samples was investigated in the culture of LLC-PK1 cells infected with the TGEV.

The composite preparation of IgG from adult donors/IgM from umbilical cord blood showed higher anti-coronavirus activity when used in LLC-PK1 cell culture than when the composite preparation from immunoglobulins IgG from adult donors/IgM from the venous blood of parturients was used in such a system (Fig. 3).

The criterion for evaluating the inhibitory activity of antiviral drugs in *in vitro* systems is the selectivity index (SI) of the samples and a decrease in the infectious titer by 1.5—2.0 LgTCD50. Therefore, the table below presents the summarized results of studies on the determination of EC50 when administering the studied immunoglobulin preparations.

The data of EC50 indicators when determining the antiviral activity of samples in the LLC-PK1 cell culture on the coronavirus model of transmissible gastroenteritis of pigs are shown in Table 1.

As a result of an experiment on the study of the antiviral effect of the samples on the experimental model of the transmissible gastroenteritis of pig's coronavirus in the LLC-PK1 cell culture, it was revealed that the studied samples effectively inhibit the reproduction of the porcine coronavirus by 2.0—4.0 Lg TCD50, SI — 250.146. The most significant antiviral effect in the TGEV-affected cell culture of the LLC-PK1

Table 1. **EC50 indicators for determining the antiviral activity of drugs in LLC-PK1 cell culture on the TGE coronavirus model**

Sample	CC50 mcg/mL	EC50 mcg/mL	SI	Inhibition of infectious titer, Lg TCD50
Total preparation of IgM from sera of venous blood of women in labor	800	25	32	3
Total preparation of IgM from sera of umbilical cord blood of newborns	800	25	32	3
Total commercial preparation of human immunoglobulin G	6880	27.5	250.18	4
Composite preparation of total IgG of donor venous blood/ total IgM of donor venous blood of women in labor	3440+400	26.25	146.28	2
Composite preparation of total IgG of donor venous blood/ total IgM of umbilical cord blood of newborns	3440+400	26.25	146.28	4

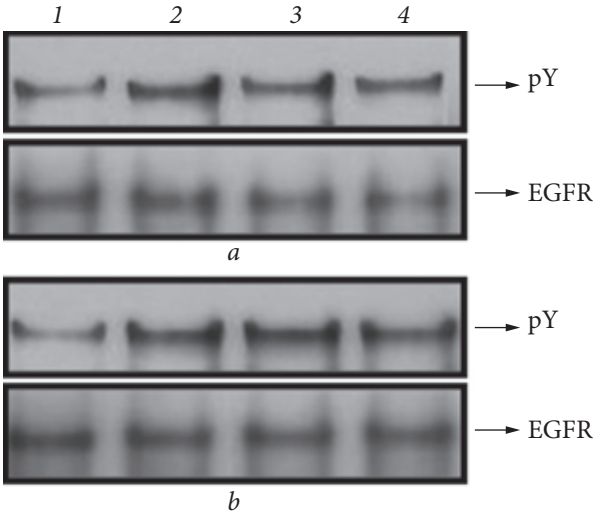

Fig. 4. EGFR autophosphorylation of LLC-PK1 cells under the action of 50 ng/mL epidermal growth factor (a) and TGEV virions (TCID50) (b): 1 — control without adding agents; 2 — induction time 30 min, 3 — 60 min, 4 — 120 min

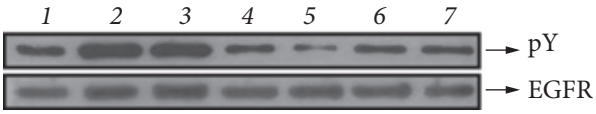

Fig. 5. Autophosphorylation of the EGF receptor in the LLC-PK1 cell line, detected by the Western blot analysis using anti-phosphotyrosine and anti-EGFR antibodies with the addition of the studied infection agents in various combinations. (Cells were incubated in serum-free medium RPMI-1640 for 60 minat 37 °C): 1 — control, not added with agents or virus; 2 — EGF (50 ng/mL) — positive control; 3 — TGEV (TCID50); 4 — IgM of the mother in labor (0.8 mg/mL); 5 — umbilical cord IgM (0.8 mg/mL); 6 — IgM of the mother in labor (0.8 mg/mL) + TGEV (TCID50); 7 — umbilical cord IgM (0.8 mg/mL) + TGEV (TCID50)

line was shown by a composite preparation of a mixture of IgMs obtained from the umbilical cord blood of newborns and a total preparation of immunoglobulins G of adults, which inhibits the reproduction of TGEV by 4.0 Lg TCD50.

The LLC-PK1 culture of epithelial cells of pig kidney tubules, which we decided to use to study the changes that may occur at the level of EGFR phosphorylation when affected by the TGEV, is a convenient tool for conducting the experiment due to the high content of the receptor on the membrane surface of these cells. Therefore, such a model makes it possible to detect changes in EGFR phosphorylation using the Western blot analysis.

During the control test of receptor activation, it was found that changes in the increase in receptor phosphorylation occurred both when the specific ligand of the EGF from the submandibular glands of the mouse and TGEV virions were added (Fig. 4). The similarity of the results for the phosphorylation of the EGF receptor during the induction of EGFR phosphokinase by the studied agents at the same intervals of time is observed.

One of the tasks of the research was to find out the effect of total preparations of immunoglobulin M on the internalization of the TGEV in the LLC-PK1 cell line. It was important to find out whether there are changes in the phosphorylation of EGFR when adding total IgM preparation in the absence of TGEV into the cell mixture, taking into account that the addition of the virus alone to the system led to almost the same increase in EGFR phosphorylation as when adding EGF directly to the system (Fig. 5). The identity of the concentrations of the applied samples was proven by the visualization of the same β-actin signals during the Western blot analysis.

It should be noted that combined preparations of human immunoglobulin M did not lead to an increase in EGFR phosphorylation, and therefore, probably, to the activation of the abovementioned receptor.

Data obtained from the statistical processing of phosphotyrosine signals using the Western blot analysis showed that upon adding a total preparation of immunoglobulins M from the umbilical cord blood of newborns, the signal decreased by almost 60% compared to the signal when the same number of cells were infected

with TGEV in the absence of the IgM system. At the same time, the reduction of the EGFR phosphorylation signal and the probable internalization of the virus into cells were more effective when adding the IgM preparation obtained from the umbilical cord blood of newborns compared to the IgM preparation from the venous blood of mothers in labor (Fig. 6).

There was a statistically significant difference in the averages between cultures treated with EGF (50 ng/mL) and TGEV (TCID50), IgM of mother in labor (0.8 mg/mL); umbilical cord IgM (0.8 mg/mL); maternal IgM (0.8 mg/mL) + TGEV (TCID50), and umbilical cord IgM (0.8 mg/mL) + TGEV (TCID50) ($p < 0.001$). Treatment of the LLC-PK1 cell line with maternal IgM (0.8 mg/mL) and maternal IgM (0.8 mg/mL) + TGEV (TCID50) resulted in statistically significant difference in EGFR phosphorylation as well ($p < 0.05$). However, the difference between treatment with umbilical cord IgM (0.8 mg/mL) and umbilical cord IgM (0.8 mg/mL) was found to be statistically insignificant.

Discussion. This study provides new insights into the molecular mechanisms elicited by TGEV infection in the LLC-PK1 cells line and definition of the potentional role of the human IgM in prevention of such infection processes.

It has been found that the addition of individual preparations of human immunoglobulin M to the culture of TGEV-infected cells of the LLC-PK1 line increases the survival of the line and can potentially suppress the possibility of virus penetration into the cell. The total preparations of IgM obtained from human venous blood of adults and from umbilical cord blood of newborns can affect the internalization of TGEV in the LLC-PK1 cell line.

It is known that composite preparations of highly purified human immunoglobulins are used for therapy and prevention of certain diseases in the world. The above-mentioned commercial samples are, for the most part, enriched with

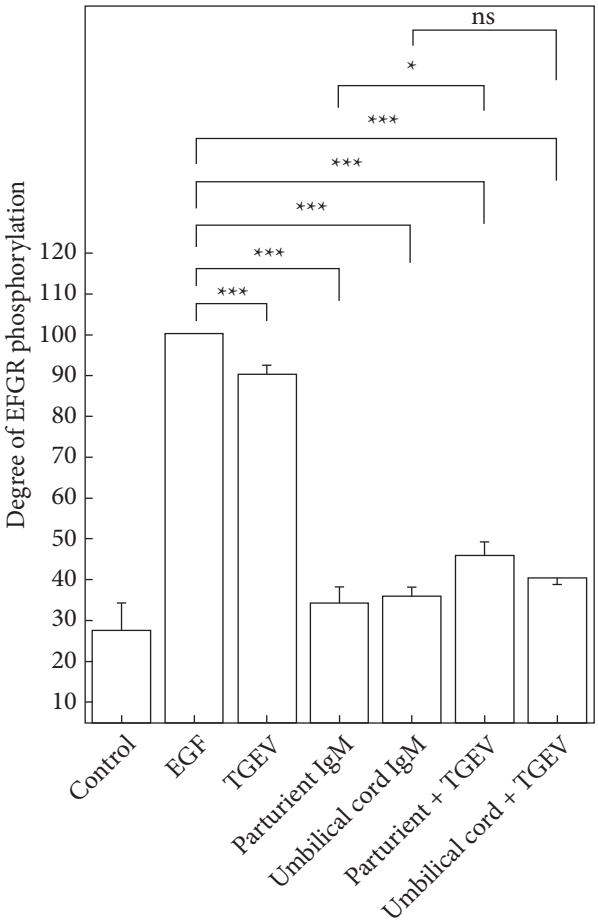

Fig. 6. Statistical treatment of EGFR phosphorylation signals in LLC-PK1 cells after pre-incubation with the following compounds: Control (without adding compounds or virus to the cell line; EGF (50 ng/mL); TGEV (TCID50); IgM of the mother in labor (0.8 mg/ mL); umbilical cord IgM (0.8 mg/mL); maternal IgM (0.8 mg/mL) + TGEV (TCID50); umbilical cord IgM (0.8 mg/mL) + TGEV (TCID50). Results of three replicates are presented. Data are expressed as the average ± outliers. *$p < 0.05$, ***$p < 0.001$, ns — no statistically significant difference

total immunoglobulins G. There is also a small number of variants on the commercial market consisting of a mixture of human immunoglobulins of different classes (IgG, IgM, and IgA).

In the course of the work, we decided to create a composition of the total human IgG/IgM preparation, where IgMs would act as a total prepara-

tion obtained from the umbilical cord blood of newborns, or a total IgM preparation obtained from the venous blood of mothers in labor. Anticoronavirus activity of such composite samples was investigated in the culture of LLC-PK1 cells infected with TGEV.

Addition of total preparations of IgM to the LLC-PK1 cell line affected with TGEV led to an increase in the cell survival in this culture, and therefore, it is possible to assume that such preparations could have an antiviral effect and be used in the future as therapeutic agents. It was therefore decided to find out the probable mechanism of prevention of cell culture virus damage when using such drugs. When conducting experiments for the direct neutralization of TGEV with total preparations of IgM, no positive result was found (data not specified). Therefore, we proposed an alternative mechanism for preventing the TGEV entry into the culture of the LLC-PK1 line cells when total preparations of IgM are added to the culture medium. There are data that one of the mechanisms for the virus penetration into the host cell is the interaction of certain (specific) viral proteins with the membrane receptors of eukaryotes.

Also, it has been established that the studied samples statistically reliably inhibit the reproduction of the TGEcoronavirus in the LLC-PK1 cell culture line.

According to the researchers' preliminary findings, the signaling pathways regulating the internalization of the receptor-virus complex into the cell may be diverse, but have common and similar functional and mechanical features between different families of viruses. There is evidence that the epidermal growth factor receptor (EGFR), a member of the ERBB family, is involved in the functioning of the life cycle of several viruses [45, 46]. Its involvement in the internalization of viruses into the cell and their transport into the endosomal space were confirmed for the hepatitis B virus (HBV) [46, 47] as well as for gastroenteritis viruses [48, 49].

Others have reported that the EGFR may play a role in the internalization of coronaviruses through interaction with protein S. Transmissible gastroenteritis virus (TGEV) is an alphacoronavirus that infects intestinal epithelial cells, causing severe, potentially fatal diarrhea in piglets. Its internalization into cells is achieved due to clathrin- and caveolin-mediated endocytosis. Then it binds to EGFR, promoting sequential clathrin-mediated endocytosis [50]. After the S spike protein of TGEV binds to the EGFR the phosphoinositide-3 kinase (PI3K) pathway is activated. EGFR is a MAPK pathway that correlates with F-actin reorganization, which is important evidence for the involvement of EGFR in endocytosis of coronavirus [51].

An interesting fact is that various respiratory viruses induce EGFR activation, which can lead to suppression of interferon-1, a regulatory inhibitory factor that regulates interiron λ activation, which, in turn, contributes to a decrease in antiviral protection in the epithelium of the respiratory tract [35, 48].

Thus, EGFR can be not only a portal for virus entry, but also participate in suppressing the host's immune response.

An obvious decrease in the phosphorylation signal (EGFR activation) when human IgM total preparations are added to the mixture of cells affected by the TGEV can be a direct evidence of its effect on preventing the interaction with the EGFR cell receptor and internalization in the cells of the LLC-PK1 line. The explanation of the more effective action in reduction of the EGFR phosphorylation signal by the total IgM sample, obtained from umbilical cord blood, can be the assumption that the avidity of a certain pool of immunoglobulin M depends on the heterogeneity of antibodies in the serum specific to a certain antigenic determinant.

The results of the conducted studies give grounds for drawing the following **conclusions:** total IgM preparations obtained from both the umbilical cord blood of newborns and

the venous blood of mothers in labor can affect the internalization of the TGEV in the LLC-PK1 cell line. Further research of umbilical cord IgM is a promising direction for the development of effective and universal antiviral agents. Neonatal IgM has a number of advantages compared to other antibodies: leveling of the significant mutational capacity of viruses due to the impossibility of adapting to the many variable regions of the Fab of heterogeneous IgM, increased aggregating capabilities, and the ability to bind viruses even inside the cell. To perform the work, an original model of virus-cell interaction was tested and applied, which makes it possible to accurately assess the effectiveness of inhibitors of virus internalization. One of the important advantages of this model is its safety. In addition, a similar concept can be effectively applied to other viruses that use EGFR to enter the cell.

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СУМАРНІ ПРЕПАРАТИ ІМУНОГЛОБУЛІНІВ М НОВОНАРОДЖЕНИХ ТА ДОРОСЛИХ ІНГІБУЮТЬ ІНТЕРНАЛІЗАЦІЮ ВІРУСУ ТРАНСМІСИВНОГО ГАСТРОЕНТЕРИТУ В КЛІТИНАХ LLC-PK1

Імуноглобуліни М (IgM) — це еволюційно найдавніший клас антитіл у вищих еукаріотів. Пул цих антитіл з'являється у людини одним із перших і починає синтезуватися на ранніх стадіях неонатального періоду розвитку плоду. Більшу частину репертуару IgM новонароджених складають так звані «природні» чи «наївні» антитіла, що синтезуються організмом без зовнішньої антигенної стимуляції. Окрім класичних функцій імуноглобулінів людини, а саме розпізнавання антигена та ініціація вроджених імунних відповідей, антитіла даного класу виявляють різноманітні неканонічні функції. Такими є дія антитіл як агоністів/антагоністів різних рецепторів, розщеплення антигена за рахунок каталітичної активності IgM, пряма інактивація патогенів за відсутності ефекторних клітин і молекул тощо. **Метою роботи** було дослідити та порівняти противірусну активність сумарних препаратів IgM новонароджених та дорослих, отриманих з сироваток пуповинної та венозної крові, на моделі клітинної лінії LLC-PK1 інфікованої вірусом трансмісивного гастроентериту (TGEV). У ході досліджень було вирішено ще дослідити вплив сумарних препаратів IgM на зміну передачі сигналу в рецепторі епідермального фактора росту як одного з механізмів інтерналізації TGEV при інфікуванні клітин-мішеней. **Методи.** Високоочищені препарати сумарних IgM дорослих чи новонароджених було отримано за допомогою методів послідовного сольового фракціонування та афінної хроматографії. Для дослідження впливу IgM людини на інтерналізацію вірусу та взаємодію останнього з рецепторною системою клітини-хазяїна в даній роботі було використано модель взаємодії TGEV з клітинами лінії LLC-PK1 та дослідження зміни фосфорилювання рецептора епідермального фактора росту даних клітин при інфікуванні вірусом. Ступінь цитопатогенної дії вірусу визначали візуально за зміною морфології клітин. Середню інфекційну дозу для TGEV в культурі клітин лінії LLC-PK1 розраховували за допомогою методу Реда-Мюнха. Аналіз змін у фосфорилюванні рецептора епідермального фактора росту проводили за допомогою Вестерн-блот аналізу. **Результати.** Встановлено, що додавання сумарних препаратів IgM змінює ступінь ефективності TGEV-інфекції лінії клітин LLC-PK1 та модулює рівні фосфорилювання даних клітин. **Висновки.** Препарати сумарних IgM, отримані як із венозної крові дорослих людей, так і з пуповинної крові новонароджених, можуть впливати на інтерналізацію TGEV в клітинній лінії LLC-PK1. Для дослідження впливу нативних сумарних препаратів IgM на інтерналізацію вірусу в клітину застосовано та апробовано оригінальну модель вірус (TGEV) — клітинна лінія (LLC-PK1). Отримані дані можуть бути корисними в подальших дослідженнях для кращого розуміння процесів розвитку імунної системи новонароджених.

Ключові слова: *імуноглобуліни М, вірус трансмісивного гастроентериту, пуповинна сироватка крові, венозна сироватка крові дорослих, рецептор епідермального фактора росту.*