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PERSPECTIVES OF BIOTECHNOLOGICAL DEVELOPMENT IN KAZAKHSTAN IN TERMS OF MONOCLONAL AND OTHER RECOMBINANT ANTIBODIES AND VACCINES AGAINST SARS-COV2

Kazakhstan became one of the few cohorts of countries that were able to produce its one vaccine against the COVID-19 virus. This fact showed the way of new development paths in the biotechnological direction, especially, since the current situation in the world. Kazakhstan's location allows its biotechnological development to attract not only academic but also investment interests to make everything possible for producing not only vaccines against the SARS-COV2 virus but also to make biotechnological oriented drug development and antiviral drug production. Also, this article gives the generalized view on current clinical success in combating COVID-19 using novice approaches in biotechnological advancement like humanized IgG 'Xenomice' technology in hybridoma technology – REG N10987, produced from transgenic mice and SARS-CoV-2-infected patients [1-3] as well as in human recombinant IgG derived from monoclonal B-cells via Phage display- CT-P59 scFv phage display library generated from cells of a convalescent SARS patient [4]. Along with 'classical monoclonal IgG LY-CoV555, human Antibody gene cloning of B cells from a COVID-19 patient [5]. All these three 'antivirals' are already used and approved by the FDA (U.S. Food and Drug Administration) clinically and demonstrate trustworthy therapeutic effects. The biggest upper hand of these approaches is that they can be used not only against the COVID-19 virus but also against various receptor-dependent disorders like lupus or some types of cancer and/or malignant tumors. Last three decades, two main approaches or methods became headliners in research and clinical implementation Hybridoma (B-cell-fusion with 'immortal' myeloma cells), and recombination technologies – bound with phage display technologies.

Key words: Vaccines, Virology, monoclonal antibodies, NGS (next generation sequencing) Receptor binding domain, RBD-inhibiting, hybridoma, phage display, recombinant antibody, neutralizing immunoglobulin, transgenic mice, monoclonality, humanization.

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SARS-COV2-ге қарсы моноклоналды және басқа рекомбинантты антиденелер мен вакциналар тұрғысынан Қазақстандағы биотехнологиялық даму перспективалары

Қазақстан COVID-19 вирусына қарсы өзінің жалғыз вакцинасын шығара алған санаулы елдердің біріне Қазақстан COVID-19 вирусына қарсы өз вакцинасын шығара алған санаулы елдердің біріне айналды. Бұл факт, әсіресе әлемдегі қазіргі жағдайды ескере отырып, биотехнологиялық бағыттағы даму жолдарының перспективалық бағыттарын көрсетті. Қазақстанның орналасуы оның биотехнологиялық дамуына тек академиялық қана емес, сонымен қатар инвестициялық мүдделерді тартуға, SARS-CoV2 вирусына қарсы вакциналарды өндіру үшін ғана емес, сонымен қатар биотехнологиялық бағдарланған дәрі-дәрмектерді әзірлеу және вирусқа қарсы препараттарды өндіру үшін қолдан келгеннің бәрін жасауға мүмкіндік береді. Сонымен қатар, бұл мақалада трансгенді тышқандардан және SARS-CoV – 2 жұқтырған пациенттерден алынған Reg n10987 гибридомдық технологиясындағы гуманизацияланған Ig "Ксеномышь" технологиясы сияқты биотехнологиялық жетістіктер саласындағы жаңа тәсілдерді қолдана отырып, COVID-19-мен күресудегі қазіргі клиникалық жетістіктерге жалпыланған көзқарас берілген [1-3], сондай-ақ фаг дисплейі – CT-P59 арқылы моноклоналды в жасушаларынан алынған адамның рекомбинантты IgG-де. SARS бар сауығып келе жатқан науқастың жасушаларынан жасалған scFv фаг дисплей кітапханасы [4]. "Классикалық моноклоналды IgG LY-CoV555"-пен қатар, COVID-19 пациенттің В жасушаларының адам антиденесінің генін клондау [5]. Осы үш "вирусқа

қарсы препараттың" барлығы FDA (АҚШ-тың Азық-түлік және дәрі-дәрмек басқармасы) клиникалық түрде қолданады және мақұлдаған және сенімді емдік әсерлерін көрсетеді. Бұл тәсілдердің ең үлкен артықшылығы-оларды тек COVID-19 вирусына ғана емес, сонымен қатар қызыл жегі немесе кейбір қатерлі ісіктер және/немесе қатерлі ісіктер сияқты рецепторға тәуелді әртүрлі ауруларға қарсы қолдануға болады. Соңғы үш онжылдықта екі негізгі тәсіл немесе әдіс зерттеулер мен клиникалық іске асыруда басты рөл атқарды: гибридома (в жасушаларының "Өлмейтін" миелома жасушаларымен бірігуі) және фаг дисплей технологиясымен байланысты рекомбинация технологиялары.

Түйін сөздер: Вакциналар, Вирусология, моноклоналды антиденелер, NGS (келесі ұрпақ секвенциясы) рецепторларын байланыстыратын домен, РБД-ны тежейтін, гибридома, фагтарды көрсететін, рекомбинантты антиденелер, иммуноглобулинді бейтараптандыратын, трансгенді тышқандар, моноклоналдылық, ізгілендіру

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Перспективы биотехнологического развития в Казахстане в части моноклональных и других рекомбинантных антител и вакцин против SARS-COV2

Казахстан стал одной из немногих стран, которые смогли произвести свою собственную вакцину против вируса COVID-19. Этот факт указал перспективное направления путей развития в биотехнологическом направлении, особенно с учетом современной ситуации в мире. Расположение Казахстана позволяет его биотехнологическому развитию привлечь не только академические, но и инвестиционные интересы, сделать все возможное для производства не только вакцин против вируса SARS-CoV2, но и биотехнологически ориентированной разработки лекарств и производства противовирусных препаратов. Кроме того, в этой статье дается обобщенный взгляд на текущие клинические успехи в борьбе с COVID-19 с использованием новых подходов в области биотехнологических достижений, таких как технология гумманизированного IgG «Ксеномышь» в гибридомной технологии – REG N10987, полученный из трансгенных мышей и пациентов, инфицированных SARS-CoV-2 [1-3], а также в рекомбинантном IgG человека, полученном из моноклональных B-клеток посредством фагового дисплея – CT-P59. Библиотека фагового дисплея scFv, созданная из клеток выздоравливающего пациента с SARS [4]. Наряду с «классическим моноклональным IgG LY-CoV555», клонирование гена человеческого антитела B-клеток пациента с COVID-19 [5]. Все эти три «противовирусных препарата» уже используются и одобрены FDA (Управление по контролю за продуктами и лекарствами США) клинически и демонстрируют заслуживающие доверия терапевтические эффекты. Самым большим преимуществом этих подходов является то, что их можно использовать не только против вируса COVID-19, но и против различных рецептор-зависимых заболеваний, таких как волчанка или некоторые виды рака и/или злокачественные опухоли. За последние три десятилетия два основных подхода или метода стали хедлайнерами в исследованиях и клинической реализации: гибридома (слияние B-клеток с «бессмертными» клетками миеломы) и технологии рекомбинации, связанные с технологиями фагового дисплея.

Ключевые слова: Вакцины, вирусология, моноклональные антитела, NGS (секвенирование нового поколения), рецептор-связывающий домен, RBD-ингибирование, гибридома, фаговый дисплей, рекомбинантное антитело, нейтрализующий иммуноглобулин, трансгенные мыши, моноклональность, гуманизация

SARS-COV-2 represented the great challenge worldwide for health care systems in 2020 and revealed the poor readiness to treat the infected patients in properly effective way to take the pandemic under control both in terms of treating and preventing as well. The immediate response on Covid19 outbreak was to develop the effective and safe vaccines that could tread the health care systems at least to plateau of infection rates and ideally to form so called 'collective immunity' during the first period of pandemic [6]. Kazakhstan's scientist in 2022 sequenced two local SARS-COVID2 genome variants, one is complete and the second one near-complete Genome, namely: Virus Strain, Variant B.1.1, Sampled from Kazakhstan and Variant B.1.1.7 Virus Strain Isolated in Kazakhstan. All these works were performed in Republican State Enterprise on the right of economic management

«Research Institute for Biological Safety Problems» (RIBS) of the Ministry of Health of the Republic of Kazakhstan by the research groups that were involved in designing first Kazakh vaccine – QazVac [7,8]. The best way to develop the fighting strategy against SARS-CCOV-2 viral spreading- is to understand how single stranded RNA positive sense coronavirus enables its entry into host cell [9]. The term 'CORONA' (crown) stands for the clear characterization of COVID-19 virus that consist of single stranded positive sense RNA genome inside the viral membrane with Spike proteins that allow viruses to insert the genome into the host cell cytosol. To make it happen, the spike (S) protein must bind to its receptor, angiotensin-converting enzyme 2 (ACE-2), and mediate subsequent membrane fusion (Walls A., 2020). The reason for the COVID-19 Variants concern is adaptation capacities or mutation rates in COVID-19 genome, especially, the structural proteins regions – S-proteins, and Spike proteins, up to 90% of all mutations or variants forming [6]. To spread viruses, multiply by copying their genome over and over. Through this molecular copying, the original strain becomes 'imperfect' and these imperfect versions of the SARS-COV2 virus are termed as 'variants' Usually, these sings of genome infirmity or mutations don't change the viral behavioral pattern biology. Sometimes, these so-called mutations make current variants weaker than original strains. However, in very rare occasions, mutation can change the virus in some important ways. It could become more infectious or more able to hide from or avoid the immune system. The more a virus is allowed to replicate unchecked or not inhibited, the more chance it has to accumulate these rare beneficial mutations. That opportunity for viral evolvement can occur when viruses are allowed to spread quickly through a population or they encounter a host that is less likely to repel the viral infection [9,10]. If a particular set of mutations makes a variant more successful, it might become more prominent than the other strains or 'imperfect' copies, and that is when it gets noticed some of them could be termed as variants of concern, such as P.1 (Brazil), B.1.351 (South Africa) and P.1.1.7 (UK) – strains/ variants [11]. Mutations are changing the properties of particular virus types through populations and time that give them the upper hand. Some variants are fast spreading and there are some hints that certain mutations could start to weaken or even evade natural and vaccine-driven immunity [6, 9, 12]. The D614G Mutation known as 'DOUG' spread wildly in the early periods of pandemic and can be seen in roughly all variants. It affects the spike protein that enables a virus to penetrate the host cell. A mutation in a genome changes one amino acid for another and makes the new variant more infectious than the original virus. There are also many other variations of mutation in spike protein that seriously improve its original properties [13,14,15,16].

Receptor-binding protein (RBD)

Viral spike protein is glycolyzed, S1-Domain, ACE-2-recognition, Furin, S2-Domain, TMPRSS2, cell and viral membrane fusion.*

The viral infection of SARS-COV-2 begins with RBD and consists of two subunits S1 and S2 respectively. They are non-covalently associated subunits. The S1-subunit binds to ACE-2 and S2-subunit anchors the S2-protein to the membrane. The S2-subunit possesses the fusion peptide and other molecular machinery needed to mediate membrane fusion upon invasion of a new host cell, so the viral genome can enter the cytosol [8,17].

After contact with the spike protein, Furin cuts off accurately the outer part of the spike protein called the S1 domain, releasing the inner core of the spike protein called S2 domain which also gets cut by transmembrane serine protease 2 (**TMPRSS2**) [18]. After these, spike protein unfolds itself and anchors into the host cell membrane. Thus, the viral and host cell membrane starts to fuse, allowing the viral genome to enter the host cell cytosol (a ribosome meets the viral RNA and initiates to translate its genetic code. It results long protein chain containing non-structural proteins (NSPs). NSPs are capable to cut the neighboring chains. First, they release short NSPs which are capable to grab onto a ribosome and occupy it in such a way that the grabbed ribosome can read only the viral RNA and not own host cell messenger RNA (mRNA). From this very beginning phase, we can say that infected host cell starts to be virus building factory thanks to control over the cell translation machinery [18]. Meanwhile, nsp3 cuts other 'neighbors' before it sets itself free. It worth to mention that the production of NSPs stops due to pseudoknot in the viral RNA on purpose to prevent the remaining RNA which codes for proteins involved in viral genome replication from passing through the ribosome, saying it differently to separate nsp-encoding from structural protein synthesis: (S-Spike, N-Nucleocapsid, M-membrane, E- Envelope- proteins) [18]. The further following NSPs that are embedded into the endoplasmic reticulum membrane cause it to curve and that forms the structure called a double membrane vesicle or DMV. The DMV is responsible in a host cell for creating a safe enclosed environment for the viral genome to be cop-

ied. Inside the DMV, the newly created NSPs build up both types of viral RNAs, full length and a set of shorter RNAs strands – subgenomic RNAs(sgRNA) that are designed to create new viral particles. The subgenomic RNA exists in the cytosol through the nsp pores in the DMV these short sgRNAs return to a ribosome and it gets translated on purpose to make four structural (main) proteins that will constitute the new SARS-COV-2 viruses. The nucleocapsid proteins have a very essential role both inside the host cell and outside it because it holds together newly replicated genome RNA and inside the viral 'husk' (envelope and membrane) [19].

Glycolyzed-means that Spike protein or S-protein has glycosylation on its surface that allow it to keep itself discrete for host immune cells and most mutations of concern come to S-protein or glycoprotein known as D416G [18].*

Immunoglobulin (IgG)

Immunoglobulin G (IgG) is one of the inexhaustible proteins in human blood and constitute adjacent 10-20% of plasma proteins. Human IgG could be a part of the five classes of immunoglobulins. Immunoglobulins or antibodies constitute mainly the humoral immune system and neutralize the agents of antigen of interest. In most mammals IgGs get produced and matured in B-cells (lymphocytes) to be specified against antigen [19]. The variable domain with two indistinguishable Fab parts associated to a steady domain serve particular capacities of the IgG [19]. The 'Fab' region enables the binding interactions with antigens, however, the 'Fc' region interacts with accessory molecules to trigger the mediation of indirect effector functions, like antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC). Some animal models implied Fc- mediated effector cell recruitment functions as responsible for tumor-targeting antibodies, also they are involved in infectious diseases by carrying out efficient pathogen clearance [20]. The neutralizing antibodies (NMAbs) that we are discussing in this review have some common aspects and features:

- They are all IgGs
- All human, humanized or gained from the blood of human COVID-19-infected patients of minor or moderate conditions.
- Renominated or/and monoclonal
- Target: Receptor binding protein (RDB)

Nevertheless, all three of them represent different technologies and methods with comparable cost-effective and clinical-related aspects, and which is most effective or expensive depends on either equipment or skilled employees engaged in biomedical activity.

CT-P59-NMAb characterizes itself as a competition- agent with ACE2 in binding with RBD and as it was mentioned earlier the scFv phage display library was implemented to gain this neutralizing antibody from the patients [4].

Neutralizing antibody-REG N10987 is gained from the transgenic mouse that is incapable of producing mice antibodies and produces human IgGs only. This approach represents many advantages like productivity and pure yield; however, it could be improved by next-generation hybridoma technology that would improve not only the quality of antibodies of interest but also the quantity of antibodies. Unlike, the previous antibody, the Xenomouse antibody is clinically confirmed in blocking the binding of ACE2 to the RBD [18]. The monoclonal neutralizing IgG LY-CoV555 is designed to interfere with the binding of RBD to cell receptor ACE2. It is a product of IgG-gene cloning of B cells from a COVID-19 patient [5]. To sum up the introduction part it makes sense to repeat the main points and direction of this review article. First and foremost, all these neutralizing antibodies gained and oriented to clinical use and human well-being to fight effectively (therapeutically) the SARS-COV-2 infection in minor and moderate illness stages. Secondly, three human and monoclonal antibodies were produced by three different approaches and to some extent, in the levels of biotechnological advancement ways with one common result – to get effective, safe, and relatively cost-friendly monoclonal antibodies-based drug items. Last but not least, even though all three antibodies aimed to target the ACE-2- RBD, they are effective in their way: to compete to bind, to block the binding, and to interfere with binding, respectively.

CT-P59-Neutralizing antibody

CT-P59 is a strong binding IgG with a high affinity for RBD with a KD value of 27 pM with a clear chemical feature: CT-P59 causes complete steric hindrance with ACE-2 receptor by blocking substantial areas of the ACE2 interaction regions, and further mitigation of SARS-COV-2 infection both in-vivo and in-vitro, therefore, inhibits SARS-COV-2 virus spreading [4]. The S-protein – D416G – mutations from various SARS-COV-2 isolates worldwide demonstrate the highest interest in vaccine and antiviral drug development. The mutations of this S-protein enhance and improve the cellular entry and subsequent viral transmission and its rep-

lication. Some mutations of viral D416G- Spike proteins make SARS-COV-2 more adaptive and accelerate the infecting capacities of particular variations of SARS-COV-2, distinguished as 'strain' [5]. It was learned that the overwhelming part of the ACE2 blocking CT-P59-like antibodies adopt a similar orientation when bound to RBD. Each of these antibodies belongs to the immunoglobulin heavy-chain variable region genes (IGHV) 3 germline and is the most frequently used IGHV gene among the known SARS-CoV-2- neutralizing antibodies [4,21]. To sum up, the CT-P59 mAb (monoclonal antibody) gained from the PBMC (Peripheral blood mononuclear cells) SARS-COV-2 negative patients and after the fresh mRNA-isolation, it was immediately converted into cDNA via commercial reverse transcriptase set for Phage display library generation and RBD-specified- antigen epitope- CT-P59 IgG variable region (VL and VH). The single chain variable fragments (scFvs) needed to be made by linking VL and VH fragments and directly cloned into a phagemid vector, **pComb3xSS**, for library construction [4,21]. This approach offers us relatively fast but highly specified and effective mAbs- production that neutralizes SARS-COV-2 infection effectively competes with other antibodies and does not lose its special affinity on RBD of ACE-2. This monoclonal antibody is recommended for use in combination with remdesivir and dexamethasone (corticosteroid that suppresses carefully the immune excessive reaction) both to prevent COVID-19 replication and its further spreading.

Phage display technology

The relatively old but robust and reliable technology that allows researchers to adapt the in vitro findings and principles such as tests and screening run into in vivo models as a new drug for instance [22]. The term library in this topic plays a critical role because cDNAs gained from PBMC mRNAs or DNA samples for encoding proteins must run screening match procedures to sort out whether affinity or epitope reaction (antigen) of protein of interest would bring the desired result. One of the most widely spread library constructs is based on the use of filamentous phage, a virus that is found in Escherichia coli [24]. Phage display has clearly demonstrated to be an outstanding technique for the interrogation of libraries containing millions or even billions of different peptides or proteins. One of the foremost effective applications of phage display has been the isolation of monoclonal antibodies using large phage antibody libraries [25-26]. The single chain fragment (scFv) belongs to the basics of phage display methodology. It consists of the VH and VL domains fold correctly (both stabilized by an intramolecular disulfide-bridge) and pairing to form a functional scFv [27,29]. The scFv usually gets produced through the fusion of the coding sequence of the antibody variable (V) regions encoding a single-chain Fv (scFv) to the N-terminus of the phage minor coat protein pIII using a phage vector based on the genome of fdtet [28]. So, through infecting the E. coli-strain with nonlytic filamentous phage, fd, or M13, and its genome encodes on the bacterial the single stranded circular plasmid genome and produce the virion proteins that then released in media.

Monoclonal neutralizing antibody- IgG- REG N10987

REG N10987 is yet another successful example of 'IgG- engineering' using transgenic mouse spleen or PBMC to get human or humanized, monoclonal IgG with high antigen specificity without the need to immunize a human. The generation of mAbs against the SARS-CoV-2 spike protein (altered through mutation of glycoprotein- D416G) by using combinatorial phage-display libraries from PBMC of COVID-19-recovered patients became 'the mainstream' in therapeutic research for finding new ways to take under the control the never-ending mutation of viral RBD [29]. The phage display is now one of the most spread techniques to 'discriminate' or choose the right Fabs or ligands of monoclonal antibodies from millions or even billion combinations of RBD epitope from so-called libraries. In vivo, studies on mammals showed and show the prophylactic as well as therapeutic effects of such modified monoclonal antibodies [29]

Neutralizing IgG LY-CoV555

LY-CoV555 is relatively well known monoclonal human IgG that mostly was used in combination with other neutralizing antibodies like REG N10987 in clinical trials. Since there was a need to neutralize the SARS-COV-2 virus 'for sure', the clinical trials used so called cocktails of many neutralizing agents that strive to bind on viral RBD with strong affinity, demonstrating the higher therapeutic outcome than the solo trials of individual mAbs types. Interfering with the binding of RBD to cell receptor ACE2 is its main purpose as drug and the more thoroughly was run so called either phage display or other cDNA-based libraries the higher the affinity and more diverse the Fab's repertoire for better RBD -epitope recognition [30].

Transgenic gain of IgGs and Hybridoma technology

Xenomice technology ground principles rest on transgenic technologies of transferring human genes in embryos of model animals, such as mice. First and foremost, the insertion of IgG's genes (human light and heavy chain genes) into mouse embryo stem cells needs to be performed carefully on a mouse individual and it is needed to inactivate the mouse IgG genes in another specimen, resulting in the first individual can produce both human along with murine antibodies, whereas another mouse is not able to produce murine antibodies and start to breading this pair till a Xenomouse germline is produced that can generate human antibodies only in first F1-generation. The Xeno-Mouse germline then could be used for lymphocyte producers as PBMC or spleen biological source for further manipulations of various implications [31]. It is important to note that to succeed the blastocyst injection, the further steps must be respected before and after aggregation-platting takes place:

- ES- compatibility (embryonic stem) line for the germline with the method of chimera production
- ES cell lines are recommended to be derived from 129 inbred agouti mouse strains
- The F1 generation fathered by a germline transmitting chimera has to be examined for the presence of the transgene or genome modification, since ES cells are normally heterozygous for such a modified allele, only 50% transmission will be observed within F1-generation
- One of the most optimal choices for an embryo donor can be used usually an outbred strain of albino mice, because their purchase price and maintaining is reasonable, provide satisfying embryo yields, especially, after superovulation, and the chimerism can be easily identified by the coat color and eye pigmentation characteristics [32].

The transgenic mice method or it is termed as 'knockout' mouse is a powerful tool in hands of researchers due to two reasons at least. First, in 90s and early two thousand it revolutionized biotechnology feasibilities to identify of such a rare event as the recombination between a target vector and its homologous sequence within the target genome that theoretically made possible to manipulate the murine genome in that way to modulate in the mice phenocopies human diseases [33]. Secondly, the further decades showed that using mice as spleen and PBMC-donors after intensive and highly selective antigen-specific immunization gave new frontiers in biomedical research, allowing us to produce human proteins like immunoglobins relatively cheap and fast combining it either with PEG (Polyethylene Glycol) -driven or fusion hybridoma technology or with more advanced BCT (B-cells targeting) – fusion associated hybridoma technology.

Hybridoma

To fuse intact cells with each other was one of the greatest breakthroughs last several decades in biological science. The purpose of cell fusion was not clearly understood at the beginning but the fact of hybridization of cells and especially their separate genetic materials – nucleus was far more promising. The second greatest challenge was to keep a fused cell biologically intact and therefore three main hybridoma technologies were invented: the first with vector as virus (HVJ), the second with chemicals as PEG- cell membrane 'opener', third through electrofusion thanks to dielectric nature of cell membrane. The great challenge was to increase the sensitivity as well as the productivity of highly potent hybridized B-cells, to get more intact and immortal B-cells yield and their products – monoclonal antibodies via novel and cutting edge – BCT (B-cells targeting) technology than the previous three approaches could offer [34]. Among technologies and approaches, it is profoundly important to mention what tools need to be used to get each of these four approaches effective and to some extent sophisticated, such as phage display, YAC (yeast artificial chromosome), fast and reliable sequencing, etc. [32-33].

The main idea of hybridoma technology consists of fusing or hybridizing valuable but not infinite antibody-producing B-cells from the spleen of a mammal with almost immortal myeloma cells which the term – hybridoma stands for. That allows to get the profound yield of monoclonal antigen-specific Igs from the limited numbers of cells. The first documented successful practice of artificial gain of monoclonal Igs (immunoglobulins) via fused mRNA synthesizing cells (murine spleen) with myeloma cells was made by Köhler G, Milstein C. in 1975[34] with the help of HVJ or Sendai-virus. This first successful manipulation was run under the HAT (hypoxanthine-aminopterin-thymidine) selective medium and the phenotypic link between the V and C regions [29]. Interestingly, in this paper [35], the somatic fusion of sensitized B lymphocytes with myeloma cells to generate hybridoma cells secreting mAbs was made by Sendai virus giving the reference link to [36], wherein 1965 The hybrid cells from the human and murine cells were successfully derived with Sendai virus or HVJ without knowing that they initiated the first steps of transgenic and

monoclonal (in some extent humanized) antibody synthesis era outside the human body.

Hemagglutinating virus of Japan (HVJ) or inactivated Sendai virus-fusion

The hemagglutinating virus of Japan was the very first conducted to cause cell fusion as the basis of cytotechnologies [34]. Hemagglutinating virus particles – virus envelope containing the HN-glycoprotein, that enables the clear exhibition of blood cell agglutinating activity were used to fuse the cells of interest. The cells that are needed to be fused (via virus agglutinating cells) possess the HVJ receptors on the cell membrane and cause cell fusion efficiently enough to detect the targeted fusion. The protocol [37] shows a relatively simple laboratory setup to ease the cell fusion, however, the viral-receptor fusion does have many practical drawbacks, and the most profound of them is the viral genome interference into fused cells therefore many laboratories prefer to avoid the hybridization in research purposes, and only as training and teaching technique that clearly demonstrate the cell-to-cell fusion possibility in biologically driven and systemized way. As we discussed earlier the successful viral cell entry of SARS-COV-2 is strongly bound to spike glycoprotein recognition too.

Hybridoma: PEG-driven fusion

In 1974 *Vicia hajastana Grossh* – Anatolian peas were fused with Pisum sativum L- the sowing peas were the first plants and biological models that were fused with the help of Polyethylene Glycol (PEG) [35]. The cells' protoplast-fusion was made possible non-specifically with adhesion between the free protoplasts from the same species, different species, and even genera. The fusion was improved by enrichment of the PEG solution with calcium ions [36]. Interestingly, the Ca^{2+} enrichment in aerobic conditions was necessary during the Hemagglutinating virus fusion too [37].

The semi-solid media protocol shows one of the handiest ways to produce hybridomas via PEG-fusion without the help of electrofusion or other techniques. The most advantageous point of this approach is a capability to isolate slow and fast-growing colonies from each other that ensures firmly the high monoclonality, allowing us to escape the sloth subcloning procedures. Thus, the minimum equipment is needed to embrace the satisfied results in yield of MAbs of interest as much as purity and specificity of antibodies producing machinery. This [34]. protocol heavily relies on 'Clone cell – HY hybridoma cloning kit' that consists of five main steps. The step 3 characterizes itself in our case as most important, because the myeloma- and splenocytes- fusion takes place under PET driven conditions. Most commercial kits require to conduct the serum free manipulations otherwise PEG will not be able to fuse to cell membranes and fusion frequency will fall dramatically. PEG is contained in all medium solutions to ensure fusion where step one or step two is bound on cellular preparations that can and recommended be run simultaneously [34,38]. Importantly to not, that PEG-must be added and held only for 15min incubation in a 370C water bath and then PEG must be washed out for further 10-14 days incubation under 370C and 5%CO2 with a petri without lid on the top in the middle of the culture dishes with lids filled with sterile water to mature the cell cultures during the incubation period [34,38]. The discussed protocol provides us with an information about forming the cell colonies that will enable us to provide with productive cell suspension with further four days incubation and assays to be sure what antibodies type is going to be yielded. The fusion step independently what kind of approach is going to be applied, whether it is PEG, HVJ, electrofusion or B-cell targeting, must take into consideration some principles:

1) HAT-media selection stage (hypoxanthine-aminopterin-thymidine): Replication ways- Salvage pathways.
2) During fusion, apart from hybrid cells of interest other cell fusion types could take a chance to form: fused plasma cells (splenocytes), fused myeloma cells as well as unfused cells of both types.
3) Cell colonies of survived hybridoma cells after two weeks of incubation can have different properties and survival rates, especially, if we consider a clinical approach where HVJ – approach is not applicable at all due to the discussed reasons above.

HAT-Media -Selection

HAT media is a milestone of Hybridoma technology, the headliner of succeeding the monoclonality and productivity. During the incubation the (mostly carefully mashed) splenocytes cell count ˜ 1*108cells and myeloma cells not less than the cell count ˜ $2*10^7$cells [32,35,37] must run the fusion procedures under the chosen technique and artificial media [38-39]. During the incubation, the selection appears, one type of cell dies out others survive by forming colonies, fused myeloma, and splenocytes – hybridomas. In HAT- media the so-called de novo pathway of new nucleotides from

provided sugars and amino acids for replication machinery in daughter cell proliferation cannot be performed due to the presence of aminopterin, which inhibits the dihydrofolate reductase [34,39].

Main principles of cellular features during two weeks of incubation:

- Salvage pathway is only available for daughter cell proliferation in HAT-media
- Salvage pathway 'recycles' the degraded nucleotides thanks to HGPRT (hypoxanthine-guanine phosphoribosyltransferase) enzyme that relies on hypoxanthine and thymidine as a precursor.
- Plasma cells or splenocytes do possess HGPRT-enzyme-driven replication but myeloma cells do not.
- Due to the short life span of splenocytes, they will not survive after a 14days incubation period and myeloma cells are not capable of growing in HAT-media because of the absence of HGPRT enzyme and blocked de novo pathway due to aminopterin.
- Hybrid–immortal monoclonal antibodies synthesizing cells (colonies) are present and the cancerous cells are excluded due to the absence of required grow-friendly conditions

Hybridoma: Electrofusion

The electrofusion in 'classical' hybridoma technology is the most technically sophisticated and requires not only skilled operating staff but also thoroughly well-tuned equipment during the procedures as well as after, cleaning and rinsing the apparatus [40]. Unlike PEG-mediated cell fusion, E-fusion (Electrofusion) provides faster results, and no 10-14 days incubation period is needed. PEG-driven fusion is still in practice, however, even the handful of commercial kits requires 18-21 days till the whole cycle is over [34, 41]. The second point that seriously repels the researchers from using PEG is that the side-product of PEG-cell membrane interaction is the generation of extremely cytotoxic H202-build, one of the oxygen reactive species (ORS) both inside the cell and outside that is hard to ignore on experimental results and some fluctuations from one run to another one appears [41]. The E-Fusion, however, does not have such a burden on experiments. First and foremost, E-fusion conducts the electricity (DC (direct current) as well as AC (alternating current)) to align and fuse cells. As it was mentioned before the cell membranes in general both in plants and animals are dielectrics, thanks to these properties, versatile capacities could be used the AC brings cells into contact, whilst the DC-pulses enable the cells to be fused, moreover, the alternating current preserves compression during the running experiments [39,42,40,41].

In light of this advantageous versatility, the E-fusion technique can be included in:

- Nuclear transfer
- Embryo manipulation
- Hybridoma production
- Plant protoplast fusion

Interestingly, the first successful PEG-driven cell fusion was -performed on plant protoplasts in 1974 [36], the electrofusion finds even here its upper hand over PEG, not solely in hybridoma production respectively. Yet, the most obvious advantages of E-fusion in comparison to PEG is the efficiency of hybridoma production, up to 10 folds, presenting, lower time of production, instead of waiting 10-14 days in selection media alone till only hybrid cells survive (not subcloning included), the e-fusion provides almost 100% fusion rates [41]. Several hours instead several weeks of experiments conducted, in hybridoma production, show us not only the fast solutions but also reliable and clear outcomes that could be trusted and reproducible.

Human hybridomas

It was only a matter of time before human tissue became the next object of so-called 'immortalization' through hybridoma fusion. So, in 1980 the very first attempt took place in the USA [42]. The myeloma cell line was fused with removed spleen cells from the patients suffering from untreated Hodgkin's disease to get highly antigen-specific monoclonal antibodies. According to [43], the cell fusion was run under PET and left in HAT media for some time. HAT-resistant hybrids grew out within 8-14 days, but incubation in HAT medium was continued for at least 3-4 weeks respectively. The main result of this publication was to confirm that human-human hybridomas are possible to generate, namely: 'The U-266 human myeloma cell line is incubated in the presence of 8-azaguanine, and a rapidly growing, 8-azaguanine-resistant, hypoxanthine-amethopterin- thymidine (HAT) medium-sensitive mutant line, U-266ARI' (Yew C., 2016: 225-33, pp. 5429). Already in the 80s, the transgenic interactions: from murine myeloma cell lines to human lymphocytes were bound with one crucial problem – the human cells' chromosome damage that led to the instability of cell cultures [43-44]. Any hybridoma manipulations are generally orientated to gain satisfactory yield rates of human or humanized IgG, including human–human hybridomas. Nevertheless, mono-species cell fusion shows better monoclonal antibody production, however antigen

sensitization on humans and human tissue could be ethically troublesome to conduct worldwide [43].

Among already discussed hybridoma IgG-production techniques, either animal (mostly mice) spleen cells or splenocytes or human tissue were required to isolate the needed cell suspension that leads to serious health conditions or to death by removing mice spleen. Driven by ethical and progressive urges, new techniques are already available. Instead of using splenocytes, nowadays thanks to the development of new biotechnological methods, the blood samples or the PBMC (plasma) is enough to isolate mRNA for compilatory DNA (cDNA) generation which in turn could be used for further experiments. The new era of monoclonal antibody production without animal tissue and animal sacrifices. The first patent on humanized antibodies was claimed in the USA, in 1996 [34]. The clinical application of murine or other non-human monoclonal antibodies represents various difficulties and the most obvious of them is T-cell activation, which seriously downgrades the efficiency of the therapeutic potential of many monoclonal antibodies-based drugs like OKT3 -for prevention of rejection symptoms, anti-CD3 antibody, OKT3 is derived from murine monoclonal antibodies that have the specific epitope recognition immune suppressive properties [37]. Already in 80s before the antibody's humanization took place, the murine and other mammalian monoclonal antibodies properties were discovered [38]. Thus, the intermediate solution was found – chimeric antibodies. The variable regions of murine antibodies genes were combined with human constant antibodies regions genes, resulting the better immunological neutrality as well as body tolerance like six times longer circulation time, and demonstrated significantly less immunogenic reactions than the murine protein parts [43,45-46].

There have been two main directions in biotechnological ways to produce monoclonal antibodies since the middle 1970s. The phage display technology strongly relies on the transcription and translation machinery of e-coli bacteria [47-48,]. The hybridoma technology is mostly bound by spleen cell isolation from the immunized mammals and thanks to new advancements in transgenic methods, human monoclonal antibodies could be provided by mice, rabbits, etc. [48-49].

Hybridoma: BCT-driven fusion

Since BCT (B-cell targeting) also known as PEF (pulsed electric field – method) or even SST (stereo specific targeting) technology nowadays is most epitope-specific and antigen-sensitive technology without hampering low molecular molecules like sugars, etc. involved in cell fusion step; there are few open-access articles on this topic and only fractured information is available to make some firm statements about its effectiveness and productivity, only principles and general experiments results, claiming the better results as ever was registered before in monoclonal antibodies synthesis.

The ever first publication on first BCT [50] was made already in 2006-2007. The most advanced and efficient technological edge of hybridoma. This approach includes three main steps to follow: 1) 'antigen-based preselection of B lymphocytes' 2) 'formation of antigen-selected B lymphocyte and myeloma cell complexes' 3) 'selective fusion of B-cell–myeloma cell complexes with electrical pulses' [50]. In nut shell, this technique requires both in vivo immunizations, as well as in vitro. A young mouse must run at least 3 immunizations (human insulin in case of [50] intervals within 15 days with adjuvants or immune boosters to get highly dense concentration of B-cells [50]. In vitro, immunization exposed on extracted splenocytes according to (Davis C., n.d.). As mentioned, step I and step II as well the preselection stages of B-lymphocytes and Myeloma cell complexes proceedings. During the B-cell targeting the insulin-avidin conjugates (antigen-avidin) were added to the spleen cell suspension. The Biotinylation of myeloma cells is very important to build the cell complexes since biotin has strong and specific interactions with antigen-bound avidin (Ag-Av), the B-cells and myeloma cell complex with antigen specificity (Ag-Av) forms with NHS-biotin proceeded myeloma cells (M-bio) the B-Ag-Av – bio-M -complex that is ready to be fused via PEF-method to get fused cells (BM) [50-51].In modern science, there is no so-called 'one–sided' approach or versatile formula for only one technique and this stereo-specificity or single-cell attachment of the antigen-selected B lymphocytes to myeloma cells was achieved thanks to the B-cell targeting in a combination of PEF-method. Interestingly, the method is so effective that even 5%PEG-mediated fusion showed higher rates of fused cell and cell survival and lower cell toxicity [52].

Artificial chromosomes

Apart from hybridoma, phage display (display library techniques), and humanization biotechnologies (transgenic mice), artificial chromosomes like YAC (yeast artificial chromosome) or BAC (bacterial artificial chromosome) became a

significant alternative for other widespread genome editing techniques (recombinant clones generating).

YAC (yeast artificial chromosome)

According to the basics of biology, the eucaryotic genes (mammalian) are generally unstable in bacterial cloning vectors like plasmids, conventional bacteriophages, or even cosmids. These three procaryotic systems often underrepresent the eucaryotic gene sequences or delete some critical regions due to relatively limited cloning capacities, starting from 10kb (plasmids) up to 50kb (cosmids) and of course bacteriophage 100-150kb [52]. Yeast chromosomes, however, can incorporate thousands and sometimes millions of base pairs, allowing the researchers to work on full-scale profound genomes and essential coding regions of genes of interest in mammals [52]. Already in 1994 [52], YAC demonstrated advantageous properties in cloning large regions of DNA. YAC was a great milestone in cloning technologies and examining the big DNA -regions up to 2000kb for genes and active DNA sites [52, 53].

The components of YAC

- Two copies of a yeast telomeric sequence
- The yeast centromere
- The yeast ARS (autonomously replication system)
- Origin of replication (bacterial Ori)
- Multiple cloning site (MCS)
- Selectable markers (for bacteria as well as for yeast) (Ramsay M., 1994: 181-201).

The YAC technology consists of many steps and represents some complicated principles of preparation as well as the running procedures. In the first stage, the YAC vector is propagated as a circular plasmid inside the bacterium, utilizing the bacterial Ori sequence [53-54]. Secondly, the YAC vector must be isolated from the bacterial host for downstream processes that are needed for further DNA purification [55,56]. Thanks to the YAC construct, the researchers can make big YAC libraries in genome studies but in our case replicate the GOI (gene of interest) of organisms that have big expressing sequences that could be used in transgenic manipulations of humans, mice, and even plants. [55-57]. As with any biotechnological approach, it has also some drawbacks as only one vector occurs in one yeast cell despite the fact the yeast cell division (mitosis) runs relatively fast but not fast enough as plasmid replication in bacteria because hundreds of plasmids (vectors) can be replicated continuously per one bacterium. In addition, bacterial vectors like plasmids and cosmids are more stable than the YAC [54].

So, we have already discussed that all vectors can be used to modify many techniques, starting from Sendai virus (Hybridoma), plasmids and cosmids (BAC – bacterial artificial chromosomes), and YACs. Indeed, in a nutshell, nowadays researchers have two main directions. The first is recombination and phage display priorities that allow studies to focus on so-called 'featuring' the functions that are of interest like novel nanobodies or transgenic organisms with the help of which a particular gen product can be generated like in transgenic mice producing human IgGs [56]. The second pathway's foundations heavily rely on hybridoma technologies and their improvement.

Active immunity

Vaccine types: QazVac, Sputnik (inactivated)

In Kazakhstan, the most popular vaccines were QazVac and Sputnik, especially, at the beginning of the pandemic. Both belong to the group of so-called inactivated vaccines with a shoot procedure pattern. Both had and have relatively high concentrations of antigen to ensure active immunity within several weeks. According to WHO (World Health Organization), by 29th April 2023, 38 355 605 vaccine doses had been administered and 12 443 364 individuals got vaccinated with at least one dose, among them, QazVac and Sputnik took the major numbers.

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Conclusion

All three clinically approved drugs are based on monoclonal (humanized) antibodies or IgGs show a therapeutic effect on SARS-COV2 infection their neutralization gives us a powerful medicine based on adaptive immunity product that saves time, subsequently lives to engage yet another very effective and safe antiviral drug. A long way had to be passed till the true potential of monoclonal antibodies could be revealed and a new era in biotechnology was initiated. The recombinant and/or conformation-specific monoclonal IgGs that were selected in phage display and in their various phage display libraries make monoclonal antibodies an extremely precise tool in scientific studies as well as in drug development [59]. The memory B-cells taken from COVID-19 patients are the main foundation of recombinant monoclonal antibody production through the phage display selection [60-62]. The hybridoma technologies however require the splenocytes from mouse or other mammalian spleen to be proceeded and animals often die from this type of surgery. The IgGs synthesizing B-cells (memory B-cells, splenocytes) are the main target of both technologies that provide us with highly effective and sensitive monoclonal neutralizing humanized antibodies. Hybridoma technology has been showing price and time efficiency, especially, the latest methods like electrofusion assay, and seems to be more biologically friendly and reliable towards antibody production rates and quality. However, the phage display shows more progressive development directions sparing animal tissue from extraction, still in terms of cost and time effort, it is still seriously inferior to hybridoma-orientated research and practice.

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