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REVIEWS
AND THEORETICAL ARTICLES

Genome-Wide Mutagenesis in Mice: In Search for Genes Regulating Immune Responses and Inflammation

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Abstract—Genome-wide mutagenesis induced by *N*-ethyl-*N*-nitrosourea (ENU) results in efficient introduction of mutations into the genome of mammalian spermatogonial cells. This feature is being used to generate animals with phenotypes associated with defects in various functional systems. In particular, using this methodology, it was possible to identify molecular mechanisms of immune responses, identify genes that regulate the development of various organs, etc. This minireview covers genetic studies of immunological and inflammatory reactions in mice using ENU mutagenesis, which led to important findings concerning the regulation of critical signaling pathways of innate and adaptive immunity.

Keywords: *N*-ethyl-*N*-nitrosourea, genome-wide mutagenesis, forward genetics, signaling, immunogenetics

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INTRODUCTION

The methodology of random genome-wide mutagenesis for studying the relationship between phenotype and genotype was initially tested on fruit flies and nematode worms, which, in particular, made it possible to generate the first map of the *Drosophila* genome [1]. Many early studies used various types of ionizing radiation as a mutagen. However, the mammalian body has effective mechanisms capable of protecting spermatogonia from the effects of various mutagens [2, 3]. For this reason, it was necessary to find a sufficiently effective mutagen to study the role of individual genes in the development of certain phenotypic traits.

In the 1970s, Russell discovered that chemical mutagenesis using *N*-ethyl-*N*-nitrosourea (ENU) caused mutations in spermatogonial cells of mice with a frequency of ~150×10^{-5} per locus, which exceeded the effectiveness of mutagens, such as X-rays and chlorambucil [4–6]. Note, that spontaneous mutations in mice associated with errors due to of replication and repair systems occur with a fairly low frequency (~5×10^{-6} per locus).

ENU is an alkylating agent that transfers its ethyl group to oxygen or nitrogen radicals in the DNA molecule, namely, to N-1, N-3, and N-7 adenine atoms, O-2 and N-3 cytosine atoms, N-3, O-6, and N-7 atoms of guanidine, and O-2, N-3, and O-4 atoms of thymine, as well as phosphate groups of the DNA backbone [7]. The highest mutation rates are observed in premeiotic spermatogonial stem cells. Studies have shown that ENU predominantly causes single nucleotide substitutions by modifying the A/T base pairs: in 44% of cases, these are A/T → T/A substitutions; in 38%, A/T → G/C; in 8%, G/C → A/T; in 5%, A/T → C/G; in 3%, G/C → C/G; and in 2%, G/C → T/A. It is estimated that 64% of mutagenesis events lead to missense mutations in the protein product, 26% lead to mRNA splicing errors, and 10% lead to nonsense mutations [8]. Thus, ENU mutagenesis has become one of the effective methods of "direct genetics" aimed at identifying genes responsible for certain phenotypes [9].

This minireview is devoted to genetic studies of the regulation of immunological and inflammatory responses using genome-wide ENU mutagenesis in

mice, which have been implemented in three large international projects.

REGULATION OF THE WAYS OF TRANSMISSION OF SIGNALS OF INNATE IMMUNITY AND INFLAMMATION

In the laboratory of B. Beutler, where a mutation in the toll-like receptor 4 (TLR4) gene was previously identified as the cause of lipopolysaccharide (LPS) tolerance in mice [10], ENU mutagenesis was used to study the regulation of innate immune response and inflammation. For four years, using defective TNF production by blood cells as a screening parameter in vitro, 56 mutant lines with defects in innate immune reactions were identified. Mutations in 17 lines were mapped to a single chromosomal locus on the chromosome, and for 12 of them, the mutation responsible for the phenotype was identified [11]. In some cases, the identified genes and mutations confirmed the results obtained by other authors (in particular, by methods of "reverse genetics").

So, when studying mutant mouse strain *Lps2*, which was characterized by insensitivity to stimulation of both LPS and dsRNA (while maintaining the response to other TLRs ligands), a MyD88-independent signaling pathway was discovered [12]. Mutation *Lps2* is the result of a deletion of one base pair in the distal region encoding the TLR activator of the IFN gene (*trif*). TRIF is required for both TLR3 and TLR4 signaling and is an important adapter molecule involved in the synthesis of IFN-β downstream of TLR4. Microarray studies showed that about half of all LPS-induced proteins are at least partially dependent on TRIF. In addition, it turned out that TRIF is the only adapter molecule for TLR3, mediating the activation of NF-κB and MAP kinases [13, 14].

In the line of mice with the phenotype *Triple D* (*3d*), TNF production was disrupted in response to stimulation of TLR3 with poly (I:C), TLR7 with resiquimod, and TLR9 with unmethylated DNA oligonucleotides containing a CpG motif, but a normal response to stimulation with LPS, lipoteichoic acid, and di- and tri-acylated bacterial lipopeptides [15]. In addition, homozygous mouse line *3d* also had a defect in the presentation of foreign antigens (there was no cross-presentation and no presentation through MHC class II) and were distinguished by increased sensitivity to infection with mouse cytomegalovirus (MCMV), which activates innate immunity through the axes TLR3 → TRIF and TLR9 → MyD88. The mutation causing this phenotype was mapped to exon 9 of the gene *UNC93b1* encoding the UNC-93B protein and caused changes in position 412 (H412R), which led to conformational changes in this protein [16]. Later it was shown that wild type UNC-93B, unlike H412R, physically interacts with TLR3, 5, 7, 9, and 13, is localized in the endoplasmic reticulum [17, 18], and controls the transport of TLR7, 9, 11, 12 and 13 from the endoplasmic reticulum [19, 20].

A more severe course of MCMV was observed in the strain of mice with the mutation *CpG1*. When sequencing the coding region *tlr9* at position 1496, a T → C substitution was found, corresponding to the amino acid substitution L499P. This led to the loss of the response of the mutant TLR9 receptor to stimulation with CpG-ODN [21]; i.e., the mutation caused impaired activation of the MyD88-dependent signaling pathway.

Several more mutant mice were selected with a phenotype affecting MyD88 signaling. Thus, in mice *Pococurante* (*Poc*), there was no MyD88 signaling, except for signals emanating from the TLR2/TLR6 heterodimer. Specifically, the signal was not induced by zymosan and lipoteichoic acid, but there was a normal response to MALP-2 or PAM2CSK4, which can signal both through TLR2/TLR6 and TLR2. In another mutant line *Insouciant* (*Int*), a point mutation in the TLR6 ectodomain (V327A) canceled signaling from zymosan, lipoteichoic acid, and MALP-2, but not from PAM2CSK4. In mice *Lackadaisical* (*Lkd*), in contrast, normal signaling through MyD88 was observed, with the exception of signals from TLR7 and TLR9, which were markedly reduced. It turned out that *Poc* and *Lkd* are missense mutations in the gene of adapter protein MyD88. Mutation *Poc* (I179N) changed the amino acid on the surface of the TIR domain of MyD88, while the mutation *Lkd* (Y116C) affected the structure of the oligopeptide chain between the death domain and the TIR domain. Presumably, some TLR2/TLR6 and TLR2 ligands (MALP-2 and PAM2CSK4) induce conformational changes in the receptor, which somehow promote the association of MyD88 with the receptor complex. Although most TLRs associate with MyD88 such that both the BB loop and the Poc site are included in the signaling interface, diacylated lipopeptides (MALP-2 and PAM2CSK4) can trigger another form of activation. Thus, analysis of these mutant lines showed that MyD88 has two different modes of intracellular signal transmission [22, 23].

In addition, several other mutant mouse strains showed reduced responses to various bacterial inducers.

So, the *Oblivious* (*Obl*) phenotype was characterized by decreased TNF production in response to lipoteichoic acid of gram-positive bacteria and to MALP2, which are specific ligands of the heterodimeric TLR2/TLR6 complex. *Obl* homozygotes showed increased susceptibility to infections of *Staphylococcus aureus* in vivo. The *Obl* phenotype was mapped to a single locus on chromosome 5, where five candidate genes had previously been annotated. All candidates were sequenced at the cDNA level, which made it possible to identify a nonsense mutation in the distal part of the coding region of the gene *cd36* [11], although

the CD36 function was not previously associated with TLRs and immunity [24].

Macrophages of homozygous mice of *Heedless* (*Hdl*) line produced TNF in response to lipid A activation and LPS without O antigen, but did not respond to LPS consisting of all three regions (lipid A, central oligosaccharide, and O antigen). Moreover, the mutation *Hdl* blocked TRAM-TRIF-dependent signaling in response to LPS and inhibited the activation of the TLR2/TLR6 heterodimer by specific ligands. This mutation was mapped to chromosome 18 within the gene *cd14* and was associated with the deletion of 83 amino acids from the C-terminus that formed the second leucine-rich LRR domain of CD14. Thereby, it was revealed that CD14 is required for LPS-induced recruitment of TRIF and TRAM and subsequent activation of IRF-3; in addition, CD14 is also required to activate the TLR2/TLR6 complex [22, 25].

Phenotype *PanR1* was characterized by the absence of biological effects of TNF in response to any activation of TLR receptors. This mutation was mapped at the very *tnf* locus and was a missense allele (P138T), as a result of which the mutant TNF trimer could not efficiently bind to the p55 TNF receptor. It is interesting that mice *PanR1*, in contrast to mice with total TNF deficiency, had normal development of lymphoid organs, including Peyer's patches, and normal development of B cells in the marginal zone of the spleen. Presumably, a low level of TNF activity persisting in homozygous mutants *PanR1*, but not in knockout mice, was sufficient for the development of the lymphoid system [22, 26].

Finally, in mouse of *Feckless* (*Fls*) line, NF-κB was not activated in response to dsRNA, but IFN-β production was initiated. It was shown that *Fls* could interact with TRAM in an independent way, which led to activation of transcription factor IRF-7 [22]. Sequencing revealed a missense mutation T → C at position 82712061 on chromosome 10. The mutation resulted in the replacement of tryptophan with arginine at amino acid 296 of the HCFC2 protein. This protein forms complexes with the transcription factors IRF2 and IRF1, facilitating their binding to the IRF element of the gene *tlr3*, after which HCFC2 releases these IRFs [27].

STUDY ON REGULATION OF ADAPTIVE IMMUNE RESPONSES

In the laboratory of C. Goodnow, genome-wide ENU mutagenesis of C57Bl/6 mice was carried out, and in the third generation, 20 mutant lines of mice with various immunological abnormalities were obtained, including mice with the complete absence of T cells or some of their subpopulations, deficiency of T and/or B cells, T-cell hyperactivity, and hypergammaglobulinemia [28]. Moreover, 11 lines of mutant mice had defects in the development and differentiation of T cells [29].

So, the *Plastic* (*Plstc*) phenotype was characterized by the development in F_1 animals of leukemia/lymphoma at the age of four months, which indicated the dominant nature of the mutation. The mutation was mapped to chromosome 11 in the gene of transcription factor Ikaros, where a point replacement A → G was discovered at position 572, which led to the substitution of arginine for histidine at codon 191. This replacement disrupted the quaternary structure of the N-terminus of zinc fingers 3 of Ikaros, which are responsible for binding to DNA [30]. Ikaros was previously shown to be involved in the maturation of T and B lymphocytes and NK cells [31]. ENU-induced allele $ikaros^{plastic}$ led to defects in the formation of other types of blood cells, including erythrocytes, granulocytes, and macrophages. Biochemical studies established that the allele $ikaros^{plastic}$ eliminates the ability of Ikaros to bind to DNA, but does not affect the ability to form dimers with other proteins. Interestingly, in this case, change in only one base pair resulted in a more severe phenotype than deletion of the entire gene [30].

In addition, the mutant line *Twimp* (*Twp*) was characterized, where the hypomorphic allele of the T cell signaling adapter Slp76 retained its functions in vascular morphogenesis, but not in the development of T lymphocytes. For example, the role of Slp76 in T cell differentiation was partially limited and there was no inhibitory effect on T cells, as a result of which paradoxical autoimmunity and allergies developed in animals [29]. The mutation was a T → G substitution in intron 12 of the gene *lcp2*. Analysis of cDNA of *lcp2* from splenocytes *Twimp* revealed the presence of both a wild-type transcript and an aberrant transcript without exon 12. In such mice, the selection of $CD4^+Foxp3^+$ T cells in the thymus was sharply reduced; however, their number in the periphery of the immune system was normal. A sharp increase in proliferation from a limited pool of thymic lymphocytes could have led to depletion of the regulatory function and a decrease in CD25 expression on peripheral $Foxp3^+$ $Lcp2^{twp/twp}$ cells [32].

In several strains of mice, various abnormalities in subpopulations of T and/or B cells were identified. So, the *Unmodulated* line of mutant mice was characterized by small changes in the levels of circulating subpopulations of T and B cells, but a significant decrease in their activation under the influence of certain stimuli and subsequent production of antibodies. This was due to a single T → A substitution resulting in the L298Q amino acid substitution in the CC domain of CARMA1/CARD11, a member of the MAGUK family. CARMA1 serves as a scaffold for the assembly of a signaling complex of other proteins across several domains for protein-protein interactions: CARD, CC, PDZ, SH3, and GUK. Since CC domains are involved in folding, dimerization, or multimerization

of proteins through the formation of bundles of intertwined α-helices, the replacement could prevent the assembly of high-molecular-weight signaling scaffolds of higher order or could interfere with binding to another protein. The biochemical consequences of the mutation were the complete loss of phosphorylation of the critical adapter, Bcl10, which is linked to the NF-κB signaling cascade, and a decrease in the activation of the NF-κB and JNK signaling pathways upon receptor stimulation [29]. Thus, the *Unmodulated* allele revealed an additional role of the CARMA1 protein in the inhibition of signaling activation cascades.

In addition, in mouse of *Xander* (*Xdr*) line, a mutation was found in the gene encoding the transcription factor NF-κB2, which was located in the intron and caused aberrant RNA splicing, resulting in the formation of a nonfunctional protein NF-κB2. The analysis showed that this mutation causes a deficiency of B cells, which manifests itself after the T1 maturation stage in the spleen, leading to a significant reduction in the follicular population and the population of the marginal zone in the spleen, lymph nodes, and peripheral blood. Thus, it was found that the accumulation of these B-cell populations is the primary function of NF-κB2 [33].

Analysis of another mouse strain *Sanroque* defined by ENU mutagenesis [29] helped identify a new gene *roquin*, encoding the RING-type E3 ubiquitin ligase, which plays an important role in preventing the development of autoimmunity. This enzyme is unusual in that it contains an RNA-binding type of zinc finger and is localized in cytoplasmic granules that control the stability and translation of mRNA. This gene is highly conserved, starting with invertebrates, but previously nothing was known about its function. Mutation in gene *roquin* uncovered a new mechanism for inhibiting the development of T cells by repression of the ICOS receptor, which controls their differentiation into a subpopulation of follicular T-helper cells [29, 34].

RESEARCH ON PHENOTYPES ASSOCIATED WITH SPONTANEOUS INFLAMMATION

Goodnow Lab and Hrabé de Angelis Lab participated in the immunological part of a large-scale ENU screening, the main purpose of which was to study the developmental defects of the immune system and defects in immune effector functions [35]. In this project, several new mutant mouse strains were identified that developed spontaneous inflammation.

So, the mouse line *Ali5* was characterized by dominant spontaneous swelling and inflammation of the paws; in addition, 50% of the mice had an abnormally high ratio of T cells to mature B cells. The mutation was mapped to chromosome 8 in exon 27 of gene *plcg2* and led to a single amino acid substitution of glycine for aspartic acid with at position 993 (D993G) of the protein phospholipase C gamma 2 (Plcg2). It was found that, in response to the activation of the B-cell receptor (BCR) in stimulated cells, the production of inositol-1,4,5-trisphosphate (IP3) and calcium increased [36]. It was later found that replacing the negatively charged residue D with G after activation facilitates the interaction of PLCγ with the membrane via stimulation of EGF or binding to Rac [37].

The *Ali14* line had similar characteristics to the line *Ali5* described above—spontaneous swelling of the paws and redness of the ears. This mutation was semidominant and was also mapped to the same gene *plcg2* on chromosome 8, but in exon 16, and represented an AT → GC substitution in the spPH domain. This led to the substitution of cysteine for tyrosine at position 495 [38] in the spPH domain, which directly or indirectly contributed to the formation of the PLCβ2 inhibitory loop. Thus, mutations of the type *Ali14* can mimic conformational changes caused by interaction with regulatory molecules, thereby bypassing the need for phosphorylation of tyrosine residues required for activation [37].

Mice of phenotype *Ali18* at the age of seven weeks were characterized by redness and swelling of the toes of the hind paws [39]. The missense mutation (A → G) corresponding to this phenotype was mapped to position 1506 of the gene encoding the Fgr protein, a member of kinases of the Src family. This mutation caused the D502G amino acid substitution at the terminal end of the Fgr catalytic domain. Thus, it turned out that Fgr tyrosine kinase is an intracellular signaling molecule involved in the pathogenesis of autoinflammatory bone diseases. Dysregulation of Fgr protein conformational changes leads to osteomyelitis in both mice and humans [40].

Finally, the mouse line *Lupo* (*Lp*) at the age of six weeks spontaneously developed chronic inflammation of the paws and skin. This mutation in the coding region of the locus *mayp/pstpip2* on chromosome 18 led to the I282N amino acid substitution of the MAYP protein [41]. MAYP is macrophage-specific and regulates macrophage morphology and motility in response to signaling via CSF-1R. MAYP controls the organization of actin into membrane structures, including regulating the formation of filopodia, and also interacts with proteins that regulate actin polymerization [42]. Low MAYP levels are associated with increased production of proinflammatory cytokines by macrophages, leading to tissue necrosis and bone destruction [41].

CONCLUSIONS

From 1997, a number of large-scale projects of genome-wide ENU mutagenesis were implemented on the basis of several laboratories in different countries, which differed in screening criteria. Within the framework of these projects, a lot of new information

Table 1. Lines of mice with immunological abnormalities obtained using ENU mutagenesis

Line name	Phenotype	Chromosome	Gene	Mutation	References
Lps2	Insensitivity to LPS and dsRNA stimulation	17	*trif*	Deletion of one base pair in the distal region	[12–14]
Triple D (*3d*)	Disruption of TNF production	19	*unc93b1*	H412R	[15–20]
CpG1	Severe MCMV	9	*tlr9*	L499P	[21]
Pococurante (*Poc*)	Activation of TLRs by MALP-2 and PAM2CSK4, but not zymosan and LTA	9	*myd88*	I179N	[22, 23]
Insouciant (*Int*)	Activation of TLRs by PAM2CSK4, but not zymosan, LTA, and MALP-2	5	*tlr6*	V327A	[22]
Lackadaisical (*Lkd*)	Reduction of signals from TLR7 and TLR9	9	*myd88*	Y116C	[22]
Oblivious (*Obl*)	Increased susceptibility to *S. aureus*	5	*cd36*	Nonsense mutation in the distal region	[11]
Heedless (*Hdl*)	Disrupted TNF production	18	*cd14*	Deletion of 83 amino acids at the C-terminus	[22, 25]
PanR1	Reduced effects of TNF	17	*tnf*	P138T	[22, 26]
Feckless (*Fls*)	Decreased sensitivity to dsRNA	10	*hcfc2*	W296R	[22, 27]
Plastic (*Plstc*)	Development of leukemia/ lymphomas	11	*ikaros*	H191R	[30]
Twimp (*Twp*)	Paradoxical autoimmunity and allergies	11	*lcp2*	T → G in intron 12	[29, 32]
Unmodulated	Changes in circulating subpopulations of T and B cells	5	*carma1/card11*	L298Q	[29]
Xander (*Xdr*)	Deficiency of B cells	19	*nfκb2*	–	[33]
Sanroque	Spontaneous autoimmunity	1	*roquin*	–	[29, 34]
Ali5	Spontaneous swelling and inflammation of the paws	8	*plcg2*	D993G	[36, 37]
Ali14	Spontaneous swelling of the paws and redness of the ears	8	*plcg2*	Y495C	[38, 37]
Ali18	Development of redness and swelling of the toes of the hind feet	4	*fgr*	D502G	[39]
Lupo (*Lp*)	Chronic inflammation of the paws and skin	18	*mayp/pstpip2*	I282N	[41, 42]
Paradox (*Prdx*)	Tolerance to LPS/D-gal, but susceptibility to lethal TNF/D-gal test	Undetermined	Undetermined	Undetermined	[46]
Fearless (*Frls*)	Tolerance to both LPS/D-gal and the lethal TNF/D-gal test	Undetermined	Undetermined	Undetermined	[45]

was obtained about the role of specific genes in the regulation of immune responses; however, some phenotypes have remained undeciphered, in particular, owing to the laboriousness of genetic mapping. In the last decade, with the advent of new generation sequencing, the study of mutant lines obtained in the early 2000s was continued. The new methods have significantly simplified the procedure for finding mutations, which, together with the development of CRISPR/Cas9 technology, makes it possible to more effectively determine and verify the role of mutations in the formation of a phenotype [43, 44].

In 2015, at the facilities of Lobachevsky State University, the first Russian ENU mutagenesis project was launched to search for neurobiological and immunological phenotypes. To date, five strains of mice with an immunological phenotype that were insensitive to model septic shock [45, 46] were selected, as well as 13 strains of mice with neurobiological phenotypes (in particular, microcephaly, predisposition to epilepsy and impaired motor functions) [47]. Currently, a search is under way for mutations leading to the indicated phenotypic abnormalities (Table 1).

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare no conflict of interest.

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