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Research article

CHARACTERIZATION OF INTOR:SWISS ALBINO MICE ADOPTED IN THE INSTITUTE OF VIROLOGY, VACCINES AND SERA – TORLAK, BELGRADE IN THE EARLY TWENTIETH CENTURY

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The Institute of Virology, Vaccines and Sera Torlak was established in 1927, while the first vaccine was produced in the Institute in 1930. Vaccines production implies using experimental animals, including mice, in in-process controls. The laboratory mice which have been in use in Torlak Institute from the very beginning belong to Swiss albino outbred stock. This stock, which has been in use for more than 80 years contains a large number of mice maintained at all times, was recently named Intor:Swiss. Biological characteristics of Intor:Swiss stock, are presented in this paper for the first time. Taking into account the presented characteristics, the Institute Torlak`s Swiss mice are suitable for use in pharmaceutical studies, vaccine development research and basic research, as well as in toxicological studies. The publication of data on the Intor:Swiss mice represents a contribution to the international scientific community, since it offers the possibility for obtaining an additional outbred mouse stock for research.

Key words: laboratory animals, outbtred, Swiss mice, IgG2c.

INTRODUCTION

It is generally known that outbred mice are used in toxicology, pharmacology, fundamental biomedical research, and generally in biological research and applied studies. It has been calculated that in the period of 3.5 years (2002-2005), 33% of publications in which mice were used referred to outbred stocks [1]. This percentage did not include the unpublished commercial studies. Having in mind such a large

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presence of mice in research, it is very useful and important to know the characteristics of different outbred stocks in as much detail as possible.

Outbred colonies are genetically undefined; i.e. no two individual outbred mice from a population are the same. They are bred in a manner that allows maximum heterozygosity in the colony. When there is a need for a human population analogue in research, e.g recessive mutations that may affect experimental results, genetic background favours outbred mice. Further advantage of using these stocks is that the mice are less expensive due to their relatively longer lifespan, heightened resistance to diseases, and higher fecundity with respect to the inbred strains.

The most commonly used outbred mice stock is Swiss albino. However, Swiss mice from different sources may differ extensively because the long separation from the original stock. This is also the case with Swiss albino mice that have been bred in the Institute Torlak for more than 80 years. They have been used for toxicology studies during vaccines production and as general-purpose mice in many fields of research. Biochemical, physiological, biological and immunological characteristics of these mice have been well studied over time, which provided a solid basis for the data collected and presented in this paper. The biological properties presented herein provide a better insight into the possibility of using the stock in different Torlak Institute studies, but also in other institutions focused on investigation of diverse biological and biomedical phenomena.

MATERIALS AND METHODS

Animals

Intor:Swiss laboratory mice described in this paper were bred in the Institute of Virology, Vaccines and Sera – Torlak. Male and female mice of all ages were observed.

One part of the presented data (phenotype characteristics) was collected by observing the colony over many decades of vaccine production control in the Institute. The second part of the presented results (food consumption, body weight, relative organ weight, biochemistry, hematology and organ weights) was obtained from analysing the control group of mice in the preclinical studies of influenza vaccine, while the antibody subclasses analysis was performed on the sera of experimental group of animals from the same study. Each group consisted of minimum 50 male and 50 female mice.

To get a lymphocyte profile in the spleen we formed two additional groups, one with ten male and one with ten female mice, aged eight weeks.

All experiments were carried out according to the rules established by the Serbian Animal Welfare Act and Regulation on the Implementation of animal experiments and approved by the Animal Institutional Care and Use Committee at the Institute of Virology, Vaccines and Sera – Torlak. The approval for using laboratory animals in the influenza preclinical studies was adopted by the Veterinary Directorate (Ministry of

Agriculture and Environmental Protection) under the number: 323-06-03742/2012-05.

All animals were kept in groups of 10 animals per cage and fed with standard food and water ad libitum in conventional conditions in the animal house. Humidity, temperature and light/dark cycles were maintained at 55% ±5%, 21 ±2 °C and 12/12 h, respectively. Polygamous (5 + 1) mating system was applied in the colony.

Biochemical and hematological analysis

Determination of biochemical values was performed using a semi-automated spectrophotometric analyzer (Vet evolution-Biosis, Italy) at the Department of Equine, Small Animal, Poultry and Wild Animal Diseases, of the Faculty of Veterinary Medicine in Belgrade. Blood required for biochemical tests was collected from the adult mice (~14 weeks) by intracardiac puncture method performed under deep anaesthesia induced as described above.

WBC, differential leukocyte and RBC counts were determined in-house. The blood samples for hematological analysis were taken from the tail vein. WBC count [2] was performed by making a suitable blood dilution in Turk`s solution (3% acetic acid in distilled water with 0.01% gentian violet) and then counted using a Neubauer hemocytometer. Differential white cell count test was done by preparing peripheral blood smears for each animal, after which the leukocyte formula was determined under light microscope. Smear preparations were fixed in methanol for 2 min and stained for 15 min. in May-Grunwald-Giemsa stain [3]. This consisted of one drop Giemsa per milliliter McJunkin-Haden buffer at pH 6.4. One hundred leukocytes were counted under oil immersion and designated as either lymphocytes, monocytes, neutrophils, eosinophils or basophils, according to nuclear morphology described by Rygaard and Povlsen [4]. For RBC count [5], blood samples were diluted in Hayem's solution (2.5% Na_2SO_4, 0.5% NaCl, 0.25% $HgCl_2$) and erythrocytes were counted under the light microscope using the Neubauer haemocytometer [3].

Immunization and sera preparation

For the analysis of antibody response, male mice (8–10 weeks old) were injected intramuscularly in each caudal thigh with either 50 μl of seasonal (composition of 2011–2012 season) split virions (Sanofi-Pasteur, Lyon, France). The vaccine incorporated A/California/7/2009 (H1N1), A/Perth/16/2009(H3N2) and B/Brisbane/60/2008 (B) influenza virus strains and contained 10 μg (3.33 μg of each virus strain) of the virus surface protein HA.

The mice were bled from retro orbital sinus under ketamine–xylazine anesthesia (ketamine, 80 mg/kg Ketamidor, Richter Pharma AG, Wels, Austria; xylasine, 8 mg/kg Xylased, Bioveta, Ivanovice na Hané, Czech Republic) four weeks post immunization. The blood was allowed to clot for 1 h at room temperature. Sera were separated by

centrifugation at 3000 rpm for 30 min, heat-inactivated at 56 °C for 30 min and stored at −20 °C.

Indirect ELISA for the detection of influenza virion specific IgG isotype

The sera samples were examined for influenza virus strain-specific IgG subclasses by ELISA [6]. Briefly, split influenza virions used for coating ELISA plates were produced at the Institute of Virology, Vaccines and Sera – Torlak, Belgrade. Nunc MaxiSorp™ ELISA plates (Nunc, Roskilde, Denmark) were covered (50 µl/well) with influenza split virions (2.5 µg/ml HA in PBS) by overnight adsorption (4 ºC). Blocking (200 µl/well of 1 % w/v BSA/PBS 2 h at room temperature) and all subsequent steps in the ELISA were followed by washing with 0.05 % (v/v) Tween 20 in PBS (four times, 200 µl/well). Appropriately diluted (1:250 in 1 % w/v BSA in PBS) sera samples were incubated at room temperature (1 h, 50 µl/well).

Ag-specific sera IgG binding was detected by peroxidase-labeled anti-mouse IgG1, anti-mouse IgG2a, anti-mouse IgG2b and anti-mouse IgG3 (Jackson ImmunoResearch Laboratories Inc., WestGrove, PA, USA). Antibodies of IgG2c subclass were detected by biotin-labeled anti-mouse IgG2c (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA), followed by incubation with ExtrAvidin-peroxidase (50 µl/well, 1 h at room temperature). All reagents were used in accordance with the manufacturers' instructions. O-phenylenediamine/H_2O_2 (OPD) system was used to visualize Ag–Ab interactions. Absorbance was monitored at 492 nm and 620 nm ($A_{492/620}$).

Detection of spleen lymphocytes subpopulations by flow cytometry

Spleens of eight-week old Intor:Swiss mice were isolated, trimmed of all excess tissue and macerated in a complete RPMI 1640 supplemented with 5 % fetal calf serum (FCS). The pellets obtained after centrifugation at 400xg for 10 min. were resuspended in residual supernatant and erythrocyte-depleted by incubation (1 min) in RBC-lysing buffer Red Blood Cell Lysing Buffer Hybri-Max™. After centrifugation at 400xg for 10 min, splenocytes were washed three times in 5 % FCS/RPMI 1640. The cells were finally diluted in 2% BSA/PBS with 0.1% sodium azide to $1x10^6$ cells/ml (hemocytometer counting). The lymphocyte population was analyzed by flow cytometry, using antibodies directed against CD3ε (145-2C11, Hamster IgG, FITC) and CD19 (PeCa1, Rat IgG2a, PE). Antibodies were purchased from Immunotools (Immunotools, Friesoythe, Germany) and were used at an amount of 4 µl per million cells. After incubation for 20 min (4 °C, dark), the cells were washed twice with 1 ml of 2% BSA/PBS/ 0.1% sodium azide vortexed and centrifuged at 400 x g for 10 min. Spleen lymphocytes populations were analyzed with FACVerse (Becton Dickinson, Mountain View, CA, USA), from a lymphocyte gate.

All chemicals were purchased from (*Sigma*-Aldrich Chemie GmbH, Steinheim, Germany) unless otherwise indicated.

RESULTS

Mice phenotype

Phenotypic characteristics of Intor:Swiss albino mice in the Institute Torlak are similar to the phenotypic characteristics of Swiss mice of the commercial breeders (e.g. Crl:CFW [7], Crl:CD18, Hsd:ICR [8], and SW [9] mice). They have short white fur and red eyes, moderately large ears and tail with little hair (Figure 1). Adult body length (nose to base of tail) is 7.5–10 cm and tail length is about 5–10 cm. They have placid temperament, unless several males are in one cage. They manifest normal characteristics of all categories of common behaviors: a) maintenance behavior (grooming, eating, drinking, nesting); b) investigative/exploratory behavior (climbing, digging, chewing, sniffing); and c) social/sexual behavior (huddling together, grooming one another, scent/territorial marking, aggression, defense, sexual behavior and social interactions).

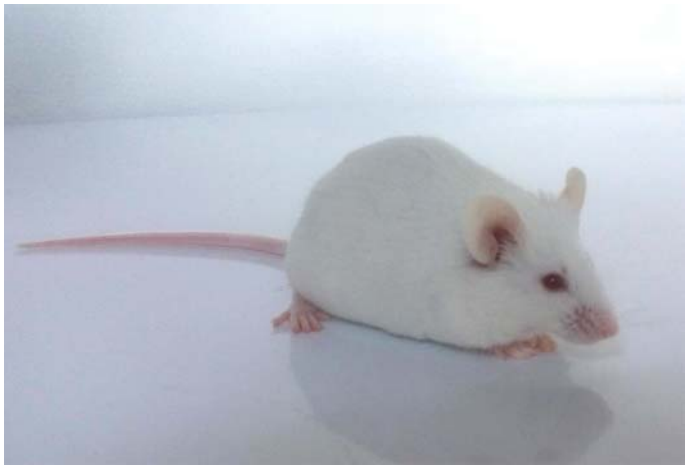

Figure 1. Photograph of an Intor:Swiss mice form the Institute of Virology, Vaccine and Sera – Torlak

Reproductive and maternal characteristics of the Intor:Swiss mice are excellent. Prior to and after giving birth, females particularly elaborate nests. Neonatal cannibalism, generally considered to be stress induced, and mortality rate among newborn litters are at an extremely low level. The rate of "spontaneous" deaths in the colony is ~1.5%. These and other biological data for mice in our colony are given in the table below (Table 1).

Table 1. Main biological characteristics of Intor:Swiss mice

Sexual maturity:	**5 to 8 weeks**
Breeding size:	30 g
Breeding age:	40-45 days (6-7 weeks)
Estrus cycle:	4 to 5 days; spontaneous
Gestation:	19– 21 days
Average litter size:	6-8 pups (primipara 4-6, multipara 9-11)
Birth weight:	0.9-1.5 g
Weaning weight	14 g
Adult weight	31-33 g (females), 34-40 g (males)
Time between two litters	30 days
Productive breeding life:	~7-8 months
Average lifespan	1.5-2 years

Serum biochemistry and hematology

The standard biochemical and hematological parameters of Intor:Swiss mice were determined from the serum and full blood collected from males and females, and are presented in the Tables 2 and 3, respectively.

Food consumption, body weight and relative organ weight

As relevant data used to describe laboratory mice, food consumption, body weight increase during growth, and relative organ weight of adult individuals were determined. As much as measuring these parameters might seem trivial and unsophisticated, it has been shown in practice that they are very sensitive indicators of general condition in mice, so we specify them for our mice.

Food consumption data were collected from groups of mice which were controls in the preclinical studies of influenza vaccine (groups of 50 female and 50 male mice). Measure of food pellets was done every day during the experimental period of 40 days for each cage (ten mice per cage) and presented as average of 24-hour consumption for individual mice. For adult females average quantity was: 3.69 g ± 0.18 g, and for males 5.2 g ± 0.25 g. The same animals monitored for food consumption were also monitored for the body mass increase (Figure 2).

Relative organ weight was determined as a percentage ratio between organ weight and body weight (Figure 3). The weight of organs representative for toxicology studies was measured. These organs were: liver, lungs, heart, kidneys, thymus, spleen, brain, and testicles and ovaries.

Table 2. Biochemical parameters of Intor:Swiss mice

Analyte Unit	Males	Females
Alanine Aminotransferase (ALT) U/I	77.5±4.8	72.6±5.2
Albumin (ALB) g/dl	3.3±0.3	3.4±0.3
Alkaline Phosphatase (ALP) U/I	112.7±9.3	136.4±12.3
Aspartate Aminotransferase (AST) U/I	92.6±4.6	103.84±7.5
Bilirubin (TBIL) mg/dl	0.269±0.03	0.3±0.03
Calcium (Ca) mg/dl	9.12±0.7	9±1
Chloride (Cl) mmol/l	117.6±2.9	118.7±3.8
Cholesterol (CHOL) mg/dl	88.8±3.5	100.4±5.8
Creatinine (CREAT) mg/dl	0.57±0.06	0.51±0.05
Gamma-glutamyl transferase (GGT) U/I	<5 U/I	<5 U/I
Glucose (GLU) mg/dl	71.35±1	63.96±1
Phosphate (PHOS) mg/dl	8.7±0.9	9.16±0.7
Potassium (K) mmol/l	7.14±0.6	6.76±0.6
Protein (TP) g/dl	5.2±0.6	5.56±0.9
Sodium (Na) mmol/l	138.8±0.4	139.36±0.4
Triglycerides (TRIG) mg/dl	128.3±43.3	174.3±51.3
Urea (URE) mg/dl	15.4±5.2	12.94±2.4

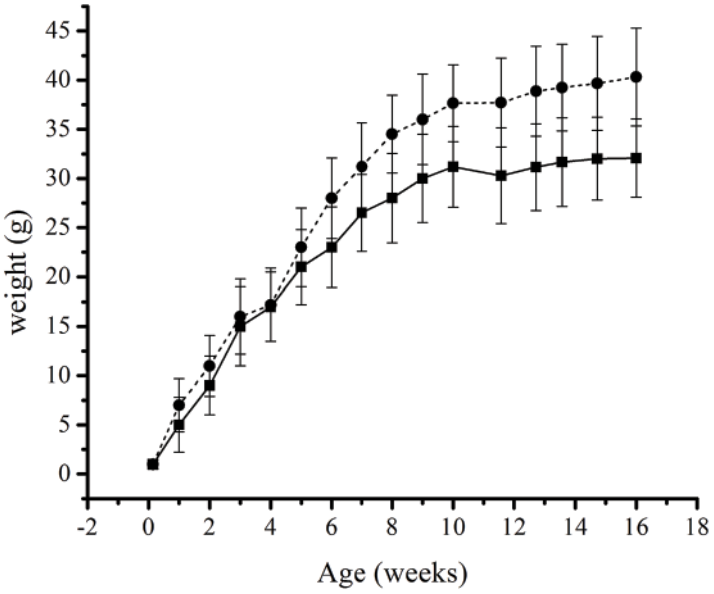

Figure 2. Intor:Swiss mice growth curve. The results for male (-•-), and female mice (-■-) ± S.E. are presented.

Table 3. Hematological parameters of Intor:Swiss mice

	Males	Females
Total WBC (count x10^6/ml)	16.02 ± 1.5	12.04 ± 1.2
Lymphocytes (%)	78.19 ± 4.3	78.19 ± 4.3
Monocytes (%)	5.96 ± 2.2	5.95 ± 2.2
Neutrophils (%)	11.01 ± 3.6	11.00 ± 3.6
Eosinophils (%)	4.61 ± 3.9	4.61 ± 3.9
Basophils (%)	0.03 ± 0.1	0.03 ± 0.1
RBC (count x10^9/ml)	9.67 ± 1.2	8.54 ± 1.4

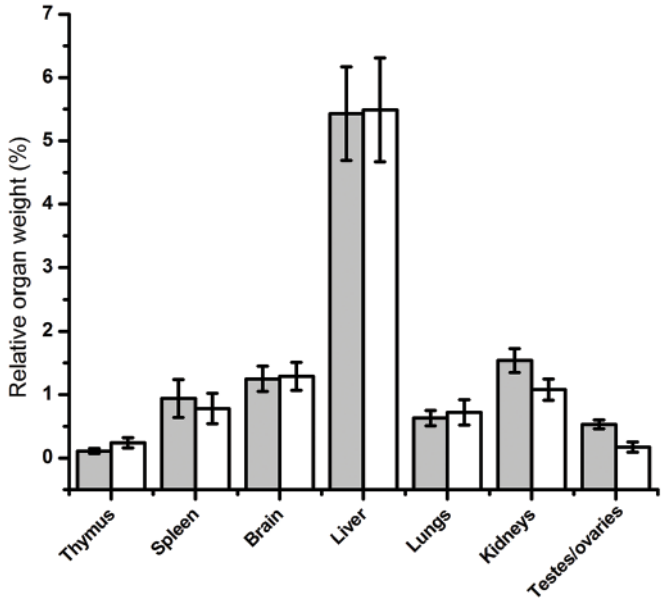

Figure 3. Intor:Swiss mice relative organ weight. Gray columns represent males, and white represent female mice. Plotted values represent the mean value ± S.D.

IgG isotypes in Intor:Swiss mice after immunization with influenza split vaccine

Figures 5 and 6 shows the expression of A H1N1 specific IgG2 antibodies after the immunisation of Intor:Swiss mice with influenza vaccine. The antibodies of all IgG2 subclasses (IgG2a, IgG2b and IgG2c) were detected. IgG2a and IgG2b antibodies, with various production intensities, are found in all tested animals. On the other side, the production of IgG2c antibodies was induced only in 10% of mice.

Similar results were obtained for all three tested viral antigens.

Lymphocyte subpopulations in Intor: Swiss mice spleen

We have determined the presence of T and B lymphocytes in the spleen of the ten non-immunized male and ten non-immunized female mice, and the results are shown on Figure 4.

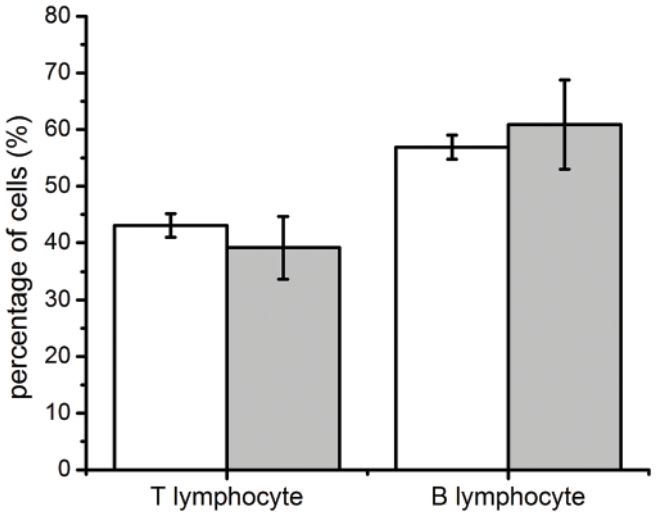

Figure 4. T and B lymphocytes in spleen of male (gray columns) and female (white columns) Intor:Swiss mice. Analysis of splenocytes was done by flow cytometry. Plotted values represent the mean value ± S.D.

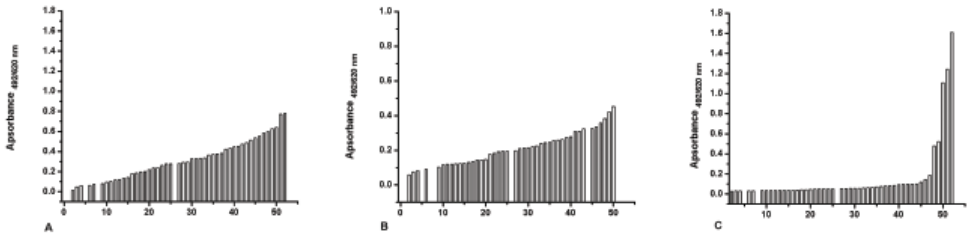

Figure 5. Distribution different isotypes of IgG2 antibodies specific for influenza antigens in group of fifty observed mice. The sera of immunized animals were analyzed by ELISA and the graph shows the measured absorbance. A) – antibodies of IgG2a isotype; B) – antibodies of IgG2b isotype; C) – antibodies of IgG2c isotype.

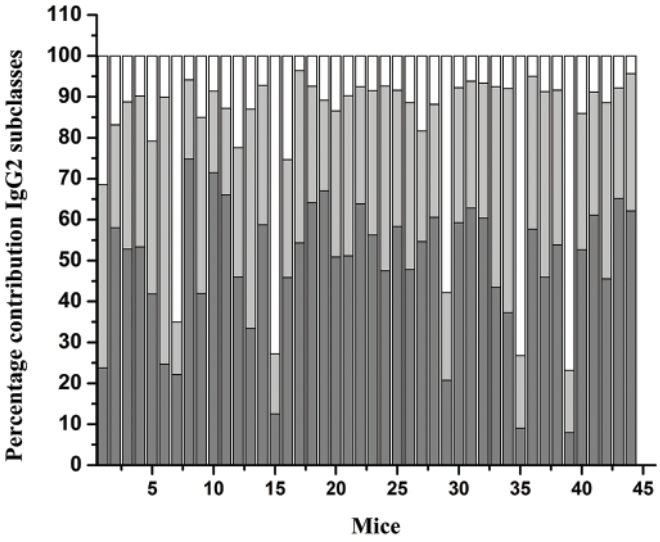

Figure 6. Elicited percentage of IgG2 isotypes in every individual mice after influenza immunization. White – antibodies of IgG2c isotype; Light gray – antibodies of IgG2b isotype; Dark gray – antibodies of IgG2a isotype. represent female mice. Plotted values represent the mean value ± S.D.

DISCUSSION

As already mentioned, in the Institute of Virology, Vaccines and Sera Torlak have used Swiss mice for a long time, probably from the 30's of the last century. In order to make our stock known to the general scholarly public, we have registered the name of the stock in the Mouse Genome Informatics (MGI) database [10] and presented data that we have collected over time.

The story about origin of Intor:Swiss mice is not precisely known. In general, all Swiss mice were named after Switzerland, the country of their origin. They all descended from animals bred at the Centre Anticancereus Romand in Lausanne, at the beginning of the 20th century. Most outbred mice have precisely that common origin. From Lausanne colonies, 2 males and 7 females were imported to the Rockefeller Institute for Medical Research in New York by Clara Lynch in 1926 [11]. These mice were the foundation for animals designated as Webster. Today's commonly available non-inbred Swiss stocks descend from Lynch's original mice, more (Swiss Webster mice - Rockefeller) or less directly from Webster's colonies (ICR mice, NIH Swiss mice) [12].

However, it is little known that Swiss mice from Lausanne actually originated from France, from a colony of about 200 animals bred at the beginning of the 20th century in the Pasteur Institute in Paris, from where they were transferred to Switzerland [1].

Unfortunately, there are no written data about the origin of the breeding stock of the animals in the Institute Torlak. However, the fact is that a designated building for

breeding and keeping of laboratory animals in Institute Torlak was constructed in 1930, [13] and that Swiss mice have been continuously bred at the Institute ever since (or even earlier). At that time, the experts who took part in founding the Institute in Belgrade went to a study visit to the Pasteur Institute in Paris. Based on the statements of senior colleagues, it is assumed that these experts were the ones who brought the mice from the Pasteur Institute breeding stock. If that is true, the Swiss mice bred in Institute Torlak descend, to a large extent directly from the mice from Paris. Unfortunately, we could not find any written evidence in Belgrade or Paris to support this statement, and without additional proof we cannot exclude the possibility that Intor:Swiss mice originate from Lausanne.

A very important matter when breeding an outbred mice stock is maintaining a sufficient level of heterozygosity. Since the extent of genetic variation depends on the previous history of the breeding stock, it is interesting whether or not the heterozygosity of the Intor:Swiss stock was preserved during the past 80 years of breeding in Institute Torlak. This genetic variation can range from almost zero, when a closed colony has been maintained with small numbers of animals for many generations, or has experienced any genetic bottleneck (such as being reduced to small numbers in one generation), to a level that we meet in populations of wild mice [14]. The mouse stock maintained in the Institute Torlak has had a very large number of animals throughout all these years since the Institute was established. The records of the number of laboratory animals date from 1961 and show that the colony size has been maintained at 2,000 to 5,000 animals. The system of random breeding has been applied all the time, not selecting the most closely related animals. Since the minimum number of pairs required to maintain the maximum heterozygosity is 25 pairs, [1] we can freely assert that the mice maintained in Institute Torlak are an outbred and not a closed colony. Even in the period before the recording system was introduced, the number of crossed pairs did not fall below the number of 25 (personal communication).

According to the literature, some level of inbreeding is unavoidable, although it will be negligible in such large colonies. However, the extent of the genetic variation will not be known without a specific genetic investigation [15]. Such genetic analyses of outbred mice have only recently been established [14,16]. It appears that for outbred mice, a genetic profile of heterozygous markers needs to be established for each particular stock, and this genetic profile needs to be monitored throughout the existence of the colony [17]. Such characterization of Intor:Swiss stock could open up the possibility of participating in the project Mouse Genome Database (MGD), the international database resource for laboratory mice. This could also make it possible to use this stock for better understanding drug or vaccine effects on the body in a physiological or pathological condition [14,18]. In addition, it is possible that, by determining genome characteristics and comparing them with the MGD data base, the assumption about our stock being one branch in the genealogy tree of commercially available outbred stocks would be confirmed or rejected [11].

Undoubtedly, despite the appropriate colony size and breeding method, certain selective pressure is exerted on the animals bred for generations in artificial conditions, i.e. animals in stock respond to any environmental changes that might occur. For example, mice from most outbred stocks weigh more than mice from inbred strains as a result of elimination of smaller animals born in the breeding colonies through generations. Similarly, the mouse colony in the Institute Torlak has fully adapted over several decades to the existing conditions and to an extent changed stock characteristics. This could result in that today's stock characteristics deviate to a larger or smaller extent from the ancestral stock characteristics or from the earlier published data referring to the same colony. Therefore, new characterization of Swiss mice and comparison with the commercial stocks are more than advisable.

In this paper, we have described biological, biochemical and hematological characteristics of Intor:Swiss mice. Comparing to the data available for commercial stocks, we did not record any significant difference in any of analyzed characteristics (morphology, growth curve, food consumption, reproductive characteristics, life span, WBC and RBC counts, leukocyte count, biochemical characteristics of the serum). Although sporadic differences were noted, they were not statistically significant. Barring all of the above in mind, we can conclude that Intor:Swiss stock has characteristics very similar to other outbred stocks, possibly originating from the same ancestral Swiss colony.

Given that the characteristics of the immune system may become critical in the design of certain preclinical studies, we have analyzed the presence of T and B lymphocytes and profiles of IgG subclasess (Figure 4, 5 i 6). It is already known that laboratory mice, including outbred stocks, [19,20] secrete IgG1, IgG2a, IgG2b and IgG3 isotypes in response to protein antigens. Genetic and structural analysis [21] of the outbred Swiss Webster mice genes encoding constant region of immunoglobulin subclasses have shown the presence and expression of two allelic forms of the *Igh* gene: *Igh-1a* allele, which codes IgG2a, and *Igh-1b* allele, coding IgG2c. IgG2b is coded by a separate gene, *Igh-3* [22]. New papers [23] have shown that lymphocytes of Swiss Webster mice may secrete, along with IgG2b, either only IgG2a or IgG2c, or both IgG2a and IgG2c subclasses. IgG2a and IgG2c most likely have different immune profiles in mice due to their divergence of the protein sequence. After immunization with influenza vaccine, Intor:Swiss mice secrete virus specific antibodies of IgG2a, IgG2b and IgG2c subclasses, which is another similarity between our and other outbred stocks. The production of IgG2a and IgG2b subclasses was induced in all animals in the colony, but with different intensities. On the other hand, IgG2c antibodies were present in only 10% of tested animals (Figure 5). Comparing the presence of individual IgG subclasses in the analyzed sera (Figure 6), it is clear that IgG2a and IgG2c subclasses are mutually suppressed.

The results presented in this paper characterize the stock designated as Intor:Swiss and show that it is very similar to the standard commercial stocks. Taking that into account, and based on the extensive experience of working with these mice in Institute Torlak,

we consider the Intor:Swiss stock suitable and reliable, for the experiments requiring mice of outbred genetic background.

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Authors' contributions

IZ conceived the study, created its design and prepared the manuscript. VP participated in the design of the study and performed the statistical analysis. KM and RM carried out the immunoassays, FACS analysis and leukocyte count. IR and IA performed immunisation, sera preparation and all weight measurements. JK was in charge of the welfare of animals and described the biological characteristics of mice. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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KARAKTERIZACIJA INTOR:SWISS SOJA ALBINO MIŠEVA DONETOG U INSTITUT ZA VIRUSOLOGIJU, VAKCINE I SERUME - TORLAK POČETKOM XX VEKA

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Institut za Virusologiju, vakcine i serume Torlak, osnovan je 1927., a prva vakcina u Institutu proizvedena je 1930. Proizvodnja vakcina je složen proces koji između ostalog podrazumeva i korišćenje eksperimentalnih životinja u kontroli samog procesa. Laboratorijski miševi koji su od samog početka bili u upotrebi u Institutu Torlak, pripadaju Swiss albino outbred soju. Ova kolonija je u upotrebi više od 80 godina i sve vreme se sastoji od velikog broja jedinki što omogućava očuvanje genetske raznolikosti, pa samim tim i outbred karakteristika. Ovi miševi su odnedavno registrovani pod imenom Intor:Swiss, i njihove biološke osobine su u ovom radu prikazane po prvi put. Swiss miševi Instituta Torlak pogodni su za upotrebu u farmaceutskim studijama, za razvojno istraživanje vakcina, osnovna istraživanja i toksikološka ispitivanja. Zbog svega navedenog Intor:Swiss miševi predstavljaju još jedan pogodan animalni model za ispitivanje lekova i vakcina.