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Effects of low and high dosage diphtheria toxin on macrophage ablation in ITGAM mice

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Table of Contents

1. Abbreviations

ALT – Alanine aminotransferase

ANOVA – Analysis of variance

AST – Aspartate aminotransferase

BALB/c - Bagg Albino (inbred research mouse strain)

Cd11B- Cluster of differentiation molecule 11 B

DT – Diphtheria toxin

DTR – Diphtheria toxin receptor

HB-EGF – Heparin-binding epidermal-growth factor

ITGAM – Integrin, alpha M (complement component 3, receptor 3 subunit)

KCl – Potassium chloride

KH_2PO_4 – Monopotassium phosphate

LUC – Large unstained cells

MONO - Monocytes

NaCl – Sodium chloride

Na_2HPO_4 – Disodium phosphate

PBS – Phosphate buffered saline

RT-PCR – Reverse transcriptase polymerase chain reaction

WBC – White blood cells

2. Introduction and aim of the study

Over the last few decades, modern technology has opened the door to a new world of possibilities within the field of medical research. Genetic engineering allows scientists to modify an organism's genome by introducing or deleting DNA segments which code for certain genes. Further developing of this technology has lead to the production of laboratory animals which may be subjected to conditional depletion of certain cell types. This is a widely used approach in research probing the immune response. The ability to create an artificially induced selective immunosuppression is inevitably useful in further artificial infection studies. For instance, transgenic mice which initially have been submitted to specific cell removal have been used in model studies of immune mediated diseases. To ensure the validity of research based on toxin mediated targeted cell removal, only the particular cell type to be studied should be eliminated from the animal. Optimal toxin dosage would be high enough to completely deplete the target cell type, but low enough to exert limited effects on other cellular systems or physiological processes.

In the following thesis, I have studied the effect of diphtheria toxin (DT) on genetically modified mice. The aim of the study was to determine the dosage of diphtheria toxin needed to deplete macrophages in integrin alpha M (ITGAM) mice. The toxin was administered in two different concentrations. We also investigated the short term effect of the toxin mediated cell depletion on bodyweight and feed consumption. Some of the previous research based on mice that have undergone specific cell type ablation suggest that the diphtheria toxin may not just affect the target cell type, but could in fact change the cellular and cytokine environment, hence making it difficult to draw definite conclusions (Roberts et al., 2015). In order to improve the precision of future research, it would be beneficial to examine closer the connection between toxin administration and change in cellular environment.

3. Survey of literature

Thirty years ago a research group succeeded in creating the first transgenic animals produced by microinjection of a genetically engineered construct into the pronucleus of a mouse zygote (Hammer et al., 1985). In medicine, recombinant DNA technology has been used to create so called "knock out" mice. In these mice a specific gene of interest is inactivated, thus by observing the consequences of its absence, the function of this gene can be better understood (Sadava et al., 2013). Furthermore, transgenic animals have proved a useful tool for studying specific cell populations *in vivo*. A frequently used method for such studies is by modifying the target cell type into expressing a toxin receptor. While the expressed protein itself is not toxic, it can potentiate the effect of an injected toxin (Saito et al., 2001). Following toxin administration, the target cell populations will be depleted. Hence by monitoring the animal's response to various stressors in the absence of certain cell types, the function of these cells is illustrated. For instance, macrophage ablated mice have been used to study physiological processes, an example of which includes a study on wound healing conducted at Johannes Gutenberg University in Mainz, observing the "Effects of Diphtheria Toxin-Driven Lysozyme M-Specific Cell Lineage Ablation on Wound Inflammatory, Angiogenic, and Contractive Processes" (Goren et al., 2009).

Figure 1. Transgenic mice from the Jackson Laboratory (Source: https://www.jax.org/jax-mice-and-services)

3.1. ITGAM mice

A transgenic organism is created by introducing a specific foreign gene segment into embryonic cells. Initially tested on mice, DNA microinjection into the male pronucleus of a zygote remains the principal method for production of transgenic laboratory animals. The injected DNA segment is incorporated into the organism's genome after entering the nucleus and can be transferred to its offspring (Maksimenko, 2013). Further steps of inbreeding result in animals homozygous for the particular gene sequence.

Due to their genome being easily manipulated, their low maintenance cost and rapid reproduction rate, mice are the premier animals used to create disease models for biomedical research (Figure 1). In contrast to other living organisms such as flies or worms used to study the cell cycle and developmental processes, mice possess the same complex physiological systems as humans and other mammals. For this reason, they are frequently used in research probing the immune system, cardiovascular and skeletal system as well as the endocrine system (Spencer 2002). In 2001, Saito et. al created transgenic mice for a genetic cell ablation trial by microinjecting the DNA construct for the Human Heparin Binding EGF-like Growth Factor into fertilized mice embryonic cells (Figure 2). Human HB-EGF is the protein that functions as a binding site for diphtheria toxin. Hepatocytes were chosen as the model target cell type, as their destruction can be easily monitored by measuring serum levels of aspartate aminotransferase and alanine aminotransferase. The study showed that mice expressing the human HB-EGF precursor under the control of the hepatocyte–specific albumin promoter rapidly developed hepatitis after administration of a small amount of diphtheria toxin (DT). The experiment demonstrated that cell removal using the combination of DT and its receptor is a successful method for conditional cell ablation (Saito et al., 2001).

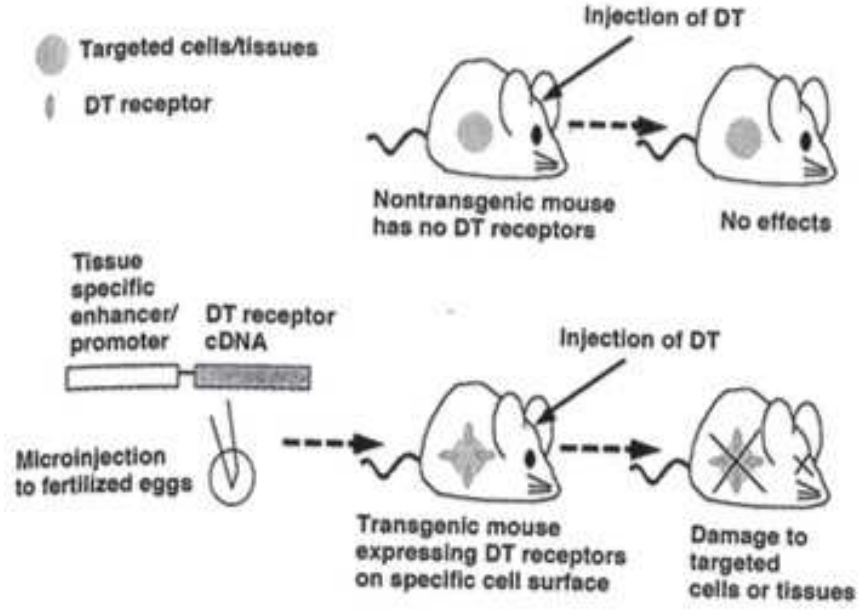

Figure 2. Production of mice expressing the diphtheria toxin receptor (Source: Saito et al., 2001).

In our study we worked with genetically modified transgenic mice imported from the Jackson Laboratory in Maine, USA. Founded in 1929, the Jackson Laboratory is a non-profit organization specializing in genetics and genomics. Pioneering the use of transgenic mice in biomedical research, the institute is well known for establishing the mouse as a primary research model for human disease. Today the institute remains one of the major suppliers of laboratory mice to the world's scientific community, as well as managing own research projects in areas such as cancer, diabetes, immune system and neurological disorders (Spencer 2002).

Similar to the study performed by Saito et al. (2001), our mice contained a transgene insert consisting of a primate diphtheria toxin receptor and green fluorescent protein controlled by the human integrin alpha M (ITGAM) promoter (CD11B). Macrophages carrying this receptor on their surface are targeted and destroyed by the diphtheria toxin. Thus, after exposure to the toxin, these mice experience a transient selective depletion of macrophages in various tissues. Cell death is expected in both dividing and fully differentiated cells (Stoneman et al., 2007). Circulating polymorphonuclear cells should not be depleted by the toxin, even though they carry the CD11B receptor. By day 4 post administration the Macrophage level should have normalized.

3.2. Corynebacterium diphtheriae

Corynebacterium diphtheriae is an aerobe, Gram-positive, club shaped bacillus capable of toxin production (Murphy 1996). Its main habitat is limited to the upper respiratory tract of infected hosts and superficial skin lesions. Humans are the only known reservoirs of the bacterium, and transmission occurs commonly by droplet infection from respiratory secretions (Parija 2014). Although classically considered to be a strict human pathogen, cases of disease have been reported in cattle (Corboz et al., 1996), and horses (Leggett et al., 2010).

In its classical form, diphtheria presents as a respiratory disease, affecting mainly children or other immunocompromised individuals. Bacterial colonization and subsequent toxin secretion is responsible for the development of disease, with symptoms generally including sore throat, malaise and fever. A characteristic layer of necrotic cells, debris and fibrin covering the mucous membrane of the throat is typically associated with the infection. The pseudo membrane covered lesions may also extend into the lower respiratory system, thus restricting the airflow. In severe cases obstruction of the airways and a secondary bronchopneumonia may lead to hypoxia and death. Occasionally, the cervical lymph nodes are involved, causing a distinct swelling of the neck (Murphy 1996). Furthermore, the toxin is sometimes absorbed into the circulation and hence transported to distant tissues such as the central nervous system and the myocardium, kidney and the adrenal glands (Guaraldi ALM et al., 2014). Consequently, life threatening systemic conditions including congestive heart failure and paralysis may develop.

By injecting purified toxin into experimental animals and observing their symptoms as far back as in late 19th century, the suspected link between the toxin and clinical disease was confirmed. Based on this discovery, diphtheria became one of the first diseases that was overcome through mass immunization with toxoid (Murphy 1996). Interestingly, the bacterium is only capable of producing toxin after being lysogenized by a particular bacteriophage. The virus provides the bacterium with the toxin producing gene (Holmes 2000).

3.3. Diphtheria toxin

The diphtheria toxin (DT) itself is a single, globular protein comprised of two subunits; an enzymatically active subunit DT-A which is responsible for the cytotoxic effect, and a cell receptor binding subunit DT-B, both with a fixed set of amino acids (Guaraldi ALM et al., 2014). Already in 1959, Strauss and Hendee suggested that the toxin interferes with the incorporation of methionine during protein synthesis, causing cellular death (Strauss and Hendee 1959). After entering the cell by endocytosis, the DT-A subunit is translocated to the cytosol where it inactivates a target protein by catalysing the adenosine diphosphate ribosylation (Emata et al., 2000). However, the toxin does not present an immediate effect, and a minimum of 10 hours to several days are needed to observe the result regardless of dosage (Collier 1975).

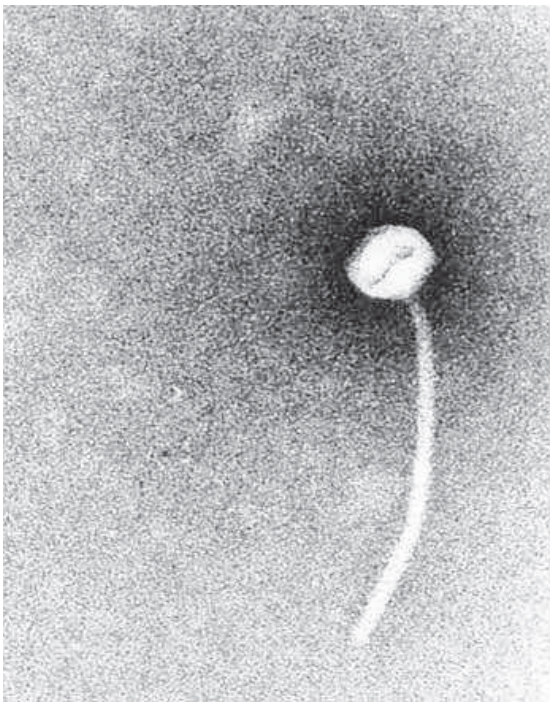

Figure 3. Electron micrograph of corynebacteriophage ß carrying the tox gene (Source: http://intranet.tdmu.edu.te.ua/data/cd/disk2/ch032.htm)

The toxic dose of diphtheria toxin in a susceptible animal such as the guinea pig is $6x10^{-5}$ mg. In comparison this confers to more than 3 million times the toxicity of strychnine. The only bacterial toxins more potent than diphtheria are those of shigalike producing *E. coli* and *Clostridium botulinum* and *C. tetani* (Todar 2012). Lethal dose of DT in humans is less than

100 ng/kg bodyweight when administered intramuscularly. In mice however, more than $1,6^{10}$ mg per kilo bodyweight is needed to kill the animal (Gill 1982).

While genetic cell ablation has long been a widespread approach for studying cell populations *in vivo*, a 2015 study conducted in collaboration between Arizona State and North Carolina University reported on problems related to the DT treatment. Their objective was to investigate the importance of alveolar macrophages in the immune response to a *Francisella tularensis* infection in the form of a live vaccine strain. They found that the DT caused disturbance in other cellular systems as well as the targeted macrophages. The researchers discovered that the diminishing macrophage numbers affected the whole cytokine and chemokine milieu of the lung, and thus potentially also the innate and adaptive immune response. Several of the mice died shortly after administration of the toxin due to neutrophilic pneumonia. The neutrophil influx in both the lung and spleen was likely caused by the toxin administration and not by the *Francisella tularensis* infection. This was demonstrated by a nonmanipulated group of mice which, upon injected with the live vaccine strain, quickly cleared their infection. Due to the complex set of changes following the DT administration, the immune response with or in the absence of alveolar macrophages could not be appropriately distinguished, suggesting more research is needed on the unexpected effects resulting from injection of the toxin (Roberts et al., 2015).

3.4. The diphtheria toxin receptor

The intramembranous heparin binding EGF-like growth factor (HB-EGF) has been recognized as the binding site for the DT-B, thus functioning as a toxin receptor in susceptible animals. Rats and mice have shown to be less susceptible to the toxin, requiring concentrations of three orders of magnitude greater per unit body weight than for instance humans or rabbits, in order to get a similar response (Saito et al., 2001). The increased tolerance of rats and mice to DT is explained by differences in the cell surface receptor of the HB-EGF protein, rendering DT unable to bind to murine cells (Naglich et al., 1992). Due to the fact that mice naturally do not express a functional Diphtheria Toxin Receptor (DTR), genetically modified mice displaying tissue specific expression of DTR are widely used in immunology research to examine the effect of an acute depletion of certain cells targeted by DT (Probst et al., 2005).

3.5. Macrophages and their role in infections

The diverse groups of cells mediating the body's immune response are collectively termed leukocytes or white blood cells. Circulating through the blood and the lymphatic system, they are quickly transported to sites of tissue destruction and infection. Based on morphology and functional characteristics leukocytes are divided into several subsets. The different cell groups all originate from common hematopoietic stem cells but develop along separate pathways in response to internal and external signals. Monocytes and macrophages belong to a subgroup called the mononuclear phagocyte system, which plays a key role in the innate immune response (Geissmann et al., 2010).

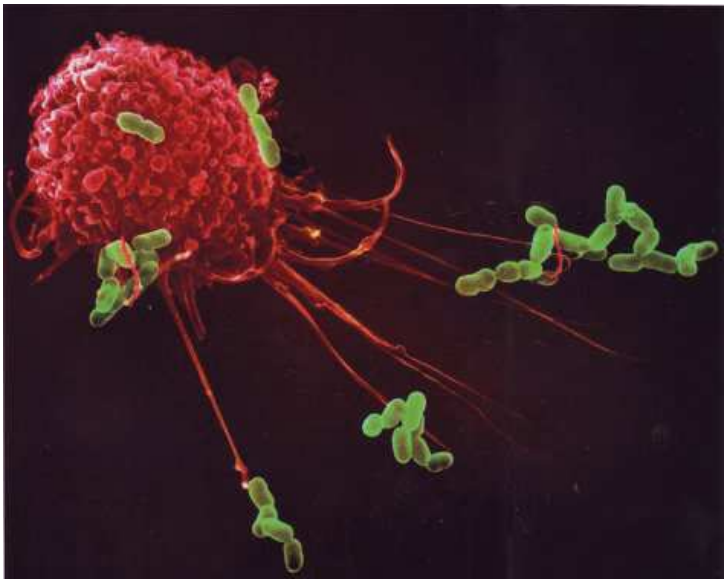

Figure 4. Scanning electron micrograph of a macrophage engulfing bacteria (Source: http://biotechin.asia/2015/05/07/origin-of-macrophages-paves-the-way-for-better-therapeutic-design-against-diseases/)

Circulating monocytes originate from myeloid progenitor cells in the bone marrow termed macrophage colony forming units. These precursor cells develop into immature monoblasts, which in turn give rise to pro-monocytes and ultimately monocytes. The fully mature monocytes are then released into the circulation (Mosser and Edwards 2008). Monocytes are equipped with Toll-like receptors enabling them to recognise invading pathogens and inflammatory chemokines released from damaged cells (Kono and Rock 2008). In case of inflammation, activated monocytes are among the first responders. Their main functions include secretion of cytokines, phagocytosis of damaged or infected cells and neutralisation of toxic molecules. Furthermore, in response to inflammatory stimuli monocytes are able to

migrate out of the blood vessels and enter the surrounding tissues, where they differentiate into macrophages (Geissmann et al., 2010).

Macrophages are specialized phagocytic leukocytes found in virtually all tissues and organs in the animal body. They include, among others, the Kupffer cells in the liver, the alveolar macrophages in the lung and the microglial cells of the central nervous system. Ever since Metchnikoff received the Nobel Prize for his description of phagocytosis in 1908, immunologists have attempted to clarify the specific role of macrophages in the host defence system (Nathan 2008).

It is now evident that macrophages form an important part of the innate defence mechanism by serving as immune effector cells. They remove harmful substances from the host organism by engulfing and digesting bacteria (Figure 4), foreign particles and dead cells (Mosser and Edwards 2008). Their high plasticity allows them to migrate through different body compartments, where they assume various functions and phenotypes based on biochemical signals received from the local microenvironment. Thus the production of pro-inflammatory mediators, cytokines and cell surface antigens may differ between macrophages in different tissues, even in response to identical stimuli (Lotze and Thompson 2005).

In addition, macrophages are essential in maintaining normal tissue homeostasis. They are responsible for the continuous degrading of aged erythrocytes, a vital metabolic process which ensures recycling of haemoglobin. Moreover, they remove cellular debris generated from tissue repair and clear cells that have undergone apoptosis (Mosser and Edwards 2008). To investigate the significance of monocytes/macrophages in atherosclerosis, scientists at the University of Cambridge carried out a study in 2007 using CD11B Diphtheria Toxin Receptor Transgenic Mice. They found that a dosage of 10 ng/g of DT reduced the peripheral blood monocytes by 88.5 ± 4.9 %. They also found that an increase of the DT concentration to 15 ng/g had no significant effect on further reduction of monocytes. Furthermore, total granulocyte count was not affected by DT, even though CD11B is expressed in neutrophils, which make up the majority of granulocytes (Stoneman et al., 2007).

3.6. Large unstained cells

The term “large unstained cells” (LUC) is commonly used to describe a classification of cells which are counted only by certain haematology analysers. Although the size and staining characteristics of these cells fall between the five major leukocyte groups, they presumably represent large lymphocytes or monocytes. Typically, the absolute large unstained cell count reflects the total lymphocyte count, and pronounced changes are rarely seen (Sahota et al., 2013). In mice, large unstained cells make up between 0.5 – 3 % (HTTP1) of the normal population of white blood cells. Reactive, activated or atypical lymphocytes or mononuclear cells are associated with an increase in LUC (Smith and Jarecki, 2011).

4. Materials and Methods

The aim of our study was to investigate the effects of low and high dosage (10 ng/g and 40 ng/g) of diphtheria toxin on the depletion of certain leukocytes in genetically modified ITGAM mice after subcutaneous injection of DT. We also investigated the short term effect of the toxin mediated cell ablation on feed consumption and bodyweight. Our research primarily concentrated on the changes in the cell population of monocytes, and large unstained cells. The effect of the toxin treatment was determined by measuring the levels of white blood cells, monocytes and large unstained cells in peritoneal fluid samples and blood smears collected from experimental animals. Cytological examination of the peritoneal fluid was achieved with ADVIA 120 (Laser Haematology Analyser, Bayer Co.). Blood smears were evaluated under light microscope with 100x magnification.

4.1. Experimental animals and experimental design

In this study 27 mice were included, obtained from the Jackson Laboratory in Maine, USA. The mice homozygous for the transgene did not show any gross abnormalities in appearance or behaviour. They were 3 months old, of normal size and fully viable. The specific transgene expression was detectable with RT-PCR analysis of bone marrow macrophages.

Table 1. Distribution of animals in experimental groups

Experimental groups	Bodyweight, survival, and feed consumption	
	Peritoneal fluid and blood smear analysis	
Control BALB-C	3 animals (female)	
Control ITGAM	5 animals (3 male and 2 female)	3 animals (female)
10ng/g	4 animals (2 male and 2 female)	4 animals (female)
40ng/g	4 animals (2 male and 2 female)	4 animals (female)

The mice were divided into 4 groups; a BALB/c control group of 3 animals, an ITGAM control group of 8 animals, a low toxin group (10 ng/g DT) of 8 animals and a high toxin

group (40 ng/g DT) of 8 animals (Table 1). The BALB/c is a strain of inbred albino mice widely used in biomedical research. The BALB/c strain progenitors were developed by Halsey Bagg back in early 1900s and is today a preferred strain for research in immunology and infectious diseases due to its increased susceptibility to various infections and its pronounced response to immunization (Potter 1985). The BALB/c mice were included in our study as negative control. The ITGAM control group was included to evaluate whether their cytological pattern remains normal in absence of DT exposure. The last two groups consisted of ITGAM transgenic mice treated with 10 and 40 ng/g diphtheria toxin respectively.

Prior to the experiment the animals were submitted to a three-day acclimatization period during which proper air quality was ensured through artificial ventilation, ambient temperature was kept at 28±3 °C, and a 12 hour light-dark cycle was applied. These conditions were maintained throughout the experimental period. The mice were randomised according to bodyweight and sex, and housed in groups of the same gender in polycarbonate containers of 15x30 cm (Figure 5). Colour marking was applied to distinguish between mice in different treatment groups. Each container was bedded with high quality wood shavings and equipped with a water dispenser. Feed and water was available *ad libitum*. The investigation was authorized by the Hungarian Animal Welfare Committee of the Szent István University Faculty of Veterinary Science of Hungary.

Figure 5. Experimental mice were kept in sterile polycarbonate containers, feed and water was available *ad libitum*

4.2. Experimental procedure

The experiment took place over a course of five consecutive days in June 2015 at the Animal House of the Department of Pharmacology and Toxicology. Diphtheria toxin stock solution was prepared from 1 mg of solid powder (Sigma Aldrich, St. Louis, USA, 100% purity) The powder was first dissolved in 1 ml phosphate buffered saline (PBS, including 8.0 g/L NaCl, 0.2 g/L KCl, 1.42 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4). The solution was then diluted in three steps with PBS resulting in a concentration of 2 µg/ml, and transferred to Eppendorf tubes. Final prepared toxin solution was stored at - 20 °C and thawed directly before application. Previously thawed Eppendorf tubes were discarded as the freeze-thawing process decomposes the toxin and thereby reduces its activity.

Table 2. Summary of the daily routine during the experimental period

Day 1	Day 2	Day 3	Day 4	Day 5
Body weight measurement	Body weight measurement	Body weight measurement	Body weight measurement	Body weight measurement
Feed measurement	Feed measurement	Feed measurement	Feed measurement	Feed measurement
Clinical examination	Clinical examination	Clinical examination	Clinical examination	Clinical examination
Observation of activity	Observation of activity	Observation of activity	Observation of activity	Observation of activity
Subcutaneous injection with DT or Salsol	-	Euthanasia of 16 animals	-	-
-	-	Sampling of peritoneal fluid and blood	-	-

A single injection of toxin solution or saline was administered on Day one (Table 2).The BALB/c and ITGAM control groups were treated with 0.4 ml of Salsol injection (0.9 % NaCl), while 10 ng/g and 40 ng/g DT were administered to the two test groups respectively. Calculating with an average bodyweight of 20 g, a concentration of 10 and 40 ng/g resulted in a 200 ng and 800 ng dosage, which corresponded to 0.1 and 0.4 ml volume of toxin solution. Although intraperitoneal injection is considered the primary route of administration, the toxin was administered subcutaneously in order to avoid causing damage to peritoneal cells.

A summary of the daily routine is presented in Table 2. Bodyweights were recorded daily for the next four days using a digital torsion scale. In addition, average feed consumption in each group was registered based on the weight of the remaining pellets compared to the previous day. Clinical examination of the eyes, fur and general body condition was performed every day together with observation of appetite, grooming, escape efforts and overall activity.

Figure 6. Sampling of peritoneal fluid from euthanized animals

Sixteen (16) animals were euthanized with ether inhalation on Day 3 while the remaining eleven (11) were monitored until Day 5. *Blood smears* were prepared by obtaining one drop of blood from *vena jugularis,* properly dissipated and air dried. Smears were then submerged into concentrated methanol and air dried again. Following this procedure, smears were stained with Diff-Quick (Reagens Kft, Budapest, Hungary) quick staining solution according to label instructions. Finally, the smears were examined under a light microscope with 100x magnification, and 30 fields of view were included in cell counting. In preparation for sampling of *peritoneal fluid*, 5 ml of refrigerated PBS was injected into the peritoneal cavity with a 23-gauge syringe. Promptly following this procedure, aspiration of 0.5 ml of peritoneal fluid was performed using the same syringe (Figure 6). Samples were transferred to Eppendorf tubes, placed on ice and immediately transported to the "Állatorvosi Hematológiai és Onkológiai Központ" Laboratory. Further analysis was completed with ADVIA 120 Laser Haematology Analyzer (Bayer Co.).

4.3. Statistical analysis

A two-way Anova was used to perform a comparison of white blood cell counts both between treatment groups and within each group. A p-value below the confidence level of 0.05 indicates a statistical significance between the factors and the dependent variables, suggesting that one or two factors influenced the results (Table 5).

5. Results

5.1. Blood smear

Results of the blood smear evaluation are summarised in Table 3. The analysis revealed no significant difference in monocyte and white blood cell counts between the different groups, indicating that the original number of cells was not adequate for a reliable statistical comparison.

Table 3. Blood smear evaluation; Distribution by percentage of different leukocyte populations in all four treatment groups

Groups	Cell number/10 field of view	Lymphocyte %	Neutrophil granulocyte %	Monocyte %
Control BALB/c	0.1	100	0	0
	0.3	100	0	0
	0.1	100	0	0
Control ITGAM	1.3	75	0	25
	1	100	0	0
	1	100	0	0
	1	100	0	0
10 ng/g DT	1	100	0	0
	1	100	0	0
	1	100	0	0
	1	100	0	0
40 ng/g DT	3.3	70	20	10
	4	91.7	0	8.3
	3	77.8	0	22.2
	1	100	0	0

5.2. Peritoneal fluid

The levels of monocytes, white blood cells and large unstained cells present in the peritoneal fluid of the four experimental groups after conclusion of the trial was determined. Results of the individual cell count are summarised in Table 4, which shows the proportion of the different cell types in G/L (giga per litre). The means and standard deviations of the different groups are indicated in Table 5.

Table 4. **Peritoneal fluid evaluation;** Distribution of cell types in G/l (giga/litre) in the different groups after termination of the experiment

Cell types	Control BALB/c			Control ITGAM				10 ng/g DT				40 ng/g DT			
	CB1	**CB2**	**CB3**	**CI1**	**CI2**	**CI3**	**CI4**	**10_1**	**10_2**	**10_3**	**10_4**	**40_1**	**40_2**	**40_3**	**40_4**
White Blood Cells	2,2	7,3	2,2	0,6	0,5	1,1	1,3	0,9	2,4	1,6	1,5	0,5	0,5	0,5	0,5
Monocytes	0,3	0,3	1,1	0,3	0	0,2	0,1	0	0	0	0,3	0	0	0	0
Large Unstained Cells	0,1	0,2	0,2	0,3	0,2	0,1	0,2	0,7	0,1	0,1	0,1	0,3	0	0	0

Table 5. Mean scores (G/L) and standard deviations of the leukocyte cell counts in the different groups

Cell types	Control BALB/c		Control ITGAM		10 ng/g		40ng/g	
	mean	SD	mean	SD	mean	SD	mean	SD
WBC	3,90	2,940	0,88	0,386	1,60	0,616	0,50	0
MONO	0,57	0,460	0,15	0,129	0,08	0,150	0	0
LUC	0,17	0,060	0,20	0,082	0,25	0,300	0,08	0,150

In the BALB/c control group the mean white blood cell count was 3.90 G/L, the monocytes and large unstained cells 0.57 and 0.17 G/L respectively (Table 5). The transgenic control group presented average white blood cells and monocyte counts of only around 25 % compared with the BALB/c group. However, when looking at the positively skewed distribution (see Table 1) and taking this into account, the ITGAM control group is closer to 50 % of the BALB/c. This is also indicated by the relatively large standard deviation. The large unstained cell numbers remained fairly similar between the two control groups. The

quantity of white blood cells in the low concentration (10ng/g) DT group was 1.60 G/L, which is 2.3 G/L less than in the BALB/c and surprisingly double the amount of the ITGAM control. Number of monocytes in the low dosage DT group was 53 % compared to the ITGAM control group and only 14 % compared to the BALB/c mice. (See table 2 for exact values) The number of LUC showed a slight increase compared to both control groups. Finally, in the high concentration (40 ng/g) DT group, the amount of white blood cells was calculated to only 12% of the volume in control BALB/c. There was no quantifiable amount of monocytes. The volume of LUC was 50 % compared to BALB/c.

A two-way ANOVA was performed to evaluate whether our results were statistically significant. Results are summarised in Table 6. A p-value below the confidence level of 0.05 indicates a statistical significance between the factors and the dependent variables. In our experiment, the *treatment groups* (rows) and the *cell types* (columns) represented the factors, and the measured amount of the different cells represented the dependent variable. P-value for the first factor *treatment groups* (rows) was 0.356, suggesting that treatment group did not have a significant effect on the amount of cell types. This might be explained by the low number of subjects. The second factor *cell types,* on the other hand, did have a significant effect on the measured cell count, with a p-value of 0.0005. This indicates the amount of cells noticeably depended on the cell type measured, which is true considering the target monocytes were completely depleted compared to the other two cell populations.

Table 6. Summary of results of the two-way ANOVA without replication

TWO-WAY ANOVA WITHOUT REPLICATION						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	15,90133333	14	1,135809524	1,159552747	0,355659982	2,063540829
Columns	19,6	2	9,8	10,00486145	0,000526749	3,340385558
Error	27,42666667	28	0,97952381			
Total	62,928	44				

5.3. Bodyweight and feed consumption

The bodyweight of each mouse was measured every day for 3 or 5 days. Results are summarised in Table 7. Data on orange background indicate mice that were euthanized on Day 3 for peritoneal fluid and blood analysis. Data on green background represent mice that were excluded from the cytological investigation, but were included in the bodyweight and feed consumption measurement. Both bodyweight and feed intake were reduced by the end of the trial period, with the exception of the BALB/c group which showed an overall increase in the mean bodyweight. From Day 1 to Day 5 of the experiment the mean bodyweight increased by 0.9 grams in the BALB/c group, and decreased by 1.6 grams, 0.9 grams and 1.5 grams in the ITGAM control group, the low concentration DT group and high concentration DT group respectively. Interestingly, the biggest deviation was seen in the ITGAM control group. The ITGAM control group also demonstrated an overall reduction in feed consumption, from 3.2 grams on Day 1 to 2.9 grams on Day 5. In the other groups the feed intake was reduced by 0.1-0.13 grams.

Table 7. Day by day bodyweight and feed consumption measured in grams for each group, including daily mean score and standard deviation. Orange background indicates animals euthanized on Day 3 for blood and peritoneal fluid evaluation. Green background indicates animals retained for bodyweight and feed monitoring

	Day 1	*Day 2*	*Day 3*	*Day 4*	*Day 5*
Control BALB-C					
	21,3	20,6	22,2		
	20,1	20,3	20,9		
	18,9	19,2	21,0		
	21,0	20,6	20,8		
Mean weight	20,3	20,2	21,2		
Weight SD (±g)	1,069	0,665	0,655		
Average feed consumption (g/mouse)	3,2	3,3	3,1		
Control ITGAM					
	17,3	17,8	19,0		
	20,0	19,7	20,4		
	23,4	23,7	23,8		
	22,0	22,4	22,7		
	21,7	21,8	21,9		
	18,0	18,4	17,7	17,2	17,4
	19,8	19,0	18,5	18,3	18,9
	19,0	13,6	19,0	19,2	19,5
Mean weight (g)	20,2	19,6	20,4	18,2	18,6
Weight SD (±g)	2,091	3,174	2,201	1,002	1,082
Average feed consumption (g/mouse)	3,2	3,3	3,1	3,3	2,9
10 ng/g					
	23,0	20,5	21,2		
	24,5	17,0	17,4		
	19,3	23	22,5		
	17,6	24,5	24,7		
	19,8	20,2	20,6	20,1	19,0
	20,5	19,8	18,5	18,6	20,8
	19,0	18,0	19,4	20,7	18,5
	20,6	19,8	19,6	19,4	20,0
Mean weight (g)	20,5	20,4	20,5	19,7	19,6
Weight SD (±g)	2,231	2,439	2,323	0,906	1,028
Average feed consumption (g/mouse)	3	3	2,8	2,9	2,9
40 ng/g					
	24,5	20,3	20,7		
	24,0	18,6	19,4		
	20,4	23,5	23,5		
	17,9	22	22,7		
	17,9	17,3	19,5	17,5	18,5
	18,6	17,5	18,4	19,0	18,9
	17,5	17,5	18,0	18,4	17,8
	17,4	18,5	18,3	18,4	18,0
Mean weight (g)	19,8	19,4	20,1	18,3	18,3
Weight SD (±g)	2,923	2,313	2,072	0,618	0,497
Average feed consumption (g/mouse)	2,83	2,91	2,83	2,9	2,7

6. Discussion

The results of the negative control groups BALB/c and ITGAM were compared with those of the treated groups to evaluate the concentration of diphtheria toxin needed for conditional cell ablation in transgenic mice expressing a functional DT receptor. Both control groups showed similar values of large unstained cells, while the amount of white blood cells and monocytes in BALB/c were increased by twofold and threefold compared to the transgenic control. The group treated with 10 ng/g toxin showed a marked decrease in monocyte amount with a mean value of 0.08 G/L compared to the 0.57 G/L in the BALB/c. Monocyte population in the group treated with 40 ng/g was not computable, indicating successful depletion.

Saito et al. (2001) worked with three different strains each showing varying levels of expression of the human heparin binding EGF-like growth factor. He injected diphtheria toxin in 4 different concentrations; 5 ng/g, 50 ng/g 500 ng/g and 5μg/g. Although we used a different strain in our study, I have chosen to compare our results with those of Saito's Tg16 strain, as it represented a moderate level of receptor expression. For comparison I will evaluate whether his observations at 5 and 50 ng/g toxin concentration corresponds to our 40 and 10 ng/g concentration. In Saito's study, intramuscular injection of 500 ng/kg resulted in a rapid increase in serum AST and ALT 12 hours after injection, and death within 60 hours post administration. When 50 ng/g toxin was injected, serum level of AST and ALT started to rise at 50 hours post injection, reached a peak at 72 hours and then returned to basal level by 120 hours (Saito et al., 2001). This observation corresponded with our study, where the toxic effect was at peak level after 72 hours, demonstrated by the complete absence of monocytes on Day 3. In our case, sampling for further analysis was performed after euthanasia due to the difficulty of monitoring peritoneal macrophages *in vivo*. Thus the replenishing of cells is disregarded in our study. Saito et al. (2001) observed that injection with 5 ng/g had no impact on the activity of hepatic enzymes in the blood. In our study, administration of 10 ng/g toxin lead to an 88 % percent reduction in peritoneal macrophages, suggesting that the minimum amount of toxin needed to completely eliminate target cells is close to 10 ng/g. This observation is shared by scientists at the University of Cambridge who, in a study on atherosclerosis from 2007, found that a dosage of 10 ng/g of DT reduced

the peripheral blood monocytes by 88.5 ± 4.9 % (Stoneman et al., 2007). They also demonstrated that an increase of the DT concentration to 15 ng/g had no significant effect on additional reduction of monocytes, further confirming that a concentration of between 10-15 ng/g DT is sufficient for specific cell ablation.

Results from the bodyweight and feed consumption measurement demonstrated that the greatest reduction was observed in the control ITGAM mice. This indicates that rather than the DT administration and the subsequent drop in monocytes, other factors may have adversely influenced feed consumption in the ITGAM control group. The overall reduction in feed intake and bodyweight in the DT test groups may be side effects of the toxin, or it may suggest that diminishing monocyte count has a negative impact on appetite. However, neither feed intake nor bodyweight showed a consistent decreasing tendency in any of the groups. The fluctuating values combined with the low number of subjects and the short experimental phase render it difficult to draw definite conclusions. In order to obtain more reliable results, it would be necessary to monitor a greater number of individuals for a longer period of time.

In conclusion, it can be pronounced that conditional cell ablation using DT insensitive eukaryotes remains a simple and widely applicable approach, which yields reproducible results. The study demonstrated that a dosage of 40 ng/g DT causes complete monocyte ablation in transgenic mice. In addition, a reduction of target cells by 88 % was observed already at 10 ng/g DT. With this observation in mind, considering previous research showing that a concentration of 15 ng/g had no further influence on cell number, it is likely that 10-15 ng/g is sufficient for complete cell ablation. To reduce the chances of unwanted side effects in cell ablated mice participating in further artificial infection studies, keeping the DT dosage as low as possible would be beneficial.

7. English summary

Genetic engineering allows scientists to manipulate an organism's genome by introducing or deleting specific segments of DNA. Transgenic animals missing or expressing a certain gene product are widely used in biomedical research to investigate the function of a particular gene. Furthermore, transgenic animals have proved a useful tool for studying specific cell populations *in vivo*. A frequently used method for such studies is by modifying the target cell type into expressing a toxin receptor. Mice do not express a functional Diphtheria Toxin Receptor (DTR), hence genetically modified mice displaying tissue specific expression of DTR are widely used in immunology research to examine the effect of an acute depletion of certain cells targeted by DT. Following toxin administration, the target cell populations are destroyed. The experimental animals can then be used in further artificial infection studies. By monitoring the animal's response to various stressors in the absence of certain cell types, a greater understanding of the function of these cells can be obtained. The aim of the study was to determine the dosage of diphtheria toxin needed to deplete macrophages in ITGAM mice. DT was administered in two concentrations; 10 ng/g and 40 ng/g. We also investigated the short term effect of the toxin mediated cell depletion on bodyweight and feed consumption, but research concentrated on the changes in the cell population of monocytes, white blood cells and large unstained cells. The effect of the toxin treatment was demonstrated by determining the amount of the different cell types in peritoneal fluid samples and blood smears collected from experimental animals at Day 3 of the trial.

In conclusion, it can be pronounced that conditional cell ablation using DT insensitive eukaryotes remains a simple and widely applicable approach, which yields reproducible results. The study demonstrated that a dosage of 40 ng/g DT causes complete monocyte ablation in transgenic mice.

8. Hungarian summary

A génmanipuláció lehetővé teszi egy adott organizmus genomjának megváltoztatását, specifikus DNS szegmensek kivágása, illetve beépítése révén. Az orvosbiológiai kísérletek során széles körben használják a meghatározott gén által kódolt tulajdonságot hordozó, vagy azzal nem rendelkező transzgénikus állatokat, az adott gén funkciójának vizsgálata céljából.

A transzgénikus állatok továbbá rendkívül hatékonynak bizonyultak a különböző sejtpopulációk *in vivo* tanulmányozása esetében is. Az ilyen vizsgálatok során gyakran használt módszer hogy a célsejt típusát módosítják, egy toxin receptort expresszáló sejtté. Az egerek nem expresszálnak diftériatoxin-receptort (DTR), ezért a genetikailag módosított egerek, melyekben szövetspecifikus DTR expresszió jellemző, széles körben alkalmazottak immunológiai kísérletekben bizonyos sejttípusok DT általi depléciójára. A toxin beadását követően a célsejt populáció elpusztul. A kísérleti állatok így a továbbiakban mesterséges fertőzéssel kapcsolatos tanulmányokban vehetnek részt. Meghatározott sejttípusok hiányában, az állatok különböző stresszorokra adott válaszait megfigyelve, jobban megérthetjük ezen sejtek funkcióját.

A vizsgálatunk célja az volt, hogy meghatározzuk a diftériatoxin szükséges mennyiségét, ami a makrofágok kimerítéséhez szükséges transzgénikus ITGAM egerekben. A DT-t 10 ng/g-os, illetve 40 ng/g-os koncentrációban adagoltuk subcutan injekció formájában. Vizsgáltuk a toxinmediált sejtabláció rövidtávú hatását a testsúlyra és takarmány-fogyasztásra, de a kísérlet elsősorban a monocyta, fehérvérsejt és a large unstained cell (LUC) sejtpopulációk változásira összpontosított. A toxinkezelés hatását igazolja az öt napos próba után a kísérleti állatokból gyűjtött peritoneális folyadékmintákban, illetve a vérkenetekben található különböző sejttípusok mennyiségének megváltozása.

Végezetül fontos megemlíteni, hogy a a legegyszerűbben és legszélesebb körben a feltételes sejtkimerítésre használt diftériatoxinra kevéssé érzékeny eukarióták alkalmazhatóak, így ezektől várható a legjobban reprodukálható eredmény is. A vizsgálat kimutatta, hogy a 40 ng/g-os DT koncentráció súlyos monocyta ablációt okozott ITGAM transzgénikus egerekben.

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