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Future alternatives in "3Rs": Learning from history

Tohru Inoue and Yukio Kodama

National Center for Biological Safety and Research, National Institute of Health Sciences

Corresponding author: Tohru Inoue, Ph.D
National Center for Biological Safety and Research, National Institute of Health Sciences
1-18-1 Kamiyohga, Setagayaku, Tokyo 158-8501, Japan
Phone: +(81)-3-3700-1564, Fax: +(81)-3-3700-1622, tohru@nihs.go.jp

Abstract
A large decrease in the number of experimental animals utilized in testing and research was reported in the last decade (Surveyed by Expt'l Animal Soc.[1]). For rats, the numbers used in experiments in Japan were 2.09 million in 1995, 1.53 million in 1998, and 1.24 million in 2001. Thus, there was a 40% decrease in the number of rats used from 1995 to 2001. For mice, a larger decrease (58%) was also observed, from 6.68 million in 1995 to 2.80 million in 2001. These decreases were clearly due not only to the development of 3Rs (i.e., Reduction, Refinement, and Replacement of animal use) in alternative research, but also to marked changes in the focus of experimental animal biology. In the academia, animal experiments using wild-type mice have decreased in number to a large extent relative to those using genetically modified mice because of the mechanistically much reliable outcomes obtained by genetically modified mice than those from wild-type animals. Yet, biological safety studies for pharmaceutical development as well as industrial chemical safety studies utilize conventional toxicological bioassays.

Keywords: 3Rs, Claude Bernard, Bruce N. Ames, Patric O. Brown

Introduction

Historically, three scientists are recognized in relation to the history of experimental animal use; the first, the initiator of experimental animal research; the second, the first contributor to the marked reduction of the number of experimental animals used; and the third, a potential contributor, who invented an ultimate method for reducing the number of animals for future research, the gene chip technology. The use of animals in experimental studies was initiated by Claude Bernard (1813-1878), originally who was trying to put an end to human vivisections common at that time; thus, he came to be regarded as "the devil of experimental animals." The most remarkable contribution to reducing the number of experimental animals used was made by Bruce N. Ames, who rescued innumerable animals that might have been used for genotoxic carcinogenicity studies. Another contribution may be attributed to Patrick O. Brown, who invented transcriptomics, which can be used to elucidate the underlying mechanistic background of phenotypes of experimental animals; the method is considered to have eventually led to the minimization of experimental animal use. Consequently, the most essential and powerful driving force for future alternatives may be minding the 3Rs but also the promotion of basic sciences and technologies.

1. Claude Bernard – An initiator of animal experiments

Claude Bernard (1813-1878)

Bernard was born in the village of Sain-Julien in 1813, and went to Paris at the age of twenty-one. As reported, he first wanted to be a play wright, but took up medical studies on the advice of a literary person. He learned medical science from the famous Françoise Magendie, and earned his PhD after pursuing the study of gastric acids. He was appointed as Magendie's deputy professor at the college in 1847, and made seminal discoveries such as those of hepatic glycogen, vasomotor neurons, and curare narcosis. Any of these discoveries must have made him an accomplished medical scientist. Because of his scientific principles, he strictly defined *observers* and *experimenters*, critically. He called observers as those who do not alter "nature", but statically observe the ostensible world; whereas experimenters are those who purposely alter "nature" to obtain a reaction, and seek natural responses behind the phenomenal world. He strongly recommended the use of living organisms to obtain responses, and seek natural reactions behind the phenomenal world. This is the reason why he emphasized the use of vivisection in science throughout his life. In his major discourse on scientific methods, "An Introduction to the Study of Experimental Medicine" (1865), Claude Bernard described what makes a scientific theory good and what makes a scientist important and a true discoverer. Unlike many scientific writers of his time, Bernard writes about his own experiments and thoughts, and uses the first person[2].

Although Bernard was the first scientist who initiated the use of animals in experiments, his original aims at that time were to criticize physicians and to rescue humans from iatrogenic accidents due to poor and insufficient surgical treatments. However, his wife and daughter initiated the first "animal rights campaign" immediately after his death[3], because of their intense aversion to Bernard's animal studies without using anesthesia, namely, vivisection, although this is ironically the best and appropriate method of determining the response of experimental animals.

It is about a century since Bernard started a systematic education on animal experiments. Experimental studies using animals changed last decades because of not only a greater awareness about animal welfare, but also greater decreases in the need for conventional experiments. Accordingly, in 1984, the International Guiding Principles for Biomedical Research Involving Animals was established. Then, in 1985, the European Convention also established the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes.

In Japan as well also, laws for animal care were successively passed in the 1970's. The Act for Animal Welfare and Proper Administration was passed in 1973, and the Guidance Documents for Experimental Animal Maintenance and Proper Administration in 1980. The Guideline for Experimental Animal Use was established in the same year in 1980 by the Japanese Academy of Science, the Guideline for Proper Use of Experimental Animals in 1987 by the College Union, and the Extension of Animal Life and Ethics by the Japanese Academy of Science in 1996. Recently, the establishment of the Act for Animal Welfare and Proper Use in Experiment was issued in 1999. Despite these guidelines, we could not eliminate all the animal experiments at the moment. However, we are now at the turning point in the history of experimental animal use.

2. Bruce N. Ames – accomplished the most prominent alternative study –

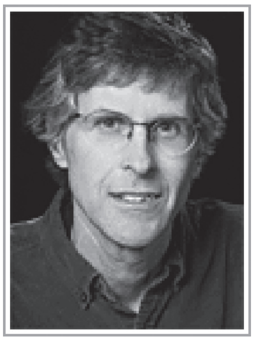
Bruce N. Ames

We now introduce a scientist who developed the revertant mutagenesis assay, Bruce Ames[4]. Ames is now a Professor of Biochemistry and Molecular Biology at the University of California, Berkeley. He is a member of the National Academy of Sciences and he was on their commission on life sciences. His publications of more than 450 led to his being among the most cited scientists.

The idea of mutation induced by chemical compounds was first described in 1944[5-6]; this was about 30 years prior to Ames' development of revertant mutagenicity assay. Chemical mutagenesis became the focus of considerable attention, because large amounts of industrial chemicals started to be used in various industries in the mid-twentieth century. Afterwards, because Ames' test enabled the detection of most mutagenic compounds, it has contributed greatly to a large reduction of the number of experimental animals used for in vivo mutagenicity bioassays. What Bruce Ames originally proposed was to use an induced bacterial gene mutation as an evaluation tool for mammalian mutagenesis. He attempted to develop a system for incorporating mammalian microsomal metabolism to the assay also by him, which is presently known as the S9-mixture[7]. It took a very long-time to establish the test system after considerable debate, because scientists at that time had to learn the difference between direct genotoxic carcinogenesis and indirect genotoxic carcinogenesis, namely, epigenetic carcinogenesis. However, after the establishment of the test system, innumerable experimental rats and mice were saved from carcinogenicity bioassay. Although Ames did not intend to save experimental animals by his invention, knowing such common rule of mutagenicity in genetics between Salmonella and mammalians made innumerable number of reduction in experimental animal use possible. Thus, from the study of Ames, our conclusion on alternative studies, is, that an essential strategy for reaching an alternative goal may be the "Development of True Sciences."

The current major interests of Bruce N. Ames are reported to be the determination of optimum micronutrient intake for minimizing human DNA damage as a preventive measure against cancer, and the study of other degenerative diseases associated with aging.[8-9]

3. Patric O. Brown – gene chip technology



Patrick O. Brown

The third person who we introduced is Patrick Brown, who invented a new methodology, that is, gene chip technology[10]. The gene chip technology and the consequent toxicogenomics[11] that he developed were supposed to rapidly minimize experimental animal use to a large extent[12] (Meeting proceedings from ECVAM-ICCVAM/NICEATM, 2006).

The establishment of the genome sequencing program in 2000 was supposed to be a strong driving force for the progress of alternative studies, particularly via toxicogenomics. All the information derived from animal experiments is incorporated in the genome expression database, that is, "computer mouse", which may be virtually used in the near future even without actual animal experiments.

The method established by Patrick Brown is "molecular microscopy", which enables the differentiation of patterns of gene expression profiles[13]. We showed sample expression profiles of genotoxic compounds studied by the consortium of International Life Science Institute (ILSI), which showed a short-term differential prediction of chemicals with DNA-binding affinity, such as cisplatin, methotrexate, mitomycin C, and chemicals with indirect genotoxicity, such as, taxol, hydroxiurea, and etoposide. Such a rapid and easy prediction may greatly contribute to the realization of essential purposes leading to the development of 3Rs.

Concerning the gene expression profiles, linear increase in dose-response relationship obtained by a conventional testing protocol may not be always applicable each other. In the presented example of microarray data after radiation exposure, because the expression levels of some genes increase with radiation dose and those of some genes decrease with increasing radiation dose, the dose-response relationship obtained by a conventional toxicological testing protocol can be assumed as the only phenomenologic outcome on the basis of one aspect. Rather, we recommend that the dose-response relationship should be considered complex, and that these combination profiles per se, may be essential biomarkers. The authors showed other sample data obtained after whole-body radiation in which one can observe dose-related expression profiles, on one hand, and dose-specific expression profilings, on the other.

Another issue that the authors introduced was age-related stochastic and probabilistic gene expression profilings, which can also be visualized in nontreated senescent mice when one focuses on their individual gene expression. By linear configuration for gene expression, one can clearly recognize that the divergent expression profiles of each individual mouse were not due to an error, but biological diversity with aging. Moreover, representative responsible genes showed clear differences between 2-month- and 21-month-old profiles, which elucidated the age-related responsible gene ontology, represented by the senescence-specific genes[14].

In the cases of experimental myeloid leukemias, spontaneous leukemias are differentiated from those of radiation-induced myeloid leukemias by their different responsible gene intensities in the line configuration of the expression gene profilings. They are also differentiated by the analysis of principal components, which are observed from the three dimensional expression. These databases are also supposed to be essential information for developing 3Rs supported by basic science.

Toxicogenomics sometimes makes the categorical border between physiology and toxicology ambiguous. Similar genes, such as those encoding apoptosis-related genes, caspases, participate simultaneously as physiologic and toxicologic parameters. Toxicogenomics sometimes changes a toxicologic paradigm. Depending on such fluctuating changes in the cell cycle genes, for example, and many other cellular functions, which may be mild or severe, the degree of oscillatory ranges differs from one another, which may be new risk factors.

Conclusion

Lastly, as we mentioned above, the use of experimental animals has, unfortunately, not been completely eliminated to date. Thus, in this regard, we would like to emphasize that "science should progress further". Certainly, one may not accept any risky drugs that have not undergone preclinical animal testing for use in one's children. On the other hand, no one may believe that animal studies will be continued for more than 4-500 years from now. We believe that experimental animals may be eventually replaced by other technical systems developed in the future, although such systems are still technologically immature to replace everything at this moment.

Table 1. Surveillance of experimental animals used in Japan.

	1995	1998	2001
rats*	2.09 (100)	1.53 (73)	1.24 (59)
mice *	6.68 (100)	— (—)	2.80 (42)

* Million / (%)
Surveyed by the Society of Experimental Animals in Japan[1].

The authors emphasized that animal testing may be eventually replaced by other new technologies, and animal testing would eventually disappear. Some people, however, believe that animal testing should be replaced immediately by other technologies; hopefully today, if not today, maybe tomorrow! These gaps may be filled by nominal driving forces such as humane animal welfare, industrial economy, and politics. However, the essential driving force for this matter may be the development of science itself, particularly by the development of "genome sciences". In other words, an elimination of animal experiments may be l'oiseau bleu (blue bird) of each scientist for the development of future science.

A recent survey by the Experimental Animal Society of Japan showed marked decreases in the number of experimental animals used[15]. As shown in the **Table 1**, the numbers of rats and mice used decreased to 59% and 42%, respectively, since 1995. The possible reason for these decreases is the obtainment of considerably clear-cut experimental results using a relatively small number of genetically modified animals, whereas unreliable experimental results are obtained with a relatively larger number of wild-type animals. These data strongly suggest the future possible reduction in the use of experimental animals.

References

1. Society of Experimental Animal: Annual surveillance of experimental animal use. Experimental Animal News 52 (5): 143-151, 2003.
2. Wikipedia; http://en.wikipedia.org/wiki/Claude_ Bernard
3. Society Against Animal Cruelty and Animal Protection established in 1854.
4. Ames BN, Lee FD, and Durston WE: An improved bacterial test system for the detection and classification of mutagens and carcinogens, Proc. Natl. Acad. Sci. USA., 70: 782-786, 1973.; Ames BN, McCann J, and Yamasaki E: Methods for detecting carcinogens and mutagens with the *Salmonella*/ Mammalian-microsome mutagenicity test. Mutation Research 31, 347-364, 1975.
5. Auerbach C. and Robson JM: Chemical production of mutations. Nature 154, 81, 1944.
6. Muller HJ: Artificial transmutation of the gene. Science 66: 84-87, 1927.
7. Ames BN, Durston WE, Yamasaki E and Lee FD: Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. Proc Nat'l Acad Sci USA. 70:2281, 1973.
8. Ames, B.N., DNA dames from micronutrient deficiencies is likely to be a major cause of cancer. Mutat Res 2001, 475: 7-20.
9. Liu, J., D. Killilea, and B.N. Ames, Age-associated mitochondrial oxidative decay: Improvement carnitine acetyltransferase substrate binding affinity and activity in brain by feeding old rats acetyl-L-carnitine and/or R-α-lipoic acid. Proc Nat'l Acad Sci U S A. 2002. 99: 1876-1881.
10. Brown PO and Botstein D: Exploring the new world of the genome with DNA microarrays. Nat Genet., 21: 33-37, 1999.
11. Inoue T, Pennie WD eds.: Toxicogenomics, Springer-Verlag Tokyo, pp. 1-11, 2003.
12. Corvi R, Ahr H-J, Albertini S, et al. Meeting report: Validation of toxicogenomics-based test systems: ECVAM-ICCVAM/NICEATM considerations for regulatory use. Environ Health Perspect 114 (2): 420-429, 2006.
13. Hirabayashi Y, Inoue T, Toxicogenomics applied to hematotoxicology. In: Joegen Borlak Ed. Handbook of Toxicogenomics-Strategies and Applications. Wiley-VCH, Verlag GmbH & Co. KGaA, Weinheim, 2005, pp. 583-607.
14. Authors: unpublished observation.
15. Society of Experimental Animal: Annual surveillance of experimental animal use. Experimental Animal News 52 (5): 143-151, 2003.