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CHAPTER 32

TOXICOLOGY: COMPLICATIONS CAUSED BY MURINE VIRUSES AND MYCOPLASMAS

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I. INTRODUCTION

Toxicology is the study of the harmful actions of chemicals on biological tissues (1). Although there has been a great deal of emphasis in recent years on *in vitro* alternatives to the use of animals in toxicology, the living animal remains the principal research tool. Alternative approaches and methods are not yet developed to the point where they can be considered as adequate total replacements (2). Rodents are the research animals of choice for many toxicology studies, particularly rats and mice because of their size, availability and life span.

As a general rule, toxicologists select parameters to be evaluated and attempt to define and understand the effects of treating rodents with chemicals at various dose levels. The animal may be considered in the simplest terms as a container of biological tissues to which chemicals are added. Observations are made and samples are taken to identify and study the changes that occur. Usually, a highly standardized rodent with minimal biological variation both at baseline levels and in response to experimental treatment is desired. Those uncontrolled variables that

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broaden baseline values, that enhance or inhibit responses or that, in combination with the test chemical, elicit new responses or prevent occurrence of expected responses are of particular concern to toxicologists because they interfere with the ability to differentiate and accurately evaluate toxicological responses.

A unique aspect of toxicology is that characterization of the biological response may be more important than learning the cause of the response. A large part of the task of toxicologists is to determine conditions under which exposure of subjects to potential poisons is safe or hazardous. To accomplish this, they must establish that a causal relationship between exposure and a biological effect exists and then must estimate the likelihood that the effect will occur under conditions of exposure. Their final analyses often have far-reaching repercussions. Toxic effects of chemicals are of great interest to the general public and to government agencies responsible for protecting its citizens from hazards over which individuals have little or no control. The results of almost all toxicology studies are subject to the most careful scrutiny because of the potential for restrictive regulations that may result. An erroneous conclusion easily could have significant medical, social, political or economic impact. Not arriving at a conclusion because results are equivocal negates the value of the study and reduces public confidence in all similar studies. Thus, any factor that may compromise experimental results must be avoided whenever possible.

It has been common knowledge for several years that some murine viruses and mycoplasmas have the capacity to cause biological alterations that can interfere with the conduct and interpretations of the results of toxicological studies. This fact is troublesome to most toxicologists and is a fitting topic for discussion. In 1983 a conference entitled "Complications of Viral and Mycoplasma Infections in Rodents to Toxicology Research and Testing" was sponsored by the Chemical Industry Institute of Toxicology. The proceedings are being published (3) and are recommended along with other pertinent reviews (4,5,6). The purpose of this presentation is not to repeat what others have already done so credibly but, rather, to attempt to provide a realistic assessment of the problem, how it is being handled, recommended management procedures and suggested research and other goals that will help improve the quality of future toxicology research and testing.

II. CAUSES OF THE PROBLEM

Of more than twenty separate viruses known to occur in laboratory rodents, only a few are of widespread concern to toxicologists because of their prevalence in commercial breeding colonies and in toxicology testing laboratories. A list of the most common murine viruses is presented in Table I. The list contains two Paramyxoviruses, three Coronaviruses and three Parvoviruses. Mice, rats, hamsters and guinea pigs can be naturally infected by two, Sendai Virus and Pneumonia Virus of Mice (PVM). Minute Virus of Mice (MVM) and Mouse Hepatitis Virus (MHV) occur naturally only in mice and Sialodacryoadenitis Virus (SDA), Rat Coronavirus (RCV), Kilham Rat Virus (KRV) and Toolan's H-1 Virus (H-1) are found only in rats.

TABLE I. Murine Viruses of Greatest Concern to Toxicologists

Virus	Group	Mouse	Rat	Hamster	G. Pig
Sendai Virus	Paramyxovirus	+	+	+	+
Pneumonia Virus of Mice (PVM)	Paramyxovirus	+	+	+	+
Mouse Hepatitis Virus (MHV)	Coronavirus	+	-	-	-
Rat Coronaviruses (RCV, SDAV)	Coronavirus	-	+	-	-
Minute Virus of Mice (MVM)	Parvovirus	+	-	-	-
Kilham Rat Virus (KRV)	Parvovirus	-	+	-	-
Toolan's H-1 Virus (H-1)	Parvovirus	-	+	-	-

TABLE II. Prevalence of Antibodies to Murine Viruses in U.S. Commercial Breeding Colonies (1981-1982)[a]

Virus	Mice	Rats
Sendai	9/20 (45%)	7/11 (64%)
PVM	8/20 (40%)	7/11 (64%)
MHV	9/20 (45%)	-
MVM	9/20 (45%)	-
RCV/SDA	-	9/11 (82%)
KRV	-	8/11 (73%)
H-1	-	2/11 (18%)

[a] Adapted from Collins, M.J. Jr. (7).

Recently reported prevalence data are summarized in Table II (7). It is clear that commercial breeding colonies are not yet free of viral contaminants. The fact that 11 of 20 mouse breeding colonies were free of Sendai, PVM, MHV, and MVM is important because it implies "clean" mice are available. The rat data are not as impressive but improvements may have been made even in the short time since 1982. It generally is accepted that isolated, well designed and managed breeding colonies can be freed of most murine viruses.

Keeping rodents free of viruses and mycoplasmas after they leave the breeder's facilities is another and more complex part of the problem. Antibodies to rodent viruses in bioassay animals and their prevalence in laboratories conducting carcinogenesis bioassays for the National Toxicology Program (NTP) during 1981-83 were similar to data for commercial breeders. Results of serologic screening of sentinel animals at six month intervals during two year studies and in experimental rodents at the end of 90 day and two year studies are presented in Table III. These figures are an update (8) of data presented previously (9). In this program, rats and mice were provided by NTP and are believed

TABLE III. Prevalence of Antibodies to Murine Viruses in Laboratories Conducting Bioassay Studies for NTP/NCI (1981-1983)[a]

	Number of Laboratories with Virus[b]			
	90 Day Studies		2 Year Studies	
Virus	Mice	Rats	Mice	Rats
Sendai	5/12 (42%)	5/10 (50%)	9/13 (69%)	10/14 (71%)
PVM	3/12 (25%)	5/10 (50%)	5/13 (38%)	10/14 (71%)
MHV	3/12 (25%)	-	8/13 (62%)	-
MVM	0	-	2/13 (15%)	-
RCV/SDA	-	5/10 (50%)	-	10/14 (71%)
KRV	-	0	-	7/14 (50%)
H-1	-	0	-	1/14 (7%)

[a] Adapted from Boorman, G.A. (8)

[b] Number of laboratories reporting positive results/ total number of laboratories conducting studies. Total number of samples submitted was 5282.

to have been free of all murine viruses with the possible exception of KRV which was known to exist at one source until 1982 (7). Sera were submitted by contract laboratories which represent a cross-section of the typical toxicology testing laboratories in the U.S. Serologic results presented indicate that Sendai, PVM and MHV in mice and Sendai, PVM and RCV/SDA in rats are common contaminants. The results probably are indicative of the current expectations one may have for keeping "clean" rodents free of murine viruses during toxicology studies in well managed modified-conventional facilities. In both mice and rats, the prevalence was slightly lower for shorter term studies, reflecting the difficulty of maintaining rodents free of infection for extended periods of time. The distribution of

TABLE IV. Distribution of Murine Virus Antibody Titers in Laboratories Conducting Bioassay Studies for NTP/NCI (1981-1983)[a]

Number of Viruses[b]	Number of Laboratories			
	90 Day Studies		2 Year Studies	
	Mice	Rats	Mice	Rats
0 [c]	5 (26,30,47, 50,50)	1 (40)	2 (20,40)	3 (30,50, 148)
1	4	4	2	1
2	3	2	6	1
3	0	3	2	8
4	0	0	1	1

[a] Adapted from Boorman, G.A. (8)

[b] Mouse sera tested for antibodies to Sendai, PVM, MHV, MVM. Rat sera tested for antibodies to Sendai, PVM, RCV/SDA, H-1.

[c] Number of samples submitted by laboratories to test in which antibodies were not detected. Numbers ranged from 20-148 as shown in parenthesis. Number submitted by other laboratories ranged from 10 to 347/ category. Total number submitted was 5282.

antibodies found in laboratories conducting bioassays is presented in Table IV. The data indicates that mice in 90 bioassays at five laboratories were free of four viruses and, at two laboratories mice were free of those viruses in chronic studies as well. One having fewer than a total of 500 animals in both groups was completely negative based on results of 50 samples. Only one of ten laboratories maintained bioassay rats free from viruses in short-term studies whereas 3 of 14 were able to keep their two year studies clean during the sampling period. One had approximately

1,000 rats on test in both subchronic and chronic studies and had no antibodies in 90 samples submitted. KRV was not included in this analysis because of the possibility that some infections were introduced with the animals. Although these data suggest that avoidance of contamination by murine viruses in the laboratory may be possible, it is the exception rather than the rule.

The omission of other murine viruses from this discussion is not intended to indicate that they are unimportant. Many do have the potential to cause significant problems if they are introduced into susceptible rodent populations being used for toxicological research. The classic example is the outbreak of Ectromelia Virus in 1979-80 (10). Fortunately, such outbreaks are not frequent occurrences. Serological evidence of Theiler's Encephalomyelitis Virus or Reovirus 3 is not uncommon in some laboratories but the prevalence and incidence usually are low and it is not obvious at this time that either represent real or potential widespread problems in toxicology studies. Murine viruses very rarely diagnosed include K Virus, Polyoma Virus, Lymphocytic Choriomenigitis Virus, Lactic Dehydrogenase Virus, the rodent Cytomegaloviruses, Mouse Adenovirus and others. K Virus appears to be near eradication (7). The others usually are limited to closed colonies or are associated with specialized types of research as either experimental models or tissue contaminants. The fact that any of the unlisted murine viruses can cause problems, even though the probability may be low, is justification to consider including them in any efforts to screen animals for adventitious agents. This is particularly true in laboratories where animals or tissues are being imported from other research facilities in the U.S. or abroad.

The incidence and prevalence of mycoplasmas is not clear. Some differences of opinion exist about the correlation between serological results and results of efforts to isolate Mycoplasma pulmonis (11,12). Recent data indicate that the prevalence of ELISA positive sera may be as high as 85 percent in both rat and mouse breeding colonies (7). This contrasts with isolation results for M. pulmonis of 4/13 (31%) in mouse colonies and 3/7 (43%) in rat colonies. Until recently, NTP did not include a test for mycoplasma in its screening battery because of the uniform absence of disease in test results. The evidence that infection may be much more common than disease has resulted in incorporation of the ELISA test. Expectations are that serologic evidence of mycoplasma infection in animals on test will not be uncommon, thus adding another agent(s) to the list for concern and further consideration.

The apparent high probability of infections by murine viruses or mycoplasmas in toxicology laboratories is not as great a threat to toxicologists as it would first appear. The diagnosis of infections in a laboratory does not necessarily mean that all studies being conducted are at equal risk to each infection or to any of them. In an update (8) of previously reported results (9) it was found that after completion of 136 ninety day bioassay studies in rats at 15 laboratories, no antibody to Sendai, PVM, RCV/SDA or H-1 were found in 87 (64%) and antibodies to a single virus were found in an additional 20 (15%). The remaining 29 studies (21%) were positive for more than one virus. Equivalent data for 106 two year rat studies completed in ten laboratories were 31 (29%) without evidence of infection, 22 (21%) with antibodies to a single virus and 53 (50%) having antibodies to multiple viruses. Of 143 ninety day bioassays conducted in mice, 100 (70%) were free of Sendai, PVM, MHV and MVM, 24 (17%) were infected by a single agent and 19 (13%) with multiple viruses. In 103 completed chronic studies 25 (24%) had no antibodies, 35 (34%) had antibodies against a single virus and 43 (42%) had antibodies to multiple viruses (8). Thus, by starting with virus free animals, it is possible to complete even long-term toxicological studies free of infection. The longer the study, obviously, the greater the risk. It is encouraging that so many studies are completed without evidence of viral infection.

It is also obvious that the threat of infection by murine viruses and mycoplasmas is real. That threat and the reality that infections do occur, whether they are diagnosed or not, constitute the cause of the problem.

III. CHARACTERIZATION OF THE PROBLEM

Traditionally, the specific research problems that result from infection by murine viruses or mycoplasmas are discussed agent by agent. Review papers previously cited and papers presented at this conference adequately describe the complications each agent can cause to different types of research efforts. The approach used here is to indicate where one or more infectious agents can interfere with the conduct and interpretation of standard toxicological experiments. References cited are intended to support examples presented rather than to be a comprehensive review of all the pertinent studies documented.

Most toxicology protocols involve administration of measured amounts of a test chemical to groups of selected species of animals via the oral or a parenteral route followed by visual observation, clinical testing, special studies and pathological examination to determine the treated animal's responses. Variables include the test substance(s); the animal species, stain or stock and sex; animal age or weight; dosage, route, frequency and duration of administration; and the type and sophistication of the observations, tests and examinations conducted before, during and upon completion of each study. Examples range from acute dose response studies based on a designated end point (frequently mortality), to highly complex toxicity studies of any length up to two years or more that may include interim as well as terminal pathological examination in conjunction with a variety of biochemical, hematological, immunological, behavioral, ophthamological, neurological and other tests and evaluations. Included are highly specialized studies in teratology, mutagenesis and reproductive toxicology. Also included are anatomical and functional studies of target organs or organ system such as the eyes, skin, lung, liver, the cardiovascular system and the central nervous system.

Each well designed toxicology study will include control animal groups containing adequate numbers for statistical analyses of the resulting data. A great deal of effort is expended in random selection of the members of control and treated groups and in managing the macro-and micro-environment to assure that all groups are equivalent and that the only treatment variable is the amount of test chemical administered. Why worry about a few infected animals? The uncontrolled variables represented by spontaneous infections, even when they are equally distributed among groups, can compromise the experiment. The degree to which results are compromised is dependent on many factors and may range from insignificant to devastating. One type of error occurs when the range of responses in infected animals is widened to the extent that real differences in responses between control and treated groups are not statistically or biologically significant. The second type of error occurs when treatment groups demonstrate differences from controls because of an interaction between the infectious agent or the disease process and the test substance. A classic example of the latter possibility is the effect that Sendai virus had on smoke inhalation studies (13). Results of a study in which infected mice were used indicated treatment related increases in mortality rates and development of adenomatous hyperplasia of the terminal bronchioles and

surrounding alveoli not found in the controls or in vaccinated animals free of Sendai. The effects could easily have been attributed to the treatment if the virus-free experiments were not performed. It is unusual to have an uninfected control group with which to compare results. In a two year inhalation study using rats exposed to methylene chloride, a significant increase in salivary gland sarcomas was detected in the high dose males (14). Clinical and serological results indicated that the rats had experienced SDA infections early in the exposure period, casting doubt on the validity of these results. It was impossible to determine whether the salivary gland tumors were a spurious result, a toxic effect of the chemical or a combined effect of the chemical and the virus.

Table V is a condensed list of the parameters that toxicologists use to evaluate biological responses. By addressing each category, it should be possible to better demonstrate the nature of the problem.

Physical observations and morbidity-mortality checks are basic to all toxicology protocols. They are conducted frequently enough to detect onset of toxic signs, beginning of recovery, time of death or a moribund state. In-life measurements of physical parameters also must be done frequently enough to detect sudden as well as gradual changes. Acute infections that are associated with overt disease and pathological changes are most likely to produce clinical signs that can mimic, obscure or enhance signs resulting from toxicity of the test substance. These include acute Sendai virus infections in mice (15), M. pulmonis infections in rats (11) and mice (12), SDA infections in rats (16) and rarely MHV infections in mice (17). In most cases, the age and genetic background of the animals and in some cases, the strain of the agent are limiting factors in the development of clinical signs. When they occur, many signs are nonspecific consisting of ruffled hair, reduced activity, huddling, weight loss or reduced rate of body weight gain and dyspnea when the respiratory tract is involved. These same signs are common indicators of toxicity. Deficiencies in body weight gain of more than ten percent in treated groups usually are considered to be a significant sign of toxicity. It is not unreasonable to speculate that the combined effect of infection and treatment can influence food consumption and conversion to a greater extent than infection alone resulting in errors in data interpretation. Infections may result in increased susceptibility to secondary infections by other viruses, mycoplasmas or bacteria (12,18,19). Chemical treatment may

TABLE V. Parameters Examined to Evaluate Toxicity Which Can be Influenced by Murine Viruses and Mycoplasmas

Physical Observations:	General Condition Motor Activity G.I. Signs Respiration CNS Responses
Physical Measurements:	Food Consumption Body Weight/Rate of Gain Organ Weight Mortality/Survival
Clinical Evaluation:	Hematology Urinalysis Serum Chemistry
Morphologic Evaluations:	Gross Pathology Histopathology Ultrastructure
Special Assessments:	Organ Function Cellular Metabolism Immunologic Alterations Carcinogenesis Behavior Reprodutive Capacity Teratogenesis Others

also enhance clinical disease (20). Either event, even if it doesn't cause death, complicates clinical evaluations of toxicity. Infection related deaths often are not attributed immediately to concurrent infections. Most are statistical events expressed as increased mortality rates or reduced survival rates. Although subsequent serological and pathological diagnoses will help identify the presence of infections, they will do little to help quantify the effects on physical responses caused by infection as compared to those by treatment.

Results of standard hematological, biochemical and urinalysis tests are evaluated in many toxicological studies to identify target organs and gain more information about

the specific effect of the test chemical. The effects that murine viruses and mycoplasmas may have on these parameters are not well documented. It is not unreasonable to presume that the susceptible host responds to these infections in more or less predictable ways depending on the pathogenesis and specific tissue tropism of the agent and the severity of the infection. For example, leucopenia is a common response to viral infection. It is also a common observation following exposure to many immunosuppressive chemicals. It seems probable that in the presence of acutely infected animals, only dramatic alterations in white cell counts induced by the test chemical may be statistically significant whereas more subtle changes can easily be overlooked.

Animals that die during the course of a toxicological study or that are killed at the end of the study usually are subjected to a complete gross and microscopic examination to describe all morphologic changes present. Like physical observations, morphologic changes associated with lesions of murine viruses and mycoplasmas may range from none to very severe and from nonspecific to pathognomonic. It is extremely important that the pathologist be familiar with the full range of lesions that may accompany infections in order to help detect the presence of the infection and to differentiate, when possible, those lesions caused by the infectious agent from other spontaneous lesions and those caused by the test substances. Toxic lesions in acute and short-term repeated dose studies are often subtle making separation of degenerative or atrophic changes and normal variation very difficult. Any change attributable to concurrent infection(s) can make the job near impossible. In susceptible adult mice, Sendai infections frequently will be associated with lesions of the respiratory tract (15). Squamoid metaplastic epithelial changes are common in reparative stages and bear a striking resemblance to carcinoma *in situ* or metastatic carcinoma (21,22). Respiratory tract lesions may or may not be so obvious in the rat (16) or in either species with *M. pulmonis*, although the latter can cause severe lesions especially when complicated with another infection (12). In mice, lung lesions are uncommon with PVM (15) and MHV (17) and nonexistent with MVM (23). MHV has been associated with relatively minor lesions of the gastrointestinal tract (24). Other strains may effect lung, liver or brain. In rats, acute infection by SDA will produce lesions of the submaxillary and parotid salivary glands and the lacrimal glands along with rhinitis and keratoconjunctivitis (16). On occasion RCV may cause a mild interstitial pneumonia and

KRV has been associated with hemorrhage and necrosis in the central nervous system. Lesions are not reported for PVM or H-1 in adult rats (16). Inconsistencies in the presence of lesions and the fact that they frequently involve target organs are disconcerting to both the pathologist and the toxicologists who, together must assess the impact concurrent infections might have on results and treatment of data. It is not uncommon that the pathologist is the first to discover the presence of an infection. In the absence of serology and also the absence of signs of disease or identifiable morphological changes, it is probable that no diagnosis will be made and the effects of the infection, if any, will be attributed to other causes.

Special assessments include many procedures performed to detect specific responses that normally are not included in the course of general toxicity testing. Some tests have become so complex that subspecialties of toxicology have evolved or just as often, toxicology subspecialties of other disciplines have developed.

Direct examination or functional evaluation of organs known or suspected of being target organs of a test chemical are frequently performed in rodents. Ophthalmalogic examination, lung, liver and kidney function tests, and cardiovascular evaluation are examples of some of these. Complications can be expected if disease related changes in any of the target organs occur concurrently with the test. Keratoconjunctivitis and the possible sequellae associated with SDA (16), the lung lesions that can occur in mice with Sendai (15) and in rats with M. pulmonis (11) and the possibility of hepatic lesions caused by MHV (17) are all potential confounding factors. Sendai infection has been found to effect the distribution and deposition of material administrated via the respiratory route and also effect clearance of the same material from lungs and the respiratory tract (25,26). MHV can modify hepatic function, e.g., decrease the rate of iron uptake and effect retention in the mouse liver (27). Also enterotropic MHV has been implicated as the cause of malabsorbtion in adult mice (24).

Similar changes usually related to organ function, are those host responses to a test chemical that occur at the cellular level. Identification of these alterations coupled with determination of the metabolic fate of the chemical are important steps in studying a toxicant. M. pulmonis infection has been associated with changes in cellular kinetics (12). Acute Sendai virus infections can effect pulmonary metabolism (28). MHV can alter hepatic tissues resulting in changes in serum biochemical activity and

effecting mitotic response to injury (24). Interferon induced by a variety of methods can cause a reduction in hepatic microsomal monoxygenase activity (29). Sendai and MHV are interferon producers (15,17) and it has been suggested (28) that infections by either or both may be accompanied by this effect. M. pulmonis inhibits interferon induction (30). This could be a complicating factor if the test material given to an infected animal is an interferon inducer.

Behavioral toxicology is the evaluation of CNS function after exposure to toxic substances. Any infection that may involve the CNS directly or indirectly could cause behavioral changes. Possibilities include MHV which can produce brain lesions (17) and M. pulmonis which may be latent in the brain although there is no evidence of CNS disease or effect on behavior (31). Subclinical SDA infection in the absence of known CNS involvement resulted in poor performance in an animal behavioral study (32). It is possible that any acute infection could have this same effect.

Immunotoxicology is the study of the immunological effects induced in the host by administration of a test substance. There is little doubt that alterations in immune responses can represent early and sensitive indications of toxicity. There is also good evidence that several murine viruses and M. pulmonis can alter immune responses in infected animals. The number of tests available to study immune function is large but, in general, alterations can be classified as evidence of immune suppression or stimulation. Sendai virus has been shown to cause immunosuppression (33,34) as has a variant of MVM although the prototype MVM apparently does not (35,36). Under certain conditions, MHV has been implicated both as an immunosuppressive agent and as an immunostimulator (37,38). KRV also can suppress or stimulate immune response depending on conditions of the experiment (39,40). M. pulmonis apparently does not produce a significant immunological effect but M. arthritidis can be immunosuppressive (41,42). M. pulmonis has been shown to be a nonspecific mitogen for rat lymphocytes (43,44). Of major importance is the fact that these alterations can occur in the absence of clinical disease. The possibility of obtaining misleading results is very real. Another significant point is the probability that some chemically induced immunologic alterations are closely related to the ability of the test chemical to produce tumors. Infection induced alterations in immune response forseeably could effect the formation of tumors in treated animals.

Carcinogenesis testing is a major field of toxicological research. Protocols are designed to identify mammalian carcinogens, estimate the potency of their effect and provide a basis for human risk assessment. In practice the conduct of these studies is probably the most complex of all toxicology endeavors. In a single study more than 500,000 data points may be accumulated which must be tabulated and analyzed accurately. It should be no surprise that difficulties occur, not the least of which are spontaneous infections. M. pulmonis was first associated with an increase in respiratory tract tumors nearly 20 years ago (45). In a classic study, tumor incidence in rats infected with M. pulmonis, Sendai virus, PVM and/or RCV were increased as compared to equivalent infections in SPF animals (46). The number of tumors per animal also were increased. All infected animals had lesions of chronic respiratory disease at necropsy. In contrast, rats infected with M. pulmonis and Sendai virus demonstrated reduced numbers of pituitary and cervico-vaginal tumors (19). Reduced incidence of pulmonary adenomas have been attributed to Sendai virus and also delayed appearance of tumors (47,48). On the other hand, either an increase or a decrease in tumors was observed in Sendai infected Strain A mice depending on the chemical that was administered (49). H-1 virus in hamsters has been associated with a reduced incidence of spontaneous tumors, of neoplasmas induced by adenovirus infection and of chemically induced tumors (50,51). It has been suggested that MHV induced proliferative activity in the liver and gastrointestinal tract could be responsible for increased susceptibility to experimental chemical carcinogenesis (24). These confounding, often conflicting results are the cause of concern because results may be clouded by an element of uncertainty. In at least one long term carcinogenesis bioassay the presence of increased numbers of alveolar and bronchiolar neoplasms in treated female mice was determined to be inconsequential because of the possibility of a co-carcinogenic effect of concurrent Sendai infections (52). To place this in perspective, however, more than 200 chemicals have been tested for carcinogenesis and reported as negative or positive by NTP with very few recognized complications related to spontaneous infections.

Reproductive toxicology is concerned with the toxic effects of a test substance on both male and female reproductive functions. It involves assessments of alterations in the reproductive process resulting from chemical insult at various stages in an animals life. The periods of interest range from the development of individual germ cells

through fertilization, gestation, birth and weaning. As in other subspecialties, there are many types of protocols for testing and evaluating both male and female responses to chemical exposure including teratology studies. There is growing evidence that rodent reproduction studies can be compromised by mycoplasmas and a few of the murine viruses. M. pulmonis can cause genital infections in rats resulting in reduced birth rates (53,54). Reportedly, such infections can reduce breeding efficiency of rats by as much as 50 percent (12). Experimental evidence of decreased implantation (55) and increased fetal resorbtion (56) in the rat also have been reported. Because these are common parameters for measuring reproductive function it is easy to understand why toxicologists should be concerned. It has been reported that the production index of a mouse breeding colony was reduced by 50 percent when the barrier was broken and Sendai virus and MHV were introduced (57). Sendai alone in rats has been associated with decreased productivity (16), reduced litter size and retarded growth (58). When given via aerosol during early stages of pregnancy, embryos were resorbed. This was suspected as being the effect of stress due to respiratory disease rather than to the infection per se (59). Reduced fertility in rats has been attributed to SDA infections (60).

A variety of protocols other than those already discussed are used in toxicology. Space does not permit a protocol by protocol assessment of the potential complications that may be associated with spontaneous infections by any of the most prevalent murine viruses and mycoplasmas. There are two, however, which deserve a separate discussion with respect to their potential problems.

Acute studies are those in which the adverse effects of a single dose of a test substance or multiple doses given within 24 hours are identified. They are not limited in scope or purpose to the much maligned LD_{50} determination. Rather, they are an extremely important group of studies intended to assess toxic potential, to aid in the preliminary prediction of hazard, to generate data for use in risk-benefit assessments and to provide information on the mechanism of acute toxic action. Well-designed acute toxicity studies usually include both lethal and nonlethal parameters. They are particularly vulnerable to experimental error resulting from concurrent infections because of the high dose/rapid response characteristics inherent in most acute study protocols and the fact that infection can easily go undetected. Physical and serologic evaluations a few days or hours before study begins may not detect the

presence of acute subclinical infections. Serologic testing rarely is performed at the end of an acute study even when survivors are not killed until several days later. Pathological examinations are often perfunctory if done at all. The results of these studies are easily biased because relatively few animals are involved and because minor effects can lead to major alterations in response. Misinterpretations of results can have an adverse effect on the next tier of studies if erroneous data is used to establish dosage or otherwise influence the design. The danger is even greater when results are used to draw specific conclusions about toxicity. It is true that chronic studies are more likely to become infected because animals are at risk for a longer period of time. They also represent larger investments in time and animals. However, lower doses, daily observations of more animals, in-life serologic testing of experimental or sentinel animals and complete pathology examination improve opportunities for detecting concurrent infection, diagnosing the agent and making rational decisions on how to proceed and how to evaluate the results. In general, spontaneous infections are a greater hazard to short-term than to long-term toxicological studies.

Carcinogenesis studies may be the most complex of toxicological studies but inhalation studies undoubtedly are the most difficult. The major problems are concerned with administration of the test chemical including generation and distribution. There are other problems as well. The lung is not only the route of administration but also is an important target organ. The lung is the site for a large number of biological mediators and a whole range of drug metabolizing enzymes. Both local and systemic responses are important. Inhalation studies are particularly susceptible to mistinterpretation when rodents are infected with those murine viruses and mycoplasmas which invade the respiratory tract. These include Sendai, PVM, MHV, RCV/SDA and M. pulmonis in susceptible species. Such infections can produce signs of respiratory distress, alter pulmonary function, mimic lesions produced by chemicals, increase mortality and increase susceptibility to secondary infections further confounding the gross and microscopic pathology. They can also effect chemical distribution and deposition and alter concentration kinetics, metabolism, immune response and morphologic effects including development of tumors. Both short and long-term inhalation studies should be monitored carefully for evidence of infection because of the very adverse impact such infections may have on results.

This litany of circumstances under which murine viruses and mycoplasmas can complicate toxicological research is far from complete. Neither is it necessarily as intimidating as it may sound. It merely serves to emphasize the importance of recognizing the threat, good experimental design, faithful conduct of protocols and adherence to a regular program of diagnostic monitoring to identify any infections that may occur.

IV. COPING WITH THE PROBLEM

In order to obtain an unbiased assessment of how toxicologists deal with the real or potential problems of murine virus and mycoplamas infections in reporting their data, all papers published during a six month period in two toxicology journals were reviewed[1]. The review included a total of 142 papers published between October 1, 1983 and March 31, 1984. The relative importance of rats and mice as research animals in toxicology was emphasized. Rodents were used in 117 (90%) of a total of 130 animal studies. Rats or mice were used in 114 (98%) of the rodent studies. Also the use of a wide variety of stocks and strains of rats (5) and mice (20) coming from many different sources (26) was clearly demonstrated. The sources consisted of 21 commercial and five institutional colonies. The duration of rodent studies from the beginning of an experiment to euthanasia of the last animal ranged from a few hours to more than two years. In this sample, short-term studies of less than 60 days predominated totaling 96 or 82 percent of those in which rodents were used.

Only four papers addressed the health status of the rodents used other than to say they were healthy and in some cases, barrier reared. Two (from the same laboratory) indicated the mice were tested for viral antibody and found to be negative before the start of the experiments. One stated that "CRD free" rats were used without specifying how this was determined. Another group of investigators used "SPF" rats immunized against and assayed for Sendai virus

[1] Toxicol. Appl. Pharmacol., 71, Vols. 1-3: 1983 and 72, Vols. 1-3: 1984; J. Toxicol. Environ. Health, 12, Vols. 4-6: 1983 and 13, Vol. 1: 1984.

and tested for mycoplasma antibody. At the end of the study there was a 10% conversion rate from mycoplasma negative to positive that was not group related. No other discussion was offered. Interestingly enough these four reports were inhalation studies reflecting, perhaps, awareness and concern of the impact respiratory infections might have on such studies. This concern apparently did not prevail throughout the research community because there were 17 inhalation studies in the group reviewed which did not address the problem in any way.

If any study was complicated by prior or concurrent infection with murine viruses or mycoplasmas it was not apparent. One concluded that no other "treatment related lesions" were noted beyond those reported and discussed implying that other lesions did exist. Another reported "focal interstitial pneumonia" in 8 out of 60 mice in total distributed between all treatment groups with no further discussion. In a third study the authors stated: "There were numerous non-neoplastic lesions observed with similar incidence and severity in control and treatment rats. They were of the types frequently occurring as spontaneous diseases and incidental lesions of aging SD rats."

It is highly unlikely that all of the studies reviewed were conducted with animals free of infection. There were too many different rodents from different sources being used at different places. The few reported attempts to screen animals for infection before or after the studies and the lack of reported results, negative or positive, suggest most toxicologists are attempting to live unobstrusively with the problem until better solutions are found. This does not mean they lack awareness or are not doing a credible job of producing valid data. Most work closely with their Director of Animal Resources (or equivalent) and the clinical and anatomical pathologists who support their work. They do their best to prevent infections and they cope with those that are diagnosed by careful assessment of the data and cautious interpretation. They do not, however, report very much information regarding the microbial status of their animals before or during an experiment even when that information is available. It has been noted that even authors specifically documenting effects of a spontaneous infection on a particular study often fail to discuss or provide data on other adventitious agents in their test animals (28). Reluctance to report positive serological results or to discuss signs of infection is understandable but failure to provide such pertinent information should not be condoned.

V. SUGGESTED APPROACHES FOR IMPROVEMENT

The obvious solution to the problem is to eliminate all contaminating viruses and mycoplasmas from research rodents. This requires procurement of "clean" animals and their protection from exposure during shipment, conditioning or quarantine and the test (research) period. We know from experience that this will not happen overnight, but that should not deter us from striving to attain the ideal by dedicated adherence to established concepts and implementation of new ideas as more knowledge is gained.

While virus and mycoplasma free rodents are available, there are windows of vulnerability. Exposure may occur in the supplier's facility if there are virus and mycoplasma free rodents in some areas and infected animals in others. Frequently, all boxes of animals are brought to one loading area for dispatch. They may be transported in the supplier's truck or by a commercial carrier, perhaps, in the latter case, mixing boxes from several sources. Finally, boxes are received at the research institution, usually at a common point where exposure is possible if they are improperly handled. The potential for disease transmission and the actual incidence during transport are unknown. Filtered boxes have not been adequately field tested. There is a need to confirm that infections are or are not transmitted to susceptible rodents in these situations and to make improvements in equipment and/or procedures where indicated.

Infections can occur at the research facility after receipt if infected rodents are already there. It was mentioned earlier that institutions vary greatly, not only in their mission, but also in their physical facilities and ability to deal with infections of rodents. Prevention and control are difficult at best but that does not justify a laissez faire attitude. Overall, conscientious observation and application of the basic principles of good laboratory practice can not be overemphasized. A scientifically sound quarantine program for incoming rodents, particularly, those without known or certified microbial status, is mandatory. The same applies to cells, tissues, body fluids and other incoming products obtained from rodents. A comprehensive health monitoring program is essential. The physical facility must be adequate and have a good preventative maintenance program. It is always more difficult to control infection in a single large laboratory with common support facilities than in one consisting of multiple small independent units. The staff should be motivated, well

trained and continually informed about the latest developments in the field of rodent diseases. Free dialogue and full cooperation between scientists and animal facility management are an absolute necessity. Introduction and use of new animals and infectious agents must be governed by acceptable protocols.

A well planned health monitoring program especially aimed at antibody detection is necessary for valid evaluation of toxicological studies. The optimum time for antibody detection in newly arrived rodents and those already on test must be determined. The principal objective is to ascertain if and when animals were exposed to infection. The persistence of maternal antibodies to different murine viruses must be known if sera of young (4-6 weeks old) rodents are to be tested. The ability of these animals to produce active antibody also must be determined. More knowledge regarding the type and persistence of antibodies in older rodents is needed as well. At present, interpretation of serological results is more difficult than performance of the various tests. The major problem that toxicologists face is to be able to rapidly obtain sensitive, specific and reproducible results. Despite recent advances in technology, there is a great deal of room for improvement, particularly for the latter. There is an urgent need for a better organized and administered quality control program for serologic testing of murine viruses with broader participation.

A key to improvements in the use of rodents in toxicological studies is open communication between the concerned toxicologists, pathologists, microbiologists and veterinarians to plan, develop and assess the progress and results of research projects. Planning should include selection of the species, strain, source and number of animals to be used, parameters to be measured and criteria for serological monitoring or infection. Other subjects to be discussed include the extent of pathological examination, use of immunization to protect against murine viruses, employment of sentinel animals and the need for interim necropsies and examination during the course of the study.

In the event evidence is found of infection by a murine virus or mycoplasma suspected of being capable of compromising a toxicological study, what course of action should be taken? This is always a difficult but critical decison. All concerned parties must confer and examine the situation. There are no set rules to follow. Reactions to positive diagnoses of murine virus or mycoplasma infections will vary from study to study and laboratory to laboratory.

In some cases, especially those where the infection is accompanied by clinical signs and pathological changes clearly attributable to a particular agent decisions may be relatively easy. More difficult to determine is the course of action that should be followed in the event of serologic diagnosis without clinical signs, significant pathological changes or other indications of disease. These results usually are obtained during routine surveillance. In either case they invariably lead to a series of questions for which there rarely are immediate or good answers:

1. Are the results valid and do they constitute sound evidence of infection?
2. How many animals are (were) involved and are there active infections now?
3. Are other studies at risk and what is the threat to them?
4. What is the probability that behavioral, physiological or pathological alterations were (are being; will be) induced by the infection that could distort the experimental results?
5. What is the probability that control animal data will be adequate to prevent misrepresentation of the results?
6. What is the probability of reinfection in a new study after termination and restart?

As more information about the research complications or lack of complications caused by both clinical and subclinical infections is accumulated it may become easier to make decisions.

Economic considerations are very important in the decision making process but they should not be limited to consideration only of investments already made and the cost of restarting the study. The value of a study can be compromised to the extent that even very high accumulated costs will not justify additional investments. The possibility of disease transmission to other studies also has economic implications. Many times the question will not be, "Can we afford to terminate a study?" but rather, "Can we afford not to terminate?"

When a decision is made to publish the results of a study in which a murine virus or mycoplasma infection(s) was diagnosed those results must be interpreted with care. Alterations in responses and the absence of alterations in responses must be examined individually for potential causes. The analyses of all available data is even more critical now than when the decision was made to proceed. Although unintentional, the presence of infection was as

much a part of the study as any other intrinsic factor and should be reported and discussed. Any qualifications that have to be made to data interpretation should be made openly without prejudice with the view of expanding the data base and furthering our knowledge of these complicating infections.

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