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CHAPTER 34

SEROLOGIC TESTS FOR DETECTION OF ANTIBODY TO RODENT VIRUSES

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I. INTRODUCTION

It was the "fate" of Dr. Wallace Rowe to have his murine tumor virus experiments repeatedly terminated due to extraneous agents in the tumor systems being studied (1). A less tenacious investigator would have given up in disgust. Dr. Rowe instead designed experiments and developed techniques that contributed significantly to our current knowledge of these agents. "In particular, development of serologic procedures has received the greatest emphasis, since these have the great advantage of cheapness, rapidity, and applicability to large-scale work" (1). A major contribution of Dr. Rowe was the development of the antibody production test, originally published with reference to polyoma virus of mice (2). Although the precise methods have changed in the intervening quarter century, serologic procedures are still the mainstay of rodent virus detection.

The purpose of this chapter is three-fold: to suggest methods for sample collection and storage, to describe briefly the basis of the most commonly used serologic

procedures and to summarize the relative merits of the tests available for selected viruses. These areas were also discussed at an earlier symposium, the proceedings of which have recently been published (3). Data supporting many statements in the following discussion can be found in that chapter.

One sentiment cannot be over-emphasized: drastic clinical action should never be taken on the basis of a single serologic test. Results should be confirmed by an independent serologic assay or, preferably, by some method of virus detection such as virus isolation or antigen detection.

II. SAMPLE COLLECTION AND PROCESSING

Whole blood, collected aseptically and without additives, is the specimen of choice. Care should be taken not to encourage hemolysis either by forcing the blood through a small gauge needle or by storing the blood too long prior to separation of the serum fraction. Serum to be tested soon after collection can be stored at 4°C. Otherwise, samples should be frozen at -20°C.

III. COMMONLY AVAILABLE SEROLOGIC TESTS

Antibody detection assays may be divided into *in vitro* tests, not involving living host systems, and *in vivo* or neutralization tests, in which antibody is detected by its ability to interfere with viral infectivity.

The older, established *in vitro* tests (complement fixation and hemagglutination inhibition) are relatively inexpensive, whereas neutralization tests are costly and time-consuming due to their biological nature. Newer *in vitro* tests require expensive equipment, but their increased sensitivity may justify their cost.

Sensitivity and *specificity* are terms frequently used to evaluate serologic methods. *Sensitivity* refers to the ability of a test to detect antibody when the sample truly contains antibody. *Specificity* is the extent to which samples that do not contain antibody are correctly classified. These concepts are illustrated in TABLE I.

TABLE I

	Test Result	
Actual Status	+	-
+	A	B
-	C	D

Sensitivity = A / A+B; Specificity = D / C+D

A. Complement Fixation (CF) Test

The CF test is applicable to the vast majority of viruses and is still widely used in the human diagnostic laboratory (4). The test is based on complement being bound (fixed) by antigen-antibody complexes and involves the competition of two antigen-antibody systems (one a sheep erythrocyte - anti-sheep erythrocyte indicator) for a fixed amount of complement. There is wide variation in CF test methodology among laboratories, so it is difficult to make inter-laboratory comparisons of results. Because of features unique to specific virus groups, no uniform procedure can be adopted for the preparation of antigens. As with all serologic tests, interpretation of the CF test can be made only with reference to the appropriate controls (4).

B. Hemagglutination Inhibition (HAI) Test

The HAI test is used only for viruses which can agglutinate erythrocytes, but is technically much simpler than the CF test. It frequently can discriminate among closely related viruses but is often complicated by the need to pre-treat sera to eliminate non-specific inhibitors (substances in serum that mimic antibody) that may yield false-positive results. Inhibitors may be eliminated by treatment of sera with receptor-destroying enzyme (RDE, neuraminidase), kaolin or periodate (5). Acetone-ether extraction is frequently used to remove lipoproteins (6), common culprits in non-specific HAI reactions. Sera occasionally contain substances which agglutinate erythrocytes and mask the inhibiting effect of specific viral

antibody (5). Prior absorption of sera with erythrocytes may remove these substances. Accurate interpretation of HAI tests is completely dependent on appropriate controls (5).

C. Neutralization Test (NT)

This bioassay may be performed in animals or in cell culture. Cell culture assays are generally preferred due to lower cost. In addition, it is desirable to reduce the use of animals, from the humane standpoint, and to reduce the potential hazards associated with the study of animal viruses in their natural hosts. Variables in the NT include the challenge dose of virus and the incubation temperature of the virus-serum mixture prior to inoculation. The latter is important because infectivity of some viruses is reduced or lost by incubation at 37°C. Tests may be quantitative, in which pocks, plaques or fluorescent foci are counted, or semi-quantitative, in which endpoints are calculated from the proportion of hosts showing signs of infection after inoculation with virus-serum mixtures. In addition, the NT may use variable virus concentrations mixed with a constant concentration of serum or a single virus concentration mixed with serial dilutions of serum. The former method is relatively insensitive for demonstrating low concentrations of neutralizing antibody and requires volumes of serum that may not be available from small species such as mice. The latter method is more sensitive and requires smaller quantities of reagents. The NT may be used in those situations requiring differentiation among strains of virus (7).

D. Indirect Fluorescent Antibody (IFA) Test

The antigen in the IFA test is cells, a proportion of which are infected with the virus of interest, that are dried onto wells of teflon-coated microscope slides. After permeabilization of the cells with cold acetone, most antigens may be stored for long intervals at -70°C without loss of antigenicity. For testing, sera are added to individual wells on the slide followed by the addition of fluorescein isothiocyanate-conjugated anti-species immunoglobulin. The cells must be extensively washed between steps to remove non-specifically bound reagents. The test is read with a fluorescence microscope and is very sensitive. An experienced reader can interpret with great accuracy the specificity of a reaction. Since each well

contains uninfected as well as infected cells (Figure 1), there is a built-in specificity control. Also, conjugates specific for different immunoglobulin isotypes are commercially available. Because infected cells are the antigen, antibody to both structural and nonstructural proteins is detected (see chapter by Tattersall and Cotmore in this volume). This may contribute to the high sensitivity of the test. There are two major disadvantages of the IFA test. First, a fluorescence microscope is required. Second, the test is relatively labor-intensive and is not practical for laboratories performing large-scale testing. It is, however, frequently used as a confirmatory test in laboratories that routinely do large-scale testing by the enzyme-linked immunosorbent assay.

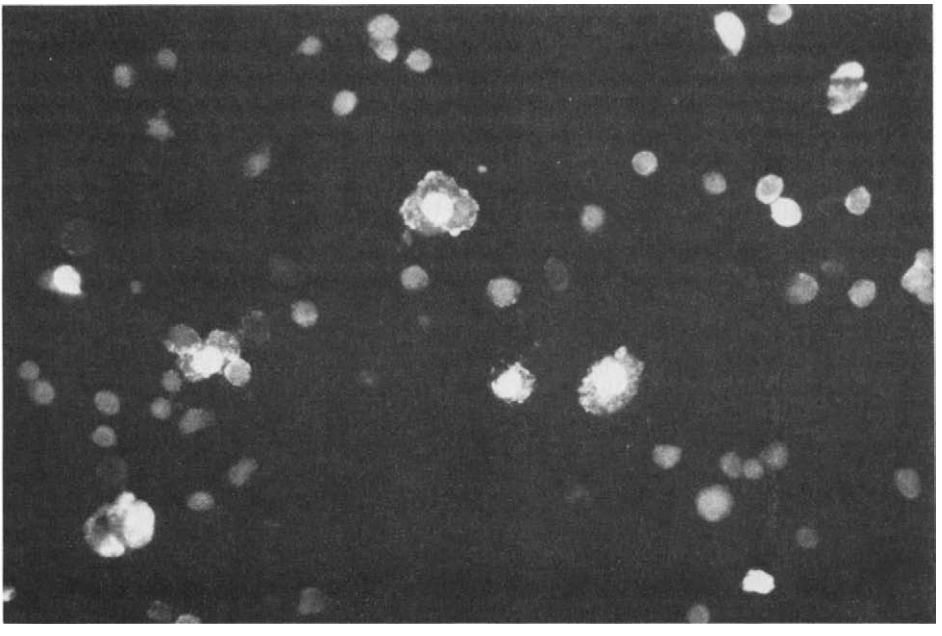

Figure 1. Photomicrograph of rat glial cells, clone C6, infected with minute virus of mice (Parvovirus). Fluorescence is seen in a proportion of cells. The inclusion of uninfected cells in preparations used for IFA represents an internal control for specificity of serum reactivity. Magnification is 800 X.

E. Enzyme-linked Immunosorbent Assay (ELISA)

The basis of this test is similar to that of IFA, except that the anti-species reagent is conjugated to an enzyme, usually horseradish peroxidase or alkaline phosphatase (8). Addition of substrate results in color development which can be assessed by eye or spectrophotometrically. The advantages and disadvantages of different enzymes and substrates have been extensively discussed in the chapter in this volume on virus isolation and identification. The ELISA is a very sensitive test for detecting antibody and can be used to distinguish among immunoglobulin isotypes. It is usually performed in microtiter plates and requires small volumes of reagents. However, plate washers and spectrophotometers, required for large-scale testing and quantitative results, are expensive. In addition, the use of purified antigen is frequently required to attain acceptable specificity. Thus, the laboratory may also have to acquire an ultracentrifuge to prepare antigens. We have recently developed a modified enzyme immunoassay for detection of antibody to minute virus of mice (9) and mouse hepatitis virus (see below). This test uses infected monolayers of cultured cells in 96-well cluster dishes as the antigen and is somewhat more sensitive for antibody detection than the IFA tests developed previously in this laboratory for those two viruses (10,11). Results are based on automated spectrophotometric readings. This modified test probably detects nonstructural as well as structural proteins. A similar test has been reported for Sendai virus (12), but it is more cumbersome since results are based on microscopic readings.

IV. QUALITY CONTROL

Whether test reagents are purchased or prepared internally, it is imperative that their quality be consistent and high. One way to insure reagent quality is to include appropriate controls in all tests. These should include at least positive and negative antigen controls and positive and negative serum controls. If the decision is made to use a commercially available test kit, that kit should contain all of these controls. Reference controls must be included in each test run and must conform to expectations in order for the test interpretation to be valid. Negative control antigens should be prepared in precisely the same fashion as the corresponding virus antigens and should contain, for

instance, the same batch of protein preservative. Testing should be performed by personnel who understand not only the technical aspects of the test(s) performed but also the theoretical basis of the assay(s). Adequate record keeping is also of great importance.

V. THE NEED FOR RAPID DIAGNOSIS

As discussed in Chapter 35 (this volume), rapid diagnosis of rodent virus infections is usually less crucial than rapid diagnosis of human infections, primarily because the strategies developed to deal with infectious disease problems are quite different for the two groups. In the rodent virus serology laboratory, a turn-around time of a week or less is commonly acceptable. However, if lymphocytic choriomeningitis virus, Hantavirus or ectromelia virus infection is suspected, rapid diagnosis is more urgent. In this case priorities may be assigned to accesssions to accelerate the testing process and provide results more rapidly to those in decision-making positions.

VI. COMPARATIVE DATA FOR SELECTED VIRUSES

A. Pneumonia Virus of Mice (PVM)

Early data (13) showed that antibody to PVM detected by CF and HAI tests developed simultaneously but that CF antibody deteriorated earlier than did HAI antibody. An ELISA was more sensitive than the HAI test for the detection of antibody arising early after experimental infection of rats with PVM (14). Based on later work, it was concluded that the NT yields an unacceptable proportion of false positive (nonspecific) reactions, although the data supporting this conclusion were not reported (15). During the course of the same study, ELISA was shown to be more sensitive than either the NT or the HAI test (15). However, in another study, a commercial ELISA kit was as sensitive as the HAI test for detecting PVM antibody in sera of mice (16, reviewed in 3).

B. Parvoviruses of Mice and Rats

The HAI and CF tests and the NT have been used to discriminate among strains of infecting rodent parvoviruses (17). The HAI test has traditonally been used for serosurveys because it is easy to perform. Sera to be tested for antibody to minute virus of mice (MVM) by HAI must be pre-treated to remove nonspecific inhibitors. If guinea pig erythrocytes are used in the HAI test, pre-treated sera may still yield reactions that cannot be confirmed using other test methods (10, reviewed in 3). Use of mouse erythrocytes reduces, but does not eliminate, this problem. IFA and ELISA have the advantage of detecting antibody to cross-reactive antigens, so MVM antigen may be used to detect antibody to the rat parvoviruses, rat virus (RV) and H-1 virus, as well as to homologous virus. The reactivity in IFA between MVM and the rat parvoviruses is not reciprocal in that the antigens of the Kilham strain of RV or of H-1 virus do not detect MVM antibody (17). An IFA test and commercial ELISA were equally sensitive for detecting MVM antibody in sera of mice experimentally infected with homologous virus (10).

C. Sendai Virus (SV)

The CF and HAI tests have been largely replaced by ELISA and IFA, which are more sensitive for Sendai virus antibody detection (18, 19; reviewed in 3). A modified enzyme immunoassay, termed an indirect immunoperoxidase (IIP) test, has been reported for the detection of Sendai virus antibody (12). Antibody titers in sera of experimentally infected mice were 10-fold higher in the IIP test than in the conventional ELISA, the HAI test or the NT. Sendai virus antibody titers in sera of conventionally housed mice ranged from 1:1000 to 1:10,000 in the IIP test, whereas the same sera had mean titers of 1:79 in the HAI test. Test sera did not require prior treatment, and antigen (infected cells) could be stored in microtiter plates for several months at refrigeration temperature (12).

D. Coronaviruses of Rodents [Mouse Hepatitis Virus (MHV) and Rat Coronavirus/Sialodacryoadenitis Virus (RCV/SDAV)]

As long ago as 1966, the sentiment was expressed that the CF test was inadequate for detection of antibody to MHV among mice infected several months prior to testing (20). It was, however, several years before more sensitive methods found their way into general use. In a comparison of an IgG ELISA with polyvalent MHV antigen to the CF test using a similarly prepared antigen, ELISA titers of mouse sera were generally 50- to 70-fold higher than CF titers (21). The same polyvalent MHV antigen has been used successfully in an ELISA to detect antibody to RCV/SDAV in rat sera (22). The cross-reactivity of the mouse and rat coronaviruses is fortuitous for the diagnostic virologist, since primary cells are normally used for growth of the rat agents. Despite extensive efforts to grow rat coronaviruses in established cell lines, only one cell line supporting RCV/SDAV growth has been identified. These cells, designated LBC, were established from a spontaneous mammary tumor of a Lewis rat and do not support MHV replication (23). An IFA test using MHV-S and MHV-JHM bivalent antigen was more sensitive than the CF test with homologus antigen for detecting SDAV antibody (11). The same IFA test was approximately equal in sensitivity to a commercial ELISA kit for detection of MHV antibody (11). A modified enzyme immunoassay, using MHV-JHM-infected cells as antigen, is slightly more sensitive than IFA for detection of antibody to the homologous MHV strain and eliminates the need to purify the detecting antigen (TABLE II; manuscript in preparation).

The NT distinguishes among MHV strains. The kinetic or timed NT is reportedly necessary to discriminate strains if the antibody is in the form of a hyperimmune reagent (24). If single-injection, early collection sera are used, MHV strains may be distinguished by a constant virus-serum dilution NT (25-27). The value of this exercise in the diagnostic setting is questionable because antigenic relationships of field isolates to prototype MHV strains are not accurate predictors of biological behaviour in the intact host (27). A Western blot procedure that identifies the viral proteins to which antibody is produced has also been reported (26). This is a powerful and sensitive tool in the research laboratory but will probably not gain widespread acceptance in the diagnostic laboratory, primarily due to cost.

TABLE II. Comparison of IFA and Modified Enzyme Immunoassay for Detection of Serum Antibody to MHV-JHM

Post-inoculation Day	SJL/J Mice		BALB/cByJ Mice	
	IFA	ELISA	IFA	ELISA
5	0/8	0/8	0/8	0/8
10	0/8	2/8 (1:20)	8/8	8/8
20	5/7 (1:10)	5/7 (1:480)	8/8	8/8

Data for three and six week-old mice inoculated intranasally with 10^3 $TCID_{50}$ of MHV-JHM are pooled. Results are given as number positive/number tested (geometric mean antibody titer). Sera were diluted 1:10 for IFA tests with bivalent MHV-S/MHV-JHM antigen and were screened at 1:20 in the MHV-JHM enzyme immunoassay prior to titration. Labelled reagents were FITC-conjugated anti-mouse immunoglobulin (Antibodies Incorporated, Davis, CA) and horseradish peroxidase-conjugated anti-mouse IgG (BioRad, Richmond, CA).

E. Ectromelia Virus

The 1979 outbreak of mousepox at the NIH and other institutions stimulated improvements in serologic tests for detection of antibody to this virus. The CF test failed to detect antibody in sera of half of the mice experimentally inoculated with the NIH isolate of ectromelia virus (29). An IgG ELISA using vaccinia virus antigen was developed and proved to be 40- to 140-fold more sensitive than the HAI test (29). IFA had the highest specificity of any of the tests used and confirmed that some false positives were detected with the ELISA (29). The HAI test had the lowest specificity of the assays compared (29) and, had no other methods been available, results from this test would have led to needless destruction of valuable colonies. The HAI test can, however, distinguish between vaccinated and infected mice. Using sera from mice infected with ectromelia virus, Buller et al. (30) showed that ELISA antibody

titers were significantly higher with homologous antigen than they were with vaccinia virus antigen. Serum ELISA titers of mice infected with vaccinia virus were equivalent in tests with ectromelia virus and vaccinia virus antigens.

When CF, ELISA and IFA were compared for detection of antibody to vaccinia virus in sera of vaccinated mice, CF and IFA detected antibody in 100% of vaccinated mice by day 10, whereas a commercially available ELISA did not detect antibody in 100% of sera until day 29 (31). Unfortunately, sera in this study were tested at a 1:10 dilution in the CF and IFA tests and at a 1:100 dilution in the ELISA.

F. Mouse Adenovirus (MAd)

Based on negative CF test results and the absence of clinical manifestations of disease, MAd is generally thought to be insignificant as an indigenous virus of laboratory mice. Recent experience in this laboratory belies that conclusion. Studies with experimentally and naturally infected rodents suggest that failure to detect MAd infections has resulted from use of a relatively insensitive test (CF) and improper antigen (the FL strain of virus). Tests of sera from experimentally infected mice showed that IFA was more sensitive than the CF test for detection of antibody to either of the known strains of MAd, MAd-FL and MAd-K87 (32). MAd-FL antigen did not detect antibody to MAd-K87 virus (32,33). A survey of mice from commercial sources showed that MAd-K87 antibody exists at low prevalence (33). Antibody prevalence was higher among conventionally housed institutional colonies. In addition, rats from four of six commercial sources tested had antibody reactive with Mad-FL, MAd-K87 or both viruses (33). These findings were confirmed by NT. These data suggest that an accurate assessment of the MAd status of laboratory rodents will require the use of two antigens used separately or in the form of a bivalent reagent.

G. Lymphocytic Choriomeningitis Virus (LCMV)

LCMV is a common contaminant of transplantable neoplasms of mice and hamsters (34). Because of the zoonotic potential of the virus, accurate and sensitive serology is of great importance. The CF test for LCMV yields a high proportion of anti-complementary and thus uninterpretable

reactions, and the NT can be unreliable because antibody titers may be low in chronic LCMV carrier mice (3). Hotchin and Sikora (35) recommend using the IFA test during the early phase of infection and the NT (micro plaque reduction) for detecting antibody during later phases of infection. In 1979, a comparison of seven methods for detecting LCMV antibody in human sera was reported (36). The authors concurred that IFA was the test of choice for rapid diagnosis soon after infection and that antibody detected by NT appears relatively late after infection. In addition, they stated that "the complement fixation test appears to be of little value for the serological diagnosis of infection with LCM virus," an opinion shared by this author. The same laboratory has developed both an IgG and an IgM ELISA for detection of LCMV antibody in experimentally infected CBA/J mice (37). Mean IgM titers were almost 1:1000 as early as five days post-inoculation (the first day tested) of 10^3 mouse ID_{50} of LCMV, and mean IgG titers peaked at greater than 1:100,000 on day 18 post-inoculation. The authors state that "titers climbed to heights not seen with any other method previously applied to this system." One advantage of IFA is that the specificity of reactions may be confirmed by absorbing suspect serum samples with virus-specific and control antigens (38,39). The absorption method was detailed in an earlier review (3). This method could be adapted for use in the ELISA. Because of the potential ramifications of epizootics, sensitive tests such as IFA and ELISA are recommended for serodiagnosis of LCMV infections.

H. Epizootic Diarrhea of Infant Mice (EDIM) Virus

IFA and ELISA are increasingly used for detection of antibody to rotaviruses. Both have been applied successfully to EDIM virus (39). Because of the cross-reactivity among rotaviruses from different species, viruses that grow more efficiently in cell culture than does EDIM virus may be used as antigen (3). An ELISA using SA 11 antigen was more sensitive than IFA using either EDIM virus or Nebraska calf diarrhea vaccine virus (39).

I. Reovirus Type 3

The current practice of monitoring for reovirus type 3 and not for types 1 and 2 seems to derive from early reports that reovirus type 1 could not be isolated from wild or

laboratory rodents and that reovirus type 2 exists in certain wild mouse colonies but not in laboratory mice (40). In contrast, reovirus type 3 infection seems to be widespread among laboratory mice (40). In addition, it can contaminate transplantable mouse neoplasms (unpublished data), confirming the suggestion of Hartley et al. in 1961 (41). The HAI test was until recently the serological method of choice for detection of antibody to reovirus type 3; however, there have been recurring questions regarding the specificity of HAI serology (reviewed in 40; 42). Other tests have been used, but CF titers are frequently very low, and the NT, which is very specific, has limited sensitivity (3). The in vitro NT has the added disadvantage of requiring 10 days of incubation prior to final reading. An ELISA, using reovirus type 3 as antigen, is very sensitive for detection of reovirus antibody (3) and would presumably detect group-reactive antibody to other reovirus serotypes. An IFA test for reovirus antibody detection is as sensitive as a commercially available ELISA and is more specific (unpublished data).

J. Theiler's Mouse Encephalomyelitis Virus (TMEV)

The HAI test has been used for serodiagnosis of TMEV, but the specificity of the test has been questioned based on the finding of HAI antibody among mice in cesarean-derived colonies with subsequent declines in the percentage of test-positive mice (42). IFA and ELISA have gained wide acceptance for TMEV antibody detection. They are approximately equivalent in sensitivity (unpublished data) and much more sensitive than the NT (3).

K. Hantaviruses

Hantaan virus and related agents can cause serious disease in man. Isolation of a Hantavirus from a transplantable rat tumor (43) suggests that, given its zoonotic potential, this virus may emerge as the rat analogue of LCMV. The IFA test for detection of antibody to Hantaviruses is as sensitive and nearly as specific as the more labor-intensive plaque reduction NT (44). IFA titers were significantly higher than NT titers. The NT has the advantage of discriminating among Hantavirus strains; however, a

newly described avidin-biotin-amplified double-sandwich ELISA also has this capability (45) and is less expensive and time-consuming.

L. Other Viruses

Selected viruses have not been discussed individually because improved techniques for serodiagnosis have not been confirmed or reported. Agents in this category include polyoma and K viruses, for which the HAI test is still commonly used, murine cytomegalovirus and lactic dehydrogenase elevating virus.

The following table summarizes current recommendations for serologic testing for selected rodent viruses. The tests are referred to in principle and the recommendations

TABLE III. Test Preferences for Selected Rodent Viruses

	HAI	CF	ELISA / IFA	NT
Adenoviruses		3	1*	2
Coronaviruses		3	1	2
Ectromelia virus	3	2	1	
EDIM virus		2	1	
Hantaviruses			1	2
LCMV		3	1	2
Parvoviruses	3		1	2
PVM	2	3	1	
Reovirus type 3	3		1	2
Sendai virus	3	2	1	
TMEV	3		1	2

* ELISA not reported.

arise from a compendium of the literature. Ranking is based on relative sensitivities, specificities, labor intensity and cost. ELISA and IFA are grouped together because they are approximately equal in sensitivity and the choice of test may depend on the number of sera to be evaluated in a single test.

VII. FUTURE PROSPECTS

During the last decade, significant improvements have been made in the serologic diagnosis of rodent virus infections. Many improvements have emanated from progress made in diagnosis of human virus infections. We must not, however, be lulled into a sense of false security. There are still agents for which tests with optimal sensitivity do not exist. In addition, the sensitivity of tests currently thought to be exquisitely sensitive may possibly be improved. The development of sensitive and specific serologic procedures is crucial for studies of the basic biology of viruses that affect virtually all facets of biomedical research involving rodents.

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Comments

Our experience in serological monitoring of rodent colonies confirms what Dr. Smith has mentioned. We want to share our results with you.

Sera from mouse and rat colonies known to be infected with different viruses were tested either by hemagglutination inhibition (HAI) test and enzyme-linked immunosorbent assay (ELISA) or by indirect immunoflourescence assay (IFA) and ELISA.

Antibody titers by ELISA for pneumonia virus of mice (PVM), Sendai virus, reovirus 3, minute virus of mice (MVM) and Theiler's mouse encephalomyelitis virus (TMEV) were approximately five dilution steps higher than titers by HAI test. However, the most important result for rodent seromonitoring was the distinct increase in positive results by ELISA in comparison to HAI results. Twenty-four of 126 sera from PVM infected colonies were negative by HAI test but were positive by ELISA. Similar results were obtained for Sendai virus, reovirus 3, MVM and TMEV infected colonies (TABLE I).

We also compared IFA and ELISA to determine if IFA can be a suitable alternative to ELISA. Sera from infected colonies were tested by both methods for antibodies to mouse hepatitis virus (MHV), TMEV, MVM, PVM, and M. pulmonis. Antibody titers by ELISA were one to two dilution steps higher by ELISA for TMEV and PVM but the sensitivity of the tests was equal in our laboratory.

Thus, in summary, the ELISA and the indirect IFA test detected up to 30% more positive reactions in naturallly infected murine colonies than did the HAI test. It is obvious that the use of these tests may improve the reliability of murine seromonitoring. Hazards for animals and bioassays can be detected earlier and smaller volumes of sera are required than for the HAI or CF tests. It will be useful to be able to use both ELISA and IFA in routine laboratory tests. This offers the possibility of confirming borderline reactions by an alternative serologic method.

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TABLE I. Comparison of Antibody Detection by ELISA and HAI Test

	No. of Sera tested	No. of Sera neg. by HAI and ELISA	No. of Sera pos. by HAI and ELISA	No. of Sera neg. by HAI and pos. by ELISA	% of Sera neg. by HAI and pos. by ELISA
PVM	126	38	64	24	19
SENDAI	169	49	64	56	33
REO 3	94	14	51	29	31
MVM	100	0	76	24	24
M. POLIO	91	50	7	34	37