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Evaluation of an Enzyme-Linked Immunosorbent Assay for the Detection of Ectromelia (Mousepox) Antibody

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Ectromelia virus, an orthopoxvirus that can cause extensive morbidity and mortality (mousepox) in colonized mice, has been epizootically responsible for serious disruption of biomedical research since 1930. The lack of a sensitive and specific serological assay for infection with this virus became apparent during outbreaks of mousepox at the National Institutes of Health, Bethesda, Md., and other biomedical research institutions in 1979 and 1980. To fill this need, we evaluated an enzyme-linked immunosorbent assay. Sucrose gradient-purified ectromelia and vaccinia viruses were compared as antigens in tests on approximately 1,000 mouse sera from experimentally infected mice and conventional colonies of uninfected mice. A statistical analysis based on the frequency distribution of the absorbance values for 152 mouse sera (free of ectromelia antibody) gave 0.22 as a value to differentiate ectromelia-positive sera from ectromelia-negative sera. When enzyme-linked immunosorbent assay results were compared with those obtained by an indirect immunofluorescence assay, the former was found to be at least 10-fold more sensitive. With the procedures employed, including the use of purified vaccinia virions as antigen, the enzyme-linked immunosorbent assay proved to be highly sensitive and specific for detecting antibodies to ectromelia and vaccinia viruses. False-positive results were not encountered. False-negative results were observed in 3% of 108 separate tests of a known positive serum. Although data indicated that ectromelia antibody can be differentiated from vaccinia antibody with homologous and heterologous antigen, this procedure probably cannot be generally used because of unavailability of ectromelia antigens.

Ectromelia virus is an orthopoxvirus which can cause severe disease (mousepox) in laboratory mice (10). Until 1980, a hemagglutination inhibition (HI) test was the principal assay used to detect antibody to ectromelia virus (2, 5). However, during the mousepox outbreak at the National Institutes of Health, Bethesda, Md., and several other biomedical research institutions in the United States during 1979 and 1980, it became apparent that the HI test was both insensitive and nonspecific (1, 5, 9, 14). Since a sensitive and specific serological assay is essential to the control and prevention of mousepox, an enzyme-linked immunosorbent assay (ELISA) was devised and evaluated for this purpose. Herein, we report results from the ELISA on approximately 1,000 sera from a variety of mouse colonies and experimentally infected mice. Both ectromelia and vaccinia viruses were evaluated as antigens. These results were compared with results from tests on some of the same sera with an indirect immunofluorescence assay.

MATERIALS AND METHODS

Cell culture. BS-C-1 (a continuous African green monkey kidney cell line) cells were used exclusively for cultivation of virus. They were grown to confluency in 150-cm^2 flasks with Eagle minimal essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin and 100 μg of streptomycin per ml (Eagle minimal essential medium–10% fetal bovine serum). New cultures were established by conventional methods.

Virus. Ectromelia virus strain NIH 79, which was used for the preparation of virus antigen, was isolated during the outbreak of mousepox at the National Institutes of Health in 1979 and 1980 (1). The starting material was liver collected from BALB/c mice 6 days after inoculation with strain NIH 79-infected chicken embryo cells. A 30% suspension of liver was centrifuged at 850 × *g* for 20 min, and the supernatant fluid was diluted to 40% (vol/vol) with sterile glycerol. Storage was at −70°C in 1-ml samples. Virus infectiv-

ity was estimated by standard methods. All work with ectromelia virus or viral antigen was carried out in a P4-level containment laboratory.

Vaccinia virus strain WR was kindly provided by B. Moss, National Institutes of Health, Bethesda, Md. A crude virus stock was prepared by infecting 150-cm^2 flasks of BS-C-1 cells with 2×10^6 PFU at a multiplicity of infection of strain WR of 0.1 in 5 ml of Eagle minimal essential medium–2.5% fetal bovine serum. After 1 h of adsorption at 37°C, 15 ml of Eagle minimal essential medium–2.5% fetal bovine serum was added to each flask, and the cultures were again maintained at 37°C. When greater than 90% of the monolayer was exhibiting extensive viral cytopathogenic effect (2 to 3 days for strain WR), the cells were harvested and concentrated by centrifugation at $850 \times g$ for 5 min. Each cell pellet was suspended in 1 ml of phosphate-buffered saline (PBS) (without Ca^{2+}) plus 0.1% bovine serum albumin (BSA) and 100 U of penicillin and 100 μg of streptomycin per ml. The suspensions were frozen and thawed twice and then stored at −70°C. Each vial of virus was sonicated in a bath sonicator for 30 s before use.

Purified virus antigen. Forty confluent monolayers (150 cm^2) of BS-C-1 cells were infected as described above with strain NIH 79 or WR at a multiplicity of infection of 0.1. The infected cells were suspended in 20 ml of 0.01 M Tris-hydrochloride, pH 9.0, at 4°C and incubated on ice for 5 min. A cytoplasmic fraction was prepared by Dounce homogenization, and the virus was purified further by sedimentation through a cushion of 35% (wt/vol) sucrose and a 15 to 30% (wt/vol) sucrose gradient (7). Purified virus was stored at −70°C in 0.001 M Tris-hydrochloride, pH 9.0. Virus protein concentration was estimated by the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, Calif.).

Experimental mouse infections. Six groups of five C57BL/6J mice under methoxyflurane anesthesia were infected with 5 to 10 PFU (in 0.05 ml) of strain NIH 79 by the footpad route of inoculation. Five days later, the first group of five mice was bled as described below. The remaining groups (2 through 6) of mice were bled at 1-day intervals. The bleeding cycle was then repeated. Experimentally infected mice under methoxyflurane anesthesia were bled from the orbital sinus into a sterile, heparin-treated Pasteur pipette. The plasma fraction was collected after centrifugation at $8,740 \times g$ for 5 min and stored at −70°C. (In certain instances, heparin treatment of the Pasteur pipette was omitted from the protocol with no significant effect on the behavior of the serum in the subsequent assays.)

Mouse vaccination. Eight groups of six female BALB/c mice each were vaccinated with vaccinia virus strain IHD-T at the base of the tail by placing a microdrop of virus at the site and stroking a bent 27-gauge needle through the site. Six mice were bled at 0, 3, 7, 11, 15, 21, 28, and 35 days postvaccination. Sera were processed as described below.

Field specimens. Mouse sera and plasma, some of which had been heat inactivated at 56°C for 30 min, were received frozen from various sources.

Control antiserum. Positive reference strain NIH 79 antisera were prepared by pooling sera from experimentally infected BALB/c mice which showed typical ectromelia pathology. Negative control sera were prepared also from other BALB/c mice which had no known exposure to ectromelia virus. These control sera were used in all indirect fluorescent-antibody (IFA) tests and ELISAs.

IFA assay. As described above, BS-C-1 cells were infected with strain WR, strain NIH 79, or no virus and harvested when there was extensive cytopathogenic effect. A cell suspension prepared in PBS was centrifuged at $400 \times g$ for 10 min. The supernatant was discarded, and 1.5 ml of PBS was added to 0.5 ml of the cell pellet and thoroughly mixed. From this suspension a volume of 0.02 ml was added to each well (7 mm in diameter) of a "printed" slide, which was air dried at room temperature and then fixed in cold acetone (−20°C) for 20 min. After further air drying at room temperature, the slides were stored at −20°C for a maximum period of 8 weeks.

To detect virus-specific antibodies, wells containing either virus or control antigen were reacted with 0.02 ml of diluted plasma for 20 min at room temperature. Unreacted plasma was removed by three 5-min washes in PBS. The immobilized antigen-antibody complexes were further reacted for 20 min at room temperature with 0.02 ml of affinity-purified fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG) at 150 μg/ml (fluorescein/protein ratio of 7) which was kindly supplied by R. Asofsky, National Institutes of Health, Bethesda, Md. Each slide received three further 5-min washes in PBS and was dry blotted with tissue paper. Slides were then overlaid with glycerin barbital buffer, pH 8.6, and examined by using a Leitz microscope with fluorescence epi-illumination (100-W ultra-high-pressure mercury lamp; PLOEMOPAK filter block K2) and a ×10-magnification objective lens. Serum was considered positive for virus-specific antibody if there was 2+ or greater fluorescence on a scale of 1 to 4 when compared with known negative and positive control sera included in each test.

ELISA. The procedure for ELISA was modified slightly from that of Murphy et al. (11). The optimal concentrations of individual reagents were determined by checkerboard titrations (13), and the assay was carried out as follows. Virus antigen (100 μl; 1 or 2 μg of protein from purified strain WR or NIH 79, unless otherwise stated) or of control antigen (100 μg of BSA) in carbonate-bicarbonate buffer (15 mM Na_2CO_3–35 mM $NaHCO_3$–3 mM NaN_3, pH 9.6; stable at 20°C for 3 months) was added to the wells of a microtiter plate (Linbro plate no. 76-381-04; Flow Laboratories, Inc., McLean, Va.) which was held at 4°C for at least 14 h and no longer than 2 weeks. Before use, unbound antigen was removed by four consecutive washes (squeeze bottle) with 0.05% polyoxyethylenesorbitan monolaurate in PBS (PBS-Tween 20). Mouse serum (100 μl) diluted in PBS-Tween 20 and 0.1% (wt/vol) BSA was added to one test well and one control well. To guard against spurious results, the outer rows and columns of the plate were not used, and preimmune and immune (strain NIH 79) control sera were assayed on every plate. After 1 h of incubation at 37°C, the plate was washed four times with PBS-Tween 20, and 100 μl of a 1:400 dilution of affinity-purified goat anti-mouse IgG (heavy and light chains) antibody conjugated with alkaline phosphatase (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) was added to each well (including the control well), followed by 1 h of incubation in a moist chamber at 37°C. The plates were again washed four times with PBS-Tween 20, and

100 μl of a 1-mg/ml solution of *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) dissolved in prewarmed (37°C) 10% diethanolamine buffer (10% [vol/vol] diethanolamine–0.5 mM $MgCl_2 \cdot 6H_2O$, pH 9.8; stable at 4°C in the dark for at least 3 months) was added to each well. After 2 h at 37°C, the concentration of yellow chromogen produced by the action of the immobilized alkaline phosphatase on the substrate was measured at 405 nm by a multichannel spectrophotometer (Multiscan Flow Laboratories, Inc., McLean, Va.) which had previously been blanked on wells containing only 100 μl of the substrate.

An assay result was accepted as valid when the negative serum control gave a corrected optical density (OD) (OD = viral antigen well minus control antigen well) of less than 0.22 and the titer of positive serum control was within a twofold dilution of 1:34,260. The test serum was scored as positive when the corrected OD was greater than or equal to 0.22 (see Results). An antibody titer was defined as the reciprocal of the highest twofold dilution of serum to be scored as positive (endpoint dilution). On occasion, antibody titers were obtained by interpolation between this endpoint dilution and the next twofold dilution.

Statistical methods. ELISA OD values of sera (1:20 dilution) from mice unexposed to ectromelia tended to be low and symmetrically distributed, and so the average of a group of such sera was expressed as the arithmetic mean, and the variability about the mean was expressed as the standard deviation (SD) or standard error (SE). Infected animals had high titers, the distribution of which was skewed to the right. The average of a group of these was expressed as a geometric mean, and the variability was expressed as a relative SE. The method of least squares was used to fit a straight regression line (see Fig. 2).

RESULTS

Specificity of ELISA. Because orthopoxviruses share extensive DNA sequence and antigenic homology (6, 8), vaccinia virus antigens have had widespread use in detecting ectromelia antibody by serological means. In preliminary tests, disrupted unpurified vaccinia virus-infected BS-C-1 cells were used as the solid-phase antigen in the ELISA. This antigen had several disadvantages. First, its use resulted in high, variable background OD readings (≧0.200) in sera known to be negative for ectromelia antibody when tested at dilutions of less than 1:100. Second, control antigen (uninfected BS-C-1 cells) prepared in a similar fashion also yielded unacceptably high background levels with both ectromelia antibody-negative and -positive sera at dilutions of less than 1:100 (unpublished data). For these reasons, a purified virus antigen was prepared (7) and used as the solid-phase antigen in the ELISA.

To evaluate the specificity of the binding of the antibody to the immobilized antigen, sera from mouse colonies with no history of mousepox were tested against both virus and a control antigen such as BSA. Because the "conventional" colonies tested consisted of mice housed in nonbarrier facilities and thus were the most representative population of colonies at risk to ectromelia infection, data from these animals were used to construct the criteria for scoring a serum positive or negative with respect to ectromelia antibodies.

Average OD values from tests on two groups of sera, each screened at 1:20, are shown in Table 1. The first group of 123 was assayed on 3 separate days early in the investigation, and the second group of 29 was assayed on 4 days quite recently. Each collection of sera was subject to variability from two sources: serum-to-serum differences within assays and assay-to-assay differences. The average OD of the group of sera assayed recently is smaller than that of the group done earlier, and the same is true of the respective SDs. These findings are consistent with an increase in assay precision as additional experience with the assay was acquired. A distributional analysis of the data summarized in Table 1 showed that less than 2% of the sera yielded an OD value of equal to or greater than 0.22. In a further test of the specificity of this criterion, sera from a population of 51 mice previously exposed to mouse hepatitis virus were tested by ELISA. The group showed a mean (±SD) OD value of 0.006 ±0.024 at a 1:20 serum dilution, and all sera were scored negative by our criterion (unpublished data).

To verify that a negative test score did not simply reflect defective reagents and that a positive result was not due to nonspecific binding of reagents not detected by the BSA control, an immune and a preimmune control serum were analyzed in each test. In each valid test, the preimmune negative serum gave a corrected OD of less than 0.22, whereas an immune serum had a geometric mean ($\times/\div$SE) titer of 1:34,260 $\times/\div$ 1.75 (data from assays on different days) and, in repeat assays, gave absorbance values which were linear and parallel over a range of six twofold serum dilutions. At low serum dilutions, the ELISA reader showed a nonlinear response at an OD of approximately 1.5 and greater.

TABLE 1. ELISA OD values of negative sera from conventional colonies

No. of sera	No. of assays[a]	Mean OD	SD	
			Within assay[b]	Among assay[c]
123	3	0.07	0.05	0.07
29	4	0.02	0.02	0.03

[a] Each assay was run on a different day.

[b] SD among sera assayed simultaneously (same day) with identical reagents on one or more microtiter plates.

[c] SD among sera assayed on different days with different preparations of reagents.

Of the 112 sera from experimentally infected mice that were initially positive by ELISA, a confirmatory IFA test and a second ELISA confirmed the initial finding without exception. However, known positive sera on repeated analysis were scored (false) negative in 3 of 108 cases.

Sensitivity of ELISA. To determine whether the ELISA test had the level of sensitivity needed to be effective for routine serological screening of mouse populations at risk, and for detecting the spread of mousepox infection in an epizootic, serum samples from C57BL/6J mice infected with strain NIH 79 were collected at different times after infection, and the sera were assayed for the presence of ectromelia antibody by ELISA and IFA test (Fig. 1). The ELISA detected a rise in ectromelia antibody at day 10 p.i., whereas the IFA titer did not begin to rise until day 13. A further comparison of ectromelia antibody titers by ELISA and IFA test is shown in Fig. 2. The slope of the regression line is 0.52 ± 0.13 (SE), which is significantly ($P < 0.001$) greater than zero. (A slope of zero would imply that there is no association between the respective levels of ELISA and IFA antibodies.) On the other hand, 0.52 is significantly less than the slope of the 45° line, which implies that, although common populations of ectromelia antibody are being measured, the relative sensitivity of measurement decreases from 60-fold in favor of ELISA at low dilutions to only 10-fold at high dilutions.

In addition to C57BL/6J mice, this ELISA has been successfully used to detect ectromelia antibody in several other strains (BALB/c ByJ, DBA/2J, C3H/HcJ, and AKRJ) of experimentally infected (injection and contact transmission) inbred mice. Furthermore, sera from a large number of naturally infected H2 congenic mice

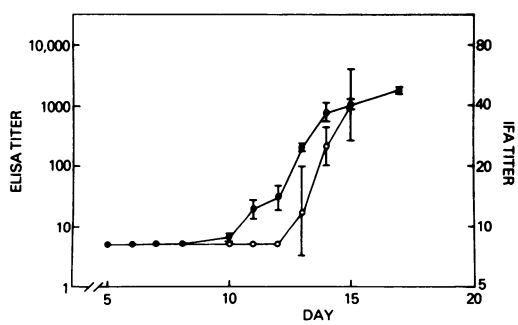

FIG. 1. Six groups of five ectromelia-infected C57BL/6J mice were bled at daily intervals commencing at day 5 p.i. At day 11 p.i., the bleeding cycle was repeated. An ELISA (●) and an IFA test (○) were performed on the collected sera (see text), and a geometric mean and relative standard error of 2 to 5 titers for each time point were plotted.

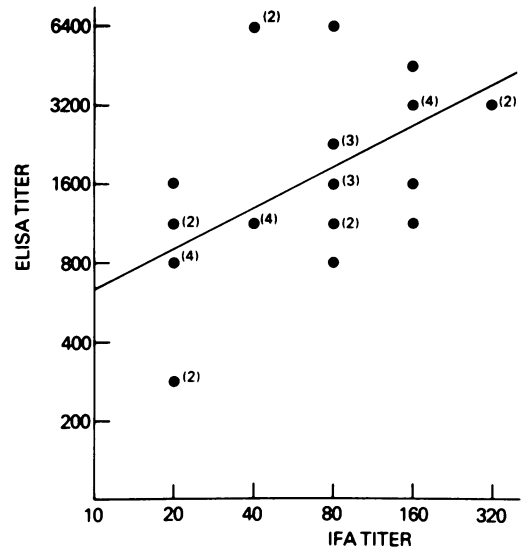

FIG. 2. Each of 34 sera from C57BL/6J mice infected as described in the text were titrated by the ELISA and by the IFA test, and the corresponding titers are plotted. The regression line was fitted by the method of least squares applied to the logged data. The slope of the line is 0.52 ± 0.13 (SE).

(on a black background), which had been scored as positive for ectromelia antibody by a number of diagnostic tests, were all confirmed as positive for ectromelia antibody by this ELISA (unpublished data).

Use of homologous and heterologous antigen to distinguish between ectromelia and vaccinia antibodies. Because the ELISA herein described cannot differentiate between vaccinia and ectromelia antibodies, surveillance for ectromelia virus in mouse colonies immunized with vaccinia virus was difficult. Thus, we tested a number of sera from mice experimentally infected with either ectromelia or vaccinia virus, comparing ELISA reactions with the respective purified virion antigens (Tables 2 and 3). As might be expected, the ectromelia antibody titer was highest when ectromelia antigen was used in the ELISA; specifically, the mean (± SE) of the 11 ectromelia antigen-vaccinia antigen titer ratios (E/V ratios) from Table 2 was shown to be 3.8 ± 0.5. This was significantly ($P < 0.01$) greater than the corresponding ratio for vaccinia antibody (1.1 ± 0.2), which produced titers to almost equivalent endpoints irrespective of which antigen was employed in the ELISA (Student's t-test).

DISCUSSION

Adequate surveillance for ectromelia virus infection in colonized mice, particularly in large colonies, is dependent on a sensitive and specif-

TABLE 2. Ectromelia antibody titers by homologous and heterologous antigens in the ELISA

Serum no.	Days p.i.	Antibody titer[a]		Ratio (E/V)[c]
		Ectromelia Ag(E)[b]	Vaccinia Ag(V)[b]	
1	17	600	300	2
2	21	2,400	400	6
3	25	1,600	800	2
4	29	4,800	1,600	3
5	34	4,800	800	6
6	39	3,200	800	4
7	42	6,400	1,600	4
8	47	4,800	800	6
9	48	4,800	1,600	3
10	63	4,800	2,400	2
11	70	3,200	800	4

[a] Sera from C57BL/6J mice experimentally infected with ectromelia virus (as described in the text).

[b] Each test well contained 1 μg of purified antigen (Ag).

[c] Mean ± SE ratio 3.8 ± 0.5.

ic serological assay that is easy to perform. Historically, the HI test has been used for this purpose (2). As a serological assay, its advantages include simplicity, a low unit assay cost, and suitability for a large-scale screening program. In addition, this test does not detect vaccinia antibody in mice that have been immunized with the IHD-T strain of vaccinia virus (2). However, the HI test is known to be relatively insensitive as compared with the IFA test and the ELISA (4, 5). During a recent outbreak of ectromelia at the National Institutes of Health and several other large biomedical institutions, it became obvious that the HI test on occasion would yield both false-positive and false-negative results (1, 5, 9, 14). An HI result was determined to be false-positive if corroborating IFA, ELISA, necropsy, or virus isolation procedures yielded negative results. Ectromelia was ruled out in one facility where 9% of 435 sera were HI positive (unpublished data). Furthermore, two additional facilities yielded 29 and 74 positive HI results, of which only 15 and 5 sera, respectively, could be confirmed as specific for ectromelia antibody. False-negative results in the HI test are also of concern. For example, in the 1979 ectromelia outbreak in Minnesota, 24 C57BL mice with characteristic cutaneous lesions of mousepox did not show a significant HI antibody (titer ≧ 20) under conditions where antibody would have been expected to be present (9). A second example of HI false-negative results in the 1979 outbreak was at the University of Utah (14). In that case, of 13 serum samples which were scored positive for ectromelia antibody by ELISA, only 6 were positive by HI test (i.e., 54% were false-negative HI results).

The ELISA was chosen over traditional IFA, complement fixation, and virus neutralization tests because it seemed to be sensitive, economical, and straightforward in its operation. Furthermore, preliminary results with an ELISA to detect ectromelia antibody had been encouraging (5). The ELISA described here used an automated microtiter reader, which was the major expense (approximately $10,000) in setting up a laboratory in which large numbers of sera needed to be screened. For more modest screening programs, this initial start-up cost can be circumvented by visually scoring the results, but with an inherent decrease in assay sensitivity (an unambiguous positive result may require an absorbancy at 405 nm of greater than 0.3 to 0.4). On a per-test basis, the contribution of the reagents to cost is approximately $0.11. The specificity of an ELISA is, to a large degree, determined by the quality of the antigen used in the solid phase; therefore, we chose to use highly purified vaccinia virus antigen which gave exceptionally low background levels with preimmune control sera. Furthermore, a purified antigen lessens the likelihood that contaminating nonviral proteins, such as fetal calf IgM from the cell maintenance medium, could react weakly with the goat anti-mouse IgG conjugate and thereby possibly affect the test sensitivity. Vaccinia virus antigen was chosen over ectromelia virus antigen because it was easily propagated in BS-C-1 cells and did not require an elaborate containment facility. We observed no ELISA false-positive results in the analysis of 112 sera when the second confirmatory test was the IFA test or the ELISA. However, it has been reported that sera from NZW and NZB mice will give false-positive reactions in the ELISA (5). False-negative results in the ELISA were observed at a frequency of less than 3%. This phenomenon probably resulted from the failure of antigen to bind to the well of the assay plate.

TABLE 3. Vaccinia antibody titer by heterologous and homologous antigen in the ELISA

Serum no.	Days p.i.	Antibody titer[a]		Ratio (E/V)[c]
		Ectromelia Ag(E)[b]	Vaccinia Ag(V)[b]	
1	21	1,000	1,000	1.0
2	28	400	600	0.7
3	35	800	400	2.0
4	35	200	200	1.0
5	35	400	400	1.0

[a] As described in the text except that BALB/c mice and strain WR of vaccinia virus were used in the experiment.

[b] Each test well contained 1 μg of purified antigen (Ag).

[c] Mean ± SE ratio 1.1 ± 0.2.

We have shown the ELISA to be at least 10-fold more sensitive than the IFA test. The significant deviation from 1.0 of the slope of the regression line (Fig. 2) may have been due to the use of purified virus antigen in the ELISA, whereas infected BS-C-1 cells were used in the IFA. The ELISA was shown to be capable of detecting ectromelia antibody by 10 days p.i. It is possible that even earlier detection times could be achieved by using goat anti-mouse IgM conjugate instead of an IgG conjugate. This reagent would be especially valuable during investigations of ongoing outbreaks of ectromelia infection.

Vaccinia virus antigen in an ELISA can specifically detect ectromelia antibody in nonvaccinated mice. However, in mice immunized with vaccinia virus, no method is as yet available to differentiate between ectromelia and vaccinia antibodies. Although it appears (Tables 2 and 3) that one can differentiate to some extent between vaccinia and ectromelia antibodies on the basis of endpoint titers on homologous and heterologous antigen, the observed overlap of the ratios of ectromelia to vaccinia antibody and the nonavailability of ectromelia antigen limit the utility of this procedure in surveillance programs. In a further attempt to solve this problem, we have isolated a non-cross-reacting specific monoclonal antibody to an abundant epitope on the surface of the vaccinia virus virion. This monoclonal antibody will be used to construct a competitive inhibition ELISA in which vaccinia virus antigen will be used. Mice that are positive for orthopoxvirus antibody in our standard ELISA but are suspected of having been vaccinated with vaccinia virus will be tested by this competitive inhibition assay.

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