Formalin-Killed Versus Heat-Killed *Legionella pneumophila* Serogroup 1 Antigen in the Indirect Immunofluorescence Assay for Legionellosis

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Indirect immunofluorescence assay titers of human sera obtained against the *Legionella pneumophila* serogroup 1 antigen (Philadelphia 1 strain) killed with 10% Formalin showed a tendency to be lower than those obtained against the reference heat-killed antigen (geometric mean titer of 194 and 370, respectively) when all other variables in the test were held constant. Test results were interpreted the same for 96% of 60 paired sera if the cutoff level used to interpret a positive test result for the formalinized antigen was lowered by one twofold dilution factor.

The indirect immunofluorescence assay (IFA) has been the reference serological test for retrospectively diagnosing legionellosis at the Centers for Disease Control since it was first used by McDade et al. in the investigation of the 1976 epidemic of Legionnaires disease (5). The test was modified subsequently by preparing the antigens from *Legionella* strains that have been cultured on artificial media, by boiling the antigens for 15 min to kill them, and by reducing the concentration of normal yolk sac (NYS) material in the antigen and in the serum diluent (10). The modified *Legionella pneumophila* serogroup 1 (Philadelphia 1 strain) antigen compared favorably with the original ether-killed antigen (10), and the test was shown to be 78% sensitive and 99% specific in testing sera from patients with pneumonia (8). The prevalence of antibodies among healthy subjects, however, varies in the United States from approximately 1 to 25% (2, 6), depending upon the population surveyed. The value of a single or sustained high titer in diagnosing a recent infection varies accordingly.

Several investigators have suggested that background antibody levels among healthy control populations can be reduced by using a formalinized IFA antigen without adversely affecting the sensitivity of the test (4, 7). It has been difficult to determine the effect of Formalin itself from the available data because other variables such as strain differences and different culture media most likely affected the results. Furthermore, the Centers for Disease Control antigen that was used for comparison had been changed during at least one of the studies (7) from an ether-killed to a heat-killed antigen. The purpose of the present study was to compare the use of Formalin with heat in preparing *Legionella* IFA antigens, to investigate the effect of washing the antigen, and to determine the best method of fixation of the antigen to the slide. Preliminary experiments showed that acetone was superior to ethanol for fixation and that antigenic stability was maintained for at least five subpassages of *L. pneumophila* serogroup 1 on charcoal-yeast extract (CYE) agar (3) from infected chicken yolk sacs. Accordingly, CYE agar-grown antigens were used throughout the experiments.

The first experiments were done to determine if the formalinized antigens could be fixed adequately to the slides. The 24-h growth of the Philadelphia 1 strain from a CYE agar slant was suspended in 2 ml of distilled water or in 2 ml of 1, 2, or 10% Formalin. The water-suspended antigen was heat killed as usual (10), and then all antigens were refrigerated overnight. The heat-killed antigen was diluted 1:100 in 0.5% NYS by established methods (10). Each formalinized antigen was diluted 1:100 in (i) Formalin (at the same concentration as the one used originally), (ii) 0.01 M phosphate-buffered saline, pH 7.6, and (iii) 0.5% NYS. The original antigen suspension in 1% Formalin was also diluted 1:100 in a 1% aqueous suspension of bovine serum albumin. Smears of each antigen were made on fluorescent-antibody slides and allowed to air dry. The formalinized antigens were then tested with the *L. pneumophila* serogroup 1 direct immunofluorescence assay conjugate (1) without further treatment, and they were tested in duplicate after they were fixed with heat. The heat-killed antigen was fixed in acetone for 15 min and then stained with the conjugate. Slides
were read with a Leitz Dialux 20 fluorescence microscope equipped with an HBO-100 mercury incident light source, an I-cube filter system, a 40× dry objective, and 6.3× eyepieces. Adherence of the cells to the slides occurred for all of the antigens that had been suspended in NYS, but was minimal for all other antigens.

In the next experiment, several concentrations of Formalin were compared in the IFA test. Antigens were prepared by killing the bacteria in 1 ml of 1, 2, 5, or 10% Formalin, as above, and were then diluted 1:100 in 0.5% NYS. Smears were air dried (no fixative was used) and tested by the IFA (10) with 23 serogroup 1-reactive human sera. Antigens prepared with 1, 2, or 5% Formalin appeared to have uneven cell surfaces compared with the 10% formalinized antigen, which nevertheless showed a slight loss of antigenic homogeneity that was not observed with the heat-killed antigen. Ten sera had the same titers against both antigens. Ten sera had titers that were one doubling dilution factor and three had titers that were two doubling dilution factors lower against the formalinized than against the heat-killed antigen.

To determine the effect of formalinization on IFA test results, paired sera from 60 patients with suspected cases of legionellosis were tested by the IFA against the 10% formalinized antigen (no acetone or heat fixation) and against the heat-killed antigen (acetone fixation, 15 min). Eighteen serum sets showed seroconversions (fourfold increase in titer to ≥128) against the heat-killed antigen. Sixteen of these also showed seroconversions (to ≥128) against the formalinized antigen. Of the remaining two sets, both the acute- and convalescent-phase sera of one had titers of 128 against the formalinized antigen, and the acute- and convalescent-phase sera of the other had titers of <64 and 64, respectively. The remaining 42 serum sets showed no seroconversion but had convalescent-phase titers ranging from 64 to ≥2,048 against the heat-killed antigen; 15 had the same titers against the formalinized antigen, 16 had titers one tube lower, and 11 had titers ≥two tubes lower. The tendency for convalescent-phase titers to be lower against the formalinized antigen (geometric mean titer = 194) than against the heat-killed antigen (geometric mean titer = 370) (Fig. 1) had no apparent relationship to the antibody specificity of the sera (determined previously as species specific or common antigen reactive) (9). A total of 46 serum sets met the Centers for Disease Control definition of positivity when tested against the heat-killed antigen; 44 (95.7%) would have been considered positive against the formalinized antigen, but only if we had lowered the criteria for positivity with the latter antigen by one twofold dilution factor (i.e., seroconversion to ≥64 or single titer of ≥128).

In a final comparison of methods, the effects of washing the heat-killed antigen three times with 2 ml of distilled water and of omitting the acetone fixation step were determined. The same IFA titers were obtained for 18 control sera (titer range, <64 to 1,024) against the control antigen, the washed antigen, and the antigen that was not fixed with acetone.

In summary, these data suggest that extensive washing of the L. pneumophila serogroup 1 antigen is unnecessary, that the presence of NYS in the antigen enhances fixation of the cells to the slides, and that acetone fixation is then unnecessary. Formalin appears to disrupt the antigenic integrity of the cell surface (as was found previously for diethyl ether) (10) and to lower IFA titers by one twofold dilution factor. However, if one then lowers the cutoff titer levels for a positive test result by one tube, results are comparable to those obtained with the heat-killed antigen (96% agreement). Both antigens bind antibodies with multiple specificities. Differences in control populations, in interpretive criteria for a positive test result, and in strains and culture conditions may have been variables that contributed to discrepant opinions regarding antigen preparation.

LITERATURE CITED