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Detection of Infectious Mononucleosis Heterophil Antibody by a Rapid, Standardized Enzyme-Linked Immunosorbent Assay Procedure

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A rapid, specific, sensitive, standardized, and reproducible enzyme-linked immunosorbent assay (ELISA) procedure has been developed for detecting the heterophil antibody associated with infectious mononucleosis (IM). The IM heterophil antibody used for the solid phase was purified from bovine erythrocyte stroma. The test uses heavy-chain-specific anti-immunoglobulin M (IgM) labeled with alkaline phosphatase and three 10-min incubations. The quantitative results correlated well with horse erythrocyte agglutination titers. Absorption tests confirmed the specificity of the ELISA reactions for IM heterophil antibodies. Neither very high levels of IgM in myeloma sera nor high levels of rheumatoid factor caused false-positive reactions. A number of probable IM cases were encountered which were positive by ELISA but negative by the horse erythrocyte slide agglutination test. Absorption studies indicated that these were true-positives for the IM heterophil antibody. The IM heterophil antibodies were confirmed to be predominantly of the IgM class, but moderate proportions of the IgG class were sometimes encountered.

The detection of immunoglobulin M (IgM) heterophil antibody response is of considerable value in the diagnosis of infectious mononucleosis (IM) caused by the Epstein-Barr virus (EBV) (9, 12). At present, the IM heterophil antibody is usually determined by the agglutination of erythrocytes from one of several mammalian species (5, 15, 24). If sheep erythrocytes are used, the specificity of the reaction must be established by a differential absorption procedure, with guinea pig kidney and bovine erythrocytes, to rule out the participation of the Forssman heterophil antibody in the reaction (14a). Horse and particularly bovine erythrocytes are rich in the IM heterophil antigen and have little or no detectable Forssman antigen. Erythrocytes from these species are therefore now widely used in agglutination or hemolytic tests for the IM heterophil antibody (29). However, the readings of these reactions are very subjective, and it is difficult to be certain of weak reactions. In those agglutination tests where absorptions and titrations are required, the assays are tedious and difficult to interpret in borderline cases (13), and reproducibility is not completely satisfactory. In addition, false-positive results have been reported with some frequency (11, 14, 21, 25, 27). Because of these problems, several investigators have used other approaches to detect this antibody, e.g., agar gel diffusion (23), latex agglutination (17), radioimmunoassay (3), and immune adherence methods (16).

Since the IM heterophil antigen has been purified from bovine erythrocytes by several investigators (4, 20, 26, 28), we have used such preparations in developing a standardized enzyme-linked immunosorbent assay (ELISA) procedure to detect this IgM antibody that uses three 10-min incubations. This method appears to offer a highly specific, sensitive, and objective alternative to determining the presence of this antibody.

MATERIALS AND METHODS

The IM heterophil antigen was purified from bovine erythrocyte stroma according to the method of Levey et al. (17), with blood obtained fresh from a local slaughterhouse. The 75% ethanol extract of stroma was used for the solid-phase antigen in most instances. Attempts to further purify the IM heterophil antigen by phosphocellulose chromatography did not improve the performance of the ELISA procedure. The solid phase consisted of small plastic disks containing isothiocyanate groups for covalent bonding (6). They were exposed to the antigen (5 μg/disk) overnight at 4°C, pH 9.6, after which they were washed and lyophilized.

Heavy-chain-specific goat anti-human IgM was isolated immunospecifically as described earlier (7) and conjugated to highly purified calf intestinal alkaline phosphatase by a modification of the method of Eng-

vall and Perlmann (2). Heavy-chain-specific goat anti-human IgG reagents were prepared similarly. To control the specificity of these reagents, disks were coated with either highly purified normal human IgG or myeloma IgM and exposed to the antibody-enzyme conjugates. The IgM was a mixture of both kappa and lambda light-chain proteins.

The ELISA assay employed the same format used in previous tests developed by our group (6–8, 18, 19). In brief, 10 μl of the test serum was diluted 1:51 in 500 μl of 6% bovine serum albumin, and to this was added an antigen-coated disk. After incubation with gentle agitation for 10 min at 37°C, the disk was washed five times with Tris-buffered saline (pH 7.2 to 8.0), and then 500 μl of enzyme-labeled anti-IgM was added. After another similar 10-min incubation, the disk was again washed five times and transferred to a clean vial. One milliliter of a fresh solution of *p*-nitrophenyl phosphate was added (1 mg/ml in 0.028 M sodium carbonate buffer, pH 9.8), and the vial was incubated for a final 10 min. The enzyme action was then terminated by adding 0.1 ml of 3 M NaOH, and the absorbance at 405 nm of each tube was read against the substrate solution as the blank. The test results were reported as the percentage of absorbance of the positive control. The latter was standardized in assembling the reagents to yield an absorbance of approximately 1.0 in the assay. Based on results described below, ≥20 was considered positive for IM heterophil antibody, ≥15 but less than 20 was equivocal, and <15 was negative.

For the absorption studies, 500 μl of the diluted test sample was preincubated with 100 μl of the absorbing antigen for 30 min at 37°C before the assay was performed. The antigens used were: aggregated human IgG, shown previously to absorb rheumatoid factor (7); native calf thymus DNA; native calf thymus deoxyribonucleoprotein (8); *Toxoplasma gondii* antigen (19); and the above-purified IM heterophil antigen from bovine erythrocyte stroma. In some cases, the bovine stroma from which the heterophil antigen was isolated was used as absorbent.

The horse erythrocyte slide agglutination test used was the Mono-Test kit, purchased from Wampole (Cranbury, N.J.). Immunodiffusion reactions were performed in IDF-1 cells (Cordis Laboratories, Miami, Fla.).

The panels of sera from IM patients and others were sent coded to Miami from Philadelphia and Madison and tested before the codes were broken. The 108 specimens from Philadelphia had been assayed for various EBV antibodies by immunofluorescence (10); the 67 specimens from Madison were assayed by an ox cell hemolysin titration (22). Other IM specimens were obtained from commercial sources, which were also tested coded. The 150 normal serum specimens were obtained from Cordis Laboratories personnel and two other local facilities. All sera were stored at 4°C.

Rheumatoid factor was assayed by an ELISA procedure previously described, using human IgG as the solid-phase antigen (7). (CORDIA RF; Cordis Laboratories).

RESULTS

All 150 normal sera were negative for IM heterophil antibodies by the Mono-Test horse erythrocyte agglutination test. They showed ELISA values averaging 2.88, with a standard deviation of 2.52. The highest normal value was 13 (Fig. 1).

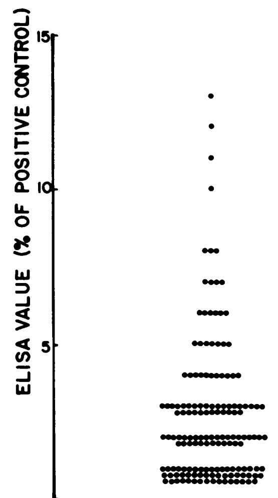

FIG. 1. ELISA results for the detection of IgM IM heterophil antibody in normal individuals. All were negative by the horse erythrocyte agglutination test. N, Number (150); M, mean value (2.88); SD, standard deviation (2.52).

Horse erythrocyte agglutination-positive sera from 79 patients with IM were tested by ELISA. All specimens but one were positive, i.e., >20 (Fig. 2). The single specimen which was not clearly positive showed an equivocal value of 18. The positives ranged from 21 to 214, with a median of 110.

The specificity of the ELISA procedure for the IM heterophil antibody was documented by several lines of evidence. One was the high degree of correlation observed between the ELISA values and the horse erythrocyte agglutination titers (see Fig. 2). The overall coefficient of correlation of the results with these sera was 0.936, $P < 0.001$, using the logarithm of the agglutination titer.

The specificity of the ELISA reactions for the IM heterophil antibody was strongly supported by the results of absorption experiments. Seven agglutination-positive sera were tested in detail, and Fig. 3 shows typical findings with four sera of varying IM heterophil antibody potency. Only pretreatment with the purified bovine IM heterophil antigen blocked the ELISA reactions. This inhibition ranged from 60 to 92% of the control values. Similar absorption tests with an ELISA-negative serum showed no artifactual effects due to the absorption procedure.

Six sera from IM patients that were strongly positive by the horse erythrocyte agglutination

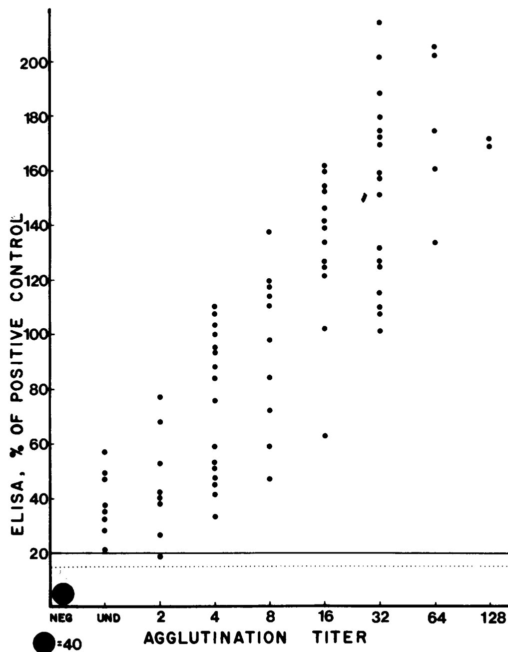

FIG. 2. Correlation of IM heterophil antibody concentrations by horse erythrocyte agglutination titrations and ELISA in 79 IM agglutination-positive patients and 40 normal individuals (negative reactors). N, Number (119); R, coefficient of correlation (0.936). The solid horizontal line represents the cut-off above which reactions are positive. The dotted line indicates the equivocal zone.

test were absorbed with the purified bovine IM heterophil antigen used in the ELISA procedure and then retested by the agglutination assay. In all cases, the horse erythrocyte agglutination test was rendered negative at the lowest dilution (1:2), whereas the control titers ranged from 1:8 to 1:32.

Sera from 14 patients were encountered that were weakly ELISA positive and horse erythrocyte agglutination test negative. At least 10 of these 14 specimens were considered to be from IM patients, based on positive IgM anti-EBV viral capsid antigen findings or ox cell hemolysin titrations. The specificities of these ELISA reactions for the IM heterophil antigen were also supported by absorption experiments with those sera still available (Table 1). In nine patients whose sera contained IgM antibodies to EBV viral capsid antigen, the IM heterophil antibody reactions were negative by both ELISA and the horse erythrocyte agglutination test.

Rheumatoid factor did not interfere with the ELISA test for the IM heterophil antibody. Sera from 15 patients with rheumatoid arthritis containing high levels of rheumatoid factor were all negative for the IM heterophil antibody. The mean ELISA value for the latter was 3.1 in these rheumatoid arthritis sera, and the highest level was 7. The rheumatoid factor levels ranged from 49 to 665 IU/ml, the mean being 255. Normal by the method used is <10.

In addition, two IgM myeloma sera containing very high levels of IgM both showed ELISA heterophil antibody values of only 1. These sera contained IgM at 1,650 and 2,400 mg/dl (normal, 39 to 117 mg/dl), and despite this, nonspecific positive reactions were not produced in the ELISA heterophil antibody test.

The use of a standard curve, as was used with assays previously developed, was considered in this ELISA test (7, 8, 19). With this goal in mind, serial dilutions of 14 positive sera of different potencies were assayed. A straight line was formed with absorbance at higher dilutions of the samples (Fig. 4). These tended to parallel each other, indicating that the useful range of such a standard curve might be limited to lower ranges of antibody concentrations, and that saturation of the antigen on the disk might occur with many specimens. A similar observation was made in the development of an ELISA procedure for rheumatoid factor determination (7), requiring use of a higher sample dilution in that assay (1:500), together with longer incubation times. In view of these findings, and the clinical indications that precise measurements of the IM heterophil antibody level might not be of great diagnostic significance (1), standard curves were not incorporated into the assay system.

Although all reports have indicated that the IM heterophil antibody is exclusively of the IgM class, tests were carried out in this ELISA system with heavy-chain-specific anti-IgG conjugate as well as the anti-IgM conjugate. Twenty-three IM heterophil antibody-containing sera were tested comparatively; in all, the overwhelming preponderance of reactivity was found with the anti-IgM conjugate. However, appreciable reactivity was found with the anti-IgG conjugate, the average absorbance seen being 11.8% of that found with the anti-IgM conjugate (range, 2.5 to 43.8%). In four instances, the IgG absorbance was greater than 20% of that noted for the IgM reaction (Table 2). Table 2 also shows the results of control tests demonstrating the high degree of specificity of the heavy-chain-specific conjugates in their reactions with disks coated with each purified immunoglobulin. Ten normal sera were similarly tested with the ELISA IM system, using anti-IgG and anti-IgM conjugates. In all of them, low equivalent and negligible absorbance values were seen for both immunoglobulin classes.

Immunodiffusion reactions were carried out with 10 sera which were potent for IM heterophil antibodies. Both the purified bovine IM hetero-

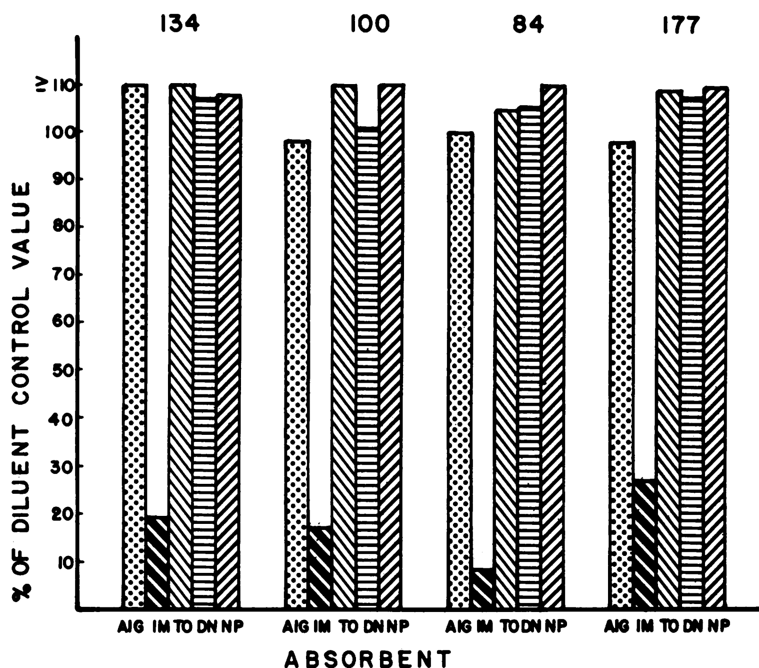

FIG. 3. Blocking experiments involving test sample preincubation with various absorbents before performing the ELISA test for detecting IM heterophil antibody. All absorbents were used at a final concentration of 0.2 mg/ml. They were: aggregated human IgG (AIG); purified bovine heterophil antigen (IM); *T. gondii* antigen extract (TO); native calf thymus DNA (DN); and native calf thymus deoxyribonucleoprotein (NP). The figures on top represent the ELISA heterophil antibody value for each serum shown.

phil antigen used to coat the disks and the preparation after further purification by phosphocellulose chromatography were used as antigens. In all cases, only a single precipitin line was formed, which merged in reactions of identity. Two immune systems were not seen in these patterns, as has been reported by others (23).

The reproducibility of the ELISA procedure for detecting the IM heterophil antibody was quite satisfactory. Five positive and one negative specimen were tested in replicates of six on three different occasions. The average within-run coefficient of variation was 5.7% for the positives; the negative showed a value of 2 in 15 of the 18 tests, and values of 1 or 3 in the others. The day-to-day coefficient of variation and interlaboratory reproducibility were equivalent.

DISCUSSION

These findings demonstrate that a simple, rapid, and standardized ELISA procedure for detecting the IM heterophil antibody is feasible, using the purified antigen from bovine erythrocyte stroma. The specificity of the observed reactions for this immune system was supported by the following evidence. (i) Positive reactions correlated with the diagnosis of the disease. (ii) The horse erythrocyte agglutination titers strongly correlated with the quantitative results of the ELISA test. (iii) Absorption experiments indicated the specificity of the assay, since only the bovine erythrocyte antigen inhibited the ELISA reactions, whereas aggregated human IgG, calf thymus DNA, calf thymus deoxyribonucleoprotein, and *T. gondii* antigen were without effect. (iv) IgM myeloma sera with extremely high concentrations of IgM did not cause nonspecific false-positive reactivity in the ELISA procedure. (v) IgM rheumatoid factor in high concentrations did not cause nonspecific false-positive reactions. (vi) Absorption of IM heterophil antibody with the purified bovine erythrocyte stroma heterophil antigen used for the ELISA procedure completely removed the horse erythrocyte agglutinating activity of the sera.

Fourteen specimens were encountered which were positive for the IM heterophil antibody by ELISA, but negative by the horse erythrocyte agglutination test. In 10 of these cases, IgM antibody reactions to the EBV viral capsid antigen or ox cell hemolysin assay indicated that the sera were from IM patients. In the other four cases, the evidence was equivocal. Absorption studies with the sera still available indicated that

TABLE 1. Blocking experiments with the ELISA IM-positive, horse erythrocyte-negative sera from IM patients[a]

Test sample	ELISA IM value after absorption with:			
	Diluent control	IM heterophil antigen	Bovine erythrocyte stroma	Aggregated human IgG
1	47	10	4	45
2	38	7	9	33
3	21	4	2	22
4	52	22	5	55
5	20	8	7	23
6	23	7	8	27
7	26	10	7	32
8	21	2	3	21
9	31	2	3	23

[a] The final concentrations of the absorbents were: IM heterophil antigen, 1 mg/ml; bovine erythrocyte stroma, 6 mg/ml; and aggregated human IgG, 0.2 mg/ml.

these positive ELISA reactions were specifically due to the IM heterophil antibody. These findings suggest that this rapid ELISA assay is more sensitive than the horse erythrocyte slide agglutination test.

Although IgM antibodies to the EBV viral capsid antigens were found in the serum from nine patients, they were negative for IM heterophil antibodies by both ELISA and the horse erythrocyte agglutination test. These findings could be due to a number of circumstances. For example, the sera may have been obtained before the development of the heterophil antibody response, or they could have been from heterophil antibody-negative cases of IM. Insufficient data were available to choose between the alternatives.

The use of heavy-chain-specific anti-IgG conjugate in the ELISA confirmed that the predominant IM heterophil antibody was of the IgM class. However, a small degree of IgG reactivity was seen with most sera; in 4 of the 23 positive specimens tested, the absorbance seen with the IgG reactions was 20 to 44% of that seen for the IgM response. Since the normal sera tested also showed no nonspecific uptake of IgG on the IM heterophil antigen disks, these findings therefore suggest that a small but appreciable portion of the heterophil antibody response in IM may sometimes be of the IgG class. It would be of

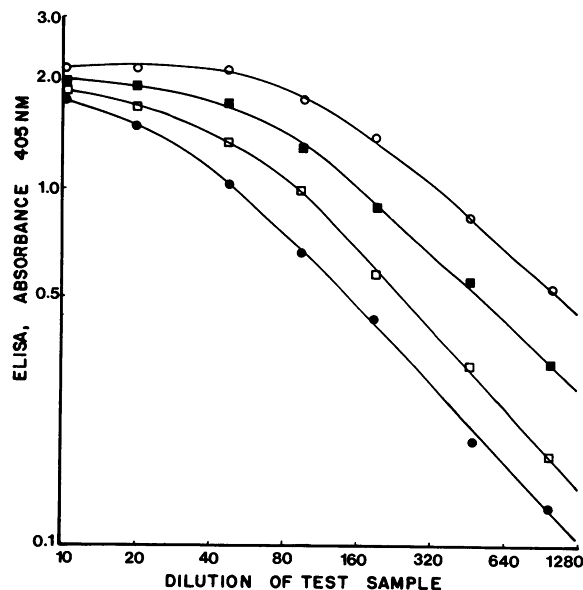

FIG. 4. Serial dilution ELISA studies with four sera of varying IM heterophil antibody potency.

TABLE 2. IgM and IgG IM heterophil antibody reactions

Test sample	Sample no.	Absorbance with:		Absorbance (%), IgG/IgM
		Anti-IgG conjugate	Anti-IgM conjugate	
IM	1	0.145	1.740	8.3
	2	0.050	0.430	11.6
	3	0.025	0.935	2.7
	4	0.045	0.885	5.1
	5	0.055	1.685	3.3
	6	0.090	0.705	13.0
	7	0.230	0.690	33.3
	8	0.270	1.305	20.7
	9	0.285	0.650	43.8
	10	0.550	1.890	29.1
Normal	1	0.02	0.01	
	2	0.02	0.02	
	3	0.02	0.03	
Control IgG disks		1.435	0.000	
Control IgM disks		0.005	1.655	

interest to know if this may be related to some clinical aspect of the illness, such as duration, severity, or prognosis.

In all cases, the immunodiffusion reactions with strongly positive sera revealed only one antigen-antibody system, which showed reactions of identity. The method of isolation of the IM heterophil antigen from the bovine stroma could account for the failure to detect two systems, as has been reported by others (23).

The reproducibility of the ELISA system was highly satisfactory, supporting the potential use of this procedure as a semiquantitative measure of the concentration of IM heterophil antibody. Individual patients may thus be tested serially as a convenient approach to following the clinical course of the disease.

ADDENDUM IN PROOF

The ELISA procedure also proved to be specific for IM with a coded panel of sera kindly supplied by F. Milgrom (University of Buffalo, N.Y.). All six sera from IM patients were strongly positive by ELISA, whereas the other 14 were negative, with low values. The latter included three sera positive for Hanganutzui-Diecher ("serum-sickness") heterophil antibodies (14a) and four lymphoma-leukemia sera, as well as seven normal sera.

LITERATURE CITED

1. **Chretien, J. H., J. G. Esswein, W. G. Holland, and C. E. McCauley.** 1977. Predictors of the duration of infectious mononucleosis. South. Med. J. **70**:437–439.
2. **Engvall, E., and P. Perlmann.** 1971. Enzyme-linked immunosorbent assay (ELISA). Immunochemistry **8**:874–879.
3. **Fletcher, M. A., T. M. Lo, B. A. Levey, and W. R. Graves.** 1977. Immunochemical studies of infectious mononucleosis. VI. A radioimmunoassay for the detection of infectious mononucleosis heterophile antibody and antigen. J. Immunol. Methods **14**:51–58.
4. **Fletcher, M. A., and B. J. Woolfolk.** 1971. Immunochemical studies of infectious mononucleosis. I. Isolation and characterization of heterophile antigens from hemoglobin-free stroma. J. Immunol. **107**:842–853.
5. **Golubjatnikov, R., J. E. Koehler, and S. L. Inhorn.** 1975. Absorbed heterophile and ox-cell hemolysin test in serodiagnosis of infectious mononucleosis. Health Lab. Sci. **12**:201–207.
6. **Halbert, S. P., and M. Anken.** 1977. Detection of hepatitis B surface antigen (HBsAg) with use of alkaline phosphatase-labeled antibody to HBsAg. J. Infect. Dis. **136**:S318–S323.
7. **Halbert, S. P., J. Karsh, and M. Anken.** 1980. A quantitative enzyme immunoassay for IgM rheumatoid factor using human immunoglobulin G as substrate. Am. J. Clin. Pathol. **74**:776–784.
8. **Halbert, S. P., J. Karsh, and M. Anken.** 1981. Studies on autoantibodies to deoxyribonucleic acid and deoxyribonucleoprotein with enzyme-immunoassay (ELISA). J. Lab. Clin. Med. **97**:97–111.
9. **Henle, W., and G. Henle.** 1973. Epstein-Barr virus and infectious mononucleosis. N. Engl. J. Med. **288**:263–264.
10. **Henle, W., G. Henle, and C. A. Horwitz.** 1979. Infectious mononucleosis and Epstein-Barr virus-associated diseases, p. 441–470. *In* E. H. Lennette and N. J. Schmidt (ed.), Diagnostic procedures for virus, rickettsial and chlamydial infections, 5th ed. American Public Health Association, Inc., Washington, D.C.
11. **Herbert, J. T., P. Feorino, and G. G. Caldwell.** 1977. False-positive epidemic infectious mononucleosis. Am. Fam. Physician **15**:119–121.
12. **Hoagland, R. J.** 1975. Infectious mononucleosis. Primary Care **2**:295–307.
13. **Horwitz, C. A.** 1979. Practical approach to diagnosis of infectious mononucleosis. Postgrad. Med. **65**:179–184.
14. **Horwitz, C. A., W. Henle, G. Henle, G. Penn, N. Hoffman, and P. C. J. Ward.** 1979. Persistently falsely positive rapid tests for infectious mononucleosis. Am. J. Clin. Pathol. **72**:807–811.
14a. **Kano, K., and F. Milgrom.** 1977. Heterophile antigens and antibodies in medicine. Curr. Top. Microbiol. Immunol. p. 43–69.
15. **Lee, C. L., I. Davidsohn, and R. Slaby.** 1968. Horse agglutinins in infectious mononucleosis. Am. J. Clin. Pathol. **49**:3–11.
16. **Lennette, E. T., G. Henle, W. Henle, and C. A. Horwitz.** 1978. Heterophil antigen in bovine sera detectable by immune adherence hemagglutination with infectious mononucleosis sera. Infect. Immun. **19**:923–927.
17. **Levey, B. A., T. M. Lo, K. E. Caldwell, and M. A. Fletcher.** 1980. Latex test for serodiagnosis of infectious mononucleosis. J. Clin. Microbiol. **11**:256–262.

18. **Lin, T. M., S. P. Halbert, C. T. Chiu, and R. Zarco.** 1981. Simple standardized enzyme-linked immunosorbent assay for human antibodies to *Entamoeba histolytica*. J. Clin. Microbiol. **13**:646–651.
19. **Lin, T. M., S. P. Halbert, and G. R. O'Connor.** 1980. Standardized quantitative enzyme-linked immunoassay for antibodies to *Toxoplasma gondii*. J. Clin. Microbiol. **11**:675–681.
20. **Merrick, J. M., R. Schifferle, K. Zadarlik, K. Kano, and F. Milgrom.** 1977. Isolation and partial characterization of the heterophile antigen of infectious mononucleosis from bovine erythrocytes. J. Supramol. Struct. **6**:275–290.
21. **Miale, T. D., J. L. Barbosa, C. T. Dellinger, and S. L. Wolfson.** 1978. Positive Monospot tests preceding the diagnosis of acute monocytic leukemia in two adolescents. Med. Pediatr. Oncol. **4**:111–114.
22. **Mikkelson, W., C. J. Tupper, and J. Murray.** 1958. The ox cell hemolysin test as a diagnostic procedure in infectious mononucleosis. J. Lab. Clin. Med. **52**:648–652.
23. **Milgrom, F., U. Loza, and K. Kano.** 1975. Double diffusion in gel tests with Paul-Bunnell antibodies of infectious mononucleosis sera. Int. Arch. Allergy Appl. Immunol. **48**:82–93.
24. **Paul, J. R., and W. W. Bunnell.** 1932. The presence of heterophile antibodies in infectious mononucleosis. Am. J. Med. Sci. **183**:90–104.
25. **Schumacher, H. R., R. M. Austin, and S. A. Stass.** 1979. False-positive serology in infectious mononucleosis. Lancet **i**:722.
26. **Schwarzweiss, H., and J. Tomcsik.** 1948. Isolation of the heterogenetic "mononucleosis antigen" from the stroma of bovine erythrocytes. Proc. Soc. Biol. Med. **68**:558–561.
27. **Smith, R. H.** 1979. Fatal adenovirus infection with misleading positive serology for infectious mononucleosis. Lancet **i**:299–300.
28. **Springer, G. F., M. H. Seifert, J. C. Adye, and A. Eyquem.** 1974. Immunization of humans with "infectious mononucleosis receptors" from mammalian erythrocytes. Clin. Immunol. Immunopathol. **2**:563–574.
29. **Stiffler, D. W., and L. E. Yang.** 1977. Serologic evaluation of rapid slide tests for the diagnosis of infectious mononucleosis. Lab. Med. **8**:6–9.