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Detection of Early Antibodies in Human Immunodeficiency Virus Infection by Enzyme-Linked Immunosorbent Assay, Western Blot, and Radioimmunoprecipitation

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A current concept of the serological response to human immunodeficiency virus (HIV) infection in humans is that antibodies to core antigens (p55, p24, and p15) are detectable earlier during initial stages of antibody production than antibodies against envelope antigens (gp160, gp120, and gp41). Comparative studies of Western blot (immunoblot), radioimmunoprecipitation assay (RIPA), and enzyme-linked immunosorbent assay (ELISA) during initial antibody production are limited to case reports and have not resolved the issue. Thirty of the 37 participants who are part of a prospective study had at least one specimen that was negative for anti-gp41 but had one or more other bands on Western blot. Twenty-seven of these 30 specimens were reactive for anti-gp120/160 in the RIPA. Of the same 30 specimens, kits from Bionetics identified 2 (7%), ElectroNucleonics 4 (13%), Abbott 13 (43%), Du Pont 25 (83%), and Genetic Systems 25 (83%). All participants had evidence of serological progression by Western blot, including a gp41 band, on subsequent visits; the ELISA kits of all manufacturers identified these later specimens with greater accuracy. These data show that the RIPA detects anti-envelope antibodies that may be not detectable by Western blot and that the production of anti-envelope antibodies approximately parallels the production of anti-core antibodies. The false-negative results by ELISA would permit transmission of HIV by blood transfusion from donors in early stages of infection. The sensitivity of licensed ELISA kits should be improved to identify antibody as soon as possible after infection.

The acquired immunodeficiency syndrome (AIDS) was first described in 1981 as a disease of homosexual and bisexual men (4) and was later found in intravenous drug users, hemophiliacs (5), and recipients of blood transfusion (6). The probable etiological agent was identified as a retrovirus and described in reports from France (2) and the United States (9). In this report, the retrovirus is referred to as human immunodeficiency virus (HIV) (J. Coffin, A. Haase, J. A. Levy, L. Montagnier, S. Oroszlan, N. Teich, H. Temin, K. Toyoshima, H. Varmus, P. Vogt, and R. Weiss, Letter, Science **232**:697, 1986). An enzyme-linked immunosorbent assay (ELISA) was developed to test serum or plasma specimens for antibodies to the retrovirus (11) and was subsequently licensed in March 1985 to screen blood donors for such antibodies. Blood that is repeatably reactive in the ELISA is, in most instances, retested by Western blot (WB) (immunoblot) as a confirmatory test (13).

The strategy for selecting antigens for candidate vaccines and for serodiagnostic reagents depends on understanding the natural history of antibody production. A widely held belief concerning the serological response to HIV infection in humans is that antibodies to core antigens (p55, p24, and p15) are detectable earlier than antibodies against envelope antigens (gp160, gp120, and gp41) during the initial stages of antibody production (5, 9, 16). In general, the WB and the radioimmunoprecipitation assay (RIPA) favor detecting anti-core and anti-envelope antibodies, respectively (17). The primacy of antibody production is therefore best described by testing sera in both systems. However, comparative studies of the two assays and ELISA kits during initial antibody production are limited to an occasional case report (R. G. Marlink, J. S. Allan, M. F. McLane, M. Essex, K. C. Anderson, and J. E. Groopman, Letter, N. Engl. J. Med. **315**:1549, 1986) and have not resolved the issue of which group of antibodies rise first.

This study set out to determine whether anti-envelope antibodies were present by RIPA in specimens that showed

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only WB anti-core reactivity. We also determined the performance characteristics of five commercially available kits. This report shows that antibodies to core proteins of HIV, as measured in the WB, and antibodies to envelope glycoproteins, as measured in the RIPA, are found simultaneously during early infection and that some commercially available ELISA kits are very insensitive for detecting these early antibodies.

MATERIALS AND METHODS

Overview. The Multicenter AIDS Cohort Study (MACS) is a prospective study of 4,955 initially healthy homosexual and bisexual men in four U.S. cities: Baltimore, Chicago, Pittsburgh, and Los Angeles. The study is designed to elucidate the natural history of infection with HIV, the etiologic agent of AIDS. Participants are seen every 6 months, at which time a detailed questionnaire is administered, a focused physical examination is performed, and clinical specimens are obtained. MACS participants were enrolled from April 1984 to March 1985. The study was approved by the Institutional Review Boards at the National Institute of Allergy and Infectious Diseases, The Johns Hopkins University, Northwestern University, the University of Pittsburgh, and the University of California, Los Angeles (UCLA). Informed consent was obtained from each study participant.

An ELISA was used by the MACS following licensure to determine the prevalence of antibody to HIV and to identify seroconverters among those who were seronegative at base line. Serological screening of the entire cohort was done by E. I. Du Pont de Nemours, Inc., with their commercially available ELISA kit. In addition, kits manufactured by ElectroNucleonics, Inc. (ENI), Bionetics Laboratory Products (formerly Litton Bionetics), and Genetic Systems were used at individual study sites for screening study participants. Confirmatory WBs were performed at Biotech Research Laboratories, Rockville, Md.

Study subjects. Study subjects were identified either by having had discrepant ELISA results or by having had a WB that showed reactivity for core antigens with minimal or no gp41 reactivity. WBs were performed on participants who had seroconverted, had discrepant ELISA results, or had been tested randomly as part of other studies in the MACS. Study subjects were also required to have sera available from at least one subsequent visit.

Procedures. Frozen samples of serum specimens from participants were sent from a central repository to The Johns Hopkins Medical Institutions for testing by ELISA with the Du Pont, ENI, and Bionetics kits, to the American Red Cross, Baltimore, Md., for testing with the Abbott kit, and to UCLA for testing with the Genetic Systems kit. ELISA and WB testing was performed in March 1986. Human T-cell lymphotropic virus type IIIB (HTLV-IIIB) is used in the ELISA kits by Abbott, Bionetics, Du Pont, and ENI; lymphadenopathy-associated virus (LAV) is used in the kit made by Genetic Systems. All specimens were coded, and tests were performed by technicians who were unaware of the code; results were tabulated before the code was broken. Assays were performed as specified by the manufacturers. Positive tests were repeated in duplicate for the Abbott, Du Pont, and ENI kits. Tests that were positive by Genetic Systems and Bionetics were retested as singletons, according to established laboratory procedures; this difference in procedure did not affect the results (see below).

To ensure uniform testing, all initial WBs were repeated at The Johns Hopkins School of Hygiene and Public Health with a kit manufactured by Biotech Research Laboratories. Sera from subsequent visits were tested simultaneously with the initial serum specimens. The WB uses disrupted HIV (HTLV-IIIB) grown in the H9 cell line as antigens for the Western blot and an avidin-biotin method to detect antibodies (S. S. Alexander, C. Tai, R. L. Ting, A. E. Corrigan, A. J. Bodner, and W. David, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, T49, p. 190). The blots were read by the naked eye under code at both Johns Hopkins and Biotech. In most laboratories, positivity is inferred from several bands in conjunction with a band at the gp41 position. A single p24 or p15 band that is weakly reactive, reactive, or strongly reactive is taken to be insufficient evidence to confirm infection with HIV. For the MACS, the following scores were assigned: 0 for a negative band, 1 for a weakly reactive band, 2 for a reactive band, and 3 for a strongly reactive band. The scores of all bands were added, and a value of ≥ 3 defined a positive immunoblot, 2 defined an equivocal blot, and ≤ 1 defined a negative blot. Equivocal blots were sometimes reclassified based on the results of serial specimens.

The contention that anti-envelope antibodies may be absent in gp41-negative specimens was tested with the RIPA. Serial specimens from participants who were shown by WB to be infected were sent to the Harvard School of Public Health for RIPA (1). Specimens were tested, and results were reported under code.

Serial specimens from three WB-confirmed seropositive and seven WB-confirmed seronegative controls were included with the test specimens of the 59 participants who were studied.

RESULTS

All positive and negative control specimens were accurately identified by each ELISA kit, WB, and RIPA. Sixteen of 59 participants were found to be totally negative (no bands) by WB. These 16 participants were selected for longitudinal testing because they had previously shown either isolated, weak p24 reactivity on a single WB blot (n = 11) or a negative blot with a concomitant positive ELISA test (n = 5). Six other subjects had one or more bands to core antigens but showed no serological progression in the WB and could not be confirmed as truly infected with HIV.

The 37 remaining subjects demonstrated progression of seroreactivity in the WB. Twenty-eight of these men were positive at their initial visit and were in an early stage of HIV infection according to their WB pattern and subsequent course (5, 9, 16). The remaining nine men were seroconverters by WB between their first and second visits; the mean interval was 209 days (range, 181 to 237 days). Each had a third visit (approximately 12 months after visit 1) that showed a fully developed WB (many bands, including gp41). The subsequent broadly positive WBs showed that earlier specimens lacking measurable antibody to gp41 did in fact represent HIV infection in each instance. In seven cases, a subsequent broadly reactive WB that included at least a reactive gp41 band was used to interpret a prior equivocal blot. Figure 1 shows representative examples of blot progression. Six of these seven were single blots that occurred just prior to the strongly positive blot. In the remaining case a sole, weakly reactive p24 band progressed to a strongly reactive p24 band 6 months later and progressed further to include a reactive gp41 band 12 months after the first blot. The weak reactivity against p24 on the first blot was considered evidence of infection at the first visit. Each of the three longitudinal serum samples from this participant was nega-

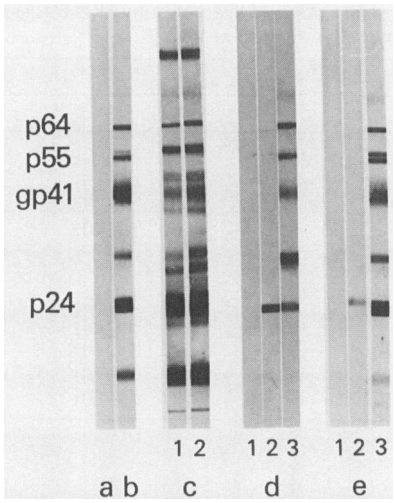

FIG. 1. Examples of blot progression. (a and b) Negative and positive controls, respectively, that are part of the WB kit. (c) Band patterns of two serum specimens from a seropositive control from the MACS. (d and e) Immunoblots from two seroconverters. Lane 2 (d) shows a strongly reactive p24 and reactive bands at p55 and p64. Lane 2 (e) demonstrates a single reactive p24 band. These sera gave negative results by ELISA kits manufactured by Abbott, Bionetics, and ENI. Lanes 3 each show clear serological progression, and these samples were accurately identified by ELISA kits from all manufacturers.

tive by RIPA, whereas in specimens from the other 36 participants, subsequent ELISAs and RIPAs were positive. This inconsistent finding raises the question of a falsely reactive WB for gp41 at the third visit (see Discussion).

Seropositives at initial visit. Table 1 presents a summary of ELISA results by kit and the corresponding reactivity of gp41 in the WB for men whose visit 1 specimens were positive by WB. Twenty-three of these 28 subjects had no detectable antibody to gp41; of these 23 sera, RIPA identified 20 (87%) as having anti-envelope antibodies (gp120/160). Du Pont and Genetic Systems kits correctly identified 18 of these 23. Each of these two kits missed one specimen that the other correctly identified as positive. The WBs of the two specimens were not perceptibly different; each was reactive for p24. Three of the remaining four blots had a faint to strongly reactive p24 band as a sole band; the remaining blot had a reactive p15 band and a faint p64 band.

Of the five participants who had a reactive gp41 band at their first visit, all were positive by RIPA and three were missed by both Bionetics and ENI kits. Both of these kits missed the same specimens, and the one specimen that the Abbott kit missed was also missed by the Bionetics and ENI kits. The degree of agreement in the missed specimens prompted an investigation of possible peculiarities in these blots. Two of the three blots had only two bands present: a strongly reactive p24 and a reactive gp41. The specimen missed by the Abbott kit was one of these. The remaining blot had the latter bands plus three reactive bands against p53, p55, and p64.

Seroconverters at visit 2. Almost identical results were seen in the visit 2 specimens from the seroconverters (Table 2). More than 70% (5 or 6 of 7) of anti-gp41-negative specimens were missed by kits from Abbott, Bionetics, and ENI. The Du Pont and Genetic Systems kits, however, identified all seven of these seroconverter specimens as positive. Bionetics and ENI kits agreed in identifying the single specimen out of seven that they determined was positive. This specimen gave a strongly reactive p24 and a reactive p53 reaction in its blot. It was reactive by the other kits as well. Note, in Tables 1 and 2, that subsequently obtained specimens showed stronger gp41 bands and that the different ELISA kits performed comparably. However, by this time many other bands had also become strongly positive and may have contributed to the similarity of reactivity in the various ELISA kits. In the RIPA, all visit 2 specimens were positive, whether anti-gp41 was present or not. Also, a visit 1 (preconversion) specimen was positive by RIPA (see Discussion).

Combined groups. Table 3 combines data for visit 1 of those who were initially positive and visit 2 of those who seroconverted. This table summarizes the performance of each ELISA kit in identifying specimens from participants with evolving seroreactivity. Reactivity in all kits was correlated with WB reactivity to gp41, especially in kits from Abbott, Bionetics, and ENI, but many other bands were present as well.

The RIPA identified anti-gp120/160 antibodies in 27 of the 30 specimens that were negative for anti-gp41 in the WB. The RIPA accurately identified all specimens that were positive for anti-gp41 in the first positive WB and all subsequently reactive WBs except one.

The criteria for a positive reaction for the five ELISA kits

TABLE 1. HIV antibody results for the RIPA and each ELISA kit compared with anti-gp41 reactivity by WB for samples from men who were seropositive by WB at visit 1

Test and manufacturer	No. (%) of samples							
	Visit 1		Visit 2			Visit 3		
	Absent (n = 23)	Reactive (n = 5)	Absent (n = 2)	Reactive (n = 10)	Strongly reactive (n = 16)	Absent (n = 0)	Reactive (n = 3)	Strongly reactive (n = 14)
RIPA	20 (87)	5 (100)	1 (50)	10 (100)	16 (100)		2 (66)	14 (100)
ELISA								
Abbott	11 (48)	4 (80)	0 (0)	10 (100)	16 (100)		2 (66)	14 (100)
Bionetics	1 (4)	2 (40)	0 (0)	10 (100)	14 (93)[a]		2 (66)	13 (93)
Du Pont	18 (78)	5 (100)	1 (50)	10 (100)	16 (100)		2 (66)	13 (100)[b]
ENI	3 (13)	2 (40)	0 (0)	10 (100)	16 (100)		2 (66)	14 (100)
Genetic Systems	18 (78)	5 (100)	1 (50)	10 (100)	16 (100)		2 (66)	14 (100)

[a] Fifteen tested.
[b] Thirteen tested.

TABLE 2. HIV antibody results for the RIPA and each ELISA kit compared with anti-gp41 reactivity by WB for samples from men who were seropositive by WB at visit 2 but seronegative by WB at visit 1

Test and manufacturer	No. (%) of samples					
	Visit 1 (all bands absent; n = 9)	Visit 2[a]		Visit 3[a]		
		Absent (n = 7)	Reactive (n = 2)	Absent (n = 0)	Reactive (n = 5)	Strongly reactive (n = 4)
RIPA	1 (13)[b]	7 (100)	2 (100)		5 (100)	4 (100)
ELISA						
Abbott	0	2 (29)	2 (100)		5 (100)	4 (100)
Bionetics	0	1 (14)	1 (50)		5 (100)	3 (75)
Du Pont	0	7 (100)	2 (100)		5 (100)	4 (100)
ENI	0	1 (14)	2 (100)		5 (100)	4 (100)
Genetic Systems	0	7 (100)	2 (100)		5 (100)	4 (100)

[a] Results are shown for anti-gp41.
[b] Eight tested.

were examined. A level 20% below the cutoff value for positive reactivity was arbitrarily selected to determine whether false-negative specimens could have been identified at a lower threshold. Lowering the ELISA threshold resulted in only a 19% overall improvement in identifying true-positives (Table 4).

Repeatability, i.e., confirmation, of true-positive reactions also was examined. With the kits of Abbott, Genetic Systems, and Bionetics, all initially positive reactions were repeatable, indicating that the single-repeat algorithm for Genetic Systems and Bionetics had no effect on the overall results (see above). The Du Pont kit gave a positive reaction that became negative on repeat testing in one WB-positive individual from visit 1. The ENI kit did this for seven participants from visit 1 and two from visit 2.

DISCUSSION

Experience with the WB is extensive because it is used to confirm reactivity in the ELISA. Antibodies to core antigens have been described as rising prior to antibodies against envelope antigens. This study demonstrates that anti-envelope antibodies are present even though they may not be detected by WB. It is also apparent that potential blood donors with early infection lack sufficient levels of anti-gp41 and other antibodies to be consistently reactive in the currently licensed test kits yet are positive by both WB and RIPA. These results extend in two ways those of Reesink et al. (10), who tested ELISA kits marketed in Europe. First, they found that, compared with WB, the kits studied were less sensitive in identifying sera from asymptomatic HIV-infected individuals than in identifying sera from AIDS patients. The authors characterized sera from asymptomatic individuals as p24 predominant and those from AIDS patients as gp41 predominant by testing the kits with serial dilutions of sera. In our study it was shown that the sensitivity of U.S. ELISA kits during the early stages of antibody production ranged from similar to to worse than that found in a study with serial dilutions. Second, the WB characterization of p24 and gp41 as predominant may be useful, but it is not altogether correct because anti-envelope (gp120/160) antibodies are present in both types of sera.

ELISA kits. The proportion of gp41-negative participants whose evolving specimens were identified in this study ranged from a low of 7% for Bionetics to a high of 83% for Du Pont and Genetic Systems. While antibody to gp41 seems to be important (J. R. Carlson, S. H. Hinrichs, N. B. Levy, M. B. Gardner, P. Holland, and N. C. Pedersen, Letter, Lancet **i**:1388, 1985), specimens that were missed in spite of reactivity to gp41 seem to indicate that higher-grade reactivity to other antigens may also be required before some ELISA kits become reactive. More intense and broader reactivity is presumably due to longer duration of infection than was present in these men when they initially became anti-gp41 positive.

An intriguing observation from this study was that specimens were not missed randomly. When one kit incorrectly identified a specimen as negative, there was usually at least one other kit, and often several others, that also produced false-negative results with the same specimen. Such consistency in these results essentially rules out a problem with one or another lot of kits as an explanation for the results and indicates a fundamental problem with the ability of certain

TABLE 3. HIV antibody results for the RIPA and each ELISA kit compared with anti-gp41 reactivity by WB for samples from men who were seropositive by WB at visit 1 or 2

Test and manufacturer	No. (%) of samples	
	Absent (n = 30)	Reactive (n = 7)
RIPA	27 (90)	7 (100)
ELISA		
Abbott	13 (43)	6 (86)
Bionetics	2 (7)	3 (43)
Du Pont	25 (83)	7 (100)
ENI	4 (13)	5 (57)
Genetic Systems	25 (83)	7 (100)

TABLE 4. ELISA kit performance after the cutoff for a positive reaction was lowered to 80% of the recommended threshold

ELISA kit manufacturer	No. of samples formerly negative but positive at 80% cutoff level		Original false-negative ELISAs (total no. from all visits)	% Improvement in sensitivity (true-positives/false-negatives)
	False-positive	True-positive		
Abbott	0	4	19	21 (4/19)
Bionetics	0	8	36	22 (8/36)
Du Pont	3	3	7	43 (3/7)
ENI	2	2	30	7 (2/30)
Genetic Systems	0	2	7	29 (2/7)
Total	5	19	99	19 (19/99)

kits to identify positive sera during early stages of antibody production.

It might be suspected that using a given ELISA kit as a primary screening device would yield results that favor that kit. The strongest evidence that such bias cannot explain these results comes from the group of seroconverters (Table 2). Specimens from these participants were selected solely because they had no or minimal reactivity to gp41 on WB. Furthermore, specimens that were selected based on discrepant ELISA results, such as those positive at visit 1, were selected from the population of men who were in the early stages of infection (estimated to be 100 participants), not from the population of strongly seropositive participants (1,880 participants). The estimate of early infection is based on the number of known seroconverters at visit 2, which was 106. The much smaller universe of participants with early infection would mean that the various ELISA kits would have to have discordance rates of approximately 50% for bias to have explained these results. Such a rate of discordance would mean that a given ELISA would have only a 50% chance of identifying a given specimen as positive if another ELISA produced a positive result. This proposition is untenable and cannot result in a highly selected, biased sample of specimens.

RIPA. Specimens from two individuals were very interesting. One individual whose antibody progression by WB took 6 to 12 months may not have been truly infected with HIV. This participant was negative at each visit by every ELISA kit tested and by the RIPA; he is the only individual whose follow-up serum samples reacted in this manner. This situation probably reflects a falsely reactive WB. The other individual had a preconversion specimen (visit 1 of a seroconverter) that was reactive in the RIPA yet negative in each ELISA tested and in the WB. It is not known whether this RIPA reactivity was falsely positive or whether the WB was falsely negative. Resolution of this issue may be available through culturing cryopreserved lymphocytes for HIV.

WB. This report demonstrates that specimens with reactivity to only the core antigens in the WB can represent infection with HIV, especially in high-risk groups. The overall proportion of true-positive solitary p24 bands cannot be estimated from this study for several reasons. First, the overwhelming majority of the cohort has not been tested by WB. This testing is proceeding. Second, the sensitivity of various WB preparations is not standardized; rates of false-positive reactivity are very difficult to compare when immunoblots are performed at different times or with different formulation procedures. In this study, 11 participants were identified as having false-positive weak p24 bands on initial blotting (with a different formulation of the WB) who were totally negative when serial specimens were tested simultaneously. Third, reactive p24 bands are found in certain population groups, e.g., multiparous women, who are probably not infected with HIV. While it is clear that false-positive p24 bands occur, it is equally clear from this study that solitary p24 bands can represent true infection and that such specimens are not being identified properly in screening procedures with the ELISA. In a related matter, it may be that anti-envelope antibodies (gp120/160) rise before anti-core antibodies. In this study, we can show only concomitant antibody occurrence because positivity was defined in terms of the WB. The few specimens in this study that were WB positive and RIPA negative may reflect antigenic variability in the envelope antigens and the relative stability of core antigens.

The proportion of individuals who lacked measurable (by WB) levels of anti-gp41 during seroconversion and the duration of this status are unknown. Published reports of studies that used frequent (weekly) blood sampling seem to show a relatively brief duration of absent gp41 reactivity (3, 8, 12). In spite of its brief duration, however, this pattern of evolution of antibodies seems to occur often. For instance, in the MACS there were 106 WB-confirmed serological converters from visit 1 to visit 2 (a period of 6 months). Of these converters, 19 showed no or weak reactivity to gp41 at their second visit. Therefore, unless individuals who are seroconverting to HIV voluntarily refrain from donating blood or plasma, it seems highly likely that such contaminated blood would be misidentified, to various degrees, by the currently licensed kits. The number of units of blood falsely identified as negative is difficult to estimate given the large number of variables and the incompletely quantified effect of self-deferral. It seems clear that the licensed ELISA kits differ markedly in their ability to detect antibodies during early infection and that the sensitivity of these kits should be enhanced.

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