Monitoring of Specific Antibodies to Human Immunodeficiency Virus Structural Proteins: Clinical Significance


Levels of antibodies to six major structural proteins of human immunodeficiency virus type 1 (gp120, gp41, p66, p31, p24, and p17) were assessed in serial samples from 22 persons with severe hemophilia (16 asymptomatic and 6 who developed acquired immunodeficiency syndrome [AIDS] or AIDS-related complex) with an automated dot blot assay using purified recombinant antigens. High and sustained levels of antibody to gp120, gp41, and p31 were found in all patients irrespective of their clinical condition for 4 to 6 years after seroconversion. In contrast, immune response to p66 and p17 was significantly lower in symptomatic patients. Over time, the levels of these two antibodies, as well as anti-p24, decreased and tended to become undetectable. Abnormal immune response and low levels of antibody to p66 and p17 are early indicators of rapid clinical progression.

Human immunodeficiency virus (HIV) infection is serologically characterized by the development of antibodies to structural and, in most cases, to regulatory gene products. For lack of appropriate methods of quantitation, antibodies are identified qualitatively by Western immuno blotting or radioimmunoprecipitation, which shows mostly antibodies to env and gag gene products. However, semiquantitation of antibodies to specific structural gene products has been performed by end point dilution with Western blot. Significant differences between asymptomatic and acquired immunodeficiency virus (AIDS) patients were found for antibodies to gp110, p65, p55, p31, p24, and p17 but not for anti-gp41. Antibodies to the core protein p24 have been further studied with more quantitative methods such as competitive enzyme-linked immunosorbent assay (ELISA), p24 antigen-binding capacity, or antigen sandwich assay because their absence or their disappearance during the natural course of HIV infection was related to clinical progression.

Antibodies to gp41 and gp120 were also semiquantified with a competitive assay or Western blot and appeared highly immunogenic and constantly detected irrespective of clinical condition. Antibodies to p27 nef, tat, sor, and rev proteins were also studied but not in a quantitative fashion and detectability ranged from 40% to 60%. Only the absence of antibody to the gag gene product, p17, or a low level of antibody to gp110 and/or p65 were found to be of prognostic interest, but no commercial assay was available to further investigate these potential markers. These studies were cross-sectional in subjects seropositive for unknown periods of time, making the differentiation between low level of antibody production and antibody decline over time impossible. Although seropositivity for antibody to HIV-1 is mandatory for the diagnosis of HIV infection, some clinical AIDS cases have been observed where no antibody to HIV was detectable by either enzyme immunoassay or Western blot. In addition, the presence of antibody to some HIV regulatory or envelope proteins was detected before seroconversion but some of these observations have not been confirmed.

The purpose of this investigation was to assess the development of antibodies to the six main structural gene products of HIV-1, env gp120 and gp41, pol p66 and p31, and gag p24 and p17, with an automated dot blot assay using purified recombinant proteins as specific capture antigens since the time of seroconversion. The observation of different antibody patterns in persons with hemophilia who remained asymptomatic or developed AIDS during the first 5 years post-seroconversion provided a new approach to the assessment of prognosis and pathophysiology of HIV disease.

Materials and Methods

A patient population of 22 males with severe hemophilia A (20) or B (2) was selected on the basis of the availability of serum samples collected before seroconversion and during the next 3 to 6 years at 6-month intervals or less. Some of these patients have been previously described. They ranged in age from 10 to 45 (median 23) and seroconverted between 1982 and 1984. Seroconversion was characterized by a repeatedly reactive commercial enzyme immunoassay (Pasteur ELAVIA-1; Diagnostic Pasteur, Paris, France) confirmed by Western blot as the presence of antibody to HIV-1 envelope and one other structural gene product. Patients were followed-up 36 to 70 months after seroconversion (mean 53, median 54 months). At the end of 1988, 16 hemophiliacs were asymptomatic and six had severe AIDS-related complex (ARC) (1) or AIDS (5). One year later, two more patients had developed AIDS and by January, 1990, five had died.

Automated dot blot assay apparatus. The MATRIX Analyzer (Abbott Laboratories, North Chicago, IL) is a self-contained assay system designed to automatically incubate, wash, and interpret reactivity to multiple analytes.

Assay specifications. Recombinant protein sequences from HIV-1 IIIB p17, p24, p31, p41, and p66 have been expressed in Escherichia coli and purified from inclusion bodies in the presence of sodium dodecyl sulfate (SDS). Recombinant HIV-1 SF2 gp120 was purchased from Chiron Corporation (Emeryville, CA). The recombinant antigens, greater than 90% pure by scanning gel densitometry and amino-terminal sequencing, are quantified, then passively absorbed onto discrete regions of bonded nitrocellulose. As a procedural control, antihuman IgG (heavy and light chain specific) (Kirkegaard and Perry Laboratories, Gaithersburg, MD) is also absorbed onto the nitrocellulose array. The nitrocellulose
array is then mounted into a plastic reaction vessel. Under standard assay conditions, the nitrocellulose-absorbed analytes capture specific human Ig and react with the reagents to produce distinct visual spots on the solid phase. The assay is performed within the reaction vessel by diluting 10 μL of serum or plasma into 1 mL of sample diluent. The reaction vessel is mixed and incubated at 35°C within the MATRIX capture specific human Ig and react with the reagents to produce washing with Analyzer for 1 hour, then washed with Tris-buffered saline. At the end of the wash cycle, 1 mL of probe, consisting of antihuman (heavy and light chain) specific antibody, coupled to long-chain biotin (Kirkegaard and Perry Laboratories) is added to the reaction vessel and incubated for 1 hour at 35°C, followed by washing with TBS. At the subsequent step, 1 mL of conjugate containing antibiotin-specific antibody, coupled to alkaline phosphatase is added to the reaction vessel and incubated for 1 hour at 35°C. After washing with TBS, 1 mL of substrate containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP) is added to the reaction vessel and incubated for 30 minutes at 37°C. HIV-specific reactivity is measured as reflectance at 660 nm, then converted to density of reflectance (Dr) according to Kubelka-Munk. Non-specific binding is subtracted, and the resulting net Dr value for each antigen is divided by an antigen-specific cutoff value. A sample to cutoff (S/Co) ratio of 1.0 or greater indicates reactivity to that particular recombinant HIV protein.

Semi-quantitative p24 antibody enzyme immunoassay. The ability of the automated dot blot assay to quantify antibody level was compared with an enzyme immunoassay designed to measure p24 HIV-1 antibody titer. In brief, highly purified recombinant p24 antigen was expressed in E coli, purified to 95%, and coated onto the solid phase. After 2 hours of incubation at 40°C with four serial dilutions of each sample (1:5, 1:25, 1:125, and 1:625), the presence of p24 antibody was detected with the same p24 antigen labeled with horseradish peroxidase. After 1 hour of incubation and repeat washing, the substrate (o-phenylenediamine) was added and 30 minutes later the color development was blocked by H2SO4. Color intensity was measured with a spectrophotometer at 492 nm. The antibody titer was derived from the intercept of the regression line fitting all dilutions and a calculated cutoff. It was then mathematically transformed into nanograms per milliliter of p24 antigen binding capacity.

CD4+ cell counts were obtained by immunofluorescence using anti-CD4 monoclonal antibody (MoAb) or by flow cytometry. Clinical examination was done by the attending physician every 6 months and the Centers for Disease Control (CDC) classification of HIV disease was used. In this study, only CDC IV a (ARC) and IV c (AIDS) are reported as end points of clinical progression. The χ2 test was used to analyze dichotomous results.

RESULTS

The two clinical groups studied were composed of six patients who developed HIV-related clinical symptoms and 16 who did not during the 3 to 6 years after seroconversion. The two groups did not differ significantly in age, date of seroconversion, or duration of follow-up and CD4+ cell count. All were initially HIV antigen negative, but over the follow-up period, 3 of 6 patients who became symptomatic, and 6 of 16 remaining asymptomatic, developed HIV antigenemia. The natural course of immune response to HIV-1 structural proteins was compared in the two clinical groups as the mean log ratio of reflectance over a cutoff established for each recombinant antigen (Figs 1 through 3). The mean ratio was calculated within intervals of 6 months. When a patient had multiple samples within each interval it was averaged so that only one value per patient per interval was used. Two clearly distinct patterns of immune response were seen depending on the antigen considered and the clinical group. Three antigens, gp120, p41, and p31, corresponding to antibodies to the external and transmembrane envelope glycoproteins and the endonuclease elicited an equally strong and sustained immune response in asymptomatic and symptomatic clinical groups (Figs 1 and 2). Significant differences between the two groups were seen with the other three antibodies to p66, p24, and p17. Antibody response to HIV-1 reverse transcriptase (p66) was significantly lower in the symptomatic group at all time points (P < .01). Beyond 30 months postseroconversion, the relative concentration of this antibody decreased progressively in the symptomatic group and was below a ratio of 25 in four of the six patients followed-up for 4 years or

![Image of Fig 1: Time course of antibodies to HIV-1 gp120 (circles) and gp41 levels (triangles) in 22 persons with hemophilia. Each data point corresponds to the mean log ratio of reflectance (Dr) in 16 asymptomatic (solid symbol) and six symptomatic (open symbol) patients in each time interval from seroconversion. Standard deviation (SD) of ratios in the symptomatic group at the various time intervals ranged from 25 to 119 and 7 to 116 for antibody to gp120 and 41, respectively. In the asymptomatic group SDs ranged between 12 and 76 (gp120) and 8 and 175 (gp41).](image1)

![Image of Fig 2: Time course of antibodies to HIV-1 p66 (circle) and p31 (triangle) levels in 22 persons with hemophilia. SDs of ratios ranged 28 to 198 and 12 to 172 for anti-p66 and p31, respectively, in the symptomatic (open symbol) group, and 88 to 160 and 39 to 175 in the asymptomatic (solid symbol) group.](image2)
within the first 2 years of follow-up. who developed symptoms had detectable p17 antibody
patients progressively developed a strong antibody re-
groups was seen with anti-pl7 (Fig 3). While asymptomatic
of antibody was assessed by comparing p24 antibody ratios
within a range of ratio 2 to 500 the
correlation coefficient between the two assays was 0.783
levels in 22 persons with hemophilia. SDs of ratios ranged
the initial immune response to
antibody titer was maintained for 2 years and subsequently
described in Fig 3, the initial immune response to
and results obtained with a more quantitative assay using a
Fig 3. Time course of antibodies to HIV-1 p24 (circle) and p17
more (Fig 2). Nine of 16 asymptomatic hemophiliacs
and p24 in two patients.
The ability of the dot blot assay to semiquantitate levels of
as shown in Fig 3, the initial immune response to
The most striking difference between the two
groups was significant at all time points
The average interval between the two peaks when
The hypothesis that the disappearance of antibody to p24
and p17 might reflect the formation of an increasing level of
specific immune complexes has been proposed. To
address this question, the relation between the immune
response development, the kinetics of disappearance of
each antibody, and the occurrence of HIV antigen was
In contrast, in five of nine patients, the peak of anti-p17 was
observed concomitantly or later than HIV antigen first
appearance. In the 22 patients studied, the maximum ratio of
p24 and p17 antibodies was reached simultaneously in
six, anti-p24 maximum ratio preceded the peak of anti-p17
in seven, and anti-p17 preceded the peak of anti-p24 in nine
patients. The average interval between the two peaks when
observed at different times was 22 months. However, over
time after the maximum antibody level was reached, variations
of p66, p24, and p17 antibody ratios varied similarly in
14 patients (both stable eight, both declining six) and
differed in eight patients. In these eight patients, variations
in anti-p66 and p17 levels were concordant in four, p66 and
p24 in two, and p24 and p17 in two patients.

DISCUSSION
In the abundant literature regarding antibody response
to HIV-1 proteins and the variations of antibody levels, two
main hypotheses have been proposed. One is the immune
complex hypothesis according to which some antibodies
(anti-p24 and p17) have a low level of detectability and tend
to decrease in concentration over time in relation to the
formation of complexes with free viral antigens. The second hypothesis stems from the fact that HIV causes
immunodeficiency and could therefore impair the mecha-
nisms of antibody production. The first hypothesis has
been supported by several lines of evidence. (1) Specific
p24 anti-p24 immune complexes have been found in p24
antibody negative samples. (2) Anti-p24 disappearance
often precedes the detection of its complementary p24
antigen. (3) Both absence of p24 antibody and presence of
HIV antigen are correlated with plasma viremia and

Table 1. Temporal Occurrence of Maximum Ratio of Antibody to p24 and p17 HIV Antigen and Symptoms in Nine Hemophiliacs Who Became
HIV Antigen Positive

<table>
<thead>
<tr>
<th>Patient</th>
<th>Maximum Reflectance Ratio</th>
<th>Time Interval From Seroconversion (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p24</td>
<td>p17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>14.4</td>
<td>0.4</td>
</tr>
<tr>
<td>14</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>18</td>
<td>4.3</td>
<td>0.6</td>
</tr>
<tr>
<td>11</td>
<td>154.8</td>
<td>32.5</td>
</tr>
<tr>
<td>15</td>
<td>19.8</td>
<td>4.6</td>
</tr>
<tr>
<td>17</td>
<td>97.0</td>
<td>255.0</td>
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<tr>
<td>19</td>
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<td>24.3</td>
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<tr>
<td>20</td>
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<td>41.0</td>
</tr>
<tr>
<td>23</td>
<td>69.0</td>
<td>109.1</td>
</tr>
</tbody>
</table>

*Patient 23 developed AIDS in 1989, 6 months after the end of the study.
clinical progression. In contrast, besides the reported decline of anti-gp120 in AIDS associated with absence of anti-p24, there is little evidence to be credited to the second hypothesis. In addition, the fact that antibody disappearance is restricted to antibodies to the two core proteins rather than to all antibody species would be difficult to explain in the context of a global dysfunction of immune mechanisms.

The data presented provide a global view of the natural history of antibodies to most HIV-1 structural proteins. Two groups of antibody seem to emerge. One group includes antibodies to external and transmembrane glycoproteins and endonuclease p31. As suggested previously by end-point dilutions on Western blot, the immune response to these antigens is strong and sustained in all patients, irrespective of the clinical outcome. However, in a previous report from the same group, a lower titer of antibody to gp120 was observed in subjects who progressed clinically from asymptomatic to ARC or AIDS. In our study, no evidence of a decline was seen in five patients with AIDS and one with ARC at the time or after clinical diagnosis (Figs 1 and 2). The discrepancy might be explained by the selection of a population of progressors who had seroconverted considerably earlier than the nonprogressor group, although both groups were asymptomatic at baseline, while our group was unselected and studied since seroconversion.

The second group of antibodies to p66, p24, and p17 is more heterogenous but is characterized by a significantly lower immune response in patients who developed HIV disease and a subsequent decline of detectable antibodies (Figs 2 and 3). This decline seems to occur soon after seroconversion for anti-p24 and later, 2 to 3 years after seroconversion, for anti-reverse transcriptase p66. In patients who did not develop HIV disease within 60 months after seroconversion, levels of antibodies to p66 and p24 tended to remain stable and antibody to p17 seemed to increase. Antibody pattern differences between patients who developed AIDS and those remaining asymptomatic can be explained by two factors: (1) the innate inability of certain patients to mount strong immune response to apparently critical antigens; and (2) the formation of immune complexes removing some antibodies. The importance of the humoral immune response to p24 antibody in the host defense against HIV has already been suggested. Our data tend to extend this hypothesis to both anti-reverse transcriptase and anti-p17. A neutralizing activity of antibody to p17 has been suggested and its absence in hemophiliacs who progressed to AIDS would be compatible with such function. However, the major body of data indicates that antibody to gp120 is the primary, if not unique, agent of neutralizing activity. The functional role of anti-p17 remains to be confirmed. The correlation between antibody variation and antigen release in circulation appears closer for anti-p24 than the other two antigens (Table 1). However, concordant variations of antibody to p66, p24, and p17 were observed in 14 hemophiliacs and of anti-p66 and p17 in 18 patients. The apparent discrepancies observed between these antibody variations, in particular to p24 and p17, can be explained by different kinetics of immune response. P24 appears to be a stronger immunogen inducing early maximum levels of antibody while p17 tends to elicit steadily increasing antibody levels over several years in asymptomatic hemophiliacs. In addition, as previously suggested, episodes of intermittent release of virus and/or viral proteins in circulation might take place. In this study, two patients who became HIV antigen positive had simultaneous fluctuations of antibody ratios to p24 and p17, suggesting underlying bursts of immune complex formation.

Contrary to the accepted concept that antibody to gp120 is the main key to containing HIV infection, our data suggest that other antibodies are critical, either directly or through mechanisms reflected in the levels of antibodies to p66, p24, and p17. The role of antibodies to HIV envelope proteins appears more qualitative than quantitative, as suggested by studies of neutralization titers or peptide binding.

Several other markers of clinical progression have been described. Among them, CD4+ cell count, levels of β-2 microglobulin and serum neopterin were shown to be strongly correlated and significantly predictive of AIDS. In a limited number of patients, the CD4 cell count of the two clinical groups were similar during the first 30 months after seroconversion (Fig 4). Later, the difference became significant and the rate of cell decline was accelerated in patients developing HIV disease.

The results of this study suggest that monitoring the levels of individual antibodies to HIV-1 structural proteins can provide early prognosis of HIV disease. HIV antigen and CD4+ cell count become predictive later in the course of the infection. The observations made in this study are derived from a relatively small group of patients with hemophilia. Similar studies should be undertaken in other risk groups where HIV is sexually transmitted or in intravenous drug abusers to confirm the data obtained in hemophilia.

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