CLINICAL RESEARCH

HIV antigen and antibody detection: variable responses to infection in the Edinburgh haemophiliac cohort

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Abstract

Sequential serum samples from 18 haemophiliac patients exposed simultaneously to human immunodeficiency virus type 1 (HIV 1) in early 1984 were tested retrospectively for serological markers of infection. Assay for total antibodies to HIV established that the time to seroconversion might be as long as 110 days after exposure to contaminated factor VIII; serum samples were also tested by Western blotting, by enzyme linked immunosorbent assay (ELISA) for specific antibodies to envelope and core proteins, and for p24 antigen by two assay systems during the two years after infection. The studies showed that five of the 12 patients for whom serum samples obtained between exposure and seroconversion were available had transient p24 antigenaemia. Although amounts of total antibody to HIV and of antibodies to envelope proteins rose continuously during the two years of the study, amounts of antibody to the core protein were variable and tended to decline in patients who became symptomatic. Two patients had persistent p24 antigenaemia that began four months after seroconversion; these patients remained asymptomatic. One patient who developed the acquired immune deficiency syndrome (AIDS) had transient antigenaemia at the time of seroconversion but failed to show any antigen for the rest of the study; progression to AIDS was accompanied by an increase in antibodies to envelope proteins.

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Much of the variability in the course of infection with HIV must represent the differences in the susceptibility of the patients to infection.

Introduction

Patients with haemophilia A are one of the groups at risk from infection with the human immunodeficiency virus type 1 (HIV 1) because they are treated with factor VIII concentrates. Infection with the virus is associated with the appearance of specific antibody that persists throughout the disease.[1] Detection of the antibody is the current method of determining past exposure to the virus and is used for screening blood donors and subjects at risk of infection. Antibody can be detected by several methods, including enzyme linked immunosorbent assays (ELISAs), radioimmunoprecipitation, and Western blotting assays with purified virus as the antigen. These tests differ in sensitivity and specificity.[2,3] Antibodies are directed against all the structural proteins encoded by the gag (group specific or core), pol (polymerase), and env (envelope) genes as well as the non-structural proteins.[4] There is a prolonged stage of infection with HIV 1, often lasting for several years, during which the patient is free of symptoms. It is not known what proportion of patients will eventually develop the full acquired immune deficiency syndrome (AIDS); laboratory tests that could predict the time course of progression in each patient would be of great clinical value.

Free HIV protein, particularly p24, a core protein, is often detected in serum samples from patients at certain stages of infection. There is evidence that transient antigenaemia occurs immediately before seroconversion.[5] A decrease in reactivity with HIV core proteins measured by Western blotting and a commercially available test for antibody against the core protein in serum samples from patients who have progressed to develop AIDS has been observed.[6-8] Immune complexes[9] and free viral antigen[10] have been detected in patients with AIDS after the loss of reactivity to p24. These findings suggest that there is a balance between the rate of viral replication and the ability of the immune system to clear the released virus.

In a previous report infection with HIV was described in a cohort of haemophiliac patients from south east Scotand who had been

maintained solely on locally produced factor VIII.[11] In most serological studies, including those in haemophiliac patients, the precise time of exposure is unknown. In this study, however, infection was acquired from a single batch of factor VIII during a short period starting in March 1984. Furthermore, the low prevalence of HIV 1 infection in Scotland in 1983, when blood for the batch was collected, makes it probable that the batch was contaminated by a single donation. The whole cohort may therefore have been exposed to a single strain of HIV 1. This fact may become important should important variations in pathogenicity or antigenicity be discovered between different isolates of HIV.

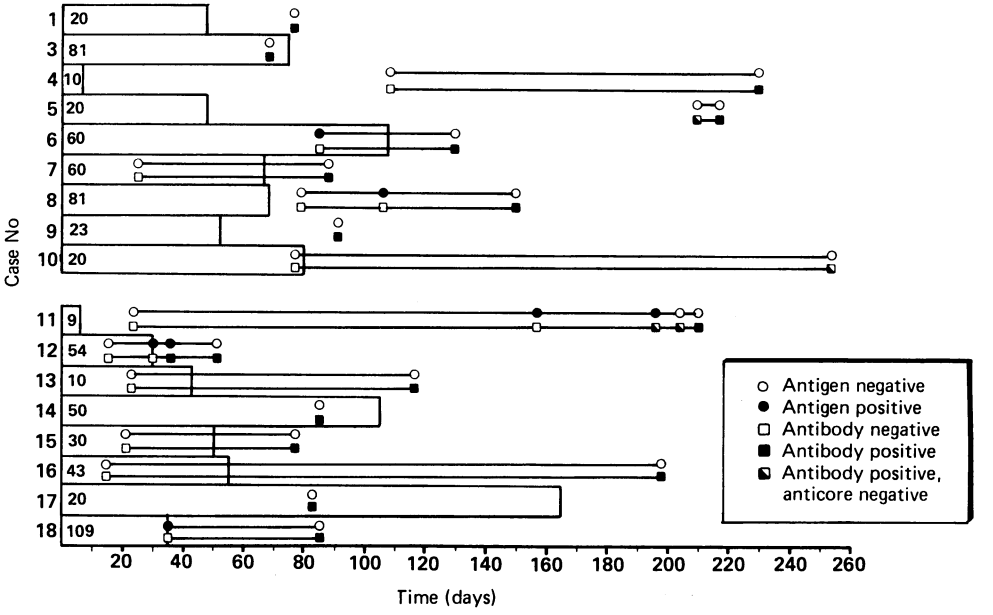

FIG 1—Relation between transfusion of factor VIII contaminated with HIV and detection of p24 antigen and antibody specific for HIV. Boxes indicate length of time for which infected batch was administered; figure in box is total number of units transfused.

Infection in the cohort was investigated by retrospective testing of stored serum samples from the patients. Antibody specific for HIV was measured by Western blotting and by an ELISA using whole virus, envelope protein, or core protein as antigen. Concentrations of antigen in serum were measured by two commercially available ELISAs. The data were compared with the clinical state of the patients to determine whether progression to AIDS or AIDS related complex correlated with any of the serological measurements.

Patients and methods

Thirty two haemophiliac patients were inadvertently transfused with a contaminated batch of factor VIII from the Scottish National Blood Transfusion Service in March 1984. There was no evidence of exposure to HIV 1 before or after this in any of the patients. Eighteen patients underwent seroconversion after transfusion, and these form the study group. One patient (case 2) underwent seroconversion after leaving Edinburgh, and although some clinical information about him was available serum samples could not be tested and he was omitted from the study. Serum samples were taken from the other patients for unrelated reasons at varying intervals from before transfusion to the time of writing; samples were stored at −20°C and tested retrospectively (up to 30 months' follow up). The concentrations of p24 antigen were measured before seroconversion and at four monthly intervals thereafter for the duration of the study. Amounts of antibody against HIV were measured in all positive samples, and amounts of antibody against envelope protein were measured immediately after seroconversion, after one year, and after two years. Antibodies against core protein were measured in the same samples, and more often in any patients whose amount of antibody fell during any part of the study. No samples were available for three patients after the first year of infection, and so they could not be included in the comparison of clinical progression and amounts of core antibody. Clinical state was determined in early 1987 and the patients divided into "well" and "unwell" groups.

Western blotting—Purified HIV 1 viral lysate (Dupont) (20 μg) was applied to the top of a band of polyacrylamide gel (12·5% acrylamide, 0·6% NN-methylene-bis-acrylamide) 17 cm wide and electrophoresis carried out by the discontinuous buffer system of Laemmli.[12] After overnight blotting the nitrocellulose sheet was blocked in 1% bovine serum albumin and 1% gelatin for 20 minutes at room temperature. Test or control serum was diluted 1/50 in phosphate buffered saline with 0·2% Tween 20 and incubated with a 2 mm strip of nitrocellulose for two hours at room temperature. The second antibody was horseradish peroxidase conjugated antihuman IgG (Scottish Antibody Production Unit) diluted 1/200 in phosphate buffered saline with Tween 20 and incubated in the same way as the first antibody. The substrate was diaminobenzidine in phosphate buffered saline, pH 7·6.

Measurement of total antibody (anti-HIV)—A direct binding assay (Dupont) was used to compare amounts of antibody. The test is a two step indirect ELISA comprising a first incubation of a 1/11 dilution of test serum in an antigen coated well and a second incubation with an alkaline phosphatase conjugated antihuman IgG conjugate, and a colour reaction test with paranitrophenol. Three serum samples with antibody to HIV 1 were diluted serially in serum samples that had yielded negative results when tested in this assay. There was an exact linear relation between the amount of antibody and optical density in the range 0-0·8. Unknown samples were tested at two dilutions (generally 1/100 and 1/1000, final test dilutions 1/1100 and 1/11 000) in the assay that included multiple replicates of the control serum that had yielded a positive titre diluted at 1/1000. Simple division of the test optical density (providing that the value lay in the range 0-0·8) by the control positive optical density gave an amount of antibody relative to the positive control. Results were expressed in units (U), with the positive control arbitrarily assigned 1000 U.

Antibody against core and envelope proteins—The Abbott confirmation (envelope/core) assay is a competition assay between antibody to HIV labelled with horseradish peroxidase and test serum at a 1/5 dilution for cloned deoxyribonucleic acid (DNA) core protein (mainly p24) and cloned DNA envelope protein (mainly unglycosylated gp41). Serial dilutions of positive samples were made in samples that had yielded negative results and tested in the Abbott envelope/core confirmation assay. Optical density readings of test samples (at two appropriate dilutions) were related graphically to a standard dilution curve of the amount of antibody in the positive control serum. The difference in antibody dilution was used to calculate a relative antibody level provided that the optical density values were in the range 0·15-0·80. Results were expressed in units (as defined above).

Antigen (p24) detection—The antigen capture ELISA (Dupont) was used for most of the study. Results were expressed in pg/ml calculated from a standard curve. Samples yielding positive results were also tested by the

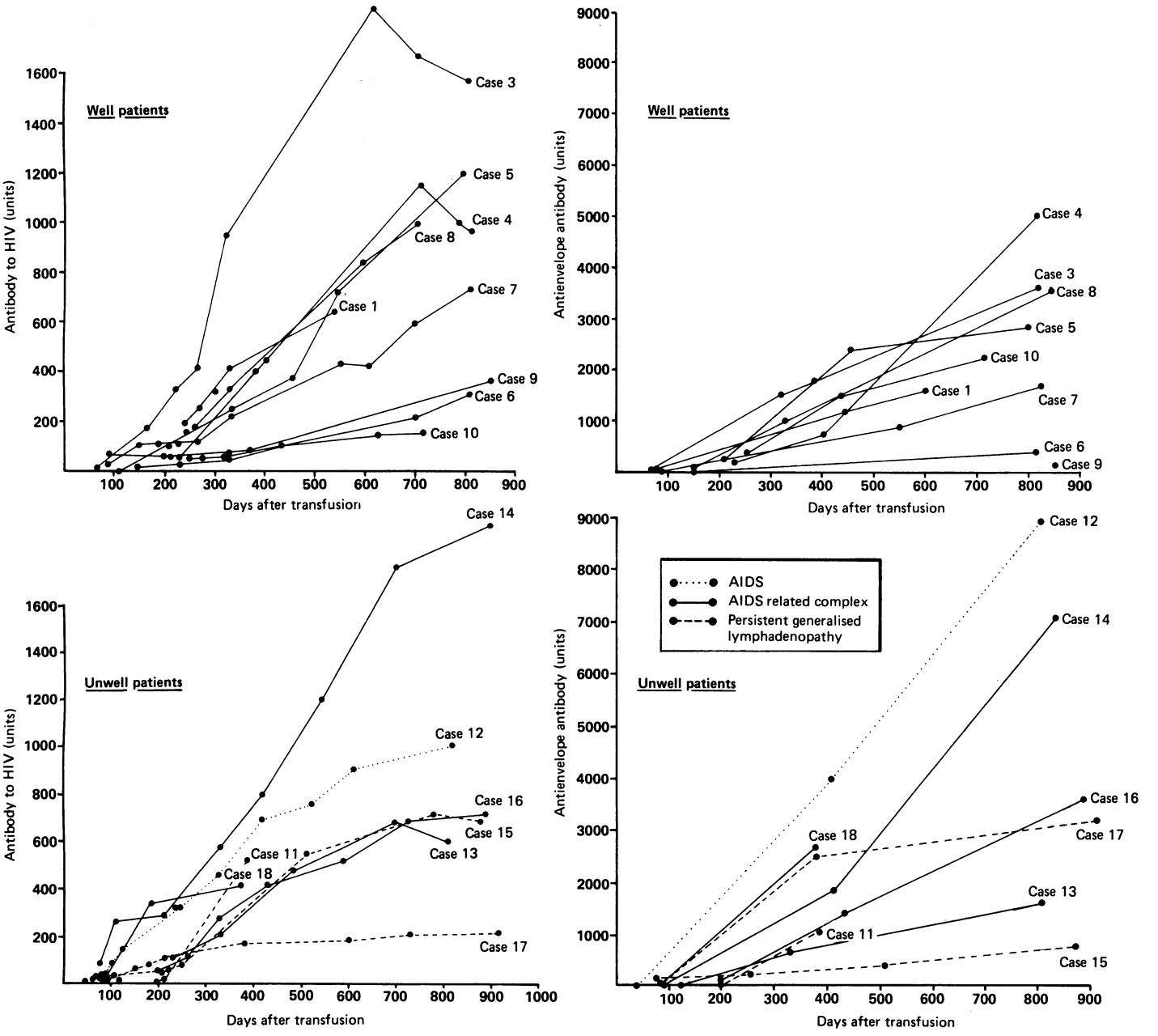

FIG 2—Antibody to HIV and to envelope protein in well and unwell patients.

Abbott ELISA with good reproducibility and comparable sensitivity. The presence of antigen in samples from two patients in whom it had reappeared after seroconversion was confirmed by neutralisation with neutralising and control antisera from Abbott, and independently by human control samples that were positive and negative for antibody to HIV.

Results

CLINICAL COURSE OF INFECTION

Although the 18 patients in the study were infected at about the same time with the same material, they differed in their current clinical state of HIV infection. Ten patients (cases 1-10) remained asymptomatic. Table I gives details of the remaining eight patients, who developed symptoms before March 1987 (cases 11-18). Two of the patients classified as well also developed splenomegaly; although this may be the result of HIV infection, splenomegaly may occur in haemophiliac patients for other reasons. There was no other evidence of progression of disease in these two patients, and so they were classified as well. The unwell group (eight patients) comprised those with AIDS, AIDS related complex, and persistent generalised lymphadenopathy.

TABLE I—*Clinical state of unwell patients*

Case No	Development of symptoms
11	Normal at 12 months; persistent generalised lymphadenopathy at 17 months
12	Disease similar to glandular fever at seroconversion; AIDS related complex at nine months, AIDS at 13 months
13	AIDS related complex at 28 months
14	AIDS related complex at 29 months
15	Persistent generalised lymphadenopathy at 11 months
16	AIDS related complex at 31 months (onset probably 25-26 months)
17	Normal at six months; persistent generalised lymphadenopathy at 12 months
18	AIDS related complex at 22 months

SEROCONVERSION

Figure 1 shows the time taken for HIV antigen and antibody to appear after transfusion of the contaminated factor VIII. Antigen was detected in serum samples from five patients before or at the time of seroconversion. The actual prevalence of antigenaemia may have been higher as, because it is a transient phenomenon, its detection depends on having a sample taken at the appropriate time. The antigenaemia seemed to last a fairly short time, the

longest periods being 35 days in case 12 and 70 days in case 8. The antigen concentration varied between patients, ranging from 20 pg/ml (case 6) to 3600 pg/ml (case 18). This latter figure was the highest observed and may have been related to the transfusion of a large amount of factor VIII (109 units) over only 35 days.

Serum samples were tested by Western blotting and for antibody against envelope protein by ELISA for the earliest signs of seroconversion. The two tests were in complete agreement about which samples did and did not show antibody. There was no evidence that antibody against envelope protein was detectable before the appearance of antibody reactivity by Western blotting in any of the patients. Western blotting was less sensitive for antibody to gp41 than the specific ELISA, as nine of the first positive samples reacted only with core proteins (p24 and pr55) in the Western blotting assay. Reactivity with gp41 in the Western blotting assay appeared some weeks later in these patients. The appearance of anticore protein reactivity was delayed when measured by the specific ELISA in two patients (cases 5 and 11). Their serum samples were, however, positive for p24 antibody by Western blotting, reflecting the difference in sensitivity of the two tests. Absence of anticore protein reactivity in the presence of serum antigen was observed in case 11 at 196 days, although one patient (case 12) showed serum antigen and anticore protein reactivity at the same time (35 days). One patient (case 10) was unusual in that the first positive and all subsequent serum samples during the two year follow up failed to react with core protein and to p24 in the Western blotting assay and were negative for p24 antigen.

The time to seroconversion after transfusion varied among individual patients. One patient (case 11) developed antibody at least 160 days after first transfusion, though antibody appeared in serum from case 12 between days 30 and 36. There seemed to be some association between the amount of factor VIII transfused and the time to seroconversion, but the varied times of sampling for each patient made this difficult to prove.

FOLLOW UP PERIOD

Figure 2 shows units of total antibody specific for HIV and antibody against envelope protein in all patients over the two year follow up period. There were consistent upward trends in both the well and unwell patients, with large individual variations. There was no statistical difference, however, in the average units of antibody to HIV between the well and unwell groups at one year or at two years after transfusion. Similarly, there was no significant difference in antibodies against envelope protein between the two groups. Within the unwell group there were no consistent differences in units of antibodies among the patients with persistent generalised lymphadenopathy, AIDS related complex, or AIDS.

Antibody to core protein was also measured by ELISA, but the same sort of upward trend was not observed in all patients. Eight patients, seven well and one with persistent generalised lymphadenopathy (case 15), showed steadily rising numbers of units of antibody to core protein over the two year follow up period, although there was considerable variation in the values finally achieved. Serum samples from all eight patients were negative for p24 antigen throughout.

Four patients showed falls in the number of units of antibody to core protein within months of seroconversion (table II). To confirm this observation the same serum samples were tested by Western blotting; those from case 12 are shown in figure 3. There was good correlation between the observed intensity of the p24 band and the number of units of anticore protein antibody in all four patients, although samples negative for the antibody as measured by ELISA often showed a weak p24 band by blot. In case 1 small amounts of p24 antigen were detected 271 days after exposure and persisted for the remainder of the follow up period. One patient (case 13) may have had small amounts of antigen on more than one occasion after seroconversion, but samples were not always reactive and were negative by the Abbott antigen test. All samples from the other two patients were negative for antigen.

TABLE II—*Patients in whom anticore antibody levels fell during follow up period*

Case No	Diagnosis								
12*	AIDS	Days after transfusion	36	101	123	243	416	607	808	1048
		Titres (units)	2·7	8·4	5·7	2·8	1·1	1·2	0	0
13*	AIDS related complex	Days after transfusion	27	117	180	330	807			
		Titres (units)	0	2·7	4·7	0	0			
17*	Persistent generalised lymphadenopathy	Days after transfusion	83	212	381	728	916			
		Titres (units)	1·7	75	15	15	0			
1	Well	Days after transfusion	77	271	475	629				
		Titres (units)	1·6	0	0	0				
		Antigen (pg/ml)	0	20	18					

*Serum samples yielded negative results to p24 antigen.

TABLE III—*Patients in whom anticore antibody levels rose after falling or fluctuated during follow up period*

Case No	Diagnosis								
14*	AIDS related complex	Days after transfusion	85	107	210	231	416	540	694	833
		Titres (units)	1·7	1·9	0·5	0	1·1	2·6	4·8	18
16*	AIDS related complex	Days after transfusion	14	198	258	327	433	887		
		Titres (units)	0	11	4	11	4	9		
8	Well	Days after transfusion	150	245	332	598	844			
		Titres (units)	1·4	1·7	0·5	7·9	8·9			
		Antigen (pg/ml)	0	35	20	80	200			

*Serum samples were negative for p24 antigen.

Two patients showed a different pattern (table III, fig 4); one (case 14) showed rising amounts of antibody to core protein after seroconversion and then a fall, becoming negative by the ELISA and unreactive with p24 by Western blotting. Antibody reappeared, however, some months later, and the amount continued to rise for the rest of the study period. A similar pattern was observed in case 8. A rising concentration of p24 antigen was detected in this patient from day 245 onward, despite the reappearance of antibody to core protein. One patient (case 16) (table III) was harder to categorise as he showed fluctuating amounts of anticore protein antibody after seroconversion and weak p24 blot reactivity, and was consistently negative for p24 antigen.

Discussion

Testing serum samples after transfusion of the implicated batch of factor VIII allowed an estimate of the incubation period before seroconversion. This turned out to be variable, ranging from less than 35 days in one patient to over 160 days in another. There was some evidence that seroconversions occurred more rapidly in

patients who were given larger amounts of the implicated batch of factor VIII. This would emphasise the association found previously between the larger amount of the contaminated batch used by these 18 patients compared with the 14 patients who remained seronegative.[11]

There was a transient antigenaemia, which was variable in both degree and duration, in at least five patients before seroconversion, similar to that reported by Allain *et al.*[6] A particularly high concentration (3600 pg/ml) was found in one patient who had been given a lot of factor VIII over a fairly short period. The delayed appearance of antibody to core protein by specific ELISA on seroconversion noted by Allain *et al*[6] was found in this study, but the serum samples were reactive with p24 on Western blotting, indicating the low sensitivity of the ELISA for the antibody. By contrast, the ELISA for the antienvelope protein antibody was more sensitive for glycoprotein antibody than the Western blotting assay.

All patients showed rises in amounts of antibody to HIV and to envelope protein during the follow up period. Five patients developed AIDS related complex and AIDS despite rising titres of antienvelope protein antibody, indicating that this antibody is not protective once patients are infected. The rise in titres may indicate continual stimulation of the immune system by viral replication; if this is the case the similar amounts of antibody in the well and unwell groups indicate a poor prognosis for patients even if they remain asymptomatic.

Although antigen could readily be detected before seroconversion, only two patients (both well) showed long term antigenaemia. This is a low prevalence compared with the results of other investigators and may reflect the short time that the patients had been infected.[10] It is notable, however, that the patient who developed AIDS remained negative for p24 antigen after seroconversion despite frequent testing. Similarly, serum samples from at least three of the four patients with AIDS related complex yielded consistently negative results over the study period.

Levels of antibody to core protein were the most variable measurement, both among individuals and with time, and they correlated to a certain extent with clinical outcome. Patients with steadily rising antibody titres were well, except for one with persistent generalised lymphadenopathy. Conversely, all patients

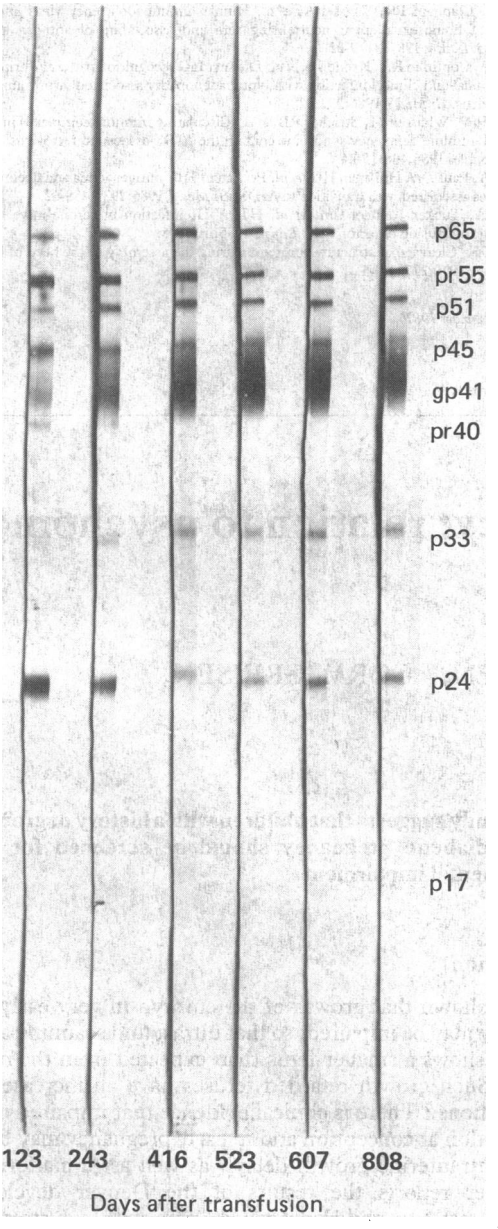

FIG 3—Reactivity of sequential serum samples from case 12 in Western blot assay.

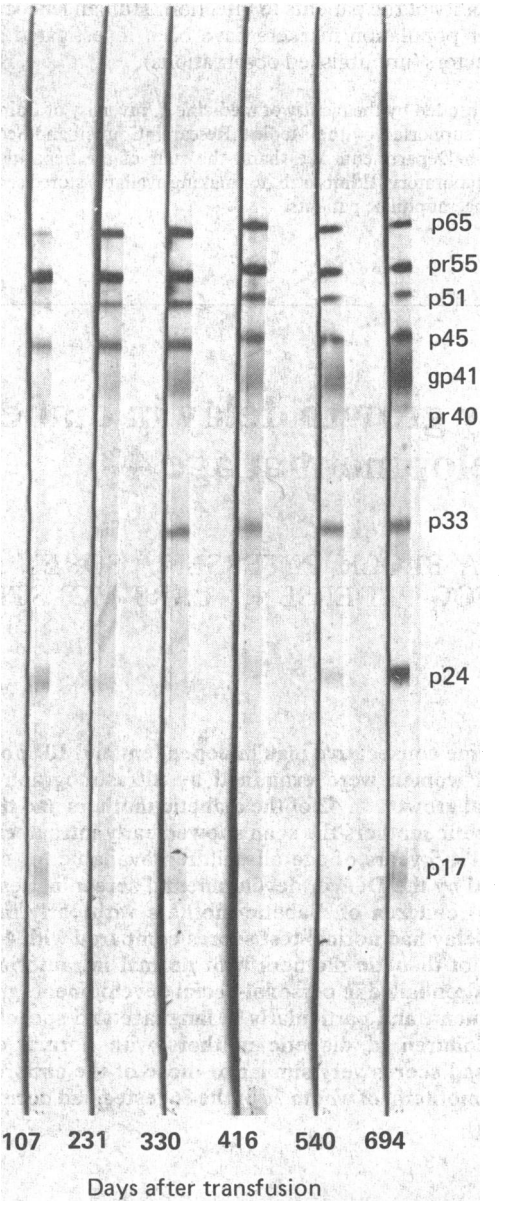

FIG 4—Reactivity of sequential serum samples from case 14 in Western blot assay.

with AIDS or AIDS related complex showed abnormal losses of the antibody. There were, however, two well patients and one with persistent generalised lymphadenopathy who also showed loss of antibody to core protein. It is certainly possible that these patients may subsequently develop symptoms, but none had any clinical evidence of immunosuppression at the time of writing. The importance of the reappearance of antibody to core protein observed in two patients is not clear; one had AIDS related complex, so it does not seem to reflect recovery from infection.

This small cohort of haemophiliac patients allowed us to study the antigen and antibody responses to HIV infection acquired at a known time from a single source. Although the infectious dose administered to each patient was different, much of the variability in the course of infection was clearly the result of differences in susceptibility of the patients to infection. Human leucocyte antigen and other population markers have been investigated as possible related factors (unpublished observations).

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References

1 Safai B, Sarngadharan MG, Groopman JE, *et al.* Seroepidemiological studies of HTLV-III in AIDS. *Lancet* 1984;i:1438-40.
2 Oldham LJ, Moulsdale HJ, Mortimer PP, *et al.* How sensitive are the commercial assays for anti-HTLV-III/LAV? *J Med Virol* 1987;**21**:75-9.
3 Reesink HW, Leslie PN, Huisman JG, *et al.* Evaluation of six enzyme immunoassays for antibody against human immunodeficiency virus. *Lancet* 1986;ii:483-6.
4 Arya SK, Gallo SC. Three novel genes of HTLV-III: immune reactivity of their products with sera from AIDS patients. *Proc Natl Acad Sci USA* 1986;**83**:2209-13.
5 Allain J-P, Luarian Y, Paul DA, *et al.* Serological markers in early stages of human immunodeficiency virus infection in haemophiliacs. *Lancet* 1986;ii:1233-6.
6 Schuepbach J, Haller O, Vogt M, *et al.* Antibodies to HTLV-III in Swiss patients with AIDS. *N Engl J Med* 1985;**312**:765-70.
7 Weber JN, Clapham PR, Weiss RA, *et al.* Human immunodeficiency virus infection in two cohorts of homosexual men: neutralising sera and association of anti-gag antibody with prognosis. *Lancet* 1987;ii:119-22.
8 Lange JMA, Coutinho RA, Krone JA, *et al.* Distinct IgG recognition patterns during progression to subclinical and clinical infection with lymphadenopathy associated virus/human T lymphotrophic virus. *Br Med J* 1986;**292**:228-30.
9 Morrow WJW, Wharton M, Stricker RB, *et al.* Circulating immune complexes in patients with acquired immune deficiency syndrome contain the AIDS-associated retrovirus. *Clin Immunol Immunopathol* 1986;**40**:515-24.
10 Lange JMA, Paul DA, Huisman HG, *et al.* Persistent HIV antigenaemia and decline of HIV core antibodies associated with transition to AIDS. *Br Med J* 1986;**293**:1459-62.
11 Ludlam CA, Tucker J, Steel CM, *et al.* HTLV-III infection in seronegative haemophiliacs following transfusion of factor VIII. *Lancet* 1985;ii:233-6.
12 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;**227**:680-5.

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Early growth delay in diabetic pregnancy: relation to psychomotor development at age 4

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Abstract

Ninety nine consecutive insulin dependent and 101 non-diabetic pregnant women were examined by ultrasonograph to assess early fetal growth. In 42 of the diabetic mothers and three of the non-diabetic mothers the scan showed early intrauterine growth delay. At 4-5 years of age all children available for study were evaluated by the Denver developmental screening test. Only 23 of the 34 children of diabetic mothers with early intrauterine growth delay had normal test scores compared with 46 of the 50 children of diabetic mothers with normal intrauterine growth. The children failed in personal-social development, gross motor development, and particularly in language and speech development. Children of diabetic mothers with normal early fetal growth had scores very similar to those of the children of non-diabetic mothers, of whom 76 of the 86 tested had normal scores.

This study suggests that children with a history of growth delay in early diabetic pregnancy should be screened for possible developmental impairment.

Introduction

We have shown that growth of the embryo in very early diabetic pregnancy may be impaired, so that ultrasound scanning at eight to 14 weeks shows a smaller fetus than expected from the menstrual history.[1] Such growth retarded fetuses have an increased risk of malformations.[2] There is clinical evidence that impaired metabolic compensation at conception and in early pregnancy may be related to early intrauterine growth delay[1 3] as well as to malformations.[4] This paper reports the results of the Denver developmental screening test[5 6] in children aged 4 years with a history of fetal growth delay in early diabetic pregnancy. The study tested the hypothesis that these children would show developmental delay.

Patients and methods

During October 1976 to February 1980 ultrasound scanning (JFP) with measurement of fetal crown-rump length was performed in 99 insulin dependent diabetic mothers (about half of all diabetic pregnancies) and 101 non-diabetic mothers (2·4% of non-diabetic pregnancies). Details of these studies have been reported.[2 7] Three fetuses of the non-diabetic mothers and 42 of the diabetic mothers were smaller than normal in early pregnancy—that is, retrospectively defined as a female fetus being seven or more days and a male fetus five or more days smaller than expected from the menstrual history.[8] In the diabetic women delivery was induced 18 to 20 days before term. All infants of the diabetic mothers and some infants of the non-diabetic mothers were admitted to the neonatal intensive care unit.

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