

## Immunological evaluation in the early diagnosis of prenatal or perinatal HIV infection

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**SUMMARY** A longitudinal evaluation was carried out of the clinical, infective, and immunological progress of 34 children (who were aged 6 to 68 months—mean 25 months at the time of writing) born to 31 mothers infected with human immunodeficiency virus (HIV), over a mean observation period of 13.4 months. Clinical symptoms, not always clearly related to HIV became apparent in 11 children, and preceding immune abnormalities were documented in two of them. In eight children culture for HIV was positive, and six of these were symptomatic. No cancers were diagnosed and none of the children died. Immune abnormalities including hypergammaglobulinaemia, IgG subclass deficiency, low serum IgA concentration, antibody deficiency, a decrease in the number of CD4+(T helper) cells, and defective cellular responses to antigens, were found in seven of the children in whom culture for HIV was positive; in two of four who had symptoms and in all four who were symptom free and in whom culture was negative for HIV but in whom HIV antibodies persisted and who were older than 15 months; and in three of nine who were symptom free and in whom culture was negative with loss of HIV antibodies.

We conclude that serological diagnosis alone may be misleading and that additional immunological assessment may help to identify affected children. Analysis of humoral and cellular responses to antigens used for vaccination such as tetanus toxoid by measurement of specific antibodies and skin testing are simple and helpful in clinical practice.

Infants born to mothers infected with human immunodeficiency virus (HIV) may acquire the virus in utero or perinatally.<sup>1</sup> In contrast with adults, in whom infection with HIV can be diagnosed simply by detection of antibodies or antigens specific for HIV, serological diagnosis in infants is more difficult because maternal antibodies specific for HIV cross the placenta. Furthermore, detection of specific IgM or IgA, or both, which could be of infant origin, may merely reflect the contact of the child with non-infectious HIV antigens crossing the placenta; it is also prone to technical problems.<sup>2</sup> In addition, the exact length of time for which maternal antibodies persist is not yet known. Thus definite diagnosis of HIV infection in infants without symptoms can, at least in the first 2 years of life, only be achieved by cumbersome techniques such as virus isolation from peripheral blood cells.

Early diagnosis of infected subjects, however, may be crucial for appropriate care and treatment. Because the number of infants born to mothers positive for HIV is increasing, and not all these

infants will be entered in studies from medical centres with accurate technical facilities for isolation or hybridisation of the virus, more information about the clinical and immunological course in these infants is needed.

From the longitudinal study of HIV in children that was started at the University Children's Hospital of Zurich in June 1986, we have selected data about 34 infants born to mothers infected with HIV to delineate further the association between clinical symptoms and immunological findings on the one hand, and the laboratory diagnosis of HIV infection on the other. The cohort in this analysis comprises more than one third of the 107 registered infants at risk of vertical transmission of HIV infection in Switzerland.<sup>3</sup>

### Patients and methods

#### SUBJECTS

Criteria for infants and children to be included into this study were maternal HIV seropositivity before

or at delivery as well as former or continuing drug abuse by the mother if HIV serology was positive only after delivery. Maternal and perinatal history were recorded following a questionnaire by review of the charts.

#### PHYSICAL EXAMINATION

The children were seen at the outpatient clinic at three monthly intervals for clinical, immunological, and virological examination. A history was taken on each occasion and on physical examination special attention was paid to dystrophy, lymphadenopathy, hepatosplenomegaly, mucocutaneous infections, confirmed bacterial or viral infections, and persistent or recurrent diarrhoea or cough. To detect early signs that the central nervous system had been affected by HIV, by other neurotropic viruses such as cytomegalovirus, or by opportunistic agents, a detailed neurodevelopmental evaluation was carried out (depending on age) with the Bayley scales of infant development, a standardised neurological assessment adapted from Prechtl,<sup>4</sup> and the Stanford-Binet test.<sup>5</sup>

#### IMMUNOLOGICAL INVESTIGATIONS

Concentrations of the immunoglobulins A, G, and M were determined by nephelometry, and IgG subclass concentrations by immunodiffusion with specific polyclonal antibodies (Central Laboratory of the Netherlands Red Cross). Infants who had been followed up since birth were immunised at the ages of 3, 4, and 5 months with diphtheria toxoid, tetanus toxoid, and inactivated polio virus vaccine. To assess the humoral immune response, anti-tetanus toxoid antibodies were measured by an enzyme linked immunosorbent assay (ELISA) before and four weeks after the last immunisation. Infants entering the study after their primary immunisation but with antitetanus toxoid antibodies below 1000 U/l were given boosters of tetanus toxoid and the antibody response was re-evaluated four weeks later. Antibody deficiency was defined as the lack of adequate antibody response (<1000 U/l) four weeks after immunisation.

Cellular immunity was assessed by measuring lymphocyte subpopulations using specific monoclonal antibodies (Becton Dickinson) for surface IgM and IgD, CD4+ (T helper cell) and CD8+ (T suppressor cytotoxic cell) markers. Phytohaemagglutinin mitogen and tetanus antigen induced lymphocyte proliferation were measured by incorporation of tritiated thymidine four weeks after either primary immunisation or tetanus toxoid booster vaccination. Candidin was used as an additional antigen when there was extensive oral thrush. Skin tests were done with tetanus toxoid and

candidin antigen using a commercial applicator (BioMerieux).

#### VIROLOGICAL INVESTIGATIONS

Serological tests for antibodies of the IgG, IgM and IgA classes specific for HIV were done by western blot based on the biotin/avidin/horseradish peroxidase system using commercial strips (BioRad or DuPont). The strips were incubated with serum diluted 1:25. Human antibodies bound to HIV proteins were detected by the addition of biotinylated goat antibody specific for the heavy chains of the respective isotypes (Vector Laboratories) and then incubated with horseradish peroxidase H (Vector). The chromogenic substrate was 4-chloro-1-naphthol. To exclude the possibility that staining of the bands was by rheumatoid factors of the IgM or IgA classes binding to IgG antibodies specific for HIV, IgG was removed from test serum by absorption to Pansorbin (Calbiochem-Behring). Detection of HIV antigen (p24) in serum was by a commercial ELISA (Abbott Diagnostics Division). HIV was isolated as previously described.<sup>2</sup> Heparinised specimens of peripheral blood were passed over Ficoll-Hypaque gradients, and  $1.5 \times 10^6$  mononuclear cells were cocultured with  $3 \times 10^6$  target cells consisting of either human peripheral blood mononuclear cells or of cord blood cells freshly isolated from normal donors seronegative for HIV. The target cells were grown for 48 hours in RPMI 1640 supplemented with 20% heat inactivated fetal bovine serum, phytohaemagglutinin (10 µg/ml; Sigma), and Polybrene (2 µg/ml; Sigma) before use. During coculture, cells were kept in RPMI 1640 containing 20% fetal bovine serum and interleukin 2 at a concentration of  $2 \times 10^4$  U/l. Cells were refed every three to four days. Culture fluids were harvested at seven day intervals, pelleted at 100 000 g, and assayed for reverse transcriptase activity<sup>6</sup> and HIV antigen. Alternatively, the patients' mononuclear cells were prestimulated for two days with phytohaemagglutinin containing culture medium, before coculture with the target cells as described.<sup>7</sup>

#### Results

From June 1986 to May 1988, 34 children (including two monozygous twin pairs and two siblings) born to 31 mothers were enrolled in the study (figure). The median age of the children at the time of writing was 25 months (range 6–68 months) and the mean observation period 13.4 months (range 3–24 months). Serology for HIV had been done for the mothers of 25 children before or at delivery. Three of the nine children whose mothers had not been tested before or immediately after delivery, were

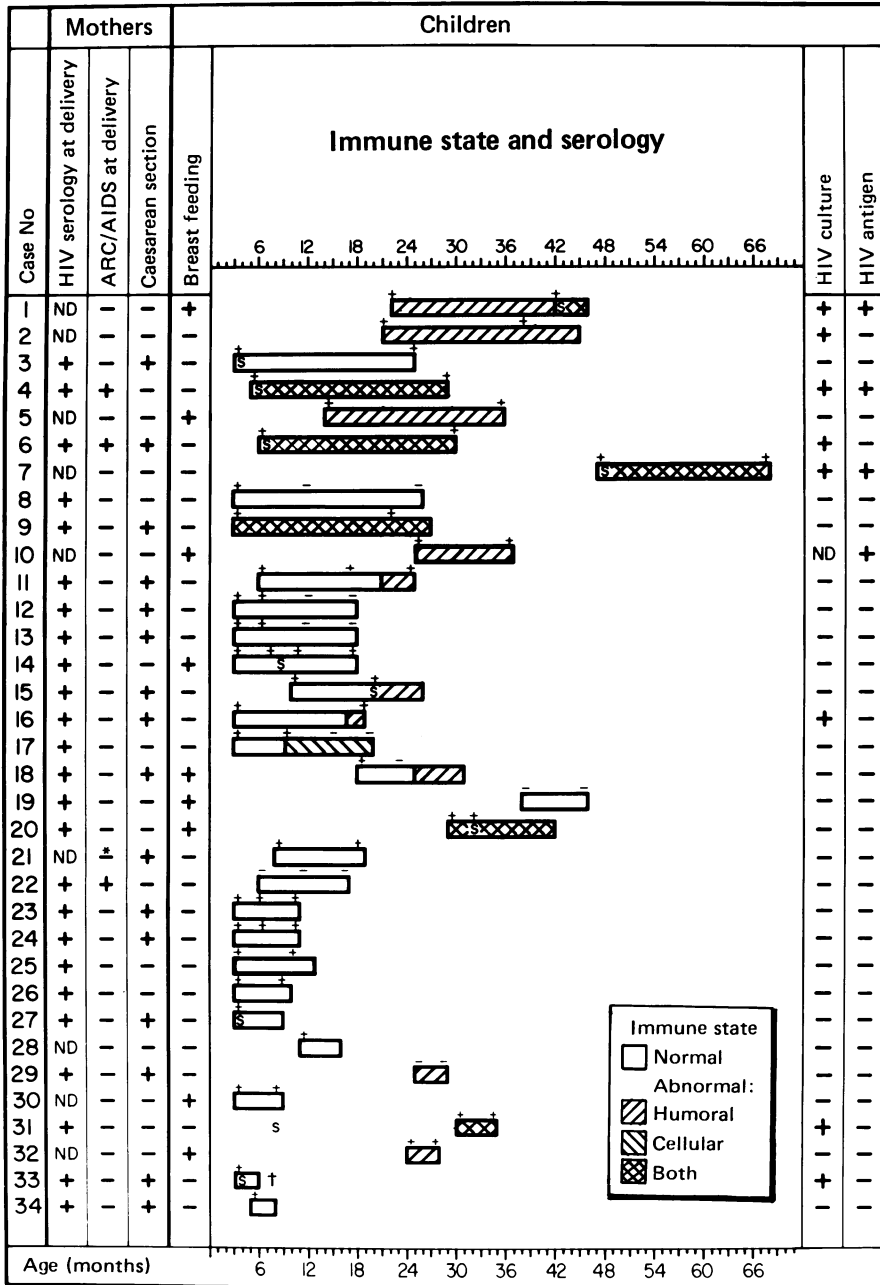


Figure Synopsis of immune state and measurements of HIV infection in 34 children. Bars indicate observation period. \*Died 28 months after delivery; †immune state not yet known; S=onset of symptoms; ND=not done.

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positive for HIV on culture (cases 1, 2, and 7), three had immune abnormalities (cases 5, 10, and 32), and three were younger than 15 months and still had anti-HIV antibodies (cases 21, 28, and 30). In one child (born to a mother who was seropositive for HIV at delivery) whom we saw first at the age of 25 months (case 19) no anti-HIV antibodies were detected. None of the 34 children received a blood transfusion.

#### MATERNAL HISTORY

Twenty nine mothers were white and two were from the Caribbean. Intravenous drug abuse before (n=16) or during pregnancy, or both (n=10), was reported by 26 of the white mothers: the remaining three had HIV-seropositive partners. Neither the Caribbean mothers nor their Swiss husbands gave histories of drug abuse or of the transfusion of blood products. Seropositivity for HIV in these two men was found only after the serological state of their wives was known. The mean age of all mothers at delivery was 25 years (range 20–35). Three mothers had the acquired immunodeficiency syndrome (AIDS) or the AIDS related complex at delivery, and died 8, 15, and 16 months later, respectively. A fourth mother who was symptom free at delivery died of *Pneumocystis carinii* pneumonia 28 months later.

#### PERINATAL HISTORY

Twenty one of the 34 children were boys and 13 girls. Their mean birth weight was 2858 g (range

1290–3950) and mean gestation 38 weeks (range 29–41). All birth weights and lengths were within the normal ranges for gestational age. Sixteen children were born by caesarean section. Symptoms of drug withdrawal were noted in nine of 13 children who were born to the 10 mothers who were intravenous drug abusers at the time of delivery. Eight infants were breast fed for a mean duration of 7 weeks (range 2–48).

#### IMMUNOLOGICAL FINDINGS

Immune abnormalities were detected in 17 of 33 children (table 1, figure). In the remaining child, a 3 month old infant (case 33), immunological evaluation had not yet been done.

Four children (cases 4, 6, 7, and 31) already had symptoms by the time of the first visit and had humoral and cellular abnormalities including hypergammaglobulinaemia (four of four) and decreased CD4+(T helper) cells (three of four). In all of them antibody deficiency and absence of an antigen specific cellular response *in vivo* and *in vitro* was registered after primary immunisation or boosting with tetanus toxoid. These four children were all positive on culture for HIV.

Three of the 13 remaining children with immune abnormalities developed symptoms during follow up: one by the time antibody deficiency was detected (case 15), and two only after hypergammaglobulinaemia, antibody deficiency, and absent *in vivo* and *in vitro* cellular response to tetanus toxoid had been discovered (cases 1 and 20). One of

Table 1 *Immunological findings*

Abnormalities of the immune system	Culture positive		Culture negative, antibody positive at last test		Culture negative, antibody negative	
	Symptoms of HIV infection*	No symptoms	Symptoms of HIV	No symptoms	Symptoms of HIV infection	No symptoms
<b>Humoral:</b>						
Hypergammaglobulinaemia	5/5	0/2	1/5	1/13	0	1/8
IgG <sub>2</sub> /IgG <sub>4</sub> deficiency in children over 24 months old	1/5	1/2	1/5	3/13	0	0/8
IgA deficiency in serum	0/5	1/2	0/5	0/13	0	1/8
Inadequate humoral response to tetanus toxoid	4/5	1/2	2/5	2/13	0	2/8
Cumulated abnormalities	5/5	2/2	2/5	5/12	0	2/8
<b>Cellular:</b>						
CD4+(T helper) cells decreased	3/5	0/2	0/5	0/13	0	0/8
No response to <i>in vitro</i> testing with tetanus toxoid†	5/5	0/2	1/5	1/13	0	1/8
Cutaneous anergy	5/5	0/2	1/5	1/13	0	1/8
Cumulated abnormalities	5/5	0/2	1/5	1/13	0	1/8

\*One child not yet investigated; †if oral thrush was present candidin was also used.

the last two was also IgG<sub>2</sub> and IgG<sub>4</sub> deficient (case 1). He was infected with HIV on culture. The other two had anti-HIV antibodies persisting beyond the age of 15 months and met the Centers for Disease Control criteria of HIV infection.<sup>8 9</sup>

Two of the 10 children with immune abnormalities and no symptoms at the time of writing were positive on culture for HIV, one presenting with IgG<sub>2</sub> and IgG<sub>4</sub> and antibody deficiency (case 2), and the other with serum IgA deficiency (case 16). In five further children anti-HIV antibodies persisted beyond the age of 15 months: two had antibody deficiencies (cases 5 and 9), the first with additional IgG<sub>2</sub> and IgG<sub>4</sub> deficiency (case 5), and the second with no *in vivo* or *in vitro* cellular response to specific antigens. Two other children had hypergammaglobulinaemia (cases 10 and 11), and the fifth was IgG<sub>2</sub> and IgG<sub>4</sub> deficient (case 32). The last three of the 10 children with immune abnormalities and no symptoms at the time of writing lost anti-HIV antibodies and presented with hypergammaglobulinaemia and IgG<sub>2</sub> and IgG<sub>4</sub> deficiency (case 18), serum IgA and antibody deficiency (case 29), and no *in vivo* and *in vitro* cellular response to tetanus toxoid (case 17).

Four of the 16 children without immune abnormal-

ities developed symptoms (cases 3, 14, 27, and 33). None was positive on culture for HIV, but two had anti-HIV antibodies persisting beyond the age of 15 months (cases 3 and 14).

#### VIRUS CULTURE

HIV infection was confirmed in eight children by virus culture from peripheral mononuclear cells. Six of them developed symptoms (cases 1, 4, 6, 7, 31, and 33) (table 2), the other two (cases 2 and 16) being symptom free at the ages of 44 and 19 months, respectively.

#### HIV ANTIGEN

Free HIV antigen (p 24) in serum was detected in three patients who were positive on culture for HIV (cases 1, 4, and 7) and in one patient aged 37 months who was symptom free (case 10), and in whom culture for HIV could not be carried out for technical reasons.

#### ANTI-HIV ANTIBODIES

Up to the time of writing two children had lost IgG anti-HIV before 12 months of age (cases 8 and 22), three between 12–15 months (cases 12, 13, and 17), and one after 15 months (case 18). In two further

Table 2 Clinical signs in the 11 children with symptoms

Case No	Symptoms	Age at onset (months)	HIV culture positive	Anti-HIV antibodies		Immune abnormality present				Age at last visit (months)
				Present	Age (months)	Humoral		Cellular		
						Age diagnosed (months)	Age diagnosed (months)	Age diagnosed (months)	Age diagnosed (months)	
1	Diarrhoea, cough, lymphadenopathy	42	Yes	Yes	42	Yes	22	Yes	42	46
3	Diarrhoea	3	No	Yes	25	No		No		25
4	Oral candidiasis, hepatosplenomegaly, lymphadenopathy	1	Yes	Yes	1	Yes	5*	Yes	5*	29
	Failure to thrive	5								
	Pneumonia, encephalopathy	6								
6	Pneumonia, hepatosplenomegaly, lymphadenopathy, failure to thrive	6	Yes	Yes	6	Yes	6*	Yes	6*	30
	Encephalopathy, oral candidiasis	10								
7	Cough	47	Yes	Yes	48	Yes	48*	Yes	48*	68
	Non-specific dermatitis	66								
14	Oral candidiasis, diarrhoea	12	No	Yes	18	No		No		18
15	Developmental delay	20	No	Yes	20	Yes	20	No		26
20	Lymphadenopathy	32	No	Yes	18	Yes	29*	Yes	29*	42
27	Spastic hemiparesis†	3	No	Yes	3	No		No		9
31	Thrombocytopenia	8	Yes	Yes	8	Yes	9	Yes	29*	35
	Lymphadenopathy	27								
33	Hyperexcitability	3	Yes	Yes	3	Not done		Not done		6

\*First visit to this unit; †probably a result of congenital toxoplasmosis, further investigation refused.

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cases the age at which they lost their antibodies is unknown because they entered the study when they were already seronegative (cases 19 and 29).

Nine children who were negative on culture for HIV (and one in whom HIV culture could not be done) had persisting IgG anti-HIV after the age of 15 months (age range at last test 18–37 months). Seven of these 10 children (cases 5, 9–11, 15, 20, and 32) had immune abnormalities, two of whom had symptoms (cases 15 and 20). Of the three children without immune abnormalities (cases 3, 14, and 21) two had symptoms (cases 3 and 14).

In all eight children who were positive on culture for HIV, anti-HIV antibodies were present. Finally, the remaining eight (cases 23–28, 30, and 34) of the 34 children were younger than 15 months and still seropositive for HIV. One of them had symptoms, probably related to congenital toxoplasmosis (case 27).

The presence of HIV specific IgM antibodies was inconsistent. They were detected in five patients who were positive on culture for HIV (cases 1, 2, 4, 6, and 16); three of them lost the IgM at ages 12, 20, and 38 months, respectively. Specific IgM antibodies were also present in eight children who were negative on culture (cases 3, 10, 11, 14, 15, 20, 25, and 30) at ages 3, 3, 5, 9, 15, 15, 25, and 32 months, respectively. In three of them the result had been negative at earlier determinations (cases 10, 11, and 15). Five of the eight children negative on culture for HIV (cases 3, 11, 14, 25, and 30) lost the IgM later.

Specific IgA antibodies against HIV antigens were found inconsistently in three children who were positive on culture (cases 1, 4, and 6) and five who were negative on culture (cases 14, 21, 25, 27, and 30).

### CLINICAL SIGNS

The symptoms and their age of onset were noted in 11 children and are summarised in table 2. Two patients had AIDS (cases 4 and 6), three the lymphadenopathy syndrome (cases 1, 20, and 31), and in six children the symptoms may well have been caused by HIV infection (cases 3, 7, 14, 15, 27, and 33). Two of these six were positive on culture for HIV, a 68 month old girl and a 6 month old boy. He was hyperexcitable at the age of 3 months when his immune state still had to be assessed. A further three of the six children were older than 15 months and met the Centers for Disease Control criteria of HIV infection because of their seropositivity.<sup>8 9</sup> The sixth child presented with spastic hemiparesis (case 31) that was most probably due to congenital toxoplasmosis (serological data not shown). His foster parents refused further investigations.

Symptoms that may not have been caused by HIV occurred in one girl with humoral and cellular immune abnormalities and a positive culture for HIV (case 7), and in three children without immune abnormalities, two of whom met the Centers for Disease Control criteria for HIV infection<sup>8 9</sup> (cases 3 and 14), and one in whom HIV infection was still not certain (case 27). At the time of writing there had been no cancers and no deaths.

### TREATMENT

Daily antimicrobial prophylaxis with co-trimoxazole (40 mg/kg/day) and ketoconazole (5 mg/kg/day) and monthly infusions of intravenous gammaglobulin (0.4 g/kg) were started in five immunodeficient children with symptoms (cases 1, 4, 6, 20, and 31) at the ages of 45, 7, 10, 34, and 9 months, respectively. The favourable effect of this regimen in the two patients with AIDS and encephalopathy has been reported recently.<sup>4</sup> After the regimen was initiated we did not note any invasive bacterial or opportunistic infections.

### Discussion

Early diagnosis of HIV infection acquired prenatally or perinatally is crucial, because regular intravenous infusions of immunoglobulins have been successful in decelerating or stopping the progression of clinical disease, and slowing the decrease in immune competence.<sup>4 10</sup> Moreover, studies with antiviral drugs such as azidothymidine are currently under way. Diagnosis of infection is, however, obscured because serological testing is confused by the specific maternal IgG antibodies crossing the placenta and persisting for a period that is still not clearly defined. To overcome this obstacle, cumbersome methods are needed (such as virus cultures or lymphocyte cultures) for the detection of HIV itself or of *in vitro* synthesis of IgG specific for HIV.<sup>11</sup>

In the present analysis we concentrated on the diagnostic value of immunological assessment in children who were at risk of acquiring HIV infection from their mothers. The nature of the immune abnormalities that we found, including hypergammaglobulinaemia, antibody deficiency, IgG subclass deficiency, and *in vivo* and *in vitro* anergy, confirms the abnormalities reported elsewhere in paediatric journals.<sup>12 13</sup> Strikingly we found immune abnormalities not only in children with symptoms who were positive on culture for HIV but also in children who were symptom free but were either positive on culture for HIV or had anti-HIV antibodies persisting after the age of 15 months. Furthermore, immune abnormalities were also detected in three children who lost anti-HIV antibodies and in whom no other reason for the abnormalities could be

found. This raises the question of whether these three children were infected or not, considering that loss of anti-HIV antibodies has been reported in children infected with HIV.<sup>14</sup>

The longitudinal design of the study enabled us to recognise the onset and the evolution of the immune abnormalities in a few cases. Based on our findings, the study population can be divided into three groups. One group comprised infants who developed symptoms of HIV within the first months of life; these infants already had immune abnormalities at the first examination. The second group comprised children in whom immune abnormalities were found but in whom symptoms, if any, emerged subsequently. These children were more likely to be infected (as proved on culture) or to have persisting anti-HIV antibodies after the age of 15 months. The third group comprised children without immune abnormalities; symptoms in these children, if any, did not seem to be clearly associated with HIV. Virus culture was always negative. Some of these children still had anti-HIV antibodies, but most had lost them.

Longitudinal assessment of immune state disclosed that humoral abnormalities—for example, hypergammaglobulinaemia and antibody deficiency, or IgG<sub>2</sub> and IgG<sub>4</sub> deficiency in children older than 24 months—usually preceded cellular immunodeficiency. In many children younger than 24 months we found IgG<sub>2</sub> and IgG<sub>4</sub> subclass deficiencies (data not shown). Because interpretation is difficult because of the lack of normal values from healthy individuals, this finding was not included in this analysis. Further immunological studies of more children must determine the consistency of this finding.

The Centers for Disease Control define HIV infection in children under 15 months by the presence of positive virus cultures, or of positive HIV serology associated with immunodeficiency and symptoms. In children older than 15 months, infection is defined by detectable anti-HIV antibodies alone. Using this definition, 19 of the 34 children studied would have to be regarded as infected. Eleven of these 19 had persisting antibodies after the age of 15 months. The negative virus cultures in these 11 children may be caused by the limited sensitivity of the method in symptom free subjects. Conversely, the high sensitivity of the western blot used<sup>2</sup> may have led to the detection of low amounts of maternal antibodies. Thus some children older than 15 months who are seropositive for HIV will not be infected, particularly those without immune abnormalities. On the other hand, loss of anti-HIV antibodies before or even after the age of 15 months does not exclude HIV infection.<sup>14</sup> Consequently, the presence of immune abnormalities

in these children may be an early indicator of infection.

In conclusion, HIV serology alone may be misleading especially in symptom free infants older than 15 months. Additional assessment of their immune state comprising detailed analysis of antibody production and cellular immune responses after vaccination, as well as assessment of IgG subclass concentrations, may help in identifying infected children. These special immune abnormalities may be the first sign of active HIV infection. Detection of immune abnormalities also provides a useful indication for the initiation of antimicrobial prophylaxis or treatment—for example, with infusions of immunoglobulins. Measurement of antibody response to tetanus toxoid and skin testing are helpful in clinical practice, particularly when virus cultures or other cumbersome techniques cannot be performed. Careful immunological follow up of children born to mothers infected with HIV is important.

We thank Professor R Gitzelmann for his support and the nursing and laboratory staff for their help. The work was supported by the Swiss National Science Foundation (grant no 3.947.87) and the Swiss Federal Office of Health.

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Accepted 21 October 1988