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RAPID *IN VITRO* DETECTION OF HIV-1-SPECIFIC ANTIBODY SECRETION BY CELLS-CULTURE WITH VIRUS ANTIGENS

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The present report describes an alternative method for in vitro *detection of HIV-1-specific antibody secretion in 24h of culture employing as stimulant of peripheral blood mononuclear cells the disrupted inactivated whole virus adsorbed onto microwells in a commercial ELISA kit plates. The results obtained from this technique have showed high sensitivity and specificity since it was capable of detecting HIV-1 infection early after birth. There were neither false-positivity nor false-negativity when blood samples obtained from HIV-1 seronegative asymptomatic individuals, and HIV-1 seropositive adult patients were analized. This rapid, low cost, simple, highly sensitive and specific assay can be extremely useful for early diagnosis of pediatric HIV infection.*

Key words: human immunodeficiency virus type 1 (HIV-1) – *in vitro* secretion of HIV-1-specific antibody – diagnosis of pediatric HIV-1 infection

Standard tests for detecting antibodies to human immunodeficiency virus (HIV) such as ELISA or Western blot are not helpful for serodiagnosis of vertically transmitted infection because maternal IgG antibodies cross the placenta during fetal life (Pizzo, 1990). The maternal IgG HIV-specific antibodies can persist for up to 15 months in infants, and for this reason seropositive asymptomatic infants under 15 months of age are classified as PO (indeterminate status – CDC, 1987).

Alternative means to confirm AIDS diagnosis in infants have been developed. Assay for anti-HIV IgM antibodies detection was used but with low sensitivity and specificity (Landesman et al., 1987). Sequential collection of serum samples soon after birth is essential as the IgM response appears at 4 weeks of age and disappears rapidly in babies (Pyun et al., 1987).

Because of the fact that IgA doesn't cross the placenta, several studies were conducted in order to detect HIV-specific IgA antibodies in sera and others body fluids. In some experiments, it is indispensable to remove IgG antibodies that compete with IgA in binding to HIV-1 antigens giving false results (Renon et al., 1990; Weiblen et al., 1990).

Definitive AIDS diagnosis in infants can be performed by polymerase chain reaction (PCR) with HIV-specific primer pairs, and culture of HIV from peripheral blood mononuclear cells (PBMC) (Mok et al., 1987; Laure et al., 1988; Rogers et al., 1989), but these techniques are available in a few sophisticated laboratories only, and are too expensive to be routinely used in developing countries.

In vitro production of HIV-specific antibodies by PBMC is a reliable tool for diagnosis as it is capable of distinguishing active antibodies production from passively acquired antibodies from mother. The conventional techniques are based on *in vitro* spontaneous antibodies secretion or achieved by polyclonal B cells activation with either pokeweed mitogen (PWM) or Epstein-Barr virus (EBV), but they require long period of culture varying from 7

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to 10 days (Amadori et al., 1988a, b; Pahwa et al., 1989).

The present report describes an alternative culture method for *in vitro* production of HIV-1-specific antibodies that employs PBMC and as stimulant the disrupted whole virus adsorbed onto commercially available HIV-1 ELISA kit microwell plates, and using the same plates for detecting the produced antibodies following the regular manufacturer's procedure, and with the advantage of antibodies secretion in 24 h (Caterino-de-Araujo et al., 1991).

MATERIALS AND METHODS

Study population – Two groups of subjects were analyzed. The patient group was composed by 8 HIV-1 seropositive adults aged from 21 to 46 years old, and 11 infants and children from 2 months to 16 years of age. The control group comprised 24 HIV-1 seronegative asymptomatic age-matched individuals (14 children and 10 adults). All HIV-1 seropositive cases were clinically evaluated and classified according to CDC criteria (CDC, 1986, 1987).

Heparinized blood of all subjects was collected and the plasmas were tested for the detection of HIV-1-specific antibodies, HIV-1-specific antigen, and the PBMC were cultured for HIV-1-specific antibodies secretion *in vitro.*

In vitro *production of HIV-1-specific antibodies* – Peripheral blood mononuclear cells (PBMC) were obtained by means of ficoll-hypaque gradient, washed 3 times and then suspended in RPMI 1640 supplemented with 10% fetal calf serum. After adjusting cell concentrations, 0.1 ml of PBMC per well were cultured onto commercial HIV-1 ELISA microtiter plates (EIA LAV – Genetic Systems, Seattle) that are coated with inactivated whole-virus antigen obtained from virus cultured in a CEM cell line. The PBMC cultures were maintained in incubator with 5% CO_2 in air and 100% humidity at 37 °C. The cultures were performed in duplicate, and in one well of every strip 0.1 ml of the cell-free-culture medium were included.

Kinetic studies were done to determine the optimal cell conditions regarding to number of cells which varied from 0.5 to 3 x 10^6 per ml, and the time of culture ranging from 1 to 8 days.

After 24, 48, 72 h, and 8 days incubation periods, the supernatants were collected, centrifuged, and stored; and the cells viability was evaluated. Then, the plates were extensively washed with phosphate-buffered saline containing Tween 20, in order to remove all PBMC. The strips were examined under inverted microscope to certify that none cells remained attached on the wells. Subsequently, the enzimatic reaction for antibody detection was performed according to the manufacturer's instructions.

Assay – HIV-1-specific antibodies were measures in commercial ELISA plates (EIA LAV – Genetic Systems, Seattle) with cultured PBMC, and with plasma specimens obtained from the patients. The standard positive and negative sera and the cell-free culture medium were tested alongside the test samples in every running. Results were expressed as the mean optical density (O.D.) of duplicate assay at 450 nm. The cutoff for plasma specimens was established by adding 0.225 (manufacturer's correction factor) to the O. D. of the median value obtained from negative control sera, and for antibody secreting-PBMC by adding 0.225 to the O. D. of the median value obtained from PBMC cultures of 24 HIV-seronegative healthy individuals. After calculation, the cutoff of 0.270 was fixed. The O. D. ratios greater than the cutoff were regarded as positive.

Western blot analysis (IMUNOBLOT WB anti-HIV-1) (EMBRABIO – Empresa Brasileira de Biotecnologia, São Paulo) were performed to compare the positive bands profiles in PBMC culture supernatant and plasma obtained from the same blood sample. Briefly, inactivated and disrupted virus provided by Genetic Systems were fractionated according to size by polyacrylamide gel electrophoresis; after transfering then to a nitrocellulose membrane. The membrane strips were incubated with the patient's specimens for the WB immunoassay. Supernatant cultures were diluted to 1:2 and the plasma diluted to 1:100 in diluent reagent (buffer solution containing non-fat milk).

All reaction was carried out according to manufacturer's instructions, and to identify specific HIV-1 proteins bands two conjugates were used (goat anti-human IgG antibodies conjugated with biotin and streptoavidin conjugated with phosphatase). The substrate was

a mixture of 5-bromo-4-chloro-3-indolyl-phosphate and nitrobluetetrazolium.

The criteria for interpretation the results was that established by the manufacturer; positive and negative control sera were included in every determination.

The presence of HIV antigen in the subjects' plasma and culture supernatants was determined with an HIV p24 ELISA system (HIV Antigen EIA – Genetic Systems, Seattle) following the manufacturer recomendations, performing the assay in 4:30 h incubation protocol. The results were regarded as positive for p24 antigen when the O. D. value of the samples was greater than the cutoff O. D. in two consecutive determinations.

RESULTS

When plasma and PBMC cultures from the control group were analyzed there was no presence of any specific antibody to HIV-1 (Table I). The cutoff for distinguishing positive from negative PBMC cultures was 0.270. A test culture was positive if the mean O. D. of duplicate measurements was greater than 0.270, and negative if the mean O. D. was lower. As internal control for reaction conditions, positive and negative serum provided in the ELISA kits and PBMC obtained from uninfected subjects were included concurrently in every testing.

The results obtained from HIV-1 infected seropositive individuals showed that their PBMC produce HIV-1-specific antibodies *in vitro* by using at least 1 x 10^6 cells per ml in culture of 24 h (Fig. 1). The antibody secretion remained almost constant over the entire culture period (Fig. 2).

There were neither contamination nor significant death of cells during entire time of culture. Cell viability after 8 days of culture was approximately 78%.

The results obtained from the patient group are presented in Table II. All adults HIV-1 seropositive classified as GII and GIII showed specific-HIV antibodies production in short term PBMC cultures of 24 h. Similar results were observed in 5 HIV-1 seropositive children aged from 8 months to 8 years old, clinically classified as P2D, and from an adolescent, classified as P1. Two P0 6-month-old

TABLE I

Determination of HIV-1-specific antibodies in plasma and PBMC cultures from 24 HIV-1 seronegative asymptomatic individuals (control group)

Subject No	Age	Results	
		Plasma O.D.[a]/cutoff	PBMC cultures O.D.[a/b]
1	46yr	0.006/0.235	0.015
2	26yr	0.016/0.235	0.000
3	45yr	0.017/0.235	0.000
4	32yr	0.010/0.235	0.000
5	30yr	0.005/0.235	0.000
6	36yr	0.007/0.243	0.025
7	26yr	0.009/0.243	0.024
8	23yr	0.034/0.277	0.047
9	57yr	0.047/0.286	0.072
10	40yr	0.039/0.268	0.026
11	2yr	0.054/0.249	0.092
12	1yr8mo	0.042/0.265	0.065
13	2yr	0.035/0.265	0.083
14	16yr	0.061/0.277	0.048
15	11mo	0.045/0.277	0.066
16	2yr	0.039/0.277	0.030
17	11mo	0.062/0.286	0.080
18	1yr2mo	0.051/0.286	0.070
19	11mo	0.052/0.287	0.054
20	8mo	0.038/0.287	0.021
21	4yr	0.060/0.272	0.066
22	5yr	0.045/0.272	0.077
23	1yr6mo	0.042/0.272	0.083
24	10mo	0.057/0.251	0.036

a: optical density values obtained from plasma and PBMC cultures using EIA LAV kits (Genetic Systems). *b:* cutoff of secreting antibody-PBMC established by adding 0.225 to the O.D. of the median value of the control cultures.

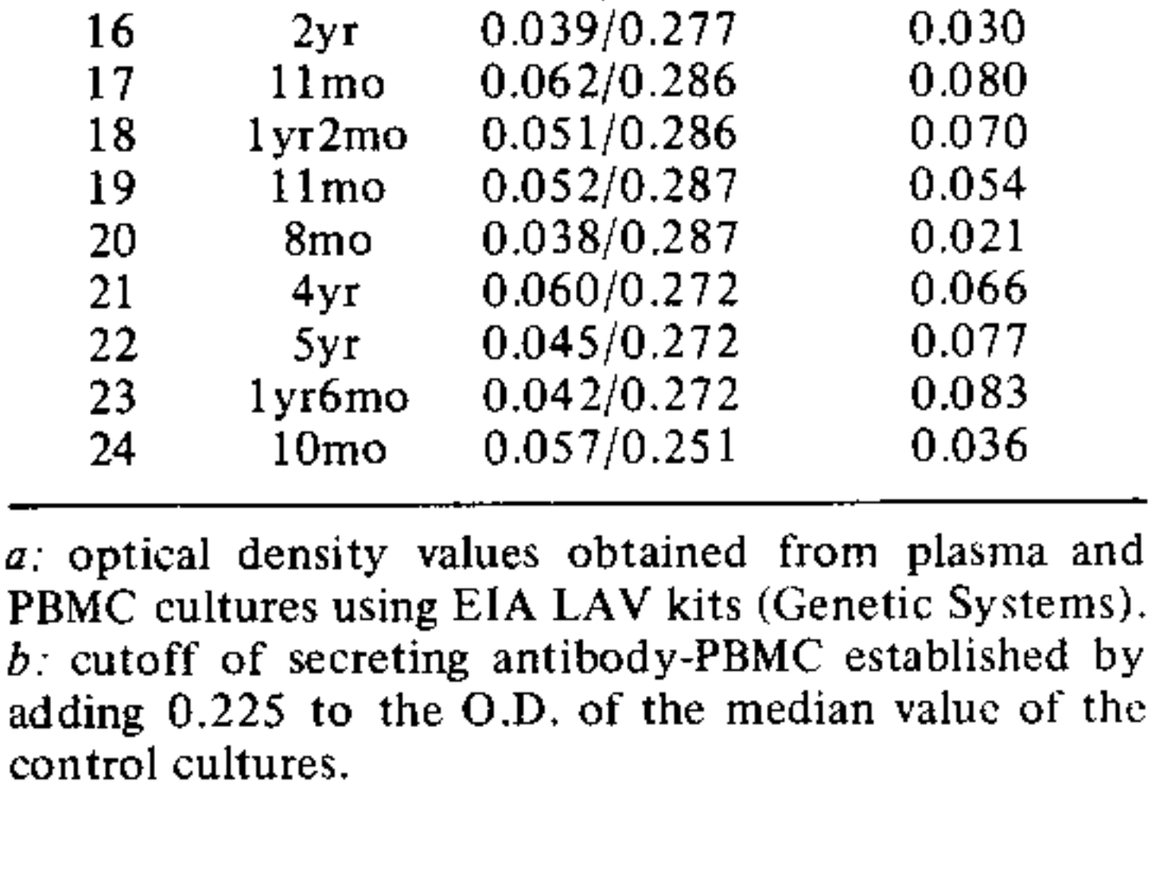

Fig. 1: illustration of the kinetic production of HIV-1-specific antibodies according to PBMC concentration at 24 h of culture. Patient 1 (○) and 3 (●) were chosen for demonstrating high and low levels of *in vitro* specific antibodies secretion. O. D. obtained using EIA LAV kits (Genetic Systems). The cutoff of 0.270 is depicted as a dashed line.

asymptomatic babies born to HIV-1 seropositive mothers were not capable of producing these antibodies *in vitro* when their PBMC were cultured. On the other hand, two 2-month-old and one 12-month-old asymptomatic babies classified as P0 showed high levels of HIV-1-specific antibody production *in vitro* (Table II).

The pattern of bands obtained from patients using IMUNOBLOT WB anti-HIV-1 with plasma specimens, and supernatant fluids obtained after 24 h of PBMC cultures stimulated with disrupted whole virus showed multiple bands in plasma (p18, p24, p31, gp41, p51, p55, p65, and gp120/160) in contrast to supernatant cultures that showed predominant reactivity against HIV-1 polymerase/endonuclease gene products (p31, p51/65) (Fig. 3).

In agreement with negative ELISA HIV-1-specific antibody results obtained from PBMC cultures of two P0 6-month-old patients (patients 9 and 10), no reactivity in Western blot analysis was observed (Fig. 4).

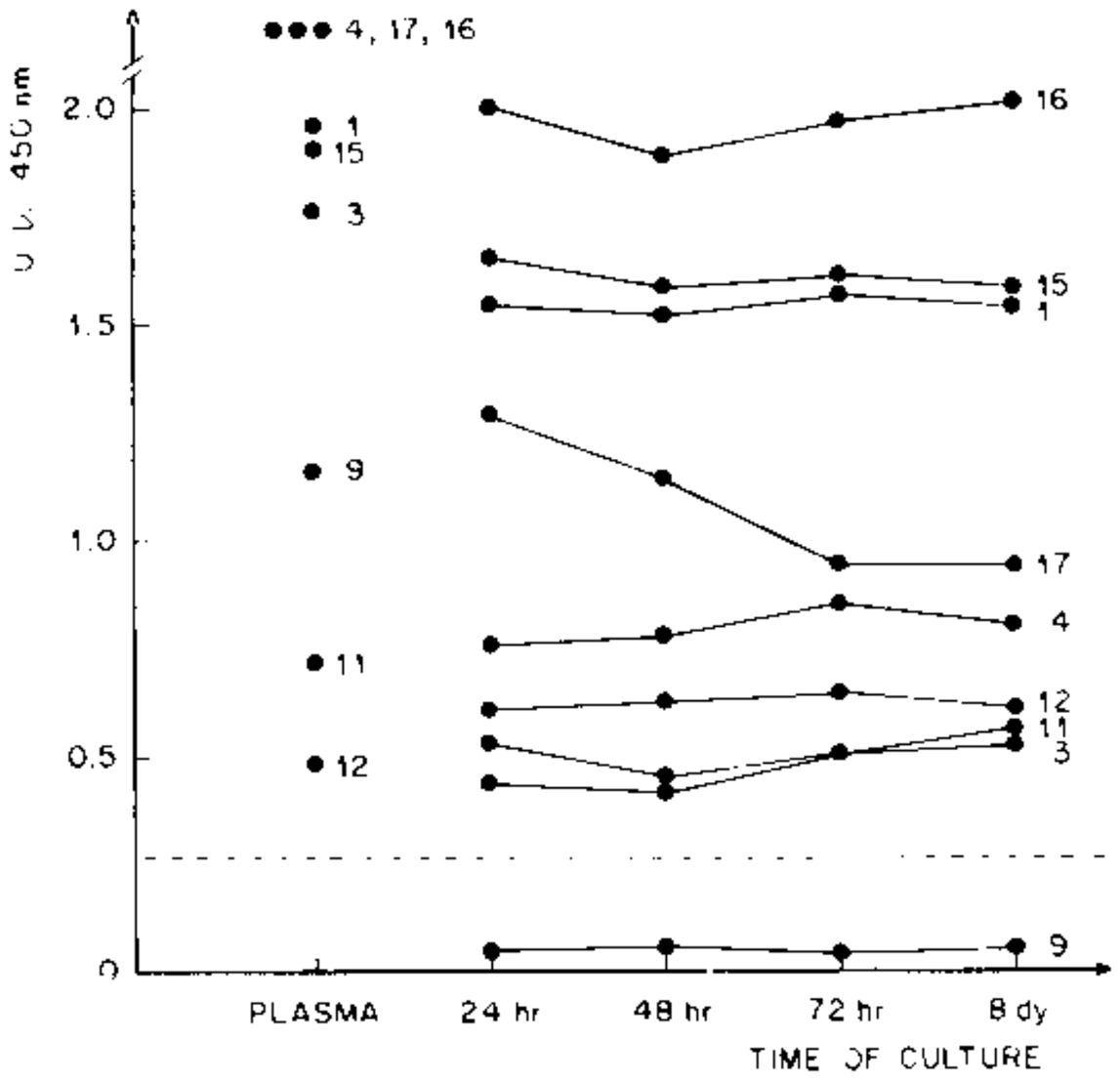

Fig. 2: HIV-1-specific antibodies secretion *in vitro* at different incubation periods. Some patients were selected according to CDC classification and age: patient 1: GII, 33yr; patient 3: GIII, 37yr; patient 4: GII, 24yr; patient 9: PO, 5mo; patient 11: P2D, 8yr; patient 12: P2D, 8mo; patient 15: P2D, 2yr6mo; patient 17: PO, 2mo. O. D. values obtained using EIA LAV kits (Genetic Systems). The cutoff of 0.270 is depicted as a dashed line.

TABLE II

Determination of HIV-1-specific antibodies and antigen in plasma and HIV-1-specific antibody secretion in PBMC cultures from patients

Patient No	Age	CDC classification	Results: HIV-1-specific antibodies		Results: HIV antigen
			plasma O.D.[a]/cutoff	PBMC cultures O.D.[a]/cutoff	plasma O.D.[b]/cutoff
1	33yr	GII	1.949/0.266[c]	1.553/0.270[c]	0.116/0.142[c]
2	21yr	GIII	2.000/0.271	0.548/0.270	0.284/0.142
3	37yr	GIII	1.790/0.286	0.454/0.270	0.082/0.142
4	24yr	GII	2.000/0.286	0.767/0.270	0.040/0.142
5	24yr	GIII	1.804/0.286	0.845/0.270	0.058/0.142
6	46yr	GII	1.140/0.254	0.507/0.270	0.079/0.142
7	40yr	GII	1.237/0.254	0.612/0.270	0.050/0.142
8	19yr	GIII	1.905/0.286	0.567/0.270	0.100/0.142
9	5mo	PO	1.183/0.286	0.041/0.270	0.095/0.142
	6mo	PO	0.487/0.286	0.097/0.270	0.038/0.142
10	6mo	PO	0.444/0.297	0.160/0.270	0.241/0.104
11	8yr	P2D	0.735/0.254	0.526/0.270	0.216/0.104
12	8mo	P2D	0.504/0.266	0.604/0.270	0.404/0.104
13	3yr	P2D	1.589/0.251	1.273/0.270	0.250/0.104
14	11mo	P2D	1.486/0.254	0.826/0.270	0.585/0.104
15	2yr6mo	P2D	1.920/0.297	1.675/0.270	0.076/0.104
16	16yr	P1	2.000/0.297	2.000/0.270	0.042/0.104
17	2mo	PO	2.000/0.275	1.270/0.270	0.080/0.104
18	2mo	PO	1.864/0.258	1.229/0.270	0.045/0.104
19	12mo	PO	2.000/0.257	1.249/0.270	0.091/0.104

a: optical density values obtained from plasma and PBMC cultures using EIA LAV kits (Genetic Systems).
b: optical density values obtained from plasma using HIV Antigen EIA kits (Genetic Systems).
c: O.D. above cutoff were regarded as positive.

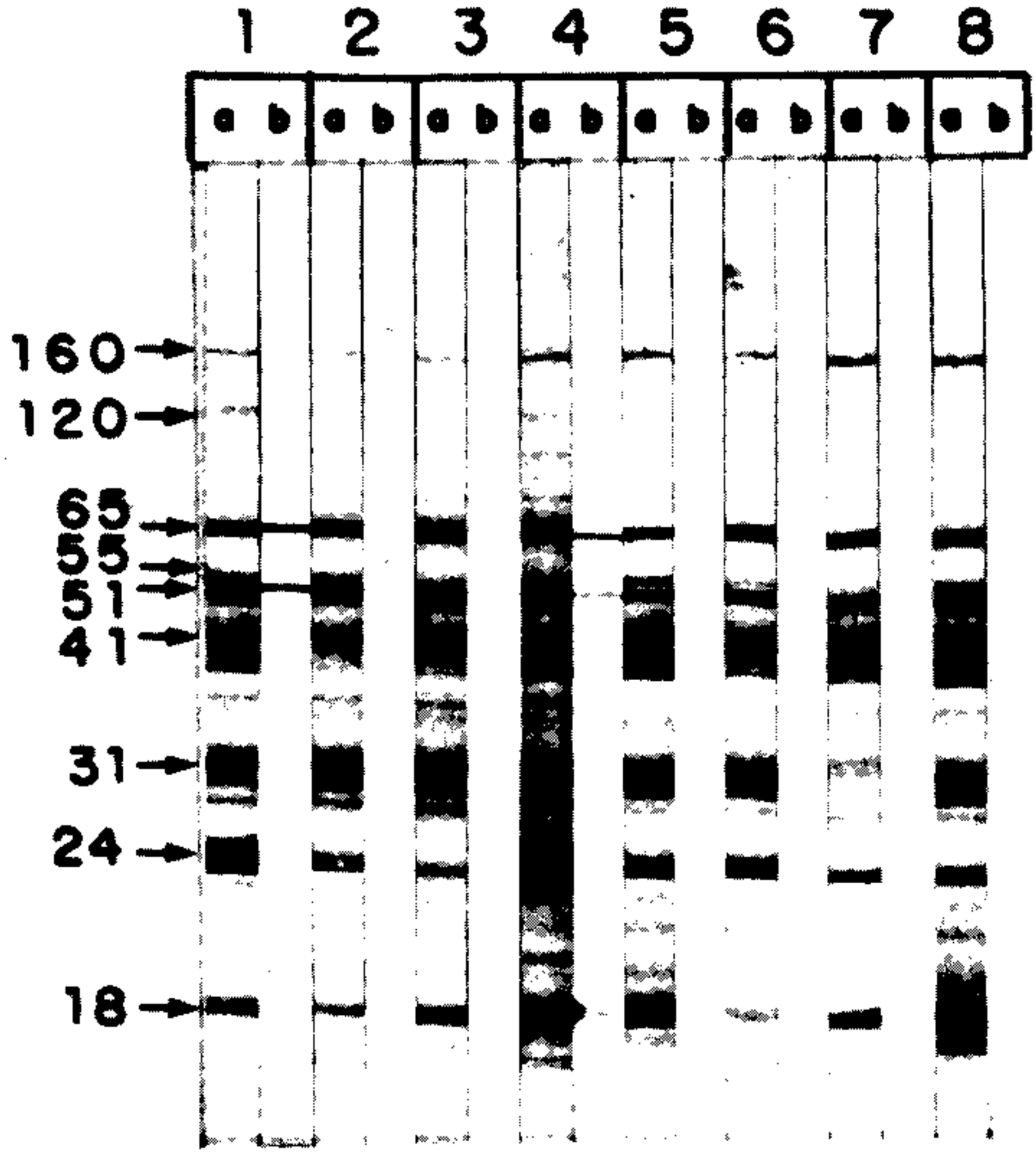

Fig. 3: comparison of Western blot analysis between anti-HIV-1 antibodies produced *in vitro* and the corresponding plasma in adults patients (1 through 8). Lane a, plasma; lane b, culture supernatant.

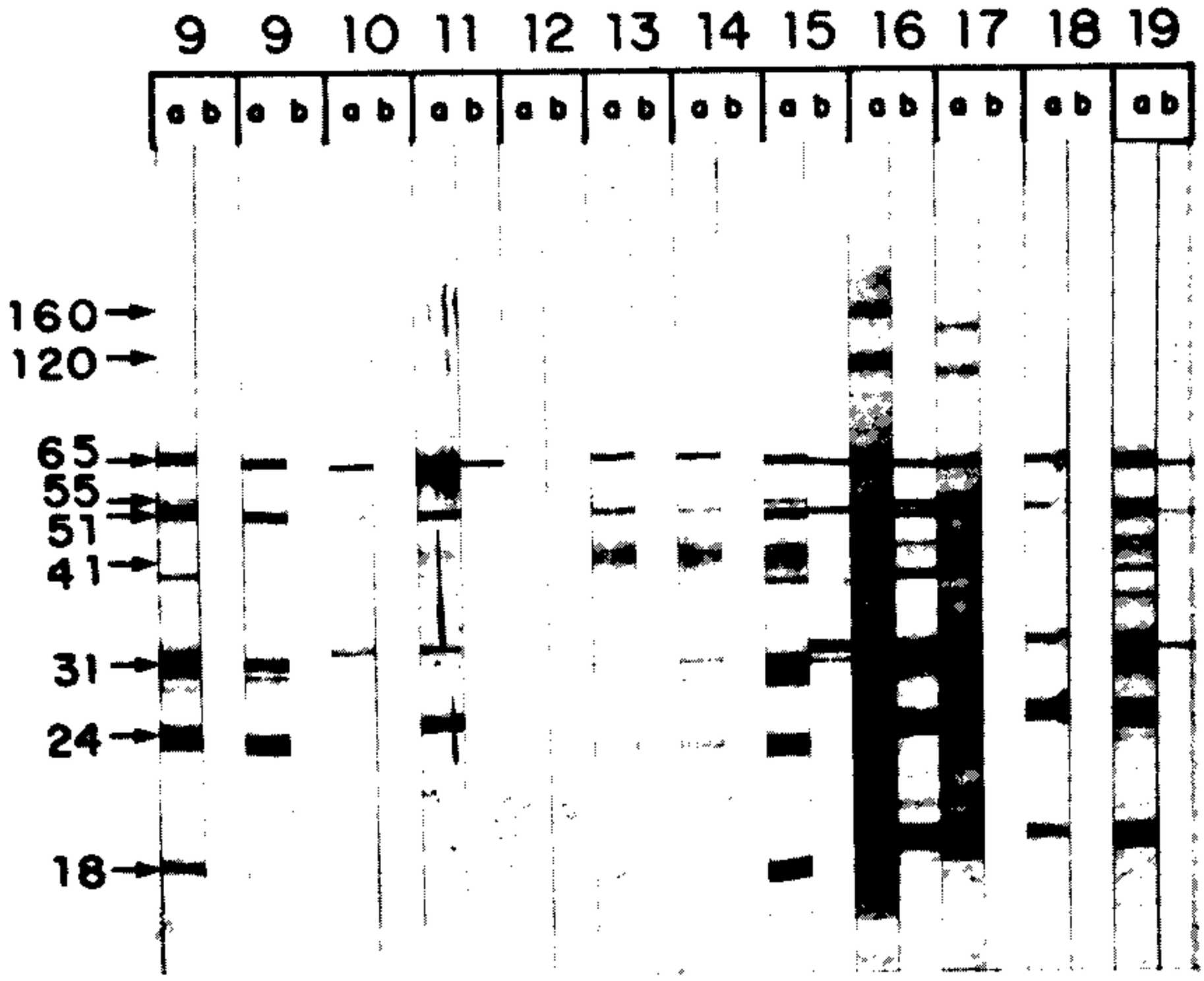

Fig. 4: comparison of Western blot analysis between anti-HIV-1 antibodies produced *in vitro* and the corresponding plasma in infants patients (9 through 19). Lane a, plasma; lane b, culture supernatant.

Plasma and supernatant fluid obtained from 24 healthy subjects, and the cell-free medium used as control failed to show any reactivity on Western blot testing.

The investigation of the presence of HIV-1 antigen in patients' plasma determined by ELISA (HIV Antigen EIA – Genetic Systems) demonstrated 6 positive cases (1 adult and 5 children) (Table II).

All supernatant fluids including culture cell-free medium obtained from cultures incubated for 24 h gave HIV-1 positive antigen results, with O. D. above 2.000. This imply in deattachment of antigens adsorbed onto plates, used as stimulant of PBMC for antibody secretion.

DISCUSSION

The difficulties involved in the diagnosis of pediatric AIDS during the first year of infant's life were due to the period of latency in HIV infection, and the persistence of maternal antibodies transmitted passively to the infant. Thus, conventional tests for detection of antibody to HIV are not reliable in children aged under 15 months (Pizzo, 1990; De Rossi et al., 1991b).

Several alternative methods for improving the diagnosis of HIV infection in children were described, as IgM and IgA specific HIV determinations (Landesman et al., 1987; Pyun et al., 1987; Renon et al., 1990; Weiblen et al., 1990), isolation of the virus from PBMC (Mok et al., 1987), polymerase chain reaction (PCR) with HIV-specific primer pairs (Laure et al., 1988; Rogers et al., 1989), and *in vitro* production of HIV-specific antibodies by PBMC (Amadori et al., 1988a, b; Pahwa et al., 1989).

The previously described techniques present variable sensitivity (De Rossi et al., 1991a, b), and many of them are notoriously difficult, labor-intensive, and too expensive to be routinely used in developing countries.

Amadori et al. (1989), Amadori & Chieco-Bianchi (1990); Jehuda-Cohen et al. (1990) reported *in vitro* secretion of HIV-specific antibodies by PBMC from patients suspected of HIV infection, in culture of long period with or without polyclonal B cell activators.

The present study presents an alternative method for *in vitro* production of HIV-1-specific antibodies by PBMC in 24 h using the disrupted whole HIV-1 virus as stimulant for B cell activation. The use of a commercially available HIV-1 ELISA kit in all steps of the procedure, from PBMC culture to the determination of the produced antibodies, it makes evident the facility in performing the method.

The technique described shows high sensitivity, since it is capable of detecting active antibody synthesis in the totality of analysed HIV-1 seropositive adults. The specificity of the test is addressed by kinetic determination, because of the consistent increase in antibody secretion in culture depending upon the cell concentration, and because there are neither cross-reactivity nor false-positivity, when the PBMC obtained from HIV-1 seronegative asymptomatic age-matched individuals and patients with auto-immune diseases (data not shown) were tested.

Pahwa et al. (1989) reported an assay system for *in vitro* HIV-specific antibody secretion employing cultures of PBMC containing EBV, PWM or cell-free medium, and assayed in a commercial ELISA kit. The best obtained results were detected after 7 days of culture stimulated with EBV, where the sensitivity and specificity were 100% in adults. In class P2 children, 23 of 24 were positive for antibody secretion, while 11 of 33 infants aged under 15 months (class P0) resulted positive. During a follow-up study for 15 months, none negative children developed symptons of HIV infection, and 6 of 11 who were positive progressed to AIDS.

The sensitivity of the present assay is 100% when HIV-seropositive adults and class P2 symptomatic infants were analized. Children under 15 months of age were capable of responding in PBMC culture producing high levels of HIV-1-specific antibodies. The method detected HIV-1 antibody secretion in 3 babies classified as P0 (patients 17, 18 and 19). In two other P0 babies (patients 9 and 10) there were no HIV-1 antibodies response with PBMC culture. Patient 9 was studied subsequently and at present time she is with 18 months of age, with undetectable antibody by ELISA and WB techniques, and she is still asymptomatic. Therefore, she is considered as uninfected child. The patient 10 presents an indeterminate WB profile in plasma and, negative *in vitro* secretion on HIV-1-specific antibody by PBMC, but positive p24 antigen in

plasma. Laboratory follow-up at 12 months of age showed no specific HIV-1 antibodies in plasma detected by ELISA and WB, and the *in vitro* antibody secretion continued giving negative results. He needs a further clinical and laboratory follow-up because PCR, culture of lymphocytes for virus isolation, and p24 antigen neutralization tests were not performed. The fact that two 2-month-old babies, class P0 (patients 17 and 18) responded in PBMC cultures may be resulted from a passive carryover of maternal lymphocytes (Amadori et al., 1990; De Rossi et al., 1991a, b). This was the case of patient 17, because the subsequent assay done when he was 9 month old gave negative result. On the other hand, patient 19 evolved to AIDS in the further 4 months of study.

There was no correlation between the O. D. obtained from PBMC cultures of patients and their clinical status. There are low and high antibodies responders independently of the stage of the HIV infection. Although only GII and GIII adult patients were tested, pediatric cases of AIDS showed relevant antibody production in PBMC culture.

The role of anti-HIV-1 antibody secretion in the natural history of the AIDS disease remain a puzzle and it was discussed by Amadori & Chieco-Bianchi (1990) in a review of B-cell activation during HIV-1 infection. The work reported by Spickett et al. (1989) emphasizes the complexity of the abnormal B cell function in AIDS, and it may explain the diversity of antibody response obtained with infected patients. The *in vitro* function of normal B cells in proliferating and producing antibody to HIV-1 after cells culture for 10 days, depends on the virus isolate, the cell line in which the virus has grown, and the normal individual B cell donor. Because of the HIV-1 itself is being capable of activating *in vivo* a high proportion of B cells, spontaneous antibody secretion occurs *in vitro*. Several investigators utilized this technique with a long incubation period of culture ranged from 7 to 14 days (Amadori et al., 1988a, b, 1989; Pahwa et al., 1989).

In this study, by using the HIV-1 as stimulant in PBMC culture, it is possible to induce specific antibodies secretion by B cells in 24 h of culture. There is no significative changes in O. D. values during the all incubation periods tested. This finding may reflect the presence of previously committed B cells in the culture which do not depend on the others supplying factors added later on. It seems likely that antibodies secreted in the first 24 h persist during the entire time of culture. This methodology gave similar results to ELISPOT (Lee et al., 1989) but with advantage of easily performing without many handling steps like following a deeply washing protocol.

In contrast to others studies that employed enriched B cell population or with depletion of CD8 cells (Amadori et al., 1989; Jehuda-Cohen et al., 1990), in this work the total PBMC obtained on ficoll-hypaque gradient is used. Therefore, it is not possible to determine whether little or no HIV-1-specific antibody production in some cases are due to the low number of pre-committed B cells to the virus and/or mediated by suppressor factor, or because the presence of CD8+ cells in the culture medium. It was possible to establish the number of 1 x 10^6 PBMC per ml (100,000 PBMC per well) as the minimal cell concentration to give detectable *in vitro* antibody response in this standardized assay. Pahwa et al. (1989) used the same cell concentration in spontaneous *in vitro* production of HIV specific antibodies, and in cultures stimulated with PWM and EBV. On the other hand, Vendrell et al. (1991) established the concentration of 5 x 10^6 PBMC per ml in culture of 24 h as the optimal conditions to detect spontaneous *in vitro* secretion of HIV-1 antibodies by PBMC from infected adults and infants.

It is indicated that a great amount of HIV-1-specific antibodies secreted *in vitro* attach onto wells coated with the disrupted whole virus, during the performance of first antibody measurement soon after the PBMC culture, as the recovered supernatants when assayed in another HIV-1 antibody ELISA plate demonstrated a drastic fall in O. D. values. These findings prove that the antibodies were previously consumed (data not shown). This observation is in agreement with the results obtained on WB. All patients' plasma showed characteristic patterns of bands on WB strips for HIV-1 infection, contrasting to the presence of only a few bands when culture supernatants were tested. In the majority of cases only the antibodies to HIV-1 polymerase/endonuclease gene products were detected.

This result contrast with those obtained from others investigators where antibodies

against gp160/120 and p24 were more frequently detected (Amadori et al., 1988a, b; Jehuda-Cohen et al., 1990). One explanation for this difference it may be the fact that a considerable amount of specific antibodies to gag and env proteins attach either on the wells or form immune complexes, since in all culture supernatants were detected high levels of free antigen, and also because of decreased antibody levels when compared with the antibody secretion in an early PBMC culture.

The possibility of producing pol gene product specific antibody during the first 24 h of culture was discarded because testing culture supernatants collected after 72 h and 8 days incubation time, they demonstrated the same results on WB assay (data not shown). Modifying the protocol of WB test using an overnight incubation during the first supernatant incubation step, no alteration in the patterns of bands were obtained.

The occurrence of antibodies to polymerase/endonuclease gene products only on WB with HIV-1 infected children samples could be also determined in clinically and epidemiologically confirmed HIV-1-infected aduls specimens, demonstrating that there are no differences on antibody response between the two groups.

These data emphasize the proposition of use of this culture technique for HIV-1-specific antibodies secretion, employing a commercially available ELISA kit for the diagnosis of suspected HIV-1 infection in pediatric cases, excluding children in the first 2 months of age.

To discard the possibility of false-positivity or false-negativity of the described method, the cells obtained from pediatric patients have kept frozen for further subsequent analysis by PCR and culture for virus isolation.

Because of the facilities in performing this technique owing to the rapidity, low cost, high sensitivity and specificity, it proves to be practical, extremely valuable and useful tool providing a relevant contribution to the diagnosis of pediatric AIDS, mainly in developing countries.

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REFERENCES

AMADORI, A. & CHIECO-BIANCHI, L., 1990. B-cell activation and HIV-1 infection: deeds and misdeeds. *Immunology Today, 11:* 374-379.

AMADORI, A.; DE ROSSI, A.; CHIECO-BIANCHI, L.; GIAQUINTO, C.; DE MARIA, A. & ADES, A. E., 1990. Diagnosis of human immunodeficiency virus 1 infection in infants: *in vitro* production of virus-specific antibody in lymphocytes. *Pediatr. Infect. Dis. J., 9:* 26-30.

AMADORI, A.; DE ROSSI, A.; FAULKNER-VALLE, G. & CHIECO-BIANCHI, L., 1988a. Spontaneous *in vitro* production of virus-specific antibody by lymphocytes from HIV-infected subjects. *Clin. Immunol. Immunopathol., 46:* 342-351.

AMADORI, A.; GIAQUINTO, C.; ZACCHELLO, F.; DE ROSSI, A.; FALKNER-VALLE, G. & CHIECO-BIANCHI, L., 1988b. *In vitro* production of HIV-specific antibody in children at risk of AIDS. *Lancet, i:* 852-854.

AMADORI, A.; ZAMARCHI, R.; CIMINALE, V.; DEL MISTRO, A.; SIERVO, S.; ALBERTI, A.; COLOMBATTI, M. & CHIECO-BIANCHI, L., 1989. HIV-1-specific B cell activation. A major constituent of spontaneous B cell activation during HIV-1 infection. *J. Immunol., 143:* 2146-2152.

CATERINO-DE-ARAUJO, A.; SANTOS-FORTUNA, E. & GRUMACH, A. S., 1991. An alternative method for *in vitro* production of HIV-1-specific antibodies. *Brazilian J. Med. Biol. Res., 24:* 797-799.

CENTERS FOR DISEASE CONTROL, 1986. Classification system for human T-lymphotropic virus type III/ Lymphadenopathy-associated virus infections. *MMWR, 35:* 334-339.

CENTERS FOR DISEASE CONTROL, 1987. Classification system for human immunodeficiency virus (HIV) infection in children under 13 years of age. *MMWR, 35:* 225-235.

DE ROSSI, A.; ADES, A. E.; MAMMANO, F.; DEL MISTRO, A.; AMADORI, A.; GIAQUINTO, C. & CHIECO-BIANCHI, L., 1991a. Antigen detection, virus culture, polymerase chain reaction, and *in vitro* antibody production in the diagnosis of vertically transmitted HIV-1 infection. *AIDS, 5:* 15-20.

DE ROSSI, A.; MAMMANO, F.; PASTI, M.; ZAMARCHI, R. & CHIECO-BIANCHI, L., 1991b. New developments in the diagnosis of pediatric HIV infections. In G. Giraldo; M. Salvatore; M. Piazza; D. Zarrilli & E. Beth-Giraldo (eds). *Biomedical and social developments in AIDS and associated tumors.* Antibiot. Chemother. Basel, Karger, *43:* 214-226.

JEHUDA-COHEN, T.; SLADE, B. A.; POWELL, J. D.; VILLINGER, F.; DE, B.; FOLKS, T. M.; McCLURE, H. M.; SELL, K. W. & AHMED-ANSARI, A., 1990. Polyclonal B-cell activation reveals antibodies against human immunodeficiency virus type 1 (HIV-1) in HIV-1-seronegative individuals. *Proc. Natl Acad. Sci. USA, 87:* 3972-3976.

LANDESMAN, S.; MENDEZ, H. & BIGGER, B., 1987. Evaluation of HIV IgM and antigen assay for determination of perinatal infection with HIV. In Proceedings of the 3rd International Conference of AIDS, Washington DC, June 1-5, 175.

LAURE, F.; ROUZIOUX, C.; VEBER, F.; JACOMET, C.; COURGNAUD, V.; BLANCHE, S.; BURGARD, M.; GRISCELLI, C. & BRECHOT, C., 1988. Detection of HIV 1 DNA in infants and children by means of the polymerase chain reaction. *Lancet, ii:* 538-541.

LEE, F. K.; NAHMIAS, A. J.; LOWERY, S.; NESHEIM, S.; REEF, S.; THOMPSON, S.; OLESKE, J.; VAHLNE, A. & CZERKINSKY, C., 1989. ELISPOT: A new approach to studying the dynamics of virus-immune system interation for diagnosis and monitoring of HIV infection. *AIDS Res. Human Retroviruses, 5:* 517-523.

MOK, J. Q.; DE ROSSI, A.; ADES, A. E.; GIAQUINTO, C.; GROSCH-WORNER, I. & PECKHAM, C. S., 1987. Infants born to mothers seropositive for human immunodeficiency virus. Preliminary findings from a Multicenter European Study. *Lancet, i:* 1164-1168.

PAHWA, S.; CHIRMULE, N.; LEOMBRUNO, C.; LIM, W.; HARPER, R.; BHALLA, R.; PAHWA, R.; NELSON, R. P. & GOOD, R. A., 1989. *In vitro* synthesis of human immunodeficiency virus-specific antibodies in peripheral blood lymphocytes of infants. *Proc. Natl Acad. Sci. USA, 86:* 7532-7536.

PIZZO, P. A., 1990. Pediatric AIDS: Problems within problems. *J. Infect. Dis., 161:* 316-325.

PYUN, K. H.; OCHS, H. D.; DUFFORD, M. T. W. & WEDWOOD, R. J., 1987. Perinatal infection with human immunodeficiency virus. *N. Engl. J. Med., 317:* 611-614.

RENON, G.; BOUQUETY, J. C.; LANCKRIET, C.; GEORGES, A. J.; SIOPATHS, M. R. & MARTIN, P. M. V., 1990. HIV-specific IgA antibodies in tears of children with AIDS or at risk of AIDS. *Res. Virol., 141:* 355-363.

ROGERS, M. F.; OU, C.; RAYFIELD, M.; THOMAS, P. A.; SCHOENBAUM, E. E.; ABRAMS, E.; KRASINSKI, K.; SELWYN, P. A.; MOORE, J.; KAUL, A.; GRIMM, K. T.; BAMJI, M.; SCHOCHETMAN, G. & the New York City Collaborative Study of Maternal Center HIV Perinatal Transmission Study Group, 1989. Use of the polymerase chain reaction for early detection of the proviral sequences of human immunodeficiency virus in infants born to seropositive mothers. *N. Engl. J. Med., 320:* 1649-1654.

SPICKETT, G.; BEATTIE, R.; FARRANT, J.; BRYANT, A.; DALGLEISH, A. & WEBSTER, D., 1989. Assessment of responses of normal human B lymphocytes to different isolates of human immunodeficiency virus: role of normal donor and of cell line used to prepare viral isolate. *AIDS Res. Human Retroviruses, 5:* 355-366.

VENDRELL, J. P.; SEGONDY, M.; DUCOS, J.; REYNES, J.; HUGUET, M. F.; NICOLAS, J. C. & SERRE, A., 1991. Analysis of the spontaneous *in vitro* anti-HIV-1 antibody secretion by peripheral blood mononuclear cells in HIV-1 infection. *Clin. exp. Immunol., 83:* 197-202.

WEIBLEN, B. J.; LEE, F. K.; COOPER, E. R.; LANDESMAN, S. H.; McINTOSH, K.; HARRIS, J. O. S.; NESHEIN, S.; MENDEZ, H.; PELTON, S. I.; NAHMIAS, A. J. & HOFF, N. 1990. Early diagnosis of HIV infection in infants by detection of IgA HIV antibodies. *Lancet, 355:* 988-990.