Acta virol. 32 : 243—251, 1988

VARICELLA-ZOSTER VIRUS IgG ANTIBODIES DURING PRIMOINFECTION IN COMPETENT AND TRANSFER FACTOR MODULATED IMMUNOCOMPROMISED HOST: COMPARISON OF THREE INDIRECT ASSAYS.

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Received August 18, 1987

Summary. — Ten patients with acute leukaemia and next three with Hodgkin's or non-Hodgkin's lymphoma, suffering from varicella-zoster virus (VZV) primoinfection, were given 1 to 2 doses of ultrafiltrate of the human leukocytes lysate (LLU) containing transfer factor (TF) activity (1 dose being equivalent to the product of 10^8 leukocytes). Only LLU administered to patients with acute lymphocytic leukaemia (ALL) at early phases of the illness (days 1 and 2) displayed a notable benefit on the clinical course of varicella. No influence upon the infection, on the other hand, was observed following LLU administration to subjects with lymphoma. The convalescent levels of IgG antibodies to VZV, as detected by indirect immunoperoxidase assay to membrane antigen (IPAMA), demonstrated no significant difference between infected competent and immunocompromised untreated and LLU treated individuals. The performance characteristics of IPAMA are compared with indirect immunofluorescence method (IFA) and non-competitive enzyme-linked immunosorbent assay (ELISA) on the same panel of specimens.

Key words: varicella-zoster virus; immunomodulation; immunosuppression; immunofluorescence, immunoperoxidase assay, enzyme-linked immunosorbent assay

Introduction

Varicella-zoster virus (VZV) is an exclusively human, widespread DNA virus, especially in the areas of moderate climate. The search for specific anti-VZV antibodies revealed high prevalence of infection, according to some authors nearly 75—100 % already in the age group of 15 years (Gershon and Projansky-Steinberg, 1981; Muench *et al.*, 1986). In the course of VZV primoinfection, which regularly unfolds in immunocompetent subjects as benign acute illness (chickenpox), the infectious virus is readily cleared from afflicted tissues, nonethe less remaining latently present in the majority of sensitive spinal ganglia.

Although rather rare, considerably serious is the generalized form of varicella spreading over the susceptible, immunologically compromised indivividuals. Cases of isolated, not seldomly lethaly progressing ailment, up to larger nosocomial infections have been observed among patients suffering from profound immunosuppression due to various pathological or iatrogenic reasons (Weller, 1983).

There is a substantial and permanent interest in reliable assessment of anti-VZV humoral immune status of these risk patients, thus promoting further therapeutically oriented research. Immune reconstitution, at least partial, in suitable cases offers certain advantages over the application of antiviral drugs, still considerably toxic. Consequently, administration of zoster immune globulin or selected immunomodulators represents the therapeutical and/or prophylactic approach of choice (Gershon, 1984).

As yet numerous methods have been studied in order to elaborate a rapid, precise and reproducible anti-VZV antibodies detection mode (Wreghitt *et al.*, 1984). In presented report we describe, in adition to indirect immunofluorescence, two sensitive and reliable enzyme immunoassays for subjective (observer's naked eye) and objective (photometric) results evaluation. The serum specimens originating from immunocompetent, as well as from immunocompromised and by LLU stimulated individuals suffering from varicella have been investigated for the presence of anti-VZV IgG. The implications for possible immunopathogenetic mechanisms involved in the disease evolution are discussed.

Materials and Methods

Patients and serum samples. The total of 96 serum specimens was investigated for the presence of specific anti-VZV antibodies; 86 in all three assays, the rest in two. The majority of samples originated from children, who's diagnosis on admission to hospital was chickenpox with associated disease of non-malignant nature (n = 17), in the next 13 patients acute leukaemia (n = 10), lymphocytic (ALL) or myelocytic and lymphoma (n = 3), Hodgkin's or non-Hodgkin's, was complicated with acute VZV infection. The patients suffering from malignancies were treated with 1 or 2 doses of LLU and were observed clinically for the progression of skin efflorescences; 4 patients diagnosed as ALL (n = 2) and Hodgkin's lymphoma, whom LLU was not given, were also included as controls.

Viral antigen. VZV was isolated and propagated according to previously described procedures (Schmidtmayerová *et al.*, 1986). Essentially, the virus was isolated from a vesicular eruption of a young boy undergoing acute chickenpox in a culture of diploid human embryo cells. The cultures were inoculated with infected cells at an input multiplicity of 0.2 PFU/cell and propagated in Eagle's minimal essential medium supplemented with 2 % inactivated calf serum (ICS) until the cytopathic effect involved 80 – 90 % of the monolayer. Thereafter the cells were thoroughly rinsed with phosphate buffered saline pH = 7.2 (PBS) and detached from the surface employing glass beads (for ELISA) or by trypsinization (for IFA and IPAMA). They were then repeatedly washed and further processed for individual serological tests.

For IFA the appropriate number of cells, approximately 5×10^4 per well, were spotted onto microscopic slides, dried and subsequently fixed for 5 min with cold acetone. In this way prepared slides were stored at −20 °C until use.

In the IPAMA we utilized dispersed cells, which were fixed on ice with 0.075 % glutaraldehyde for 1 min. Next, cold glycin was added up to 5-fold molar excess. The cell preparation was washed with PBS, adjusted to the concentration 2.5×10^5 cell/ml and dropped in 50 ul aliquots into the wells of flat bottom polystyrene microtitration plates (Dynatech). Cells were allowed to settle down for 1 hr at 37 °C, the supernatants were widthrawn and the plates were left at room tempe-

rature for another hour, untill they became completely dry. Up to the use they were stored dessicated at −20 °C.

For use in ELISA, the cells were frozen and thawed once, then sonicated on ice three times 30 seconds with 250 W sonic dismembrator (Dynatech) at maximum output. The cell debris was removed by low speed centrifugation and the protein concentration of the supernatant was determined by the Lowry method (Lowry *et al.*, 1951). The optimal coating concentration was found in box titration.

Control antigen was prepared exactly in the same manner as the viral one and was included in each of the three assays.

Indirect immunofluorescence assay. Serum specimens heat inactivated and liver powder adsorbed were diluted twofold in PBS and applied to tested and control antigens for 30 min at room temperature. After washing, swine anti-human IgG-FITC conjugate (Sevac, Prague) in optimal dilution was added for another 30 min. Finally, the preparations were counterstained with Evans blue, mounted into the buffered glycerol and evaluated. The intensity of fluorescence was scored according to predetermined fourpoint range (point 2 being recognised as positive) and results were read in terms of reciprocal of end point titer in respect to the reaction with the control antigen. Positive and negative serum was included each time.

Indirect immunoperoxidase assay to membrane antigen. The sera were diluted geometrically in PBS containing 1 % bovine serum albumine (BSA), 0.05 % Tween 20 and 0.01 % merthiolate (PBSAT). Specimens in amounts 50 µl were incubated for 30 min at 37 °C with tested antigen and control antigen. The plates were then washed three times with PBS containing 0.05 % Tween 20 (PBST). The appropriate dilution of swine anti-human IgG-peroxidase conjugate (SwAHuIgG/ /Px, Sevac, Prague) which was determined by box titration (80×) was done in PBSAT and was allowed to react with the previously formed antigen-antibody complex for 30 min at 37 °C. Final washing was performed with PBST and PBS, and the bound peroxidase was visualised by adding the substrate solution yielding an insoluble product (Haikin and Sarov, 1980). It was prepared by dissolving 4 mg of benzidine (Ferak, Berlin) in 0.5 ml acetone, diluted up to 10 ml with PBS and completed by adition of 10 ul of 33 % H_2O_2. After the development of reaction, which proceeded for 5 min at room temperature, the cells were counterstained with Safranin stain. Each plate comprised also two control sera, one positive with titre of 128 and one negative, with titre <4. The results were read in an inverted microscope at low magnification. The end point titres were defined as the reciprocal of the last dilution giving clearly distinguishable reaction product, simultaneously with no reaction in the control well.

Enzyme-linked immunosorbent assay. The partially purified antigen and control antigen were adsorbed to the wells of F type polystyrene plates (UMG, CSAV, Prague) by overnight incubation in 0.05 mol/l carbonate-bicarbonate buffer pH = 9.6 at 4 °C. The standard serum, which was the pool of herpes zoster reconvalescent sera with high titres of anti VZV IgG antibodies, as had been determined previously, was used for the construction of calibration curve. The serum specimens with unknown content of antibodies were uniformly diluted 50×, the calibration curve serum in 6^n order 50—388 000×, both in duplicates in PBS with 50 % ICS, 0.2 % Tween 20 and 0.1 % sodium azid. The plates were incubated for 2 hrs, washed three times with PBST and filled with appropriate dilution of anti IgG peroxidase conjugate in PBSAT. Following 1 hr incubation and final washing, the substrate solution (0.1 mol/l citrate-phosphate buffer pH = 5 with 0.05 % H_2O_2 and 0.05 % O-phenylendiamin dihydrochloride, Fluka) was added. Reaction was stopped after 30 min by adding 25 ul of 4N H_2SO_4 and the resulting optical density was read spectrophotometrically at 490 nm employing Minireader II (Dynatech). All incubation steps were carried out at 37 °C and the respective loaded volumes were 100 ul.

The optimal concentration of immunochemicals was determined beforehand in three-dimensional checkerboard titration of VZV antigen and control antigen, positive serum and peroxidase conjugate. The amount of anti-VZV antibodies in serum samples was extrapolated from calibration curve and expressed in arbitrarily defined units, assuming the 50× diluted standard having 288 000 U and the cut-off value being 26 U. For permanent monitoring of the test's reproducibility, the control specimen has been run in each experiment.

Lysed human leukocytes ultrafiltrate containing TF activity consisted of ethanol precipitated LLU subjected to gel filtration as described by Mayer *et al.* (1985). The product equivalent to 10^8 leukocytes was dajusted to 1 ml (dose) and administered subcutaneously.

Statistics. The statistical significance of the difference of two independent samples was determined nonparametrically by Wilcoxon-Mann-Whitney U-test. Linear regression and correlation coeficient r were used for the comparison of the pairs of serological tests.

Results

Comparison of anti-VZV IgG levels detected in IFA, IPAMA and ELISA

Altogether 43 individuals, among them 38 suffering from varicella, were included into the study evaluating IFA, IPAMA and ELISA for their capacity to detect IgG antibodies to VZV. In patients, as a rule, serial serum samples were withdrawn preferentially in the acute and convalescent period of the disease. A total of 86 specimens was comparatively investigated in all three assays, additional 10 samples were examined in only two. The interrelationship of the results gained in IFA, IPAMA and ELISA is depicted in Fig. 1 in the form of linear regression. There was a good correlation between the pairs of independent tests, the correlation coeficient being 0.88 for IFA and IPAMA, 0.70 for IFA and ELISA and 0.75 for IPAMA and ELISA.

Specificity and reproducibility of the assays

The crossreactivity with other members of the Herpes virus family was checked by testing paired sera with significant rise in anti-VZV IgG titres on herpes simplex type 1 (HSV) antigen and vice versa. In adition, adsorbtion experiments with VZV, cell associated HSV, cytomegalovirus complement fixation and control antigens were undertaken on sera positive for VZV IgG.

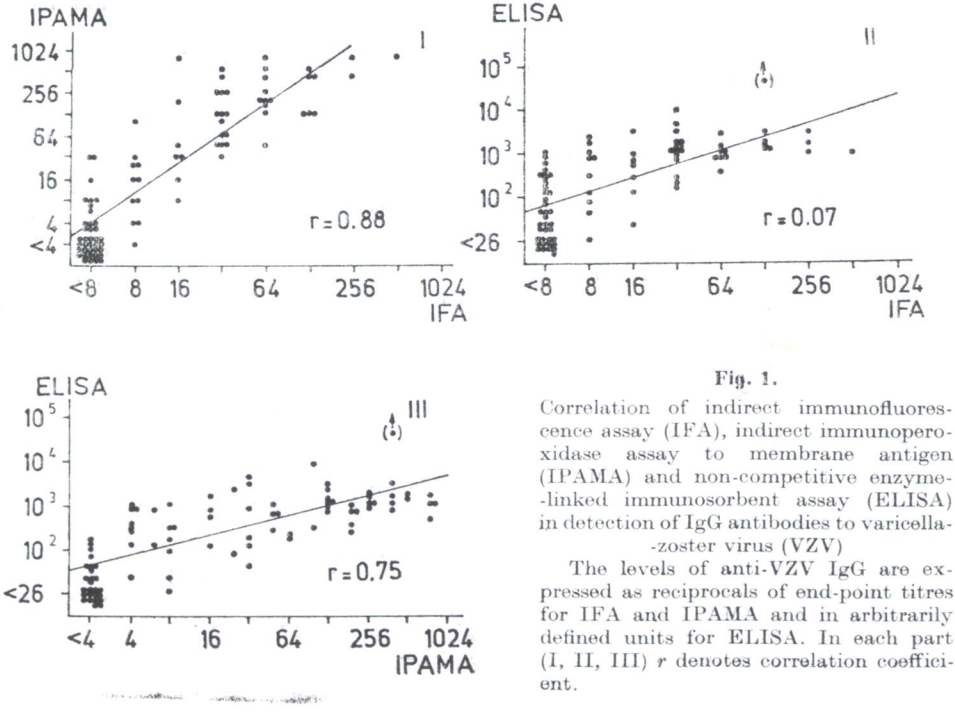

Fig. 1.

Correlation of indirect immunofluorescence assay (IFA), indirect immunoperoxidase assay to membrane antigen (IPAMA) and non-competitive enzyme-linked immunosorbent assay (ELISA) in detection of IgG antibodies to varicella-zoster virus (VZV)

The levels of anti-VZV IgG are expressed as reciprocals of end-point titres for IFA and IPAMA and in arbitrarily defined units for ELISA. In each part (I, II, III) *r* denotes correlation coefficient.

Table 1. Comparison of indirect immunofluorescence assay (IFA), indirect immunoperoxidase assay to membrane antigen (IPAMA) and non-competitive enzyme-linked immunosorbent assay (ELISA) in detection of fourfold rise of IgG antibody levels or seroconversion to varicella-zoster virus in 2 subsequent serum samples.

		A		B
ELISA	+/−	−	+	+
IPAMA	+/−	+	−	+
IFA	+/−	+	+	+
	32/35	2/35	1/35	20/20
	91.4%	5.7%	2.8%	100%

A — Not defined intervals for withdrawal of the 1st and 2nd sample.
B — 1st sample withdrawn until day 4, 2nd sample since day 7 of the illness.
+/− : either detected or not detected
\+ : detected
− : not detected

Only VZV antigen efficiently diminished the titres of specific antibodies in the homologous detection system. Under these conditions the assays proved to be specific for IgG antibodies to VZV.

The titres of control specimen in IFA and IPAMA did not surpass the range of fourfold dilution from run to run. In ELISA the values of interassay and intrassay were 20.3 % and 12.3 %, respectively.

Sensitivity of the assays

When taking into account the proportion of specimens found to be devoided of specific antibodies, upon investigation of 86 sera in all three assay, ELISA turned out to be the most sensitive test (16 % sera negative), followed by IPAMA (27 % negative) and finally by IFA with 43 % negative sera. Comparison of geometric mean titres (GMT) corresponding to positive samples supported the above stated order of sensitivty. The GMT for IFA was 34 and for IPAMA 94.

The ability of individual assays to monitor the dynamics of antibody production and thus to confirm the clinically established diagnosis by detection of at least fourfold rise in antibody titres or seroconversion is shown in Table 1. Based on samples withdrawn in the course of the disease not always rationally, all three tests gave concordant results, positive or negative, in 91.4 %. ELISA alone failed in 5.7 % and the sole IPAMA in 2.8 %. If only the specimens withdrawn in more appropriate time intervals were evaluated, (the first sample until day 4 and the second sample since day 7), all three tests proved equally reliable and scored positive in all patients in terms valid for laboratory diagnosis, as they were mentioned earlier.

IgG antibody response to VZV and duration of exanthema progression in LLU treated immunocompromised and in competent patients

Fig. 2 shows the time course of IgG antibody production, as determined by IPAMA, in individuals without malignant disease in contrast to the dynamics of anti-VZV IgG levels in patients with acute leukaemia and malignant lymphoproliferative disease (Fig. 3). All patients of the former group

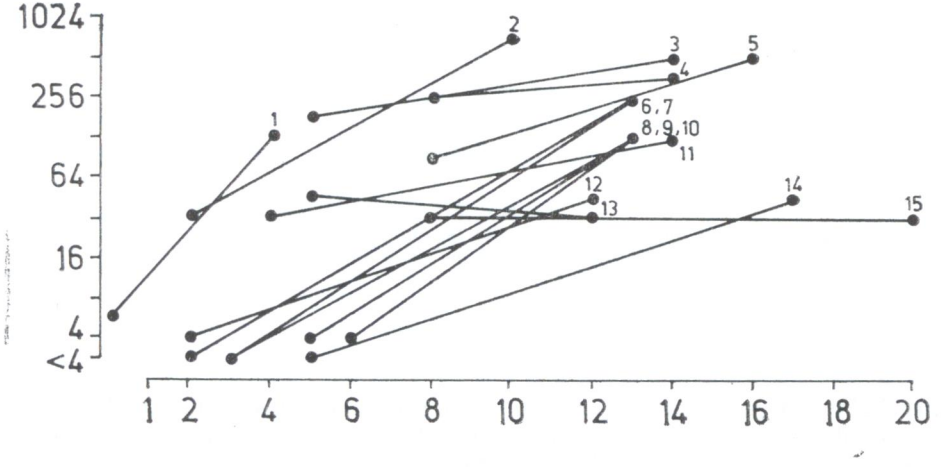

Fig. 2.

Dynamics of IgG antibody response to varicella-zoster virus (VZV) in immunocompetent subjects during varicella

Titers of anti-VZV IgG in paired serum samples (No. 1—15) were determined in indirect immunoperoxidase assay to membrane antigen. Subject No. 1 received zoster immune plasma subsequently to varicella contact. Abscissa: days of illness; ordinate: dilution reciprocals.

were given one dose of LLU except one with leukaemia, who received two doses on days 4 and 6 since occurence of skin rash. New waves of exanthema in patients with leukaemia who were LLU treated later in the disease, i.e. from day 3 to 6, were observed up to day 8. The second contingent of patients with leukaemia, which was diagnosed as ALL, was LLU treated earlier, on days 1 and 2 of illness. The overall interval during which the new efflorescences emerged, as registered in this group, was 5 days. Statistical analysis revealed that the difference in duration of efflorescences progression in the two groups of patients with acute leukaemia, which differed in time schedule of immunomodulation was significant ($P < 0.05$). Furthermore, the early onset of immunotherapy was accompanied with a marked subjective relieve.

In the cases of lymphoma the antibody response was directly comparable to that of leukaemia patients, independently on the time of LLU application. No apparent clinical benefit of LLU treatment was observed in these patients.

The antibody production in treated group with underlying malignancy was not related to the time of LLU administration and differed neither ($P > 0.05$)

from that observed in the untreated control group nor from the group with accompanying nonmalignant disease (Fig. 2), as suggested the nonparametrical analysis of antibody titres on day 7 or later on.

Discussion

In patients with cancer on chemo- or radio-therapy, in general, a heavy decline in cell mediated immunity (CMI) was observed (Ruckdeschel *et al.*, 1977). These subjects present a permissive system for foudroyant VZV infection. The important biological feature of VZV is the strict intracellular association of its replicative cycle. It is conceivable and indeed it was experimentally corroborated, that the containtment of infection resides predominantly in CMI effector mechanisms, while humoral immunity plays a minor role (Gershon *et al.*, 1979, Arvin *et al.*, 1986). The rational for therapeutical administration of LLU containing TF activity, in varicella prophylaxis of immunocompromised children has already proved its usefulness (Steele *et al.*, 1980), is to induce the antigen-specific CMI response analogous to the

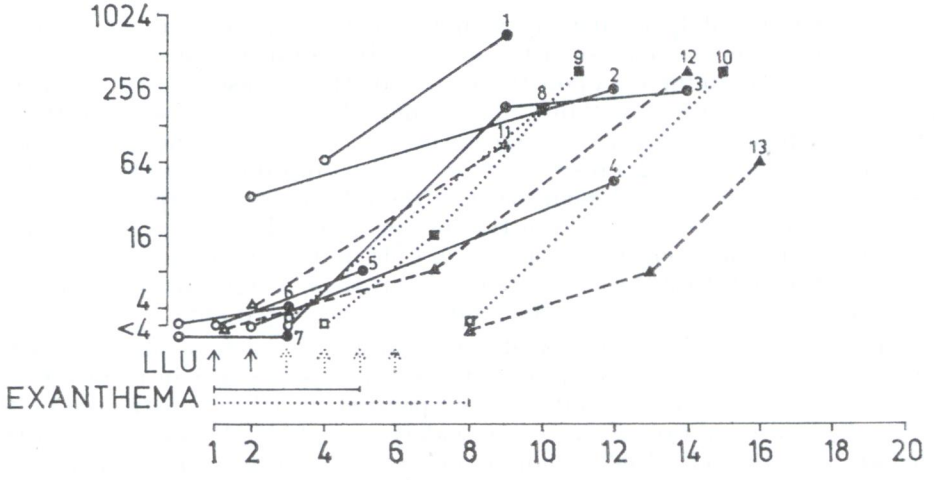

Fig. 3.

Dynamics of IgG antibody response to varicella-zoster virus (VZV) in immunocompromised subjects treated with ultrafiltrate of human leukocytes lysate (LLU) during varicella

Patients with acute lymphocytic leukaemia were given LLU on days 1 and 2 of the illness (○——●; 1—7), patients with acute lymphocytic and myelocytic leukaemia were given LLU since day 3 to 6 (□.....■; 8—10) and patients with Hodgkin's and non-Hodgkin's lymphoma were given LLU on days 2 and 5 (△— — —▲; 11—13). Full line arrows denote time of earlier (○——●; 1—7) and dotted line arrows time of later (□.....■; 8—10) LLU administration to patients with acute leukaemia. Horizontal full and dotted line bars denote duration of skin rash progression corresponding to earlier and later LLU application respectively. Abscissa: days of illness; ordinate: reciprocals of anti-VZV IgG titres in immunoperoxidase assay to membrane antigen.

immunological experience of the donor. Yet not negligible are the effects amplifying the number of existing, specific antigen-reactive lymphocytes and augmenting nonspecific immune mechanisms.

In our study LLU exhibited a clinical benefit that was linked to the timing of its administration. The progression and evolution of skin lessions was critically altered and effectivelly halted in patients with acute leukaemia in the stage of maculo-papular rash after early LLU application (1 to 2 days since the first skin efflorescences became manifested). Marked subjective improvement was evident as well. LLU is thought to accelerate the CMI events in skin lesions and to promote the cessation of viral replication in target organ. Interestingly, no effect was noticed in individuals with lymphoma, which is known to be accompanied with the profound CMI suppression. This was perhaps due to the advanced stage of malignancy and possibly low antigen specific potency of employed LLU, which was prepared from random donors. Plausibility of this explanation supports report of Drew *et al.* (1973), who observed that the dialysed leukocytes lysate from donors selected for high responsivness to VZV antigen transferred transiently the reactivity to viral antigen and phytohaemagglutinin and evoked clinical improvement in a patient with lymphoma.

The dynamics of IgG antibody response in patients with acute leukemia was similar, independently on LLU dosage. Humoral immune response had apparently little influence upon the course of the disease. Moreover, in the group with chickenpox without malignancy the greatest rise in antibody titres took place after day 4, when no new waves of exanthema appeared. The overall pattern of IgG response in immuno-compromised and -competent children closely resembled each other, although not so prompt start in the rise of antibodies was observed in the former group. Further in concordance with considerations concerning the role of CMI in immunopathogenesis of this disease is the fact, that the comparison of convalescent IgG titres disclosed no significant differences between the studied groups of patients.

When assessing the numbers of sera reactive in each of the assays for anti-VZV antibodies, ELISA proved to be the superior test in terms of sensitivity. The favourable results on comparison of ELISA with assays for antibodies to membrane antigen are widely accepted (Hacham *et al.*, 1980, Shehab and Brunell, 1983), though not equally encouraging reports had appeared (Gershon *et al.*, 1981).

The sole failure of IPAMA and one of two failures of ELISA in registering the fourfold rise of IgG antibodies was attributable to higher amounts of antibodies detected in the first samples withdrawn at day 5 of the illness, consequently smearing the difference in relation to "convalescent" ones. On single occassion ELISA showed no seroconversion, with minimal titres in IPAMA and IFA for unexplained reason.

Despite the relative reliability for rise in titres of serial samples, IFA turned out to be the least sensitive method and not adequate for qualitative studies for the presence of IgG antibodies. The necessity of visualization of whole cell antigen adjusted for antibodies to surface determinants is evident.

Due to the inherent features of each test, another spectrum of antibodies with diverse degree of sensitivity was detected. This discrepancy is discernible from Fig. 1 and is in inversed proportion to coefficient *r*. Closer in nature and performance were IFA and IPAMA, utilizing fixed cells and visual evaluation, greater diversities were with ELISA, which utilized sonicated, not solubilized antigen adsorbed to plastic solid phase and high sensitivity affording soluble chromogen.

IPAMA for antibodies to membrane VZV antigen, commonly encountered in fluorescent version, proved sufficiently sensitive and well suited as a diagnostic tool for disclosing the subjects susceptible to VZV infection. The modification in microtitration plates was further in favour of the procedure's practicability and feasibility.

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