Original article

Reliability of candida skin test in the evaluation of T-cell function in infancy

Background: *Both standardized and non-standardized candida skin tests are used in clinical practice for functional in-vivo assessment of cellular immunity with variable results and are considered not reliable under the age of 1 year. We sought to investigate the reliability of using manually prepared candida intradermal test in the evaluation of T cell function in infants during their second year of life.* ***Methods:*** *Twenty-five healthy infants were tested with manually prepared intradermal candida test. Cultured lymphocytes were stimulated with phytohemagglutinin (PHA) and gamma interferon (IFN-γ) levels were measured in the culture supernatant of stimulated and non-stimulated samples using ELISA.* ***Results:*** *The enrolled infants were 14 to 24 months old (mean 19.2 ± 3.13 months). They were 17 boys (68 %) and 8 girls (32 %). Candida skin test was positive in 17 out of the 25 infants (68%). All infants showed increased IFN-γ levels after PHA stimulation (mean ± SD: 0.83±0.29 ng/ml) compared to basal levels (mean ± SD = 0.16 ± 0.16 ng/ml). The increase of IFN γ levels after PHA stimulation ranged from 1.54 to 38 folds. Infants with positive and negative candida tests showed comparable results in terms of clinical and immunological assessment except for weight percentiles for age that were higher among candida positive group.* ***Conclusion:*** *Candida intradermal test is a cost-effective simple test for evaluation of T cell function with 70 % sensitivity in healthy infants above the age of one year.*

Keywords: Candida, IFN-γ, infants, intradermal, lymphocyte proliferation, PHA stimulation, T cell, Tuberculin.

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INTRODUCTION

The function of T cells is to recognize specific "non-self" antigens, during antigen presentation, leading to generation of specific responses that are tailored to maximally eliminate pathogens or pathogen-infected cells.[1] Measurement of cell-mediated immunity can be done both in vitro and in vivo. However, assays are plagued by standardization problems, biological variability, and technical difficulties. Most tests are expensive and highly specialized making referral to a clinical immunologist necessary.[2]

Cutaneous delayed-type hypersensitivity (DTH) is the classic in vivo test of cellular immunity which measures the recall response to an intradermal injection of an antigen to which an individual has already been exposed over a period of time.[3] A panel of four reagents can be used for DTH testing: *Candida albicans* extract, Tetanus toxoid, purified protein derivative (PPD), and Trichophyton.[4] The incidence of DTH reactions to unstandardized Candida antigens has been reported to vary from 52 - 89%, depending upon the strength of the antigen and the induration diameter required for a positive test.[5] Infants are able to respond, but the test is not considered generally reliable under the age of 1 year.[6]

DTH testing is advantageous due to its ease and low cost; it is a useful screening test in many instances of suspected cellular immune deficiency. The skin response following intradermal inoculation of an antigen is dependent on the specific memory T cells and results in a local reaction after 48–72 hours due to recruitment of mononuclear cells and neutrophils. The presence of one or more positive DTH skin tests generally indicates an intact cell-mediated immunity. However, an impairment in any step of the response pathway can lead to a negative test result. In addition, the DTH response is often suppressed during viral and bacterial infections, and even with live attenuated vaccines administration including the combined measles, mumps, and rubella vaccine. DTH skin reactivity is also suppressed by anti-inflammatory drugs such as glucocorticoids and other immunosuppressants such as cyclosporine, tacrolimus, and mycophenolic acid.[7]

We sought to investigate the reliability of manually prepared candida skin test in the evaluation of T cell function in infants above one year of age and its correlation with the in-vitro

functional assessment of T cells. The ultimate objective is to validate the use of this simple inexpensive test as a screening tool for T cell function in infants.

METHODS

This cross-sectional study comprised 25 clinically healthy infants enrolled from the Outpatient Clinic of Children's Hospital, Ain Shams University. Infants with severe lymphopenia (<1500/mm^3), or with clinical signs of infection at the time of enrollment were excluded from the study. We also excluded infants with any chronic illness including malnutrition and those receiving corticosteroid or any immunosuppressive therapy or received recent live vaccines.

The study protocol gained approval from the Local Ethics' Committee of the Pediatric department, Ain Shams University and an informed written consent was obtained from parents or care givers of each infant before enrollment in the study. All recruited infants were subjected to full history taking with special emphasis on history of infections or chronic illness, vaccination history, or any complication following vaccination. Infants underwent evaluation for growth parameters and for exclusion of the presence of infection or underlying chronic illness.

Study measurements

1- ***Complete blood counting (CBC):*** EDTA (K3EDTA) vacutainer with concentration of 1.2 mg of the anhydrous salt per ml of blood was used. The CBC was done using the automated cell counter coulter® LH 750 cell counter (Coulter Corporation, Florida, USA), with manual white cells differential count.

2- ***Lymphocyte culture with PHA stimulation and measurement of IFN-γ by enzyme- linked immunosorbent assay (ELISA) in the supernatant of cultured mononuclear cells before and after PHA stimulation.*** Heparinized, preservative-free vacutainers were used. Samples were processed within two hours of collection as described below.

- Reagents for in vitro stimulation of peripheral blood mononuclear cells (PBMCs):

Sterile cell culture RPMI 1640w/ L-Glutamine and Amphotericin B antifungal were provided by Lonza (Walkersiville, USA). Penicillin / streptomycin and Gentamycin were obtained from Sigma (St. Louis, Mo.). Fetal bovine serum (FBS) and Phytohemagglutinin (PHA) were provided by Invitrogen, Ave Carlsbad, USA. Assay Max Human IFN-gamma ELISA Kit was obtained from ASSAYPRO, SACHIN 394230 Surat, INDIA for detection of IFN-γ in the culture supernatant.

- Isolation of PBMCs and lymphoproliferation assay:

PBMCs were separated from whole blood by Ficoll-Hypaque (Amersham Biosciences) density gradient centrifugation. Cells were treated with red blood cell lysing buffer (155 mM NH4Cl, 10 mM NaHCO3, and 0.1 mM EDT**;** pH 7.4) to lyse red blood cells. PBMCs freshly harvested from the studied infants were incubated in 96-well tissue culture plates (2×10^5 cells in 0.2 ml/well) in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units of penicillin G per ml, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericin B. Cells were cultured with medium alone (without stimulation) and with stimulation by Phytohemagglutinin (PHA) (1ug/ml) and at 37°C with ٥% CO2. The cultures were incubated for 5 days.

- IFN-γ detection by ELISA:

At the end of the incubation period; cell-free supernatants were collected and frozen at -٧٠° C until cytokine levels were determined by ELISA, using AssayMax Human IFN-γ IgG semi-quantitative ELISA kit, (ASSAYPRO) (www.assaypro.com), USA, to assay the T lymphocyte function in response to the in-vitro stimulation. Polyclonal antibody specific for human IFN-γ has been pre-coated into a 96 –well microplate. IFN-γ in standards and samples is sandwiched by immobilized antibodies and biotinylated polyclonal antibody specific for IFN-γ, which is recognized by a streptavidin – peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added**.** The color development is stopped and the intensity of color is measured at optical density (450nm) with reference filter 570 nm by using an ELISA reader then the concentrations of samples were determined from the standard curve.

- As there are no well-defined age related reference ranges for serum gamma interferon level and as patients differs in their IFN-γ baseline levels as well as their levels after mitogen stimulation[8], so the following values were calculated for IFN-γ results:

Fold of increase = final value divided by the initial value

Percentage of increase= (final value - the initial value) / initial value multiplied by 100.

3- In-vivo T cell function test (Candida intradermal test):

Candida intradermal test was done by intradermal injection of 0.1 ml of manually prepared *Candida*

Albicans extract (1:100) in the volar surface of the forearm after skin sterilization with alcohol. The test reagent was prepared in the Microbiology Department, Ain-Shams University. The test was read at 48 hours and 72 hours by palpating the indurated area and calculating the average of the longest and midpoint orthogonal diameters. A positive DTH reaction consists of induration ≥ 5 mm, indicating an intact T cell function[7].

Statistical Analysis

Data were analysed using an SPSS (version 20) statistical software package under Windows 7 operating system for IBM compatible PC. Arithmetic mean and standard deviation was calculated for categorized parametric data. Comparison of different variables in various groups was done using student t and Mann-Whitney U tests. Wilcoxon signed ranks' test was used to compare multiple readings of the same variable. Spearman's correlation coefficient was used for relating non-parametric variables. A probability p value ≤ 0.05 for all tests was considered significant.

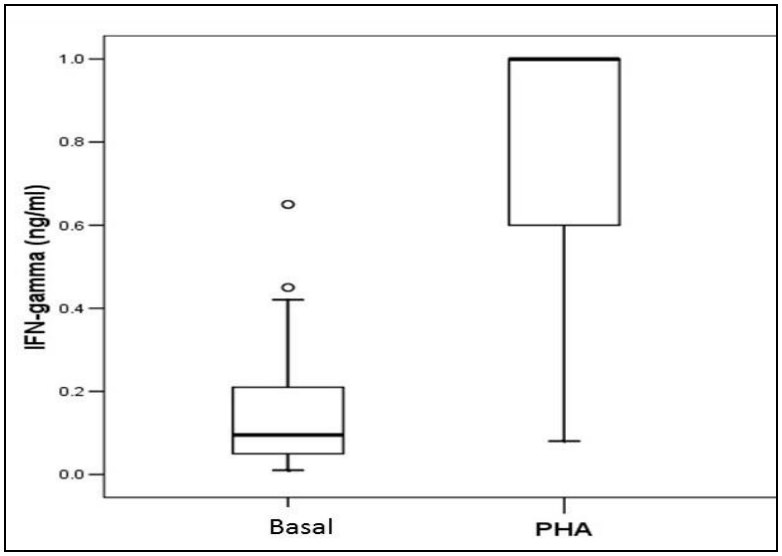
RESULTS

The age of enrolled infants ranged between 14 and 24 months (mean±SD = 19.2±3.1 months). They were 17 boys (68%) and 8 girls (32%). The weight percentiles ranged from 2.9 to 97.3 (mean ± SD = 49.86±34.1) and the length percentiles ranged from 11.3 to 95.3 (mean±SD = 45.62± 22.57).

Candida skin test was positive in 17 out of the 25 infants (68%). Among the 8 infants with negative results, two developed induration diameters of 2 mm while 6 infants had no induration at all. Induration diameters ranged from 0 to 20 mm (mean±SD = 9.84± 6.61mm). Analysis of results obtained after in-vitro PHA stimulation of cultured isolated mononuclear cells showed significant rise in the mean level of IFN-γ after PHA stimulation (mean±SD: 0.83±0.29 ng/ml) compared to basal levels (mean ± SD = 0.16 ± 0.16 ng/ml) (z=-2.088, p=0.037) **(Figure 1)**. The rates of increase ranged from 24% to 4450% (median (IQR): 564 (147-1650) %). The rise in IFN release after PHA stimulation mounted up to 1.45-38 folds.

A significantly positive correlation was observed between Candida intradermal test induration diameters and the weight percentiles for age (r= 0.5, p= 0.01**) (Figure 2)**. However, the Candida test induration diameter did not correlate significantly to IFN-γ expression before and after PHA stimulation (p values were 4.2 and 7.9 respectively)

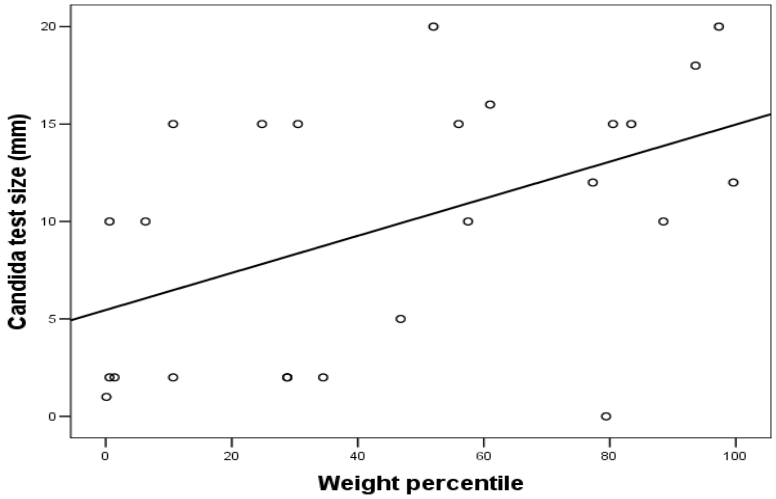
Clinical and laboratory data of the studied infants did not vary significantly with the Candida intradermal test results except for the weight percentiles that were significantly higher in the Candida skin test positive group **(Table 1).**



z=-2.088, *p*=0.037

IFN γ: interferon gamma; PHA: phytohemagglutinin

Figure 1. IFN γ levels before and after PHA stimulation



r= 0.5, *p*= 0.01

Figure 2. Positive correlation between candida test induration diameter and body weight percentile

Table 1. Variation of clinical and laboratory data according to Candida skin test results

Parameters	Candida test positive (n=17)			Candida test negative (n=8)			Comparison	
	Range	Mean± SD		Range	Mean± SD		Z/t	P
Age (months)	14-24	19.41	3.2	16-24	19.88	3.4	t=0.33	0.75
Weight percentiles	6.3-97.3	57.0	31.70	2.9-79.4	24.25	25.67	**t=2.54**	**0.02**
Length percentiles	11.3-74.5	45.96	21.77	13.6-68.8	37.02	16.70	Z=1.02	0.31
WBC (x10^3/mm^3)	7-15	11.49	2.39	5.9-16	10.97	3.10	t=0.45	0.65
ANC count (x10^3/mm^3)	1.5-10.9	4.94	2.44	1.5-8.1	3.58	2.25	Z=1.54	0.12
ALC count (x10^3/mm^3)	3.2-9.9	5.47	1.69	3.4-7.8	6.23	1.53	t=1.08	0.29
IFN γ basal *(ng/ml)*	0.01-0.42	0.162	0.141	0.03-0.65	0.152	0.207	Z=0.46	0.67
IFN γ after stimulation *(ng/ml)*	0.38-1.00	0.914	0.193	0.08-1.00	0.789	0.393	Z=0.39	0.79

ALC: absolute lymphocytic count; ANC: absolute neutrophil count; IFN γ: interferon gamma

DISCUSSION

Testing of DTH to recall antigens has been suggested as a more simple and less expensive method of measurement of T cell function, in comparison to the in-vitro proliferation assays, although being a less sensitive alternative.[2] Both standardized and non-standardized candida preparations for intradermal skin tests are used in clinical practice with variable results ranging from 23 % below the age of 1 year and up to 64 % above that age.[6-8] Our results showed that 68% of infants who underwent candida intradermal skin testing had positive results (≥5 mm) which is higher than the figure reported by Ohri and colleagues.[6] In their study, positive skin test response was elicited in 64% of individuals who were subjected to a standardized Candida albicans skin test (Candin). In the same study, there was a 27% (3/11) positive Candin response for subjects below 1 year of age. The difference is possibly due to early exposure to Candida albicans in our series that belong to the low/middle socioeconomic sector of our community. For the purpose of assessing T-cell function diagnostically, the intradermal test may be performed with any antigen to which the patient has been exposed. Examples other than Candida include tetanus and diphtheria toxoids, streptococcus, Trichophyton, and Proteus. Glucocorticosteroids and other immunosuppressive drugs may blunt the response; the reaction may also be suppressed during acute infectious illnesses. In these situations, or when the interpretation of a negative result is unclear, an in vitro method of measuring T-cell function should be used.[9, 10]

In the current study, all infants had significant response to PHA stimulation with increased IFN-γ production. Activation of T lymphocytes by antigens, alloantigens, and mitogens leads to the release of a number of lymphokines such as interleukin-2 (IL-2) and immune interferon (IFN-γ).

The production of IFN-γ following PHA stimulation varies significantly with age being lowest in cord blood lymphocytes and highest in adults. Therefore, values of such test especially in patients with suspected T cell immune defects should be assessed in comparison to normal age matched healthy controls.[11] In in-vitro T cell proliferation assays, nonspecific mitogens like PHA and concanavalin A can rapidly stimulate the majority of T cells (80 to 100 %), and these assays can be performed in 3 days. On the other hand, the use of specific antigens for testing will require antigen processing and presentation to specific T cells. These latter tests require about 5 to 7 days in culture and peripheral blood mononuclear cells from a healthy control should be evaluated in parallel for comparison. However, these tests may not be able to detect defects of T-cell interaction with other cell types that will lead to only partial impairment of the test results in response to in-vitro stimulation. Similarly, partial impairment of T cell proliferation does not necessarily indicate that the cells are incapable of generating effector responses that are sufficient for protection against many pathogens.[9,12]

In our series, except for weight centile, other clinical and laboratory parameters showed no variation according to the candida skin test results. Also, infants with positive skin test were comparable to those with negative results in terms of their IFN-γ levels before and after PHA stimulation. IFN-γ levels before and after PHA stimulation did not correlate with the candida test induration diameter. This may suggest that negative skin response to candida antigen in an otherwise healthy child should not be interpreted as immunodeficiency. A positive response to intra-dermal candida antigen injection requires uptake and processing of antigen by antigen presenting cells, their interaction with CD4+ helper T cells, cytokine production by T cells, and subsequent recruitment and activation of monocytes and macrophages. Thus, skin testing may be a sensitive indicator of intact cellular immunity, but negative results must be interpreted with caution, because an impairment in any step of the response pathway will lead to a negative response. Repetition and further investigations should be conducted before interpretation.[13] Elucidation of the value of candida intradermal test in screening for T-cell functions and judging its predictive values for intact cellular immunity in infants necessitates controlling the results by a group of age matched patients with significant T-cell defects. A study by Hossny et al,[14] concluded that a negative Candida skin test is more likely to be found (75 %) among patients with laboratory evidence of T-cell immunodeficiency in comparison to those without (18 %). However, the age categories in their series were higher than ours.

CONCLUSION

Candida intradermal skin testing could serve as an easy cost-effective test for evaluation of T cell function. Our results revealed a sensitivity of 68 % in healthy infants above the age of one year. The small sample size and lack of age related reference ranges for gamma interferon production by PHA stimulated cultured mononuclear cells were the main limiting factors in our study.

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