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Variability of sequential studies of lymphocyte blastogenesis in normal adults

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SUMMARY

Sixteen healthy adults had serial studies of delayed-type skin test reactivity and *in vitro* lymphocyte blastogenesis to several antigens over a period of 7 months. In many subjects blastogenesis varied broadly from month to month without apparent cause. Responses to all antigens usually increased or decreased together on sequential testing. Blastogenesis to coccidioidin appeared to result largely from cross-reaction with histoplasmin. Humoral factors were not demonstrably responsible for these changes. Blastogenesis rose consistently and non-specifically in subjects following revaccination to vaccinia virus.

These studies reflect the lymphocyte blastogenesis reaction as a dynamic equilibrium, subject to spontaneous variation, and responding non-specifically to stimuli such as vaccination. Whatever the causes for these changes, it is clear that serial determinations of blastogenesis response to various antigens do not carry the apparent consistency of the skin test response to that antigen, and single tests must be cautiously interpreted.

INTRODUCTION

The *in vitro* lymphocyte blastogenesis test provides one widely applied parameter for assessing the cell-mediated immune response. The competence of lymphocytes to enter blastogenesis under the influence of antigens or 'non-specific' mitogens is considered a result of immunological recognition (Mills, 1966). While specific antigens generally elicit significant blastogenesis only if the donor has been previously sensitized to the antigen, most thymic-derived (T) lymphocytes are thought to possess surface receptors for the mitogen phytohaemagglutinin (PHA) (Borberg *et al.*, 1968; Elves, Roath & Israels, 1963). Therefore, the PHA response is considered a broad index of the number of functional T lymphocytes present in a cell culture.

The delayed-type skin test has long been the standard measurement of cell-mediated immunity. The skin test has accordingly undergone extensive scrutiny in terms of its constancy of response in an individual and cross-reactivity between antigens. Transformation has been much less extensively studied in terms of either consistent response to a given antigen or cross-reactivity among antigens in a given subject. Correlation of skin test with transformation results may vary depending on the antigen, the methods used, and, perhaps most importantly, the person's immunological competence and experience (Oppenheim, 1968; Smith & Reichman, 1972; Thomas, Clements & Grzybowski, 1971).

Non-specific effects on blastogenesis have been described in several situations. Increases due to antigen–antibody complexes can occur in plasma-supplemented medium (Bloch-Shtacher, Hirschhorn & Uhrm, 1968). On the other hand, non-specific suppression of blastogenesis may occur in uraemic plasma (Silk, 1967). Furthermore, suppression of parameters of cell-mediated immunity including blastogenesis may occur in serious infections such as mucocutaneous candidiasis, tuberculosis, and histoplasmosis. Despite impairment of cell-mediated immune responsiveness in these circumstances, high titres of

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specific serum antibody are often present, and a part of the suppression has been attributed to humoral factors (Heilman & McFarland, 1966; Kirkpatrick *et al.*, 1971; Oppenheim, 1972; Schlegel *et al.*, 1970).

The present series of studies was undertaken to explore in healthy persons several aspects of lymphocyte blastogenic responsiveness. These were: (1) consistency of blastogenesis responses over a long period of time; (2) concordance of responses to different antigens; (3) correlation of blastogenesis to delayed skin test reactivity; (4) influence of plasma factors of healthy subjects on transformation; (5) effect of vaccinia re-immunization on blastogenesis; (6) relationship of thymidine uptake of unstimulated cultures to that of antigen stimulated cultures.

MATERIALS AND METHODS

Serial studies. Sixteen healthy Tennessee residents were repeatedly studied. Their ages ranged from 20 to 50. All underwent monthly venesection of 60 ml of blood, which was heparinized (20 u/ml) and used for lymphocyte transformation studies.

One time studies. Data from paired tests of lymphocyte transformation of 280 normal persons tested in this laboratory over a 5 year period were analysed to compare unstimulated to *Candida*-stimulated thymidine uptake.

Skin testing. After *in vitro* studies had been followed a minimum of 5 months, thirteen subjects were tested with histoplasmin (Parke-Davis) and then were tested with 1:100 coccidioidin (Cutter Laboratories). Tests were measured as mm of induration of 48 hr.

Vaccination. Seven healthy volunteers were vaccinated against vaccinia as part of an on-going hospital personnel immunization program. All had prior history of vaccination. All seven had vesicular then pustular reactions developing from 2 to 7 days after vaccination, and lasting up to 3 weeks post vaccination. Lymphocyte transformation was performed immediately prior to the vaccination, at a second time between 3 and 7 days post vaccination, and finally 21 days post vaccination.

Lymphocyte transformation. Lymphocyte transformations were performed according to a method reported earlier (Alford, 1973). Briefly, duplicate 2-ml cultures were prepared to contain one million cotton fibre-purified lymphocytes, and 0·2 ml of either Hanks' balanced salt solution (HBSS) for unstimulated control, or 0·2 ml of HBSS containing histoplasmin (1:10, Parke-Davis), PPD (25 μg/ml, Parke-Davis), and coccidioidin (1:10, kindly supplied by Dr Demosthenes Pappagianis). The vaccinia 'antigen' used in this study was a 1:30 dilution of commercial vaccinia vaccine (Eli Lilly) diluted in phosphate-buffered saline. This complex antigen contained bovine protein.

All antigens except vaccinia were extensively dialysed against phosphate-buffered saline before final dilution in HBSS. Dilute PHA-P (350 μg/ml, Difco Laboratories) was used as a non-specific mitogen. Cultures were in glutamine-supplemented medium TC 199 containing 20% autologous plasma, heparin (20 u/ml), penicillin (100 μg/ml) and streptomycin (25 μg/ml). Three subjects had serial paired culture sets prepared with one set containing 20% pooled heat-inactivated human serum and the other autologous heparinized plasma. Cultures were incubated for 3 days (PHA) or 5 days (antigens) in humidified 5% CO_2 at 37°C, pulsed overnight with 2 μCi of tritiated thymidine, harvested, and counted. All cultures were run in duplicate. Results are expressed as disintegrations per minute (d/min) per 10^6 lymphocytes, and as the stimulation index:

$$\text{stimulation index} = \frac{\text{d/min of antigen or PHA stimulated lymphocytes}}{\text{d/min of unstimulated lymphocytes}}.$$

No subject had lymphocyte transformations performed if he was symptomatically ill or if he had ingested any medication over the preceding 24 hr.

RESULTS

Consistency of responses

Most subjects had a broad range of response to the various antigens and to PHA. These responses widely fluctuated from month to month with no obvious cause, such as illness or medication. An example of such spontaneous changes occurring in a subject who was not vaccinated is presented graphically in Fig. 1. Detailed responses to candida, an antigen to which most normal subjects respond strongly, are depicted in Table 1. All subjects were studied at least five times, and some were studied as many as eleven times for a total of 129 studies. The mean d/min = Σd/min each study/n for all studies on any single subject. The mean response ranged from a low of 24,000 d/min to a high of 181,000 d/min; mean stimulation indices ranged from 14 to 74. In only twenty studies was the response less than 10,000 d/min to candida. Only two studies had stimulation indices less than three. The mean of all 129 studies was 71,000

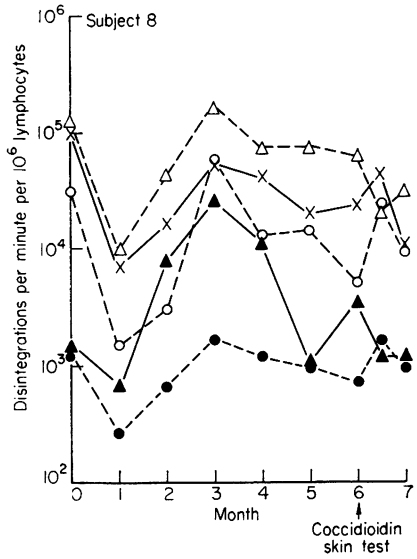

FIG. 1. Sequential lymphocyte transformation in a healthy adult: variation of response. Lymphocyte transformation response to four antigens followed over 7 months. Note marked variability of response to antigens from month to month. Negative coccidioidin skin test apparently has no effect on transformation. (△) *Candida*; (×) histoplasmin; (○) PPD; (▲) coccidioidin; (●) unstimulated.

TABLE 1. *Candida albicans* transformation response

Subject number	Disintegrations per minute per million cells (× 10^3)			Stimulation index		
	Mean	Range		Mean	Range	
		Low	High		Low	High
1	181	90	432	33	6	70
2	129	50	220	74	11	111
3	97	41	136	37	18	68
4	93	37	239	57	33	117
5	87	34	109	49	21	72
6	79	31	155	42	26	71
7	70	11	163	37	10	58
8	63	4·1	179	46	8	94
9	60	30	123	27	12	71
10	50	5·5	109	60	31	111
11	47	5·9	111	31	11	56
12	44	16	68	29	16	48
13	40	16	80	31	13	58
14	35	0·6	149	14	2	43
15	33	13	65	16	1	49
16	24	0·9	51	28	5	44

d/min with stimulation index of 38. The range of two standard deviations of the stimulation index was 5·9–70. These studies indicate a blastogenic response to candida antigen considerably higher than that reported by other laboratories, and slightly higher than reported from this laboratory for older normal subjects (Alford, 1973; Kirkpatrick *et al.*, 1971; Shannon *et al.*, 1966). Within this broad but strongly positive range, responses widely varied in each person from month to month, as indicated by the range of response.

TABLE 2. Correlation of skin test and transformation

Skin test	Subject	Skin test (mm)		Mean transformation in d.p.m. ($\times 10^{-3}$)				
		Histo	Cocci	Unstimulated	PHA	*Candida*	Histo	Cocci
Histoplasmin-positive	2	15	0	1·7	741	33	11	53
	6	10	10	2	536	79	56	8·5
	8	20	0	2·6	308	63	33	15
	9	10	0	2·4	657	60	66	89
	10	15	0	0·8	276	50	36	34
	12	15	0	1·6	429	44	64	53
	Mean			1·9	491	55	44	34
Histoplasmin-negative	3	0	0	0·9	331	24	2·9	1
	4	0	0	1·6	399	93	26	2·7
	5	0		3	496	97	21	3·8
	11	0	0	1·6	489	40	19	2
	13	0		2·6	328	33	11	5·1
	15	0		2·1	415	87	14	11
	16	0	0	1·6	489	40	19	2
	Mean			1·9	412	60	15	7
P value by Student's *t*-test Histo pos *v* Histo neg				n.s.	n.s.	n.s.	< 0·01	< 0·05

Concordance of responses

Antigenic responsiveness to the four antigens tested were usually concordant with all values rising or falling together as compared to the prior month. This is shown in months 0–4, Fig. 1. These trends occasionally broke up with respect to one or more antigens falling out of the 'pattern', as shown in Fig. 1, months 4–7.

Delayed-type skin tests and lymphocyte transformation—naturally acquired cross-reactivity

Thirteen subjects were skin tested with histoplasmin and ten were also tested with coccidioidin. Result are presented in Table 2. Mean lymphocyte transformation values in d/min for skin test positive or negative groups are similar for unstimulated, PHA-stimulated and *Candida*-stimulated cells. However, histoplasmin skin test-positive subjects had markedly greater transformation to histoplasmin than histoplasmin skin test-negative subjects (44,000 d/min *v* 15,000 d/min). Coccidioidin-induced lymphocyte transformation skin test relationships differed from those to histoplasmin. Only one subject had a measurable skin test to 1:100 coccidioidin. Nevertheless, she and five other subjects positive to histoplasmin by skin test had markedly greater lymphocyte transformation to coccidioidin than did histoplasmin-negative subjects. This indicates that the problem of cross-reaction between histoplasmin and coccidioidin, which has been observed with skin testing, is even greater with transformation and that lymphocyte transformation results with coccidioidin must be interpreted with great care in a histoplasmin skin test-positive subject. Subject 9 had a negative coccidioidin skin test, but his lymphocytes consistently transformed more intensely to coccidioidin than histoplasmin.

Influence of plasma

In most of the studies both unstimulated and stimulated blastogenic responses in 20% autologous plasma varied less than twofold from concurrent studies in 20% pooled normal human serum. A few with greater differences were equally divided among greater or lesser responses in plasma than serum.

Vaccination

Lymphocyte transformation results relating to vaccination are summarized in Fig. 2. Following vaccination, two subjects had progressive elevation of their resting or unstimulated transformation to as

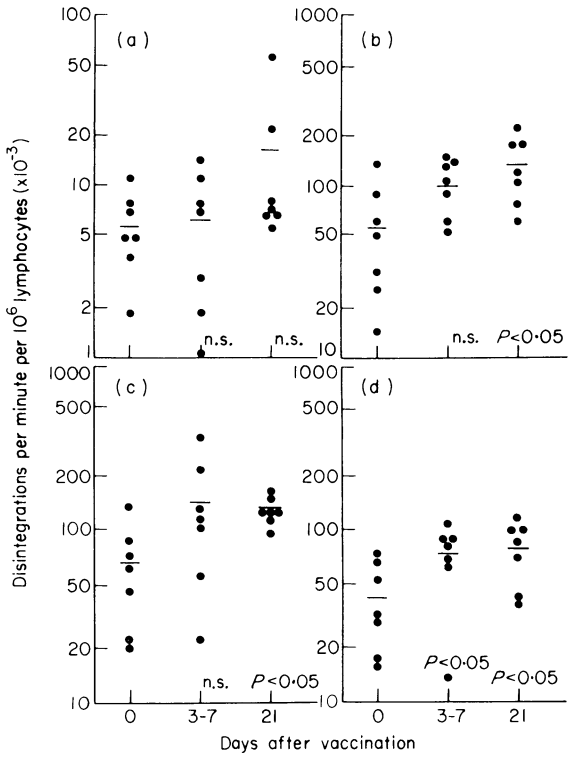

FIG. 2. Lymphocyte transformation following vaccination. Transformation response to three antigens following vaccinia revaccination. Note significant increase in response to vaccinia 'antigen' at 3–7 days, and to both *Candida* and histoplasmin at 21 days post-vaccination. (a) Unstimulated; (b) candida-stimulated; (c) histoplasmin-stimulated; (d) Vaccinia-stimulated.

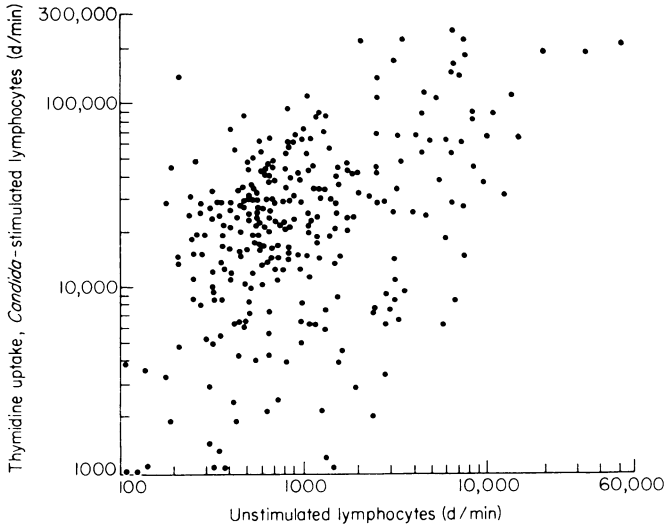

FIG. 3. Correlation between candida antigen stimulation and unstimulated lymphocyte thymidine uptake. The degree of antigen-induced transformation and the degree of unstimulated transformation show a broad positive correlation. Note that the vertical and horizontal axes are both logarithmic scales.

high as 57,000 d/min 3 weeks after vaccination. However, there was no significant increase in the mean unstimulated transformation of the entire group of subjects following vaccination. Four of the seven subjects had progressive increases in transformation to vaccinia and the two unrelated antigens, histoplasmin and candida, both at 7 days and 21 days post-vaccination. Two subjects had increases only at 21 days post-vaccination, and one subject had only a minor increase (less than double the pre-vaccination values). When compared to the pre-vaccination transformations, significant increases occurred in transformation to all three antigens by 21 days, and to vaccinia by 3–7 days.

Correlation between unstimulated and candida-stimulated thymidine uptake

Transformation test results from 280 studies of normal adults performed over a 5-yr period are depicted in Fig. 3. Approximately 46% of the values emanated from the sixteen normal subjects who were sequentially studied. The tests are considered together since they were performed by one technician with no alteration in reagents or methodology throughout the study period. Candida-stimulated counts greater than 100,000 d/min were seldom encountered in normal subjects using the methods employed herein. The positive correlation between candida antigen-stimulated and -unstimulated culture values is depicted in Fig. 3. Very high candida-induced thymidine uptake ($>100{,}000$ d/min) almost always occurred in cultures having high unstimulated thymidine uptake, whereas only two of eighteen had paired control values <2000 d/min. Sixteen of eighteen candida-stimulated cultures yielding $>100{,}000$ d/min were associated with unstimulated control values >2000 d/min.

DISCUSSION

In recent years an increasing number of assays of 'cell-mediated' immunity have been developed. Major parameters include the delayed type skin test *in vivo*, and *in vitro* tests for lymphocyte transformation, migration inhibition factors for macrophages and mixed human leucocytes, monocyte chemotactic factors, and lymphocyte-mediated cytotoxicity (David, 1973). For years the skin test to a given antigen has been recognized as being subject to cross-reactivity with related antigens; this problem has confounded interpretation of: (1) tuberculin testing in the southeastern United States (due to infections with atypical mycobacteria); and (2) histoplasmin testing in the southwestern states, the endemic area of *Coccidioides immitis* infection (Arnold, Scott & Spitznagel, 1970; Doto *et al.*, 1972; Edwards, Edwards & Palmer, 1959). In addition to the state of prior sensitization, the intensity of a skin test reaction depends on additional factors including primary or acquired immune deficiency, debilitating disease, or concurrent viral infection (Bentzon, 1953; Heilman & McFarland, 1966). However, in a healthy subject the delayed type skin test is thought to be a relatively consistent index of sensitization to specific antigens.

The lymphocyte transformation reaction has been utilized as an *in vitro* test for *in vivo* antigenic sensitization. This test may also be affected by stimuli such as antigen–antibody complexes, ingestion of certain drugs, pregnancy, and may be depressed in various immune deficiency states, following surgical or thermal trauma or during the course of viral infections, and in association with polymorphonuclear leucocytosis (Fauci & Dale, 1974; Levy & Kaplan, 1974; MacKinney & Booker, 1972; Park *et al.*, 1971; Purtilo, Hallgren & Yunis, 1972; Smithwick & Berkovich, 1969).

The present studies indicate that even in the absence of such known factors, the lymphocyte transformation reaction is remarkably variable. This is not due to intrinsic variation of the test since replicate determinations of tests upon aliquots of lymphocytes in this laboratory seldom vary more than 10%. Only two subjects were female, excluding any effect of cyclic oestrogen/progesterone levels in most subjects. The broad range of responses in our subjects was not due to clinically apparent antecedent illness, ingestion of aspirin or other medication. Plasma factors did not appear to exert a major influence on transformation as compared to a single lot of heat inactivated pooled human serum. Thus, by exclusion, this great variability in responses of a given subject appears to depend on varying reactions of each subject's lymphocytes from time to time.

Using the commonly accepted designation of a stimulation index greater than three as positive, many of our subjects studied on a single occasion would have been reported as 'negative'. Few studies were

negative to the potent candida extract used. However, blastogenic indices to histoplasmin and coccidioidin were less intense. Subjects whose mean blastogenic index was in the range of 6–15 had up to 26% of their individual studies 'negative'. These studies therefore indicate that a negative stimulation index to histoplasmin or coccidioidin bears repeating before a subject with such a test is considered 'nonreactive'. Although not presented, data for PPD are similar to histoplasmin/coccidioidin in their consistency.

Positive correlation of transformation with skin test reactivity was observed in the reaction to histoplasmin. In addition clear correlation between histoplasmin skin test reactivity and coccidioidin-induced lymphocyte transformation also occurred and was independent of response to candida or PHA. This was quite strong, and subject 9, despite a negative coccidioidin skin test, had a consistently greater transformation in response to coccidioidin than to histoplasmin. Two others, 10 and 12, had coccidioidin responses almost as intense as those to histoplasmin. The histoplasmin skin test-negative subjects had frequent, though less intense, transformation in response to histoplasmin, suggesting cross-reaction to still other antigens. This degree of cross-reaction therefore requires a circumspect interpretation of transformation to coccidioidin in a histoplasmin-sensitized subject. These studies used amounts of antigen previously shown to cause maximal transformation. Not reported here, lesser amounts of antigen reduced, but did not eliminate, cross-reacting transformation.

Appreciation of spontaneous variation in these tests is especially important in view of efforts now being made to immunologically evaluate immunosuppressed persons undergoing 'immune reconstitution' with leucocyte extracts such as transfer factor. Tuberculosis, histoplasmosis, and coccidioidomycosis may be associated with spontaneous changes in clinical and immunological reactivity. It is of utmost importance that changes attributed to attempted therapeutic reconstitution reflect more than this spontaneous fluctuation of the disease process or normal variation in lymphocyte reactivity suggested by our studies. The data presented suggest that each person has a 'general reactivity level' which may change or remain relatively constant from month to month. A subject with dramatic increases in response to one antigen usually had similar augmentation in reactivity to other antigens. This suggests that interpretation of changing response to specific antigens requires controls that include other 'non-specific' antigens.

Consistent non-specific increases in transformation occurred following re-vaccination with a live viral agent known to stimulate cell-mediated immune responses. Concurrent unvaccinated controls were not studied, leaving open a possibility that these changes could have been a response to unrecognized external influences such as occult infection by another viral agent. Against this, all subjects were studied concurrently and appeared in excellent health except for their local vaccinia reaction. Therefore, these studies suggest that vaccinia reinfection of a previously vaccinated or presumably sensitized subject is promptly followed by increased transformation, not only to the vaccine but to other unrelated antigens to which the vaccinee has previously been sensitized. This increase in a 'general level' of antigen-induced transformation responsiveness is similar to the broad but spontaneous fluctuation seen in the other sixteen subjects. This finding is of particular note since it differs dramatically from the often recorded effect of viral infection or measles vaccination in depressing lymphocyte blastogenesis (Bentzon, 1953; Mellman & Wetton, 1963; Mitchell, Nelson & LeBlanc, 1935; Smithwick & Berkovich, 1969).

Our finding correlating high candida-induced transformation values with those of high unstimulated thymidine uptake is consistent with the following interpretation. Peripheral blood lymphocytes, which in unstimulated cultures avidly take up thymidine, denote that a condition (or population) of peripheral blood lymphocytes exists at that time which predisposes to an increase in the 'general level' of antigen-induced transformation responsiveness. The factors causing this status are largely unknown. It appears that reinoculation with live vaccinia virus is one of them. The clinical implications of a general increase in lymphocyte transformability could be far reaching and need further investigation.

The lymphocyte blastogenesis reaction is indeed a complex and dynamic phenomenon. Determination of lymphocyte blastogenic response may be valid on one determination if the subject is strongly positive. If the subject is moderately positive or negative, repeat studies are warranted to be certain he is reacting to that antigen and not just showing nonspecific effect of spontaneously changing reactivity.

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