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CORRELATIONS AMONG CUTANEOUS REACTIVITY TO DNCB, PHA-INDUCED LYMPHOCYTE BLASTOGENESIS AND PERIPHERAL BLOOD E ROSETTES

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SUMMARY

Comparisons of the results obtained in a study of fifty-two patients with genito-urinary malignancies using three assays to monitor the thymus-dependent immune system (delayed cutaneous hypersensitivity to DNCB, PHA-induced lymphocyte blastogenesis, and peripheral blood E rosette-forming lymphocyte counts) yielded statistically significant positive correlations between the DNCB and PHA assays in twenty-one of twenty-eight instances, the DNCB and E rosette assays in thirteen of sixteen instances, the PHA and E rosette assays in twenty-eight of thirty-six instances, and among the DNCB, PHA and E rosette assays in ten of fourteen instances.

The results suggest that both PHA-stimulated lymphocyte blastogenesis and peripheral blood E rosette-forming lymphocyte levels provide meaningful *in vitro* correlates of cell-mediated immunity.

INTRODUCTION

The growing interest in cell-mediated immunity has been reflected in the emergence of an increasing number of assays to monitor cellular immune functions. However, attempts to compare the results obtained from different assays have often failed to yield significant correlations. This has engendered serious doubts regarding the validity of interpretations of data derived from such assays. The reasons for these discrepancies are unclear; however, it is likely that quantitative and selective defects in cellular immune function occur which can be detected by some assays but not others.

Our main purpose in this brief communication is to compare the results obtained from three assays which have been commonly used to monitor thymus-dependent immune mechanisms in a variety of human disease states: (1) the elicitation of delayed cutaneous hypersensitivity (DCH) to dinitrochlorobenzene (DNCB); (2) the proliferative response of peripheral blood lymphocytes to phytohaemagglutinin (PHA); and (3) the spontaneous

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formation of rosettes between human T lymphocytes and sheep erythrocytes (E rosettes). To provide a spectrum of cellular immune responsiveness, patients with malignancies of the genito-urinary system were studied.

PATIENTS AND METHODS

Patients

Fifty-two patients with genito-urinary tumours were studied. Nine patients were female and forty-three were male. The mean age of the patients was 56·8 years old. Tumour types represented are shown in Table 1.

All patients were tested concurrently with at least two of the three assays under consideration. The respective numbers of patients tested with each combination of assays are shown in Tables 2 and 3.

TABLE 1. Tumour types represented among fifty-two patients with genito-urinary malignancies studied

Tumour	Number of patients tested
Bladder carcinoma	23
Prostatic carcinoma	16
Renal cell carcinoma	9
Testicular carcinoma	4
Total	52

Methods

All the techniques used in these assays have been reported in detail in preceding communications; therefore they will be described here only briefly or by reference.

DNCB assay

Doses of 2000 μg and 50 μg of DNCB in 0·1 ml of acetone were applied to an area of 3 cm^2 on the upper arm and forearm, respectively, as previously described (Catalona *et al.*, 1972), and the sites were observed 14 days later for the occurrence of a spontaneous flare reaction. Previous studies from our laboratory (Catalona, Taylor & Chretien, 1972) have shown in a population of 143 healthy volunteers that 96%, including forty-seven of forty-nine subjects between 60 and 80 years old, will manifest a spontaneous flare reaction to 2000 μg of DNCB within 14 days of its application. Accordingly, for the purpose of this study, DNCB reactivity was considered normal if a spontaneous flare reaction occurred to 2000 μg of DNCB within 2 weeks of its application, and impaired if a spontaneous flare failed to occur.

PHA assay

Peripheral blood lymphocytes were separated from heparinized whole blood by sedimentation in 1% methyl cellulose after addition of carbonyl iron. Cultures containing $5{\cdot}0 \times 10^5$ thrice-washed mononuclear cells per millilitre of 90% Roswell Park Memorial Institute 1640 medium and 10% pooled human AB+serum were prepared.

Phytohaemagglutinin-P from one large batch, reconstituted in saline, and used in concentrations of 3·1, 6·25, 12·5 and 25·0 μg of protein per millilitre of culture, was added to the cultures and the cultures were incubated along with unstimulated control cultures for 54 hr. Cultures with each dose of PHA as well as control cultures were always prepared in triplicate.

Cultures were then incubated with [^{3}H]thymidine (300 mCi/mmole, 30 μCi/ml culture) for 3 hr, after which they were immediately frozen in liquid nitrogen. The acid-precipitable nucleoprotein was analysed by liquid scintillation counting and the incorporation of [^{3}H]thymidine was expressed in disintegrations per minute per culture. The reactivity of a lymphocyte population was defined as the highest reactivity recorded (regardless of the PHA concentration at which it occurred) minus the control reactivity (unstimulated cultures).

Previous studies from our laboratory (Catalona, Sample & Chretien, 1973) using this technique have demonstrated that responsiveness of lymphocytes to PHA varies with age. Accordingly, age-correlated, PHA-induced lymphocyte stimulation levels from 284 healthy volunteers were used to establish mean values and standard deviations (s.d.) of the mean for each decade represented among the patients studied. PHA responsiveness was arbitrarily defined as being low if it was less than 1 s.d. below the mean for the age group, and normal if it was greater than this value.

E rosette assay

The method used in this study is a modification (Catalona, Potvin & Chretien, 1974) of methods previously described (Brain, Gordon & Willetts, 1970; Wybran & Fudenberg, 1973).

Peripheral blood lymphocytes were separated on a Ficoll–Hypaque gradient and washed three times with RPMI 1640 medium. The final concentration was adjusted to approximately 2×10^6 cells per millilitre and 0·25 ml of the lymphocyte suspension was added to 0·2 ml of 1% (v/v) thrice-washed sheep erythrocytes and 0·1 ml pooled human AB+ serum which had been heat-inactivated at 56°C for 30 min and absorbed against an equal volume of the same sheep erythrocytes for 30 min at 4°C. Preparations were incubated at 37°C for 15 min, centrifuged at 200 *g* for 5 min and maintained at 4°C for 60 min before counting. The pellet was resuspended by tilting the centrifuge tube and one drop of 1% Toluidine Blue-O was added for staining. A minimum of 200 cells from each sample were counted and the percentage of cells forming E rosettes was determined. All samples were done in duplicate.

Previous studies from our laboratory (Catalona *et al.*, 1974) have shown that under the conditions of our assay, healthy subjects over 50 years old have a mean of 72·5% (s.d. = 7) peripheral blood E rosette-forming lymphocytes and a mean of 1506 (s.d. = 508) total E rosette-forming lymphocytes per millilitre of blood. Total E rosette counts were arbitrarily defined as being low if they were less than 1 s.d. below the mean for the controls and normal if they were greater than this value.

Statistical methods

The proportion of subjects having normal assays and the proportion having abnormal assays were determined for each assay. These proportions were used to calculate the frequency with which the assays would be expected to correlate, assuming complete independence between assays. Differences between expected frequencies of correlation and fre-

quencies of correlation actually observed were tested for statistical significance by χ^2 analysis using 2-by-2 tables (Tables 2 and 3).

RESULTS

Individual assay results (Table 2)

Seventeen of thirty patients tested or 56·7% had normal DNCB reactivity, while thirteen or 43·3% had impaired DNCB reactivity. Twenty-nine of fifty patients or 58·0% had normal PHA responsiveness, while twenty-one or 42·0% had impaired responsiveness. Twenty-two of thirty-eight patients or 57·9% had normal E rosette counts, while sixteen or 42·1% had low total E rosette counts.

TABLE 2. Individual results of three assays commonly used to monitor the thymus-dependent immune mechanism in fifty-two patients with genito-urinary malignancies

Assay	Number of patients	Normal assay		Abnormal assay	
		Number	%	Number	%
DNCB	30	17	56·7	13	43·3
PHA	50	29	58·0	21	42·0
E rosette	38	22	57·9	16	42·1

Thus, in the patient population studied, the probability of obtaining a normal assay score by chance alone was 0·567 for the DNCB assay, 0·580 for the PHA assay and 0·579 for the E rosette assay. Similarly, the probability of obtaining an abnormal assay score by chance alone was 0·433 for the DNCB assay, 0·420 for the PHA assay and 0·421 for the E rosette assay.

Relationship between DNCB and PHA assays (Table 3)

Assuming independence between assays, the expected frequency of both assays being normal by chance alone in the population studied would be $0·567 \times 0·580$ or 32·9%. The corresponding expected frequency of both assays being abnormal would be $0·433 \times 0·420$ or 18·2%. Therefore, by chance alone, assays would be expected to correlate in 51·1% of instances.

Both DNCB and PHA assays were normal in thirteen of twenty-eight patients and both were abnormal in eight of twenty-eight patients tested. Thus, the results obtained from the DNCB and PHA assays correlated in twenty-one of twenty-eight instances, a 75% frequency of correlation. When tested by χ^2 analysis, the difference between the observed and expected frequencies of correlation is significant at the 0·02 level.

Four of seven patients in whom the assays did not correlate had normal cutaneous reactivity to DNCB and low PHA responsiveness, while three had normal PHA responsiveness and impaired DNCB reactivity.

Relationship between DNCB and E rosette assays (Table 3)

Both DNCB and E rosette assays were normal in eight of sixteen patients tested and both

TABLE 3. Interrelationships among cutaneous reactivity to DNCB, PHA-induced lymphocyte blastosgenesis and peripheral blood E rosette-forming lymphocyte counts in fifty-two patients with genito-urinary malignancies, demonstrating statistically significant positive correlations among the assays

Assays compared	Numbers of patients studied	Assay results*	Expected frequency of result†	Observed results (number of patients)	Observed frequency of result	Statistical significance‡ χ^2	Statistical significance‡ *P* value
DNCB and PHA	28	DP	0·329	13	0·464	6·39	<0·02
		dp	0·182	8	0·286		
		Dp	0·238	4	0·143		
		dP	0·251	3	0·107		
DNCB and E rosette	16	DE	0·328	8	0·500	5·83	<0·05
		de	0·182	5	0·313		
		De	0·238	1	0·062		
		dE	0·250	2	0·125		
PHA and E rosette	36	PE	0·336	16	0·444	10·10	<0·005
		pe	0·177	12	0·333		
		Pe	0·244	5	0·139		
		pE	0·243	3	0·083		
DNCB, PHA and E rosette	14	DPE	0·190	6	0·428	(insufficient number of observations for statistical analysis)	
		dpe	0·076	4	0·286		
		DPe	0·138	1	0·071		
		DpE	0·138	1	0·071		
		dPe	0·106	1	0·071		
		Dpe	0·100	1	0·071		

* D = normal DNCB reactivity; d = impaired DNCB reactivity; P = normal PHA reactivity; p = impaired PHA reactivity; E = normal total E rosette count; e = low total E rosette count.

† Calculated from the respective proportions of patients in the population having normal or abnormal scores in each individual assay, e.g. (from Table 2) D = 0·567, since 56·7% of patients tested had normal DNCB reactivity, and $P = 0{\cdot}580$ since 58·0% had normal PHA stimulation; therefore, DP = D × P = 0·567 × 0·580 or 0·329.

‡ Compares the significance of the differences between expected frequencies of correlation and observed frequencies of correlation, assuming independence between assays (using 2-by-2 tables).

were abnormal in five patients. Thus, the results of the assays correlated in thirteen of sixteen instances, or 81·2%. Assuming independence between assays, the expected frequency of correlation would be 51·0%. When tested by χ^2 analysis, the difference between observed and expected frequencies of correlation is significant at the 0·05 level.

One of three patients in whom the results of the assays did not correlate had normal DNCB reactivity and low E rosette-forming lymphocyte levels, while two patients had abnormal DNCB reactivity and normal E rosette counts.

Relationship between PHA and E rosette assays (Table 3)

Both PHA and E rosette assays were normal in sixteen of thirty-six patients tested, while both were abnormal in twelve patients. Thus, the results of the assays correlated in twenty-eight of thirty-six instances, or 77·8%. Assuming independence between assays, the expected frequency of correlation would be 51·3%. When tested by χ^2 analysis, the difference between observed and expected frequencies of correlation is significant at the 0·005 level.

Five of eight patients in whom the results of the assays did not correlate had normal PHA responsiveness and low E rosette counts, while three had impaired PHA responsiveness and normal E rosette counts.

Relationship among DNCB, PHA and E rosette assays (Table 3)

All three assays were normal in six of fourteen patients tested, while all were abnormal in four patients. Thus, the results of the assays, when performed concurrently, correlated in ten of fourteen or 71·4% of instances. Assuming independence among assays, the expected frequency of correlation would be 26·6%. However, the number of observations in this group were insufficient for statistical analysis.

Two of four patients in whom the assays did not correlate manifested abnormalities of only one assay, and two patients had abnormalities of two of the three assays.

DISCUSSION

This study demonstrates that, under the experimental conditions described, three assays commonly used to monitor the thymus-dependent lymphocyte subpopulation yield results which correlate in more than 75% of instances. This finding suggests that both PHA-stimulated lymphocyte blastogenesis and total peripheral blood E rosette-forming lymphocyte levels provide meaningful *in vitro* correlates of cell-mediated immunity.

A direct correlation between PHA-induced lymphocyte transformation and DCH reactivity was previously demonstrated in patients with Hodgkin's disease using morphological criteria for blast transformation and cutaneous reactivity to common skin test antigens (Hersh & Oppenheim, 1965), and a similar relationship was demonstrated using cutaneous reactivity to DNCB (Brown *et al.*, 1967). A positive correlation also was demonstrated between PHA-stimulated lymphocyte activation using [^{3}H]thymidine incorporation and cutaneous reactivity to DNCB in patients with sarcoidosis (Sharma, James & Fox, 1971). However, results at variance with these were reported by other investigators in studies of a variety of diseases associated with defective cell-mediated immunity (Daguillard *et al.*, 1969; Han & Sokal, 1970; O'Connell, Golub & Morton, 1973).

Persuasive evidence has been advanced in support of the concept that spontaneous rosette formation between lymphocytes and sheep erythrocytes is a property of thymus-derived lymphocytes (Wybran, Carr & Fudenberg, 1972; Jondal, Holm & Wigzell, 1972). Furthermore, peripheral blood E rosette-forming lymphocyte levels have been correlated with the proliferative response of lymphocytes to PHA (Stjernsward *et al.*, 1972; Brugarolas *et al.*, 1973) and to cutaneous reactivity to common skin test antigens (Brugarolas *et al.*, 1973) (although data are not presented in the latter study).

The reasons underlying the lack of correlation in approximately 20–25% of instances in this study are unclear. However, several possible reasons for discrepant results among these assays are readily apparent. Deficiencies in antigen recognition or in the capacity to mount a normal inflammatory response would result in abnormal cutaneous responsiveness to DNCB, but may not be reflected in the PHA or E rosette assays. Furthermore, while the E rosette assay provides information regarding the relative abundance of thymus-derived lymphocytes in the peripheral blood, it gives no indication of their functional capacity. Also, recent observations (Phillips & Roitt, 1973), which suggest that PHA may be partly a B-cell mitogen in man provides another possible explanation for the lack of correlation of the PHA assay with other T cell assays. Finally, and perhaps equally important, the intrinsic

limitations of experimental techniques must be acknowledged as possible causes for discordant results among assays.

Thus, while the high frequency of correlation among the results obtained from the DNCB, PHA and E rosette assays demonstrated herein indicates the clinical usefulness of each assay, the incidence of discordant results observed underscores the desirability of utilizing multiple assays to provide a more accurate measure of cell-mediated immunity in human disease states.

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