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Relationship between cutaneous delayed hypersensitivity and cell-mediated immunity in vitro responses assessed by diphtheria and tetanus toxoids

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Cutaneous delayed hypersensitivity (CDH) testing with microbial antigens in man is thought to reflect the status of cell-mediated immunity (CMI). We have evaluated diphtheria-tetanus (DT) and tetanus (T) toxoids by comparing the CDH response with in vitro parameters of CMI: lymphocyte deoxyribonucleic acid (DNA) synthesis, leukocyte inhibition factor (LIF) in 5 immunized adults, and lymphotoxin release in 2 adults. Cord blood lymphocytes were used as controls for each assay. A dose response with both toxoids was used to compare the CDH reaction with each in vitro assay, establishing the maximum response and threshold dose which gave a positive response. All subjects had a positive CDH response to both antigens (≥ 5 mm induration at 48 hr), positive DNA synthesis (stimulation index ≥ 3), LIF release (migration $\leq 80\%$), and lymphotoxin production, while cord blood lymphocytes were usually negative to all in vitro assays. No consistent quantitative relationships between CDH reactions and in vitro CMI responses were seen. Threshold antigen dose data revealed that DNA synthesis was approximately ten times as sensitive an assay as CDH, and 10^5 times as sensitive as the LIF technique. No difference in sensitivity was noted between DT and T toxoids. Three subjects re-evaluated 16 mo after the initial study showed positive CDH and CMI responses to tetanus toxoid, although the antigen dosage required varied considerably. We conclude that the CDH response with either toxoid in the concentrations used is a good indicator of CMI in the immunized individual. Although recommended starting antigen dose is given for each assay, a dose response for each assay must be performed to adequately evaluate the CMI responsiveness to a test antigen.

The cutaneous delayed hypersensitivity (CDH) response to common microbial antigens is usually found to correlate well with in vitro lymphocyte responses to these antigens, which are thought to reflect intact cell-mediated immunity (CMI).[1, 2] However, these responses are dependent on whether or not exposure sufficient to induce immunity has occurred. This is the major problem in assessing CDH with these antigens, particularly in the young child.[3] Diphtheria and tetanus toxoids are potentially ideal test antigens because repeated immunizations with these toxoids are recommended routine medical practice. In addition, if the immunization status is in doubt, one can usually administer booster immunizations without

serious complications. We have previously shown that children immunized with DPT have a positive CDH response to diphtheria-tetanus toxoids regardless of age.[4] Factor, Bernstein, and Fireman[5] have shown a high incidence of positive CDH responses to tetanus toxoid in healthy children and adults.

In vitro lymphocyte responses to tetanus toxoid have been shown with human peripheral blood lymphocyte blast transformation[6] and DNA synthesis with whole lymphocyte populations[7, 8] and thymic-derived (T) lymphocyte, but not bursal-derived (B) lymphocyte subpopulations.[9] Two studies have compared CDH with lymphocyte DNA synthesis using tetanus toxoid.[8, 10] In general, both CDH and in vitro responses to tetanus toxoid have been positive in individuals with intact CMI and negative in those patients who had defective T lymphocyte function or who were unimmunized. However, the relationship between CDH and in vitro assays for CMI using tetanus toxoid has not been clearly elucidated in these

studies, nor have any quantitative data regarding the relative sensitivity of the assays been presented.

In order to confirm the validity of diphtheria-tetanus antigen-induced CDH response as an indicator of CMI, we have investigated the quantitative relationship between CDH and several in vitro assays that are thought to reflect CMI. We were particularly interested in the relative magnitude of each response and the threshold of antigen concentration required to give a positive reaction. Furthermore, tetanus (T) toxoid and the combination of diphtheria-tetanus (DT) toxoids were compared with respect to their immune reactivity. The assays employed include CDH, antigen-induced lymphocyte DNA synthesis,[11] the leukocyte inhibitory factor (LIF) technique,[12, 13] and lymphtoxin production.[14] Finally, in order to evaluate the reproducibility of tetanus toxoid–induced CDH and CMI with time, studies were repeated approximately 16 months after the initial evaluation.

METHODS AND MATERIALS
Subjects

Five healthy subjects ranging in age from 23 to 38 yr (mean, 30.6) included 3 women and 2 men. All had been previously skin-tested with both tetanus and diphtheria-tetanus toxoids. In addition, each had received a tetanus booster from 6 mo to 7 yr before the study. Four cord blood specimens from full-term neonates were used as controls.

Antigen preparation

Aqueous tetanus (T) and pediatric diphtheria-tetanus (DT) toxoids (Lilly) with thimerosal preservative were used. Tetanus toxoid contains 15 Lf U/ml, and DT contains 50 Lf U/ml of diphtheria toxoid and 15 Lf U/ml of tetanus toxoid. The same lot of antigen was used throughout the initial study. The toxoids were diluted in phosphate-buffered saline (PBS) for skin testing. They were dialyzed for 24 hr in PBS for in vitro studies and then concentrated with an Amicon macrosolute concentrater to approximately 200 times the initial concentration. Protein nitrogen (PN) determinations were done by the Lowry method. The PN content of the undialyzed T toxoid was 120 μg/ml; the DT toxoid contained 150 μg/ml. After dialysis, the unconcentrated T toxoid contained 138 μg/ml, and the DT toxoid contained 182 μg/ml PN. All in vitro assays refer to the dialyzed PN content. The assays to be described use dose-response curves for each toxoid. A follow-up evaluation was performed 16 mo after the initial study using a different lot number of tetanus toxoid since the original material was no longer available.

Skin testing

A 0.1 ml of a 1 : 100 dilution of each toxoid was injected intradermally, followed by measurement of erythema and induration at 24 and 48 hr. Skin tests were applied and recorded by one observer. Five mm of induration at 48 hr

was considered a positive skin test. If the 1 : 100 dilution was less than 5 mm of induration, a 1 : 10 dilution was used.

Leukocyte inhibitory factor (LIF) assay

Blood was collected in a heparinized syringe containing 250 U/ml heparin and then divided for the various assays. The LIF assay employed here is a modification of the Clausen technique.[12, 13] One part of 6% dextran (MW 234-Sigma) in pyrogen-free saline was added to four parts of heparinized blood in an inverted syringe and incubated at 37° C for 30 min. The supernatant was collected in a 50-cc tube, and an equal volume of warm (37° C) Hanks balanced salt solution (HBSS) was added. The contents were then spun for 5 min at 100 × g, and the supernatant was discarded. The cells were washed two more times in warm HBSS and resuspended at a density of 2.2 × 10⁸/ml in warm medium 199 (Gibco) with 10% heat-inactivated horse serum and 2% penicillin-streptomycin (10,000 U-10,000 μg/ml).

Fifty lambda of the cells were incubated with five lambda of warm antigen or media for 30 min at 37° C. Fresh agarose (Induboise 37) was prepared by allowing 360 mg of agarose to dissolve in 36 ml of boiling sterile pyrogen-free water and cooling to 47° C. This was followed by the addition of 3.6 ml of medium 199 (10X), 3.6 ml of heat-inactivated horse serum, and two drops of a supersaturated bicarbonated solution. Eighteen ml of agarose was pipetted into a tissue culture plate (100 × 15 mm). Holes were made with a 2.5-mm punch. Seven lambda of the incubated cell mixture were placed in each well. Each antigen dose was performed in quadruplicate. The agarose plates were incubated at 37° C in 5% CO_2 by floating them in water in a large covered tissue culture dish for 18 to 24 hr. Following the incubation period, the agarose was melted away in 80° C water and then submerged in cold water and allowed to dry. The cells were permanently fixed to the culture plates. Two cross diameters were measured in a Hyland precision viewer. The migration index was calculated by the following formula:

$$\% \text{ LIF migration index} = \frac{\text{Antigen well migration}}{\text{Control well migration}} \times 100.$$

The mean ± 1 SD migrations were determined by averaging the migration from four wells. Viabilities were performed by trypan blue exclusion.

Lymphocyte DNA synthesis

Peripheral blood lymphocytes were separated by layering whole blood over a Ficoll-Hypaque gradient. After centrifugation at 400 × g for 40 min, the cells in the interface were 95% lymphocytes and 5% monocytes. Following washing and adjustment of cell density, the cells were stimulated with the antigen for 5 days using a microculture system. The cells were then pulsed with 1 μCi of tritiated thymidine overnight and harvested using a Multiple Automated System Harvester (MASH). They were subsequently analyzed in a Beckman LS-100 scintillation counter.
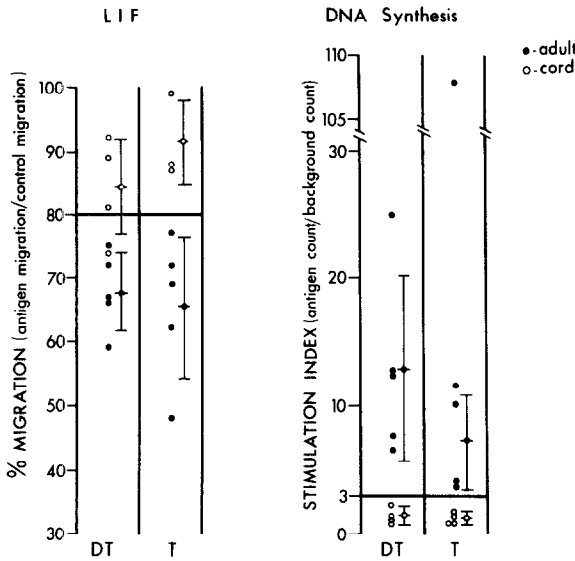
FIG. 1. Shown is the mean and 1 SD (Ⅱ) of leukocyte inhibitory factor (LIF) and DNA synthesis maximal responses in adult and cord blood lymphocytes stimulated by DT and T toxoids.

FIG. 2. The cutaneous delayed hypersensitivity (CDH) dose response is shown. A positive response is induration ≥5 mm at 48 hr. A 1:100 (weight by volume) dilution is equivalent to 1.2 μg/ml.

Lymphotoxin (LT) release

This assay utilized the method developed by Granger and co-workers.[14] One unit of LT was defined as the reciprocal of the dilution which represents 50% cell destruction.

Statistical analysis

Statistical significance for the LIF and DNA synthesis assays comparing adult and cord blood lymphocyte responses was determined by the Student t test for paired means.

Follow-up evaluation

Three of the original 5 subjects were restudied 16 mo after the initial study, utilizing both the CDH response and lymphocyte DNA synthesis with tetanus toxoid.

RESULTS
Initial studies

Maximal responses. The magnitude of CDH, LIF, and DNA synthesis responses with T and DT toxoids is seen in the 5 study subjects (Table I). A positive response for each parameter includes: ≥5 mm induration at 48 hr for CDH, ≤80% migration for LIF, and a stimulation index of ≥3 for lymphocyte DNA synthesis. One notes that T and DT give comparable responses for all parameters. Four of 5 subjects gave a positive CDH response of 1:100 dilution, while subject 5 required a 1:10 dilution. In no case was a positive CDH response associated with a negative in vitro response. No consistent correlation was seen between the magnitude of the CDH response and
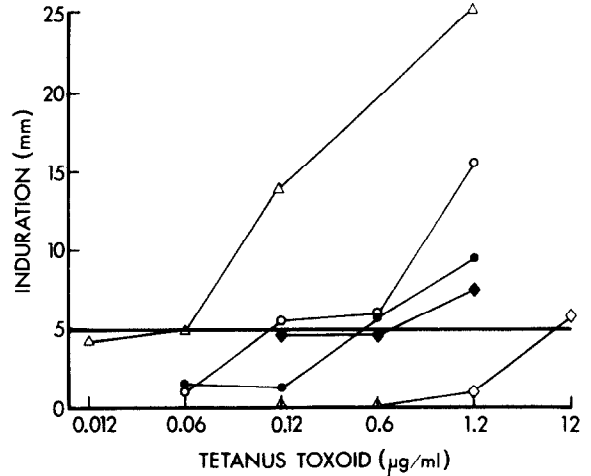
either the LIF assay or DNA synthesis. In fact, the magnitude of DNA synthesis for Subjects 2 and 5 was similar, while the skin test responses were markedly different.

Comparison of adult and neonatal responses. A comparison of adult and neonatal responses to both toxoids is seen for the LIF and DNA synthesis assays in Fig. 1 and Table I. The mean and 1 SD are shown for each group in Fig. 1. In the LIF assay, the DT toxoid gave 67.8% ± 6.1% migration for adults compared to 84.0% ± 8.1% for cord blood cells. One cord blood had a 74% migration index, which is positive, but greater than 1 SD of the adult mean. Using the T antigen, the adult cells had 65.60 ± 11.26 inhibition, compared to 91.3 ± 6.7 for cord blood lymphocytes. No migration index below 80% is seen using T antigen in the cord blood cells. The stimulation index for DNA synthesis is also shown. No cord blood response was above 3 using either antigen.

The difference between adult and cord blood responses is statistically significant (p < 0.01) with regard to DT and T in the LIF assay and for DNA synthesis.

Threshold responses. Dose-response curves were established for CDH, DNA synthesis, and LIF assays. In Fig. 2, one sees the CDH dose response for the 5 subjects using tetanus antigen (the dose responses for DT were similar). Assuming a positive response to be ≥5 mm induration, one sees considerable intersubject variability. Two subjects were positive with an antigen concentration of 0.12 μg/ml (1:1,000 dilution) while another required 12 μg/ml

TABLE I. Comparison of CDH and CMI maximal responses*

	CDH		LIF migration index		DNA synthesis	
	DT	T	DT	T	DT	T
Subject						
1	20	28	59	77	25.0 (60,368)	108.0 (56,621)
2	14	18	75	69	12.9 (84,567)	11.7 (84,945)
3	10	8	67	62	9.9 (38,408)	3.9 (30,570)
4	9	10	66	48	6.6 (88,460)	4.3 (68,993)
5	3 (9)†	3 (6)†	72	72	12.5 (56,416)	10.3 (49,740)
Neonates						
1			81	99	1.11 (2,167)	0.80 (−4,786)
2			74	87	0.84 (−1,157)	0.78 (−1,559)
3			92	88	1.37 (26,350)	1.40 (28,474)
4			89	ND	2.36 (30,679)	1.79 (17,974)

*Shown are CDH responses in millimeters of induration at 48 hr, LIF migration index in percent, and DNA synthesis in terms of stimulation index and antigen-stimulated counts minus background counts per minute of tritiated thymidine in parentheses. Positive responses include induration ≥ 5 mm, LIF migration $\leq 80\%$, and a DNA stimulation index ≥ 3.

†A 1:00 dilution of DT and T antigens was used for the CDH responses except for Subject 5, in whom a 1:10 dilution is shown in parentheses.

TABLE II. Comparison of CDH and CMI threshold responses*

	CDH		LIF		DNA synthesis	
	DT	T	DT	T	DT	T
Subject						
1	0.015	0.12	9.0×10^3	14.0×10^3	0.08†	0.06†
2	0.15	0.12	13.5×10^3	7.0×10^3	0.08†	0.18†
3	1.5	1.2	9.0×10^3	7.0×10^3	0.17	0.06
4	1.5	1.2	4.5×10^3	1.4×10^3	0.25	5.1
5	15.0	12.0	13.5×10^3	7.0×10^3	0.06	0.04

*Shown is the lowest antigen concentration (μg/ml protein nitrogen) resulting in a positive CDH response (≥ 5 mm induration), positive LIF assay (80% or less migration), and positive DNA synthesis response (stimulation index of 3 or greater). For CDH, 12 μg/ml and 15 μg/ml are equivalent to a 1:10 dilution of the undialyzed tetanus and diphtheria-tetanus toxoid antigens, respectively.

†Lowest dose tested.

(1:10). Four of 5 subjects were positive with 1.2 μg/ml concentration, and all would presumably be positive with 12 μg/ml.

The LIF dose response is seen in Fig. 3. Eighty percent migration or less is considered a positive response. The dose of antigen required to get a positive response in all subjects was 100 times the undiluted toxoid concentration of approximately 140 μg/ml (14,000 μg/ml). The range of antigen dose required for $\leq 80\%$ migration was from 1,400 μg/ml to 14,000 μg/ml.

In Fig. 4, one sees the DNA synthesis dose-response curve. A stimulation index of ≥ 3 is considered positive. Again, considerable intersubject variability is seen. All subjects were positive with an antigen dose of 1 μg/ml, while all responses were negative at 100 μg/ml protein nitrogen.

Data comparing threshold doses in μg/ml for CDH-, LIF-, and DNA-positive responses are summarized in Table II. Again, there are no significant differences between T and DT antigens in any of the assays. The LIF assay required 10^4 to 10^5 more antigen for DNA synthesis than CDH. DNA synthesis appeared to be. at least 10-fold more sensitive than CDH. Threshold doses for any one assay did not accurately reflect the threshold dose required for another assay. Subjects 2 and 5 had similar threshold doses for LIF and DNA, but Subject 5 required 100 times more antigen for a positive CDH response.

Longitudinal follow-up

Three subjects in the initial study were re-evaluated 16 mo later. In Table III, A, one sees that the threshold dose for DNA synthesis was approximately
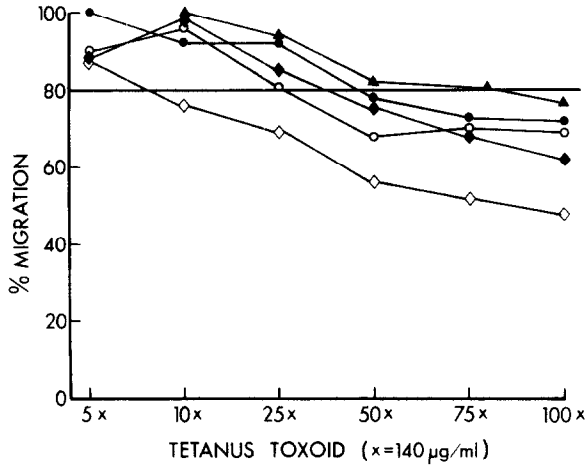
FIG. 3. Shown is the LIF assay dose-response curve; 140 μg/ml is the concentration at the undiluted tetanus toxoid. A positive response is ≤80% migration.

the same for both test periods. Although Subject 3 required 100 times less antigen for a positive response, the peak doses and peak stimulation indices did not change significantly. In contrast, CDH required approximately 20 times more antigen in 2 subjects than in the inital study, while the peak responses were similar for both periods.

Lymphotoxin assay

Lymphotoxin release was measured in two adults and two cord blood specimens following antigen stimulation with T toxoid. The adult response was 41 U (range 31 to 51 U) with an antigen dose of 1.1 μg/ml. The cord blood lymphocytes released less than 5 U with antigen stimulation, which is the same as the control background. No release was seen from the cord blood with antigen concentration up to 30 μg/ml.

Pathology

A skin biopsy of a 48-hr DT skin test response in an adult is shown. One can see the typical histopathologic characteristics of a delayed hypersensitivity phenomenon (Fig. 5).[15]

DISCUSSION

Our basic question and the reason for the present study were to determine whether or not the CDH response with T and DT toxoids was a reliable indicator of CMI. Although it has been shown that the tetanus toxoid antigen can induce an immediate wheal-and-flare response and an Arthus reaction in addition to CDH,[5] several studies strongly suggest that the 48-hr induration response truly reflects CMI. Thus, Oppenheim, Blaese, and Waldman[8] have shown that in the Wiskott-Aldrich syndrome there is a negative
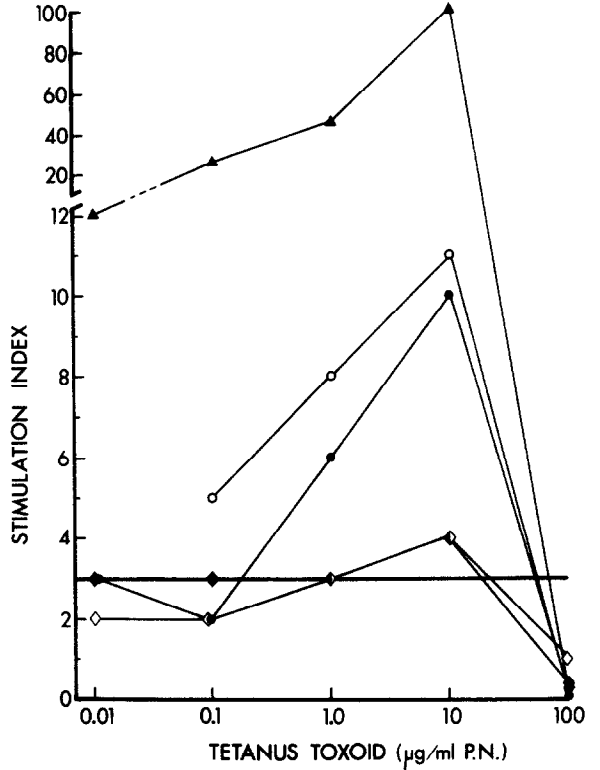
FIG. 4 Shown is the DNA synthesis dose-response curve expressed as the stimulation index (SI). A positive response is a SI ≥ 3.

TABLE III. Longitudinal evaluation of tetanus toxoid CMI

A. Lymphocyte DNA synthesis

	Threshold dose (μg/ml)		Peak dose (μg/ml)		SI (peak)	
Subject	A	B	A	B	A	B
1	0.02	0.02	6.4	1.3	108.0	40.0
3	0.06	0.02	4.8	7.6	3.9	21.0
4	2.20	0.02	20.0	5.1	4.3	6.6

B. Cutaneous delayed hypersensitivity (CDH)

	Threshold dose (μg/ml)		Peak size (mm induration)	
Subject	A	B	A	B
1	1:10,000 (0.01)	1:10,000 (0.03)	28	18
3	1:100 (1.20)	1:10 (25.00)	7	12
4	1:100 (1.20)	1:10 (25.00)	9	5

The three subjects, Nos. 1, 3, and 4, from the initial study were compared for lymphocyte DNA synthesis and cutaneous delayed hypersensitivity (CDH) on (A) 1/8/76 and (B) 5/5/77.

FIG. 5. A skin biopsy of a 48-hr DT skin test response is shown. One notes the perivascular mononuclear infiltrate typical of cutaneous delayed hypersensitivity.

CDH response in conjunction with decreased lymphocyte DNA synthesis following stimulation with tetanus toxoid. However, antibody titers to this antigen were in the normal range. Conversely, Schiff and co-workers[7] found normal DNA synthesis responses in patients with intact CMI but with X-linked agammaglobulinemia. Finally, Dean and co-workers[9] showed that DNA synthesis following tetanus toxoid incubation occurs in the T lymphocyte population and not in the enriched B cell population.[9] We have also shown an excellent correlation between CDH and in vitro CMI responses. In addition, we have histologic evidence from a biopsy specimen 48 hr after skin testing strongly suggestive of CMI.[15] The greatest danger of a screening test arises when a false-positive test occurs that would prevent the physician from pursuing the assessment of CMI further. This was not

seen in our study even with the 1:10 antigen concentration. Facktor, Bernstein, and Fireman,[5] using approximately the same tetanus toxoid antigen concentration for skin testing as our 1:10 concentration, found several normal individuals to be unresponsive at this concentration and concluded that this concentration did not produce an irritant response. Although the CDH threshold dose was similar to DNA synthesis, there was no consistent quantitative relationship between the in vivo and in vitro responses. Thus, Subject 5, with a very high CDH dose threshold, had a similar LIF and DNA synthesis threshold to Subject 2, who had a low CDH threshold. The rather poor quantitative relationship between CDH and in vitro lymphocyte responses has been noted by others.[16] In a repeated evaluation of CDH and DNA synthesis, we found that the DNA synthesis response remained

fairly consistent, while CDH required more antigen in 2 of 3 subjects. We are unaware of studies that have evaluated reproducibility of other CDH antigens over a period of time in the same subjects. Unfortunately, we could not use the same lot of antigen, so that no definite conclusions can be made regarding subject response variability.

We realize that the number of adult subjects and cord blood specimens studied was small. However, each adult subject and each cord blood specimen were evaluated over a broad range of antigen concentrations by several assays performed at the same time for comparison purposes. There was essentially no overlap between the adult and cord blood groups. One cord blood LIF response to the highest DT concentration was positive, but this was not confirmed by DNA synthesis to the same antigen. Other investigators have also noted an occasional positive in vitro CMI response with cord blood lymphocytes,[13] so that these cells may not represent the best negative controls. Unimmunized older children or adults and clearly defined immune-deficient patients may have been preferable negative controls, but these were difficult to find. In spite of these considerations, we feel our observations are meaningful because of the generally clear-cut differences between the adult and cord blood lymphocytes even at the highest antigen concentrations used. Finally, the tetanus toxoid antigen produced positive CDH and CMI responses in several subjects when studied longitudinally, so that the immune subject gives reasonably reproducible responses when studied over a period of time.

From our study, several conclusions are warranted:

1. The CDH response with T and DT toxoids is a good indicator of the presence of CMI reactivity to these antigens. Since both antigens were equivalent in immune responsiveness, we recommend the use of the T toxoid.

2. The 1:100 antigen concentration should be tried first. If this is negative, a 1:10 dilution should be tested. We have not established the maximum antigen concentration that gives a specific CDH reaction.

3. The skin test showed approximately the same sensitivity to antigen stimulation as in vitro DNA synthesis, but no consistent relationship exists between CDH and in vitro assays in all subjects.

4. The LIF assay, while being relatively insensitive, requiring a high antigen concentration, is specific, rapid, and simple to perform. A positive response should correlate with other in vitro assays, but a negative response must be pursued by more sensitive methods.

5. Finally, a dose response is necessary to ade-

quately evaluate the CMI responsiveness to a test antigen. We would recommend the following starting doses of tetanus toxoid for each of the assays employed: CDH 1:100 dilution (1-2 μg/ml); LIF assay 100 × (14,000 μg/ml); DNA synthesis (1 μg/ml).

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