Performance of Antigens Used in Detecting Delayed-Type Hypersensitivity in Adolescents Infected with the Human Immunodeficiency Virus

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We examined the performance of delayed-type hypersensitivity (DTH) antigens employing a new Candida albicans product in a human immunodeficiency virus (HIV)-infected and nonanergic adolescent population. Diameters of induration (in millimeters) for three intradermally applied antigens (C. albicans, tetanus toxoid, and mumps) were compared in a population of HIV-infected 12 to 18 year olds at study entry in a national multicenter study of HIV disease progression. CD4+ T-cell counts were measured in quality-controlled laboratories. The influence of past immunization, gender, and clinical status on antigen reactivity was evaluated with contingency table comparisons and relative risk estimation. Nearly one-half of the 123 eligible subjects were untreated, and almost three-quarters were early in HIV disease by clinical indicators. There was no statistically significant difference in reactivity by past immunization status. Candida antigen (CASTA; Greer Laboratories) evoked DTH response in a significantly higher number of males and females at every level of induration (largest P value, 0.049 for male comparisons; all P values, <0.001 for females) and in subjects with early and intermediate HIV disease at every level of induration (all P values, <0.0001) than either tetanus or mumps antigens. No two-antigen combination was as useful as all three antigens across either gender or clinical categories, although candida and tetanus was the most useful two-antigen combination at indurations of <3 mm. The superior performance of a new C. albicans antigen may extend the utility of DTH assessment in monitoring immune function.

The natural history of untreated human immunodeficiency virus (HIV) infection is marked by progressive loss of CD4+ T cell lymphocytes and a corresponding increase in the probability of cutaneous anergy. Subtle T-helper-cell immune dysregulation occurs even in early HIV infection when numbers of peripheral CD4+ T cells remain stable. Since there is a time-dependent sequential loss of T-helper-cell function that progresses from loss of response to recall antigens to the loss of lymphocyte mitogenic response (25), the ability to measure function in vivo through delayed-type hypersensitivity (DTH) to recall antigens is of potential great utility not only as an adjunct to flow cytometry and lymphocyte proliferation assays where they are available but also as a substitute where they are not.

The interpretation of the studies on DTH response in HIV-infected individuals is complicated by the lack of standardization across studies (Table 1). Populations vary from healthy volunteers (9) to HIV-positive and -negative service personnel and dependents (3, 7), medical patients of different ages (10, 12, 13, 20), and study cohorts (1, 4, 11, 19, 24). Some of these studies have used a device that allows the simultaneous application of seven antigens (Multitest CMI; Merieux) (9, 10, 13), while others employ the Mantoux intradermal method to deliver from two (4, 11), three (1, 12, 19, 24), to four or more antigens (3, 7, 20). The criterion for reactivity also varies from any palpable induration (1, 24), to skin indurations of >2 mm (9–11), >3 mm (4, 12), and >5 mm (20) in diameter. Studies (1, 4, 11, 12, 19, 24) which compare the performance of different antigens, although using the same manufacturer, have applied different criteria to define anergy.

Klein et al. (15) assessed various definitions of anergy for their ability to distinguish HIV status and level of immunodeficiency in a population of women with a median age of 36 years. By comparing antigenic performance, they determined the best induration cutoff was 2 mm at 48 to 72 h and that the combination of antigens that best distinguished women able to mount a positive purified protein derivative response from those not able to do so included the tetanus toxoid and mumps antigens.

We report here on a similar examination of antigenic performance in an HIV-infected adolescent population of females and males, employing the same antigens but substituting a new Candida albicans product as well as stratifying by a composite clinical status variable rather than CD4+ T-cell-count criteria alone.

MATERIALS AND METHODS

Subjects. Study subjects were enrolled in the Reaching for Excellence in Adolescent Care and Health (REACH) Project of the Adolescent Medicine

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TABLE 1. Studies of DTH response employing intradermal application of antigen

| Population description | CD4 profile | Antigens (manufacturers)* | Reaction | Performances | Reference |
|------------------------|-------------|--------------------------|----------|-------------|----------|
| Community based; HIV* | >400, 85%; ≤400, 8% | Tetanus (Wyeth), mumps (Eli Lilly), candida (Hollister-Stier) | Any palpable induration | Tetanus, 59%; mumps, 72%; candida, 53% | 24 |
| Community IDUs; HIV* | >350, 69%; 201–350, 23%; ≤200, 8% | Mumps (Connaught), candida (Hollister-Stier) | ≥2 mm | Mumps, 46%; candida, 53% | 11 |
| Community based; HIV* | ≥600, 23%; 400–599, 28%; 200–399, 29%; 0–199, 20% | Mumps (Connaught), candida (Hollister-Stier) | ≥5 mm | Mumps, 34%; candida, 28% | 19 |
| Clinic based (>1 site); HIV* | ≥500, 27%; 200–499, 38%; ≤200, 35% | Tetanus (Connaught and Lederle), mumps (Connaught), candida (Allermed) | ≥3 mm | Tetanus, 45%; mumps, 35%; candida, 57% | 12 |
| Community IDUs; HIV* | >500, 42%; 350–499, 22%; ≤350, 36% | Mumps (Connaught), candida (Hollister-Stier) | ≥3 mm | Mumps, 40%; candida, 45% | 4 |
| Community based; HIV* | ≥500, 30%; 200–499, 44%; ≤200, 26% | Tetanus (Connaught), mumps (Connaught), candida (Miles) | Any palpable induration | Tetanus, 37%; mumps, 39%; candida, 22% | 1 |

* All antigens were administered singly and intradermally.
* HIV* n, number of HIV-positive subjects.
* CD4+ T-cell information available for 1,069 of 1,120 subjects.
* CD4+ T-cell information available only for male subjects.

HIV/AIDS Research Network; its full methodology is reported elsewhere (21). Briefly, REACH was an observational study in 15 clinical sites of HIV disease progression in HIV-positive adolescents 12 to 18 years old who were infected through sex or drug-taking behaviors; under medical care; and enrolled between 1995 and 1999. Data in this report are from the September 1998 database lockdown. DTH assessment was a component of a larger study protocol reviewed and approved by the institutional review board at each of the participating sites. All subjects were informed of study requirements, and they gave written consent. Parental permission was obtained where required. REACH subjects have annual tuberculin skin testing and DTH assessment performed annually, which was scheduled at the 3-month study visit and in lieu of that at a subsequent visit in the future. The protocol excluded pregnant subjects from DTH measurement (n = 12).

CD4+ T-cell counts were determined at site laboratories, all of whom participated in the flow cytometry quality control program sponsored by the National Institute of Allergy and Infectious Diseases. Clinical status was assigned using Centers for Disease Control and Prevention grid criteria for symptom status (A, B, and C) versus CD4+ T-cell status (1, ≥500/mm³; 2, 499 to 200/mm³; and 3, <200/mm³), with ordering (from best to worst categories: A1, A2, B1, B2, A3, C1, B3, C2, C3) based on expert consensus opinion from the clinical investigators of the Adolescent Medicine HIV/AIDS Research Network (21). Death was added as a final category after C3. The scale was then condensed from the ordered 10-point scale to the more clinically relevant 3-point scale with the groupings early (A1, A2, B1), intermediate (B2, A3, C1), and progressed (B3, C2, C3).

Measurement of DTH response. The three antigens recommended by the Centers for Disease Control and Prevention (5) were employed in this study. The DTH antigens included CASTA, tetanus toxoid, and MSTa. CASTA (Greer Laboratories, Lenoir, N.C.) is a recently developed investigational C. albicans skin test antigen preparation whose safety and efficacy had already been tested in adult populations (8). We chose the higher of the two recommended doses in order to accomplish the DTH assessment in two clinic visits, believing that a single lot of antigen preparation was employed at all clinical sites. Other antigens were commercially available and licensed for skin testing. Tetanus toxoid (Connaught Laboratories) was formulated at a 1:10 dilution of a 4-LFU (limit of flocculation unit)/0.5 ml stock solution and · mumps skin test antigen (MSTA; Connaught Laboratories) was formulated at a 40-CFU/ml dilution. All antigens were applied in a 0.1-ml volume intradermally (Mantoux method) and read by centrally trained clinic personnel. Palpable induration was measured by length and width and recorded on a standardized form for all test antigens; induration by largest transverse diameter was used in analysis. Prior to placement of any skin test antigens, standardized routine questions were asked to ascertain previous hyperalergic responses to skin test antigens.

Subjects with measurements performed <24 h (since true induration was unlikely) or >72 h were excluded from the analysis as were subjects who were nonreactive to all three antigens since they could not contribute performance data due to their anergic status. The immunization status of subjects was ascertained through medical record review and subject report. The impact of recent immunization on DTH response in these subjects may differ from the response in subjects who were immunized to mumps or tetanus since study entry but prior to the DTH testing, a time period ranging from 3 to 9 months, were excluded since their limited numbers did not allow an adequate evaluation of their impact (n = 10).

Statistical analyses. All analyses have been based on data collected within the first 9 months subsequent to study entry; all measures are contemporaneous for a given subject. For comparison of reactivity in independent samples, the statistical test used to evaluate (r by c) contingency table comparisons for significance was the χ² test. For (fourfold (two-by-two) tables, the continuity-adjusted χ² was used and, where appropriate, the Fisher exact test was employed. Comparisons of subgroups were undertaken only when a global test of the more general hypothesis was significant at the level of P < 0.01. Comparison of antigenic responses among related samples (e.g., comparison of responses of all three antigens among males) was done using Cochran’s Q test (6, 16). The exact test was performed using StatXact4 (C. Mehta and N. Patel, StatXact4 for Windows, Cytel Software Corporation, Cambridge, Mass.). For ease of presentation, all tables provide in the header of each column or in a footnote the denominator used for deriving percentages in that column. The only exception is for viral load, which was performed using StatXact4 (C. Mehta and N. Patel, StatXact4 for Windows, Cytel Software Corporation, Cambridge, Mass.). For ease of presentation, all tables provide in the header of each column or in a footnote the denominator used for deriving percentages in that column. The only exception is for viral load, which was performed using StatXact4 (C. Mehta and N. Patel, StatXact4 for Windows, Cytel Software Corporation, Cambridge, Mass.).
specific antigen in a group of subjects with a factor putatively related to reactivity to the rate of reactivity in a group of subjects without such a factor. The confidence intervals (CIs) for RR are given as in the work by Katz et al. (14), based on large sample approximation techniques, and their validity is good for moderate to large samples. For some contingency tables presented, the CIs as approximations, may present apparently conflicting results such as statistically significant association with a CI for an RR which includes unity (2, 23).

RESULTS

DTH measurement was specified in the protocol for 207 subjects. Of these, those excluded from the analysis included 47 who did not have all three antigens applied, 18 who were anergic to all three antigens, 10 who were immunized within the previous 9 months, 5 who received nonstandard doses of antigen, and 4 whose results were not read or were read outside of the window. A comparison of the variables relevant to outcome (age, HIV type 1 [HIV-1] RNA in plasma, CD4$^+$ T-cell count, antiretroviral treatment, and clinical status) did not demonstrate statistically significant differences between those subjects included ($n = 123$) and those excluded ($n = 84$) (data not shown). The 18 anergic subjects included 15 females and 3 males, with age distributions comparable to the included subjects but a different clinical profile (56% of the subjects were in early infection, 11% were in intermediate infection, and 33% were in late infection); this population has been described elsewhere (22).

The clinical profile of the 123 eligible subjects (Table 2) demonstrated a population which was relatively early in its HIV infection with sufficient immunologic reserve (mean CD4$^+$ T-cell count = 525.5/mm$^3$, standard deviation = 256.1) and adequate viral suppression (55% with $\leq$10,000 viral copies/ml). Nearly half of this study population was untreated. Significant differences in the distribution of CD4$^+$ T-cell counts did exist between males and females; male subjects appeared to be older than female subjects with marginal statistical significance.

When the differences in nonreactivity rates were examined by gender (Table 4), the only statistically significant difference to emerge was for the mumps antigen, with males were more likely than females to be nonreactive at every induration point below 5 mm. The candida antigen performed best in both males and females. Candida antigen evoked a DTH response in a significantly higher number of male subjects than tetanus toxoid antigens at all levels of induration (largest $P$ value, 0.049) and at all levels of induration for female subjects (all $P$ values, <0.0001); Candida antigen evoked a DTH response in a signifi-

| Parameter | No. (%) of subjects | $\chi^2$ | df | $P$ |
|-----------|---------------------|---------|----|-----|
| Age range (yr) | | | | |
| 14–17      | 52 (55.9) |           | 3.36 | 1 | 0.07 |
| 18–20      | 41 (44.1) |           |       |   |     |
| CD4$^+$ T-cell count/µl | | | | |
| $\geq$500  | 50 (55.6) |           | 5.28 | 1 | 0.02 |
| <500       | 40 (44.4) |           |       |   |     |
| HIV-1 plasma RNA levelb | | | | |
| $\geq$400  | 21 (28.0) |           | 0.54 | 3 | 0.91 |
| 400–1,000  | 28 (37.3) |           | 11 (42.3) | |     |
| 1,000–20,000| 8 (10.7) |           | 2 (7.7) | |     |
| $>20,000$  | 18 (24.0) |           | 5 (19.2) | |     |
| Clinical stagec | | | | |
| Early      | 70 (75.3) |           | 1.99 | 2 | 0.37 |
| Intermediate| 18 (19.3) |           | 9 (31.0) | |     |
| Advanced   | 5 (5.4) |           | 2 (6.9) | |     |
| Antiretroviral therapy | | | | |
| None       | 46 (49.5) |           | 4.87 | 3 | 0.18 |
| Monotherapy, no Pld | 7 (7.5) |           | 1 (3.3) | |     |
| Combination, no P | 21 (22.6) |           | 5 (16.7) | |     |
| Combination with PI | 19 (20.4) |           | 12 (40.0) | |     |

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a Note: missing values are not included in the calculation of percentages; viral load values are missing for 18% of eligible subjects.
b HIV-1 RNA level in plasma (copies per milliliter) is determined in a central laboratory for study subjects by nucleic acid sequence-based amplification (NASBA; Organon Teknika Corporation).
c See Materials and Methods for explanation of clinical staging criteria.
d PI, protease inhibitor(s).
TABLE 3. Difference in nonreactivity rates to DTH testing with tetanus or mumps antigen among adolescent subjects by immunization status with the corresponding antigen

| Immunization and cutoff point (mm) | No. (%) reactive in immunization group | RR* (95% CI) |
|-----------------------------------|----------------------------------------|-------------|
|                                   | Nonimmunized                           | Immunized   |
| Tetanus**                         |                                        |             |
| 1                                 | 44 (46.8)                              | 10 (34.5)   | 1.36 (0.5–2.3) |
| 2                                 | 44 (46.8)                              | 10 (34.5)   | 1.36 (0.5–2.3) |
| 3                                 | 48 (51.1)                              | 13 (44.8)   | 1.14 (0.7–1.8) |
| 4                                 | 51 (54.3)                              | 16 (55.2)   | 0.98 (0.7–1.4) |
| 5                                 | 54 (57.5)                              | 17 (58.6)   | 0.98 (0.7–1.4) |
| Mumps**                           |                                        |             |
| 1                                 | 35 (36.5)                              | 11 (36.7)   | 0.99 (0.6–1.7) |
| 2                                 | 37 (38.5)                              | 12 (40.0)   | 0.96 (0.6–1.6) |
| 3                                 | 42 (43.8)                              | 15 (50.0)   | 0.88 (0.6–1.4) |
| 4                                 | 51 (53.1)                              | 16 (53.3)   | 1.00 (0.7–1.5) |
| 5                                 | 57 (59.4)                              | 17 (56.7)   | 1.05 (0.7–1.5) |

* No RR estimate reached statistical significance.

** Note: subjects with recent immunizations were excluded only if the particular antigen was recently administered or immunization status was unknown. The sample sizes are thus slightly different from those in other tables.

DISCUSSION

The need to identify a simple but clinically relevant technology to assess general immune function and monitor immune reactivity in HIV-infected patients in areas where more sophisticated and expensive technologies are unavailable has been noted, and the case has been made for DTH testing to be that technology (26). Critical to the effort, however, is the requirement to identify antigens that do not depend on established population immunization programs, have the capacity to detect accurately and consistently all individuals with intact DTH response across ages and genders, and discriminate clinical status in HIV-infected individuals.

This is the first examination of the performance of antigens in eliciting DTH response in a population of HIV-positive adolescents. The need to rigorously define this study population has resulted in a number of exclusions, primarily related to missing data for one or more of the three comparison antigens. No statistically significant differences in characteristics predictive of antigen performance were seen between those included and those excluded. As in the previous examination by Klein et al. (15), we have compared the performance of the three antigens most commonly used to assess DTH and applied criteria similar to those in their evaluation.

Unlike in their report, however, we present information on adolescent males as well as females, although we have information on HIV-positive subjects only. We have also excluded subjects who were nonreactive to any of the antigens (i.e., the anergic subjects) in order to more clearly evaluate the capacity of the antigen to evoke a reaction in individuals able to respond. Further, we specifically examined the effect of immu-

TABLE 4. Difference in nonreactivity rates by varying cutoff points of induration to DTH testing with various antigens among adolescent subjects by gender

| Cutoff point (mm) | Tetanus antigen | RR (95% CI) | Mumps antigen | RR (95% CI) | Candida antigen | RR (95% CI) |
|------------------|-----------------|-------------|---------------|-------------|-----------------|-------------|
|                  | No. (%) nonreactive | RR (95% CI) | No. (%) nonreactive | RR (95% CI) | No. (%) nonreactive | RR (95% CI) |
| 1                | 12 (40)          | 0.9 (0.5–1.5) | 16 (53)       | 1.6 (1.0–2.5) | 3 (10)          | 0.8 (0.3–2.8) |
| 2                | 12 (40)          | 0.9 (0.5–1.5) | 17 (57)       | 1.6 (1.1–2.5) | 4 (13)          | 0.9 (0.3–2.7) |
| 3                | 13 (43)          | 0.9 (0.5–1.4) | 19 (63)       | 1.6 (1.1–2.3) | 4 (13)          | 0.8 (0.3–2.1) |
| 4                | 15 (50)          | 0.9 (0.6–1.4) | 21 (70)       | 1.4 (1.1–2.0) | 5 (17)          | 0.9 (0.4–2.1) |
| 5                | 18 (60)          | 1.1 (0.8–1.5) | 22 (73)       | 1.3 (1.0–1.8) | 5 (17)          | 0.9 (0.4–2.1) |

* A total of 30 males (M) and 93 females (F) received each antigen.

b Subjects were more reactive to candida antigen than to tetanus antigen at all cutoff points (largest P for males and females, 0.049 and 0.0001, respectively). Subjects were more reactive to candida antigen than to mumps antigen at all cutoff points (largest P for males and females, 0.0023 and 0.0012, respectively).

c Only for the mumps antigen were nonreactivity rates significantly different between males and females.
TABLE 5. Difference in nonreactivity rates by varying cutoff points of induration to DTH testing with tetanus, mumps, and candida antigens among adolescent subjects by clinical status.

| Cutoff points (mm) | Tetanus | | | Mumps | | | Candida | | |
|-------------------|---------|---|---|-------|---|---|---------|---|---|
| E | I | A | E | I | A | E | I | A |
| 1 | 34 (38.6) | 12 (44.4) | 6 (85.7) | 36 (40.9) | 9 (33.3) | 2 (28.6) | 10 (11.4) | 2 (7.4) | 2 (28.6) |
| 2 | 34 (38.6) | 12 (44.4) | 6 (85.7) | 37 (42.0) | 10 (37.0) | 2 (28.6) | 11 (12.5) | 2 (7.4) | 2 (28.6) |
| 3 | 39 (44.3) | 14 (51.9) | 6 (85.7) | 43 (48.9) | 11 (40.7) | 2 (28.6) | 13 (14.8) | 2 (7.4) | 2 (28.6) |
| 4 | 44 (50.0) | 14 (51.9) | 6 (85.7) | 48 (54.5) | 16 (59.3) | 2 (28.6) | 15 (17.0) | 3 (11.1) | 2 (28.6) |
| 5 | 47 (53.4) | 15 (55.6) | 6 (85.7) | 51 (58.0) | 19 (70.4) | 3 (42.9) | 17 (19.3) | 4 (14.8) | 2 (28.6) |

* Clinical status was defined as early (E) (*n* = 88), intermediate (I) (*n* = 27), or advanced (A) (*n* = 7).

* For antigenic comparisons, subjects were more reactive to candida antigen than to tetanus or mumps antigen at all cutoff points (in early and intermediate stages only, *P* < 0.0001).

* Only clinical category comparison nonreactivity rates significantly different for any antigen.

Employing antigens, like mumps or tetanus, which depend on existing and effective population-wide immunization programs, may only be useful in countries with the resources to implement and maintain such programs. If DTH measurement is to be considered as the technology for immunologic assessment, the more useful antigens to use would be those to which large proportions of the population are routinely exposed because the antigens are environmentally ubiquitous, such as *C. albicans* antigens. Our study has employed a new investigational preparation of *C. albicans* (CASTA; Greer Laboratories). When compared to a 1/1,000 dilution of *C. albicans* (Hollister-Stier Laboratories) administered to healthy volunteers, CASTA at a dose of 1.0 μg resulted in a higher proportion of subjects responding but with smaller and less variable induration diameters (8). Greer Laboratories recommends two doses (1.0 and 10.0 μg) of CASTA to be used in sequence. We chose one administration with the larger dose based on what was practicable in this study population.

Candida antigens in preparations other than CASTA have performed either equivalently (4, 11, 12) or comparatively worse than simultaneously applied antigens in the HIV Epidemiologic Research Study (HERS) (15) and in other populations as well (1, 19, 24). All of these study subjects come from populations, similar to our study population, which are likely to experience repeated candidal exposures. However, the rate of reactivity to candida in our subjects was consistently and significantly higher than the reactivity rates for either the mumps or toxoid antigens for both genders at every level of induration. These data suggest that CASTA is a more potent DTH antigen and thus more appropriate for use in epidemiologic studies attempting to define the prevalence or incidence of anergy. Furthermore, its performance in our cohort recommends its candidacy in assessing immune function in technology-poor areas.

Clinical status can affect subject response to DTH antigens, and it is probable that CD4+ T-cell count alone does not fully explain the occurrence of anergy that may result from immunologic dysfunction operating before the decline of peripheral CD4+ T-cell counts. We attempted to evaluate this by comparing nonreactivity rates at different levels of a composite clinical variable which incorporated both CD4+ T-cell categories as well as clinical symptoms. This was important since unlike the HERS population in which only 29% of the subjects had CD4+ T-cell counts greater than 500/mm3, 50% of the subjects in this report fell into that infection category. In direct comparison, candida was able to elicit hypersensitivity reac-

**TABLE 6. Nonreactivity to DTH testing by gender among HIV-positive adolescents: effect of varying the number and combination of antigens**

| Nonreactivity and antigens | Male (% of nonreactive subjects) | Female (% of nonreactive subjects) |
|---------------------------|---------------------------------|-----------------------------------|
| Defined as <1 mm | | |
| Mumps and tetanus | 7 (23.3) | 15 (16.1) |
| Candida and mumps | 1 (3.3) | 4 (4.3) |
| Candida and tetanus | 0 | 3 (3.2) |
| Candida, mumps, and tetanus | 0 | 0 |
| Defined as <3 mm | | |
| Mumps and tetanus | 9 (30.0) | 22 (23.7) |
| Candida and mumps | 2 (6.7) | 6 (6.5) |
| Candida and tetanus | 0 | 6 (6.5) |
| Candida, mumps, and tetanus | 0 | 2 (2.2) |
| Defined as <5 mm | | |
| Mumps and tetanus | 14 (46.7) | 30 (32.3) |
| Candida and mumps | 3 (10.0) | 10 (10.8) |
| Candida and tetanus | 2 (6.7) | 11 (11.8) |
| Candida, mumps, and tetanus | 2 (6.7) | 5 (5.4) |
Considerations significantly better than tetanus or mumps in early to intermediate HIV disease states, but candida appeared to offer no advantage to other antigens in discriminating among levels of clinical status. However, since numbers of subjects with advanced disease are limited in the REACH cohort, this study objective has been incompletely evaluated. Having adequate subjects across categories is always a challenge in small cohort studies, and the lack of subjects with advanced disease in this examination prevents us from commenting on one of the three criteria we set for the choice of a useful skin test antigen to employ in technology-poor areas, i.e., the capacity to discriminate clinical status. We do believe, however, that our data support further study of CASTA in more clinically diverse HIV-positive populations. In addition, it is equally important to study further the concordance between DTH and T-cell reactivity to CD3 in vitro. For example, anergic CD3 T-cell population, delayed type hypersensitivity skin testing, and CD4 T-cell subset phenotyping independently predict survival time in patients infected with human immunodeficiency virus. J. Infect. Dis. 172:79–87.

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