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Immunity to *Salmonella typhi*: considerations relevant to measurement of cellular immunity in typhoid-endemic regions

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SUMMARY

Experiments were performed in Baltimore, Maryland and in Santiago, Chile, to determine the level of *Salmonella typhi* antigen-driven *in vitro* lymphocyte replication response which signifies specific acquired immunity to this bacterium and to determine the best method of data analysis and form of data presentation. Lymphocyte replication was measured as incorporation of ^{3}H-thymidine into desoxyribonucleic acid. Data (ct/min/culture) were analyzed in raw form and following log transformation, by non-parametric and parametric statistical procedures. A preference was developed for log-transformed data and discriminant analysis. Discriminant analysis of log-transformed data revealed ^{3}H-thymidine incorporation rates $>3\,433$ for particulate *S. typhi*, Ty2 antigen stimulated cultures signified acquired immunity at a sensitivity and specificity of 82·7; for soluble *S. typhi* O polysaccharide antigen-stimulated cultures, ct/min/culture values of $>1\,237$ signified immunity (sensitivity and specificity 70·5%).

Keywords *Salmonella typhi* typhoid fever lymphocyte vaccine cellular immunity

INTRODUCTION

When used in studies conducted in typhoid-free regions, *in vitro* *Salmonella typhi* antigen-driven lymphocyte replication (LR) assays are more sensitive measures of prior contact with *S. typhi* than are conventional serologic procedures (Espersen *et al.*, 1982; Levine *et al.*, 1987b; Mogensen, 1979; Murphy *et al.*, 1987). In contrast, when utilized in typhoid-endemic regions, conventional LR assays yield data which are difficult to interpret (Murphy *et al.*, 1987; Rajagopalan, Kumar & Malaviya, 1982a, b). A major reason for this difficulty evolves from the method normally used to identify immune responders: the comparison of magnitude of LR expressed by individuals of unknown immune status with concomitantly assayed known immune and known non-immune controls. In regions of high endemicity, it is rare to find adults who present as non-immune (Murphy *et al.*, 1987), i.e. in endemic areas the negative control population is missing. Therefore, alternative methods must be used to define the level of LR which denotes specific acquired immunity.

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One approach is to use identical procedures and materials in non-endemic and endemic regions, with data from the non-immune group resident in the non-endemic region serving as the negative control for assays performed at both sites. We present here data on LR in response to *S. typhi*-derived antigens from groups of typhoid-immune or non-immune individuals resident in typhoid-free or typhoid-endemic regions, and develop statistical methods for defining the level of LR which denotes specific acquired immunity to *S. typhi*.

MATERIALS AND METHODS

Subjects

Fifty-eight residents of Santiago, Chile (typhoid-endemic) (Levine *et al.*, 1987a; Ristori, 1981), mean age 22·5 years $\pm$ 3·8 s.d. (30 females), and 53 adults (mean age 26·4 $\pm$ 4·5 s.d. years, 14 females) from the Baltimore, Maryland metropolitan community (typhoid-free, Marylanders) were studied. All Chileans had experienced clinical typhoid fever (confirmed by isolation of *S. typhi* from blood cultures). The mean interval from typhoid fever to participation in this study was 29·5 $\pm$ 15·2 s.d. weeks.

Of the Marylanders, 47 had no history of typhoid fever, vaccination against typhoid fever or travel to regions where typhoid is endemic. Nineteen were studied both before and 21 and 60 or 90 days after oral immunization with live attenuated

Table 1. Descriptive statistics for culled data base: mean ct/min (Standard deviation; range; number of sets of triplicates)

Group	Stimulant in Culture		
	None	Ty2	OPS
Control	1 033 (674; 105–2 850; 76)	2 471 (1 474; 163–5 791; 38)	1 077 (630; 229–2, 429; 38)
Vaccinees	730 (455; 177–2 868; 85)	8 988 (6 510; 189–24 539; 37)	2 760 (2 084; 139–8 256; 40)
Typhoid	821 (621; 99–2 918; 58)	6 921 (4 505; 999–23 667; 58)	1 092 (860; 238–4 191; 46)

S. typhi strains 541Ty or 543Ty. Data were pooled from individuals immunized by ingestion of a single 10^9 colony-forming unit (CFU) dose, a single 10^{10} CFU dose or 2, 10^9 CFU doses spaced 4 days apart. Six individuals were included who had received *S. typhi* vaccine Ty21a (Swiss Serum and Vaccine Institute, Berne, Switzerland, three enteric coated capsules containing a minimum of 10^9 CFU per capsule with intervals of 2 days between capsules) an average of $53{\cdot}3 \pm 32{\cdot}0$ s.d. weeks before blood donation. Data from an additional 144 Chileans (37 children, mean age $1{\cdot}1 \pm 0{\cdot}5$ s.d. years, 16 females, and 107 adults mean age $22{\cdot}5 \pm 9{\cdot}0$ s.d. years, 55 females) of varying immune status with respect to *S. typhi* were included in the determination of 'normal' basal LR rate. Informed consent was obtained from all of the participants in this study.

Lymphocyte replication assay

On the basis of previous results (Murphy *et al.*, 1987), LR assays of 8 days duration were performed. Cultures were maintained without antigen or with either particulate *S. typhi*, Ty2 or O polysaccharide (OPS) from *S. typhi* at concentrations of 1×10^5 bacterial bodies or 10 μg/culture, respectively. For each individual, three cultures were established for each condition (no antigen, Ty2 and OPS). The details of this procedure and the materials and antigens used are published (Murphy *et al.*, 1987; Robertsson *et al.*, 1982).

Data summary

For each triplicate, average counts per minute (ct/min) per culture of ^{3}H-thymidine was determined by standard liquid scintillation procedures. In addition, two derived values often used for expressing LR data, net ct/min/culture (Nct/min) (Levine *et al.*, 1987b; Murphy *et al.*, 1987) and stimulation index (SI), (Robertsson *et al.*, 1982) were calculated. Nct/min was determined by subtracting from the ct/min of an antigen-stimulated triplicate the ct/min of a paired triplicate maintained without antigen. SI was calculated as Nct/min for an antigen-stimulated triplicate divided by the ct/min of a paired antigen-free triplicate.

The ct/min values for each culture were log-transformed and the average of the transformed values for triplicate sets was determined ($_l$ct/min). Log stimulation index ($_l$SI) was also calculated as: $_l$ct/min for cultures containing antigen divided by the $_l$ct/min for concomitant antigen-free cultures. Robertsson *et al.* (1982) used log-transformed data to analyze the results of their LR studies of *Salmonella typhimurium* immunity in calves.

Table 2. Tests of normality of data, using Shapiro–Wilk criterion, for several measures of immune response, as measured by LR assay

Assay System	Group (sample size)	Shapiro–Wilk test statistic for normality (prob(normal))				
		Untransformed data			Log-transformed data	
		ct/min	Nct/min	SI	$_l$ct/min	$_l$SI
Basal LR*	Control (356)†	0·769 (0·001)			0·977 (0·038)	
Ty2	1. Ty2 control (38)‡	0·927 (0·019)	0·943 (0·073)	0·786 (0·001)	0·957 (0·205)	0·969 (0·462)
	2. Vaccinees (58)	0·866 (0·001)	0·881 (0·001)	0·646 (0·001)	0·984 (0·832)	0·917 (0·001)
	3. Typhoid fever (37)	0·910 (0·006)	0·905 (0·004)	0·828 (0·001)	0·897 (0·002)	0·972 (0·558)
	4. 2 and 3 combined (95)§	0·874 (0·001)	0·881 (0·001)	0·671 (0·001)	0·949 (0·003)	0·935 (0·001)
OPS	1. OPS control (38) ¶	0·924 (0·016)	0·954 (0·170)	0·718 (0·001)	0·946 (0·088)	0·955 (0·174)
	2. Vaccinees (46)	0·788 (0·001)	0·919 (0·003)	0·704 (0·001)	0·954 (0·107)	0·966 (0·356)
	3. Typhoid fever (40)	0·868 (0·001)	0·825 (0·001)	0·813 (0·001)	0·955 (0·161)	0·856 (0·001)
	4. 2 and 3 combined (86)	0·782 (0·001)	0·800 (0·001)	0·765 (0·001)	0·971 (0·222)	0·952 (0·009)

* Cultures maintained without antigen.
† Number of sets of triplicates.
‡ Cultures from non-immune Marylanders containing TY2 antigen.
§ Result when data from both immune groups were combined.
¶ Cultures from non-immune Marylanders containing OPS antigen.

Definitions

For the purpose of this manuscript, individuals resident in Maryland who had not received *S. typhi* vaccination or travelled to regions where *S. typhi* is endemic were defined as 'non-immune'. 'Immunity' was presumed for Chileans who had had *S. typhi* isolated from blood or Marylanders who had received attenuated *S. typhi*. Sensitivity was defined as the percentage of 'immune' individuals who were correctly classified as immune through the application of a specific statistical criterion (see Results). Specificity was defined as the percentage of 'non-immune' individuals who were classified as non-immune by a statistical procedure. 'Screening value' is defined as the numeric value of the response variable (ct/min, Nct/min, SI, $_{I}$ct/min or $_{I}$SI) which corresponds to the point where maximum resolution between presumed immune and non-immune groups occurred (i.e. the point of simultaneous maximum sensitivity plus specificity). Screening values are used to allow direct comparison of assays, data forms and analysis procedures.

Parametric analysis

Due to extreme right-skewness of raw ct/min, Nct/min and SI (see Results), only log-transformed response variables were analyzed using parametric methods. Each log-transformed response variable was subjected to a discriminant analysis followed by classification of observations into 'immune' vs 'nonimmune' groups. As each discriminant analysis contained two groups and a single response variable, this was equivalent to a *t*-test. For classification, as *a priori* sample sizes were set equal, the screening value became the midpoint between group means: that is, observations falling below this value were considered 'non-immune' and those above were classified as 'immune' individuals.

RESULTS

Culling of data base

It was found that occasional individuals displayed abnormally high rates of basal LR. Because we have never found, in this series of experiments or in any other series, subjects who reproducibly maintained such high levels of background LR, we concluded that these results reflected either technical problems or a transient perturbation in the individuals' mononuclear cell responsiveness. On the basis of this conclusion, we eliminated, for each individual with unacceptably high background values, all data for the experiment in which the high value occurred. To determine which values would be eliminated, we calculated the average basal ct/min for all assays (883 ± 711 s.d.; $n = 356$, 165 Maryland, 191 Santiago), and set a limit on normal values as the average plus 3 standard deviations (i.e. 3 016 ct/min). Seven individuals (2% of all cultures) exceeding this threshold were excluded. Table 1 presents descriptive statistics for the ct/min values for the culled data base.

Distribution of data

Data analyzed in raw form were not normally distributed (Table 2) due mostly to right skewness. Following log transformation, the majority of groups, especially for ct/min and Nct/min, achieved normality. Residual non-normality after transformation was due to kurtosis.

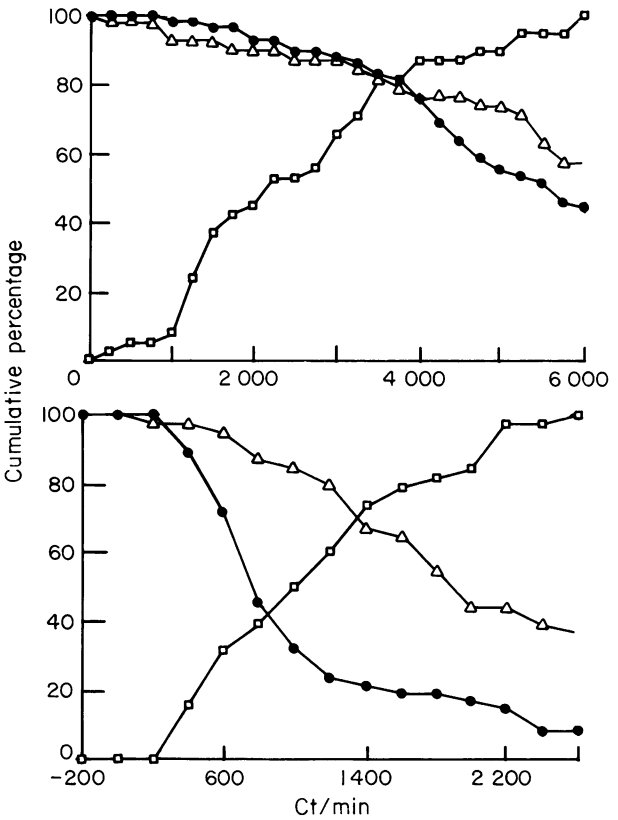

Fig. 1. Sensitivity versus specificity for data processed as ct/min for cultures stimulated with Ty2 or OPS antigens. Threshold limits were established at intervals of 250 or 200 ct/min for Ty2 (upper frame) or OPS (lower frame) assays, respectively. For each interval, the percentage of individuals correctly classified was determined and plotted. Sensitivity is presented as points marked with solid circles (●——●) for the post-typhoid fever group, or open triangles (△——△) for the vaccinated group (plotted as a decreasing function of ct/min). Specificity is denoted by points marked with open squares (□——□, e.g., the Maryland control group). The value on the x axis directly below the intersection of sensitivity and specificity curves is the screening value (see Table 2). Similar plots (not shown) were made to determine sensitivity, specificity and screening values for N ct/min, SI, $_{I}$ct/min and $_{I}$SI.

Sensitivity and specificity

Non-parametric analysis. Cumulative frequency plots of sensitivity versus specificity were made (Fig. 1). For Ty2 antigen, similar distributions of LR were found for acquisition of immunity by infection or by vaccination (Table 3). For OPS antigen, however, sensitivity was markedly greater for vaccinees than for previously infected individuals. The response variable used did not markedly affect the pattern of results.

Parametric analysis. All discriminant analyses of $_{I}$ct/min and $_{I}$SI for Ty2 antigen yielded significant differences between immune and non-immune groups ($P \leqslant 0{\cdot}0001$ for all response variables); that is, classification of observations into groups which corresponded to immune status (as defined in Materials and Methods) was better than expected from random assignment (Table 4). For OPS antigen, however, the responses of vaccinees were significantly different ($P \leqslant 0{\cdot}0002$) from those of controls, whereas responses of typhoid patients did not differ significantly from control responses ($P > 0{\cdot}80$).

Table 3. Effect of form of data presentation on sensitivity and specificity

Assay	Group (sample size)	ct/min	Nct/min	SI	$_1$ct/min	$_1$SI
Ty2	Vaccinees (37)					
	Intersection	81·2	81·2	81·1	79·9	81·1
	Screening Value*	3 485	2 601	4·34	3 429†	3·60†
	Typhoid Fever (58)					
	Intersection	81·6	84·6	79·2	77·5	75·0
	Screening Value	3 671	2 667	4·26	3 328	3·56
	Combined (95)					
	Intersection	81·6	83·5	78·9	78·4	76·6
	Screening Value	3 563	2 648	4·29	3 378	3·54
OPS	Vaccinees (40)					
	Intersection	70·6	72·8	65·7	67·8	64·5
	Screening Value	1 353	571	0·95	1 200	3·00
	Typhoid Fever (46)					
	Intersection	41·9	45·1	47·9	41·0	50·0
	Screening Value	847	153	0·26	812	2·83

Screening value (see Fig. 1) is the numerical value of the x axis directly below the intersection. Combined analysis was not conducted for the OPS assays because the vaccinated and post-typhoid groups did not yield similar results.

* Group compared with control.

† Back-transformed from natural log.

Table 4. Discriminant analysis; effect of form of data presentation on sensitivity and specificity

Assay	Group (sample size)	$_1$ct/min	$_1$SI
Ty2	Vaccinees (37)		
	Percent correct	82·7	74·7
	Screening value*	3 536†	3·51†
	Typhoid Fever (58)		
	Percent correct	82·3	72·9
	Screening value	3 370	3·59
	Combined (95)		
	Percent correct	82·7	73·7
	Screening value	3 433	3·56
OPS	Vaccinees (78)		
	Percent correct	70·5	64·1
	Screening value	1 237	3·01
	Typhoid Fever (84)		
	Percent correct	57·1	52·4
	Screening value	830	2·87

Screening value is the back-transformed value determined from discriminant analysis as the segregation point for immune and nonimmune groups. Combined analysis was not conducted for the OPS assays because the vaccinated and post typhoid groups did not yield similar results.

* Group compared with control.

† Back-transformed from natural log.

For the Ty2 antigen, the fraction of observations classified correctly ranged from 73–83%. For the OPS antigen these values were substantially lower (52–71%). These values compare favourably with the non-parametric analysis of untransformed data. For each immune group compared to the common control, sensitivity and specificity was greater for $_1$ct/min than for $_1$SI.

DISCUSSION

Inactivated *S. typhi*, when injected parentally, constitutes an effective vaccine in preventing typhoid fever (Pfeiffer & Kolle, 1896; Wright, 1896; Wright & Leishman, 1900; Yugoslav Typhoid Commission, 1957, 1962; Clasener, 1967; Levine *et al.*, 1987a). Vaccines of this composition are relatively easy and inexpensive to prepare and have been available for most of this century (Pfeiffer & Kolle, 1896; Wright, 1896; Wright & Leishman, 1900). However, in spite of the persistence of typhoid fever as a significant global health problem (Taylor, Pollard & Blake, 1983; Edelman & Levine, 1986; Levine, *et al.*, 1978), these vaccines have not become widely accepted as public health tools. A major reason for non-acceptance is the high rate of vaccine-caused adverse reactions (Ashcroft, Morrison-Richie & Nicholson, 1964; Hefjec *et al.*, 1966; Yugoslav Typhoid Commission, 1964). Attempts have been made to construct vaccines with reduced reactogenicity (reviewed in Levine, 1988); one such successful attempt was the construction of attenuated strains of *S. typhi* (Germanier & Furer, 1975). One difficulty in bringing attenuated bacterium vaccines into common use arose when it was found, through clinical trials (Gilman *et al.*, 1977) and field trials (Wahdan *et al.*, 1982; Levine *et al.*, 1987a; 1989 (in press); that efficacy was markedly affected by method of preparation and delivery. The numerous field trials (and tens of thousands of volunteers) required to test each formulation or delivery scheme (Wahdan *et al.*, 1982; Levine *et al.*, 1987a; 1989 (in press); are due, in part, to a lack of a reliable immunoassay of protection from typhoid fever as engendered by immunization.

The problem of finding a level of *S. typhi* antigen-driven LR corresponding to acquired cellular immunity to this bacterium was addressed by hypothesizing that individuals who had had known contact with virulent or attenuated *S. typhi* had acquired, as the result of this exposure, cellular immunity. The hypothesis was tested by determining the levels of *S. typhi* antigen-driven LR for immune and non-immune individuals and then ascertaining the capacity of various statistical procedures to correctly classify individual responses into immune or non-immune groups. For those procedures showing good discriminatory power, screening values, which define the border between immune and non-immune response, were determined.

Data were analyzed by non-parametric and parametric statistical procedures. Both procedures showed, for certain conditions, good discrimination of immune status. For Ty2 antigen-driven assays, these independent means of data analysis yielded essentially identical results; maximum sensitivity and specificity for the pooled typhoid immune groups was, 81·6% (ct/min data) and 82·7% ($_1$ct/min data) as determined by the non-parametric or parametric procedure, respectively. Results obtained from OPS-driven LR showed similar patterns of specificity and sensitivity when analyzed by non-parametric and parametric procedures. However, for this antigen, the vacci-

nated and post-infection groups differed in level of LR which denoted immunity.

We conclude that data analysis as $_{i}$ct/min is justified statistically and biologically and leads to good discrimination of immune and non-immune groups. Log-transformed data usually were normally distributed and thus could be analyzed using the automated parametric analysis. Further, biologically LR is a logarithmic function, and is thus better represented by data in this form. Further still, we prefer to process data as $_{i}$ct/min rather than as $_{i}$SI because each condition (i.e. antigen comparison) yielded higher sensitivity and specificity as $_{i}$ct/min, and because the derived value $_{i}$SI is a relationship between two average values and thus is affected by the combined variances of the averages. Thus, $_{i}$SI is inherently less precise than the single mean represented by $_{i}$ct/min. The values addressed in the remainder of this section pertain to analyses of $_{i}$ct/min data.

For LR assays which employed particulate *S. typhi* as antigen, vaccinated and infected individuals showed similar patterns of response, sensitivity and specificity of assays (82·7% vs 82·3%) and screening values (3 536 vs 3 370). Because of this homogeneity, we combined these groups; it is concluded that ct/min values in excess of 3 433 denote immunity to *S. typhi* with sensitivity and specificity of 82·7%.

Results obtained from LR assays driven by OPS did not provide as high resolution and were not comparable for vaccinated or infected groups. Under the best circumstances, with vaccinees, OPS-driven LR was about 10% less sensitive and specific than particulate antigen-driven LR. This difference may reflect the intrinsic sensitivity of the respective assays. Since maximum LR to OPS stimulation is 10-fold less than that to particulate antigen (Levine *et al.*, 1987b; Murphy *et al.*, 1987), it is more difficult to discriminate OPS response from background. The low maximum response to OPS may result from the limited number of epitopes in this antigen. In contrast, Ty2 antigen, which is a whole bacterium, presents multiple antigens. A second explanation is that immune response to particulate antigen may be long-lived but that response to OPS is more transient. A third explanation is that the OPS-driven LR is detecting cellular immune responses which are not linked to those detected with Ty2 antigen. There is a disunion in level of LR observed between populations which were immunized by different means. Combined sensitivity and specificity was 70·5% among vaccinees, but only 57·1% among infected individuals. This disparity may reflect differing capacities of the immunizations to generate cellular immunity to those antigens represented on OPS. From our data, it would appear that vaccination is more powerful than systemic infection in generating anti-OPS cellular immune response.

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