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In Vitro Stimulation of Human Peripheral Blood Lymphocytes by Soluble and Membrane Fractions of Renografin-Purified Typhus Group Rickettsiae

A. LOUIS BOURGEOIS,[1]* GREGORY A. DASCH,[1] AND DOUGLAS M. STRONG[2]

Department of Microbiology[1] and Department of Clinical Investigation,[2] Naval Medical Research Institute, Bethesda, Maryland 20014

Cell-free extracts of disrupted Renografin-purified *Rickettsia typhi* and *R. prowazekii* were evaluated as antigens in lymphocyte transformation assays for cell-mediated immunity to typhus group rickettsiae in 19 individuals with and 9 without histories of exposure to these organisms. Exposure consisted of clinical disease, vaccination with epidemic typhus vaccine, or occupational exposure to these agents. Both the soluble and membrane fractions of disrupted purified rickettsiae were used, and transformation of peripheral blood lymphocytes (PBL) was determined in microcultures by incorporation of [^{3}H]thymidine. Of the antigen concentrations tested (1 to 400 μg/ml), 10 μg/ml appeared to be the most satisfactory. At this concentration, PBL transformation was highly reproducible and correlated well with donor exposure and the presence of enzyme-linked immunosorbent assay anti-typhus group immunoglobulin G. At higher concentrations, PBL from both exposed and control donors often responded to a lipopolysaccharide-like component present in these preparations. Specific transformation responses to rickettsial fractions were detected in several individuals decades after infection or vaccination, indicating that both fractions contained antigens associated with persisting cell-mediated immunity in humans. Generally, stimulation indexes with the soluble fraction were slightly greater than those obtained with corresponding concentrations of the membrane preparation, and in three individuals transformation was observed only with the soluble fraction. PBL transformation to soluble fractions also appeared to have some species specificity, since PBL from individuals with documented *R. typhi* infections were more responsive to the homologous soluble preparation than to the soluble fraction of *R. prowazekii*. PBL transformation also correlated well with homologous but only poorly with heterologous enzyme-linked immunosorbent assay immunoglobulin G titers.

Recent experimental evidence suggests that anti-rickettsial cell-mediated immunity (CMI) plays an important role in defense against typhus and other rickettsial infections (17, 21, 30, 35). Studies in experimentally infected animals indicate that immunity to *Rickettsia typhi* and *R. tsutsugamushi* is cell-mediated and possibly independent of humoral antibody (21, 30). In humans, anti-rickettsial CMI, measurable by delayed-type skin hypersensitivity and in vitro lymphocyte transformation, has been shown to develop after infection or after vaccination (2, 3, 8, 17, 23, 36). Wisseman (36) has suggested that such tests may ultimately replace serological tests as correlates of immunity, since past vaccines which have elicited good CMI responses in addition to humoral antibody have provided better protection against rickettsial diseases than those that did not. However, further studies are needed before the true significance of cellular responses in humans can be established.

Studies of anti-rickettsial CMI would be greatly facilitated by the identification of specific antigens associated with this response. Since anti-rickettsial CMI in humans has been shown to persist in the absence of detectable circulating antibody (3, 8, 17), such antigens would have excellent potential as diagnostic and epidemiological tools. Furthermore, the identification of rickettsial antigens associated with persisting CMI in humans would be of particular interest since they may have potential as future candidate vaccines. With this in mind, we evaluated cell-free extracts of Renografin-purified *R. typhi* and *R. prowazekii* as antigens in lymphocyte transformation assays for CMI to typhus group rickettsiae. Because of their freedom from contaminating host cell proteins (9, 10, 13, 34; S. Halle and G. A. Dasch, submitted for publication), extracts of Renografin-purified organisms

have been shown to be a highly satisfactory source of antigens for rickettsial serologies, including the enzyme-linked immunosorbent assay (ELISA) (9, 13; Halle and Dasch, submitted for publication), but they have not been examined for their suitability in assays of CMI. In this report, we detail the specific stimulation of human peripheral blood lymphocytes by both the soluble and membrane fractions of *R. typhi*. These two fractions were used rather than total cell extracts because they differ in antigenic composition, and the crude soluble fractions of *R. typhi* and *R. prowazekii* used here contain the majority of the species-specific antigens (G. A. Dasch, J. R. Samms, and J. C. Williams, submitted for publication). The reproducibility of the specific peripheral blood lymphocyte (PBL) response to these fractions and the relationship of this response to anti-rickettsial antibody levels were also evaluated.

MATERIALS AND METHODS

Lymphocyte donors. All donors participating in this study were fully informed of its purpose before being bled. Exposure histories to *R. typhi* and *R. prowazekii* for individual donors are summarized in Table 1.

Preparation of antigens from typhus group rickettsiae. The soluble and membrane typhus fractions, used as antigens in lymphocyte transformation, were obtained from the same total French pressure cell extracts used as antigens in the ELISA for anti-typhus group antibody determinations (Halle and Dasch, submitted for publication). These ELISA antigens were prepared by passing suspensions (2 to 3 mg of protein per ml) of Renografin density gradient-purified *R. typhi* (Wilmington strain) or *R. prowazekii* (Breinl Strain) (34) in 0.01 M $NaPO_4$ buffer, pH 7.0, through a French pressure cell at 20,000 lb/in^2 twice, centrifuging the crude extract at 12,000 rpm for 15 min in a Sorvall SS-34 fixed-angle rotor to remove intact cells, and adding Formalin to 0.1% final concentration. Formalinized cell-free ELISA antigens that had been stored for more than a month at 4°C were centrifuged at 32,000 rpm for 1 h in a Spinco type 40 rotor. The resulting supernatant was collected and designated the soluble antigen. The pellet was resuspended in the same buffer containing 0.1% Formalin and designated the membrane antigen (Halle and Dasch, submitted for publication). Both antigens were stored at 4°C until needed. Protein concentrations were determined by the method of Lowry et al. (19). Before use in lymphocyte transformation assays, soluble and membrane fractions were diluted to 1 mg/ml and dialyzed for 18 h at 4°C against 500 to 1,000 volumes of Dulbecco phosphate-buffered saline. After dialysis both fractions were diluted to the appropriate working concentrations in RPMI 1640 tissue culture medium (Microbiological Associates) supplemented with L-glutamine (2 mM), gentamicin (50 μg/ml), and HEPES buffer (12.5 mM; *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (complete RPMI).

TABLE 1. *Exposure histories to typhus group rickettsiae and antibody prevalence among lymphocyte donors*

Type of exposure	Donor	ELISA antibody titer[a] *R. typhi*-IgG	ELISA antibody titer[a] *R. prowazekii*-IgG
Clinical disease	D1 (44/46)[b]	270	370
	D2 (46/46)	190	370
	D3 (61/79)	3,500	600
	D4 (74/76)	1,700	2,200
	D5 (75/79)	400	100
	D6 (75/79)	450	520
	D7 (77/79)	4,300	5,600
	D8 (78/78)	800	340
Occupational	O1 (28)[c]	210	370
	O2 (25)	1,200	1,100
	O3 (25)	540	1,600
	O4 (8)	1,400	1,000
	O5 (5)	850	700
	O6 (4)	270	190
	O7 (3)	<100	<100
	O8 (0.5)	<100	<100
Vaccination	V1 (10/67)[d]	<100	<100
	V2 (≥2/52)	<100	500
	V3 (≥2/53)	<100	150
No exposure	C1	<100	<100
	C2	<100	<100
	C3	<100	<100
	C4	<100	<100
	C5	<100	<100
	C6	<100	<100
	C7	<100	<100
	C8	<100	<100
	C9	<100	<100

[a] Donor's ELISA IgG titers as of January to May 1979; IgG titers ≥500 are considered indicative of previous specific antigen stimulation.

[b] Approximate year of infection/last year of known exposure.

[c] Years of exposure to typhus group rickettsiae.

[d] Approximate number of vaccinations against epidemic typhus/year of last vaccination.

Titration of typhus group antibody levels by microplate ELISA. Immunoglobulin G (IgG) endpoint serum titers against *R. typhi* and *R. prowazekii* total French pressure cell extract antigens (above) were determined in the microplate ELISA as described elsewhere (9; Halle and Dasch, submitted for publication). The reciprocal of that serum dilution with an ELISA optical density of 0.25 (4 standard deviations above the mean of normal serum controls) was defined as the endpoint. With single sera, titers ≥500 were regarded as positive (Ab+), those between 100 and 500 were suspect positives (Ab±), and those <100 were considered negative (Ab−) for antibody against typhus rickettsiae.

Lymphocyte transformation assays. Venous blood from donors was collected in preservative free heparin (100 U/ml) (Upjohn) and diluted with an equal volume of complete RPMI. After centrifugation through Ficoll-Hypaque (7), the lymphocyte-rich band was collected, washed twice in complete RPMI, resus-

pended, and counted with a Coulter Counter (model ZBI, Coulter Electronics). For lymphocyte transformation assays, lymphocytes were suspended at a final concentration of 2×10^6 cells per ml in complete RPMI supplemented with 20% human AB plasma.

Transformation assays were performed in 96-well, round-bottomed microculture plates (Linbro IS series, Bellco Glass) as previously described (32). Each well received 0.1 ml of a lymphocyte suspension and 0.1 ml of complete RPMI or complete RPMI containing various concentrations of rickettsial antigens, *Escherichia coli* lipopolysaccharide (O111:B4) (LPS) (Difco), or phytohemagglutinin-P (Difco) (0.2%). Phytohemagglutinin-P was included to insure that PBL were viable and capable of mounting proliferative responses in vitro. Unless otherwise specified, all plates were incubated at 37°C in a 5% CO_2-humidified atmosphere for 5 days. Eighteen hours before harvesting, 20 μl of complete RPMI containing 1 μCi of [*methyl*-^{3}H]thymidine (1.9 Ci/mmol, Schwarz-Mann) was added to each well. Cultures were then harvested onto glass-fiber filter paper strips (H. Reeve Angel & Co. Inc., grade 934 AH) with a multiple automated sample harvester (15). After harvesting, the paper strips were dried, and the disks were punched out, placed in polypropylene scintillation vials (Bio-vials, Beckman) containing 2 ml of complete LSC (Yorktown), and counted in a Searle 81 liquid scintillation counter (Searle Analytic, Inc.). Transformation results are expressed as the mean disintegrations per minute ± standard error for quadruplicate or triplicate cultures and as stimulation indexes (SIs) calculated as (mean disintegrations per minute of cultures containing antigen or mitogen)/(mean disintegrations per minute of control unstimulated cultures). SIs were considered significant if they were >3 (approximately the 0.01 level of significance).

RESULTS

Exposure to typhus group rickettsiae and antibody prevalence among lymphocyte donors. The in vitro lymphocyte transformation response to *R. typhi* and *R. prowazekii* fractions was studied in 19 individuals with varying histories of exposure to typhus group rickettsiae and in 9 without such exposure. Lymphocyte donors were grouped on the basis of their past histories of exposure to typhus group rickettsiae, consisting of clinical disease, known occupational exposure to these agents, or vaccination with killed epidemic typhus vaccine (Table 1). Eight donors had histories of clinical typhus infections (D1 to D8). Diagnosis was based on demonstration of a rising anti-rickettsial antibody titer as measured by either the complement fixation or ELISA techniques (10, 13; Halle and Dasch, submitted for publication). Serological results and individual exposure histories suggested that five donors (D1, D3, D4, D5, and D8) had been infected with *R. typhi*, and one (D2) had been infected with *R. prowazekii*. In two donors (D6 and D7) the typhus agent responsible for infection could not be identified with certainty. Three donors (D1, D4, and D6) in this group had received the two-dose primary immunization with killed epidemic typhus vaccine. All illnesses were laboratory acquired, and at the time of this study, five of nine donors still maintained highly significant levels (ELISA IgG titers ≥ 500) of circulating antibodies to *R. typhi* or *R. prowazekii* (Table 1). The remaining four donors had IgG titers in the 100 to 500 range. Although ELISA titers in this range are inconclusive when based only on the analysis of a single serum sample (Halle, personal communication; Halle and Dasch, submitted for publication), they were considered to represent persisting anti-rickettsial antibody, because of the past clinical and serological histories of these individuals.

Eight donors (O1 to O8) had no clear history of typhus-like illness, but all had experienced extensive laboratory occupational exposure (Table 1). Exposure among these individuals was generally limited to *R. typhi* and *R. prowazekii* strains, although donor O1 also had extensive exposure to spotted fever group rickettsiae. In addition, donors O1, O2, and O3 had also received multiple injections (>2) of typhus vaccine, whereas O4 had a single vaccination. Current ELISA titers among occupationally exposed individuals tended to increase with their years of exposure (Table 1). Based on exposure histories and previous serological results, IgG titers in the 100 to 500 range were again considered significant for donors O1 and O6 (Table 1). Donor O6 had exhibited an IgG titer >500 in a single earlier serum specimen. Two donors (O7 and O8) in this group were consistently Ab− (ELISA IgG titers <100).

Exposure in three donors (V1 to V3) consisted of vaccination only with commercial typhus vaccine received 12 to 27 years previously, donor V1 received 10 doses of vaccine, and V2 and V3 received at least 2 doses. The ELISA test detected serological evidence of prior vaccination in two donors (V2 and V3).

Nine donors (C1 to C9) with no known exposure to these agents and negative for antibodies against *R. typhi* and *R. prowazekii* were included as controls (Table 1).

Effect of rickettsial antigen concentration on PBL proliferation. The concentrations of rickettsial-soluble and membrane fractions required to give maximum specific stimulation of [^{3}H]thymidine uptake in cultured PBL were determined. These fractions differ markedly in their content of species-specific typhus antigen (Dasch et al., submitted for publication) and in preliminary experiments were more satisfactory than total cell extracts or whole cells in

stimulating PBL proliferation. Ten donors with high levels of circulating anti-typhus group antibodies (ELISA IgG titers ≥500 to either *R. typhi* or *R. prowazekii* or both) (Ab+) and four controls (C1 to C4) (Table 1) were tested against rickettsial fractions at several concentrations (1 to 400 μg/ml), and transformation results were evaluated after 5 days of culture. Representative responses for Ab+ and control donors are shown in Table 2. All Ab+ donors responded to rickettsial fractions at concentrations of 400 or 100 μg/ml. However, three of four controls, to a lesser extent, also responded to these concentrations (compare donors C2 and C4 in Table 2). At lower antigen concentrations (1 to 10 μg/ml), all Ab+ donors responded to both fractions except V2, an individual last vaccinated against epidemic typhus in 1952 who did not respond to either fraction, and O3, who had both occupational and vaccination exposure and responded to the soluble fraction only (Table 2). In contrast, none of four controls responded at these concentrations. Transformation responses at a concentration of 1 μg/ml were significantly lower (Table 2). Seven of eight Ab$^+$ donors tested still exhibited significant stimulation with the soluble fraction, but only four of a subset of six individuals responded to the membrane preparation at this concentration. Generally, SIs with the soluble fraction were slightly greater than those obtained with the corresponding concentration of the membrane preparation (Table 2). SIs did not shift significantly when tests were repeated 1 to 6 months later (Table 2). Three Ab+ donors and three controls were also tested against similar concentrations (1 to 400 μg/ml) of the soluble fraction of *R. prowazekii*. Similar responses were observed (data not shown).

Proliferative response of control donors to *R. typhi* fractions and *E. coli* LPS. Nonspecific cellular responses to rickettsial preparations have been reported previously (8, 36). We observed a similar phenomenon with high concentrations of both fractions (100 to 400 μg/ml) (Table 2). Further analysis of the responsiveness of controls to *R. typhi* fractions suggested that stimulation was due to the presence of an LPS or endotoxic-like component in these preparations. The three control donors mentioned above who responded to high concentrations of rickettsial fractions (e.g., donor C2 in Table 2) also responded strongly to *E. coli* LPS, whereas the nonresponding control (C4) did not. Dose-response curves for representative control donors (C2 and C4) to *R. typhi* and *E. coli* LPS are shown in Fig. 1. Similar response curves for a typical Ab+ donor, D3, are included for comparison. Extension of these studies to five additional controls (C5 to C9 in Table 1) yielded essentially the same results, with one exception. Control donor C9 responded to the soluble fraction of *R. typhi* at both high (100 μg/ml; SI = 5.5) and low (10 μg/ml; SI = 5.0) concentrations. C9 did not respond to the membrane fraction at

TABLE 2. *Transformation response of Ab+ donors and controls to R. typhi fractions*

Donor	Date	Control	RTS[a] (μg/ml)				RTM[b] (μg/ml)			
			400	100	10	1	400	100	10	1
D3	11/78	12 ± 1[c]	491 ± 42	634 ± 43	423 ± 28	158 ± 17	402 ± 30	492 ± 23	410 ± 17	175 ± 19
			(40.9)[d]	(52.8)	(35.3)	(13.1)	(33.5)	(41)	(34.2)	(14.6)
	5/79	14 ± 5	ND[e]	648 ± 58	474 ± 37	237 ± 24	ND	469 ± 50	395 ± 28	125 ± 10
				(46.3)	(33.8)	(16.9)		(33.5)	(28.5)	(8.5)
O3	2/79	7 ± 1	ND	72 ± 20	50 ± 5	20 ± 1	ND	47 ± 4	16 ± 2	8 ± 1
				(10.2)	(7.1)	(2.4)		(6.7)	(1.5)	(1.1)
O5	1/79	35 ± 4	882 ± 75	752 ± 20	487 ± 31	316 ± 30	470 ± 56	562 ± 59	363 ± 35	343 ± 38
			(25.2)	(21.1)	(13.9)	(9.1)	(13.4)	(16.1)	(10.4)	(9.8)
	6/79	53 ± 4	ND	711 ± 78	781 ± 35	684 ± 70	ND	848 ± 16	760 ± 41	681 ± 63
				(13.4)	(14.5)	(12.9)		(15.8)	(14.3)	(12.8)
V2	2/79	10 ± 1	ND	40 ± 5	25 ± 4	21 ± 3	ND	35 ± 5	21 ± 2	12 ± 3
				(4.0)	(2.5)	(2.1)		(3.5)	(2.1)	(1.2)
C2	1/79	25 ± 2	496 ± 31	238 ± 31	57 ± 5	30 ± 5	248 ± 35	89 ± 6	34 ± 3	34 ± 4
			(19.8)	(9.5)	(2.2)	(1.7)	(9.9)	(3.6)	(1.4)	(1.4)
C4	11/78	16 ± 2	14 ± 5	27 ± 4	27 ± 3	29 ± 4	19 ± 1	41 ± 7	24 ± 2	23 ± 1
			(0.9)	(1.7)	(1.7)	(1.8)	(1.2)	(2.6)	(1.5)	(1.5)
	2/79	21 ± 4	ND	56 ± 5	38 ± 3	42 ± 6	ND	41 ± 6	24 ± 2	31 ± 5
				(2.6)	(1.8)	(2.0)		(2.0)	(1.1)	(1.5)

[a] Soluble fraction of *R. typhi*.
[b] Membrane fraction of *R. typhi*.
[c] Disintegrations per minute × 10^{-2} ± standard error.
[d] SI.
[e] ND, Not done.

the lower concentration. In contrast to other control donors, C9 responded to *E. coli* LPS over a broad range of concentrations (10 to 500 μg/ml), and this sensitivity may account for the responsiveness at the lower concentration of the soluble fraction. However, the possibility of exposure in the donor's native country cannot be excluded. This donor has recently immigrated to the United States from a country in Southeast Asia where serological evidence indicates that infection with typhus group rickettsiae is quite common (Bourgeois, unpublished data).

Eight of ten Ab+ donors discussed in the previous section also exhibited varying degrees of PBL stimulation with *E. coli* LPS (data not shown). In these individuals, response curves for LPS were similar to those of C2, whereas curves for *R. typhi* fractions corresponded to those of D3 (Fig. 1).

The presence of an LPS-like component in these preparations was also suggested by positive reactions in the *Limulus* amoebocyte lysate assay and mouse B-cell mitogenicity (Bourgeois et al., manuscript in preparation).

Proliferative response to rickettsial fractions in exposed donors with inconclusive or negative anti-rickettsial serologies. A dose of 10 μg/ml was selected to study specific proliferative responses in nine additional individuals who had experienced exposure to typhus group rickettsiae and had ELISA IgG titers <500 (Table 1). Six (D1, D2, D5, O1, O6, and V3) had IgG titers in the 100 to 500 range (Ab±), whereas three (O7, O8, and V1) had titers <100 (Ab−). In some cases exposure to typhus group rickettsiae was remote and limited (Table 1). Table 3 summarizes the transformation results of these nine donors, as well as the previously discussed Ab+ and Ab− controls. All six Ab± donors responded to the soluble fraction, and five responded to the membrane fraction. Of the three Ab− exposed donors, one (V1) responded to both fractions, and two (O7 and O8) responded to none. SIs for Ab± and Ab− exposed donors were significantly lower than SIs for Ab+ donors. The mean (soluble fraction) for Ab+ donors was 15.5 ± 2.8, whereas values for Ab± and Ab− exposed donors were 9.6 ± 1.3 ($P < 0.05$) and 2.7 ± 0.6 ($P < 0.01$), respectively. The mean SI for control donors, including C9, was 1.94 ± 0.4. Comparison of mean values for controls and exposed donors by the Student *t* test (33) yielded *P* values <0.01 for both Ab+ and Ab± groups. However, differences between means for exposed Ab− individuals and controls were not significant ($P > 0.10$). When SI were compared on the basis of exposure (groups shown in Table 1), mean values (soluble fraction) were highest for individuals with histories of clinical illness (15.7 ± 3.3) and lower for occupationally exposed (9.7 ± 2.2) and vaccinated individuals (5.8 ± 2.6). Mean values for both the disease and occupationally exposed groups were again highly significant when compared with controls ($P < 0.01$). However, differences between means for vaccinated individuals and controls were not ($P > 0.10$). SIs with the membrane fraction were generally comparable, but slightly lower.

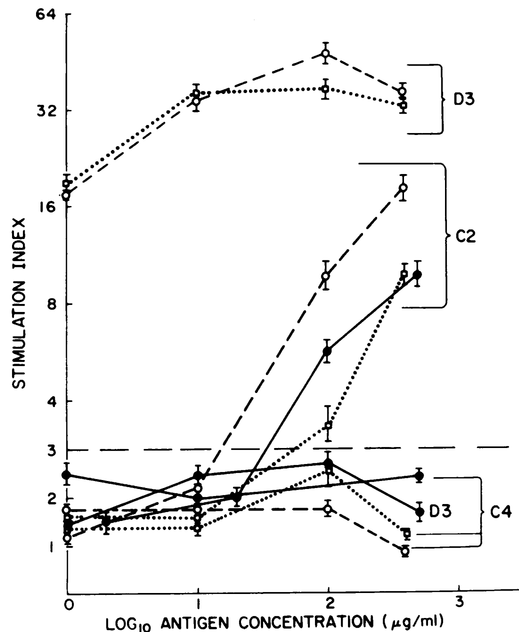

FIG. 1. *Dose-response curves of lymphocyte donors to varying concentrations of R. typhi fractions and E. coli (O111:B4) LPS. Donors are from Table 2. SIs for soluble* (○) *and membrane* (□) *fractions of R. typhi and E. coli LPS* (●) *are shown. Vertical lines represent the standard error of the mean of quadruplicate cultures.*

When all data on SIs for the 19 exposed donors (Table 3) were plotted against their respective ELISA IgG titers to *R. typhi*, the linear regression showed a strong correlation for the soluble ($r = 0.84$, Fig. 2), and membrane fractions ($r = 0.79$, not shown). When values for donors infected with *R. prowazekii* (D2) or exposed exclusively to this agent (V1, V2, and V3) were deleted, the regression lines (not shown) were not significantly different from those calculated with all the data. In contrast, only a weak correlation existed between donors' IgG titers to *R. prowazekii* and their responses to these fractions (soluble fraction; $r = 0.52$, membrane fraction; $r = 0.36$) (not shown).

PBL SIs in two weak responders (D2 and V1) and in two nonresponders (O8 and V2) (Table

TABLE 3. *Summary of PBL transformation to R. typhi fractions*

ELISA antibody	Donor	SI[a]	
		RTS[b] (10 μg/ml)	RTM[c] (10 μg/ml)
Ab+	D3	35.3 ± 2.3	34.2 ± 1.3
	D4	17.5 ± 2.1	ND[d]
	D6	9.6 ± 0.3	6.2 ± 0.5
	D7	20.1 ± 0.8	13.1 ± 0.8
	D8	14.8 ± 1.3	10.1 ± 1.1
	O2	15.2 ± 0.6	15.2 ± 1.7
	O3	7.1 ± 0.7	1.5 ± 0.3
	O4	18.8 ± 1.6	14.5 ± 0.6
	O5	14.5 ± 1.2	14.3 ± 1.0
	V2	2.5 ± 0.4	2.1 ± 0.3
	Mean	15.5 ± 2.8	12.3 ± 3.2
Ab±	D1	12.5 ± 1.3	12.0 ± 0.8
	D2	4.2 ± 0.3	5.3 ± 0.6
	D5	11.7 ± 0.8	9.0 ± 0.3
	O1	11.0 ± 1.6	6.0 ± 0.6
	O6	7.6 ± 0.7	6.6 ± 0.9
	V3	10.8 ± 1.1	2.0 ± 0.1
	Mean	9.6 ± 1.3	6.8 ± 1.4
Ab−	O7	2.1 ± 0.2	2.5 ± 0.1
	O8	2.1 ± 0.2	2.0 ± 0.3
	V1	4.0 ± 0.5	5.0 ± 0.3
	Mean	2.7 ± 0.6	3.2 ± 0.9
Ab− (controls)	C1	1.3 ± 0.3	1.8 ± 0.2
	C2	2.2 ± 0.2	1.4 ± 0.1
	C3	1.1 ± 0.1	0.9 ± 0.1
	C4	1.7 ± 0.2	1.5 ± 0.1
	C5	1.2 ± 0.1	ND
	C6	2.2 ± 0.2	1.1 ± 0.1
	C7	1.1 ± 0.1	1.9 ± 0.1
	C8	1.4 ± 0.1	ND
	C9	5.0 ± 0.4	1.2 ± 0.2
	Mean	1.9 ± 0.4	1.4 ± 0.1

[a] SI ± standard error of the mean; PBL transformation to the soluble or membrane fraction of *R. typhi* (10 μg/ml) yielding a SI >3 is considered indicative of CMI to typhus group rickettsiae. For donors tested more than once, only the highest SI is shown.

[b] Soluble fraction of *R. typhi*.

[c] Membrane fraction of *R. typhi*.

[d] ND, Not done.

3) were increased two- to threefold by extending the period of lymphocyte cultivation from 5 to 7 days (data not shown). This procedure had little effect on donor O7, control donors (C1, C3, and C4), or donors responding strongly at 5 days (D7 and D8). Differences in the time course of the transformation response to *R. typhi* fractions in these late responders may reflect relatively low numbers of circulating PBL sensitized to rickettsial antigens.

Specificity of the proliferative response to rickettsial fractions. As evidenced in Table 1, infection with *R. typhi* or *R. prowazekii* stimulates the formation of antibodies with pronounced cross-reactivity to the heterologous organism. Analysis of sera from typhus patients by a battery of serological tests (complement fixation, indirect fluorescent antibody, and ELISA) frequently gives no clear indication of the typhus agent involved, or in the case of vaccinated individuals serological results may actually be misleading (10, 12, 13, 25, 26; Halle and Dasch, submitted for publication). The specificity of the lymphoproliferative response to other soluble antigens is well documented (18, 27). With this in mind, the specificity of the proliferative response to the species-specific antigen containing soluble fractions of *R. typhi* and *R. prowazekii* (Dasch, Samms, and Williams, submitted for publication) in five confirmed typhus infections and in three donors with exposure to both agents (Table 4) was compared with their ELISA results (Table 1). In those cases in which the species responsible for infection was identified, the SI to the homologous strain was almost invariably higher, although the results with the 100-μg/ml concentration must be regarded with caution, because of the relatively high LPS content (Table 4). Donors D1, D3, D4, and D8 were *R. typhi* infections, whereas D2 was infected

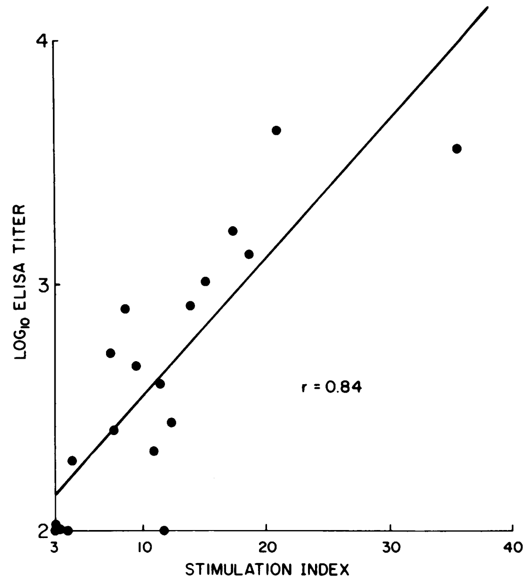

FIG. 2. *Correlation between ELISA IgG titers against R. typhi and lymphocyte transformation responses to the soluble fraction of R. typhi. ELISA IgG titers were obtained with total cell extracts of French pressure cell-disrupted R. typhi, whereas SIs were obtained with only the soluble fraction at 10 μg/ml. The solid line is the linear regression of log ELISA IgG titer and SI; r is the correlation coefficient.*

with *R. prowazekii*. Transformation results in three donors (D2, D3, and D8) were consistent with their current anti-rickettsial antibody titers and clinical histories (Table 4). However, in two donors (D1 and D4) transformation results exhibited greater specificity than their respective serologies, possibly because both these individuals had received typhus vaccine before their infection with *R. typhi*. As noted by others (12, 25, 26), the specificity of the antibody response of vaccinated individuals who are subsequently infected with endemic typhus is directed against the vaccine antigen rather than *R. typhi*. In the three donors with exposure to both rickettsial agents, 2 donors (O4 and O5) responded preferentially to the *R. typhi* fraction, whereas O6 responded equally well to both soluble preparations (Table 4). IgG antibodies in all three donors exhibited slightly stronger reactivity toward *R. typhi* antigens.

DISCUSSION

Stimulation of human PBL by antigens of the typhus and spotted fever group of rickettsiae has been reported by previous investigators (2, 8, 23). Although the antigens employed in previous studies, ether-extracted yolk sac suspensions of typhus rickettsiae (8) or sucrose gradient-purified *R. rickettsii* (2, 23), differed from the membrane and soluble fractions employed here, both nonspecific (8) and specific (2, 8, 23) PBL stimulation was seen with these antigens. Sufficient chemical and immunological evidence has been obtained now to conclude that typhus rickettsiae have an LPS with endotoxic activity, as do most other gram-negative bacteria. Smith and Winkler (31) have provided chemical evidence for 2-keto-3-deoxyoctulosonic acid in *R. prowazekii*, and Schramek et al. (28) have described the preparation of a hydrophobic LPS with endotoxin-like activity by phenol-water extraction of both *R. typhi* and *R. prowazekii*. These studies complement observations in older literature by Olitzki et al. (22) on endotoxic reactions to rickettsial vaccines in rats, by Bendich and Chargaff (4) that the well-known Weil-Felix reaction is due to a cross-reaction between typhus antibodies and the LPS moiety of *Proteus* OX-19, and by Wisseman et al. (36) who recently found positive skin reactions to antigens derived from *R. typhi* and *R. prowazekii* in control subjects. Unlike mouse B-lymphocytes (24), human PBL do not consistently exhibit a polyclonal mitogenic response to LPS, but significant LPS stimulation of human lymphocytes has been observed by other investigators under certain conditions (16, 20, 24). Although yolk sac contaminants were present in the antigens used by Coonrod and Shepard (8), these contaminants were not believed to contribute to the nonspecific PBL proliferation that they observed. Possible nonspecific synergistic effects of yolk sac contaminants are unlikely with the highly purified antigens employed here (10, 13,

TABLE 4. *Specificity of the PBL transformation response to soluble rickettsial fractions*

Infection	Donor	ELISA titer ratio[a]	Antigen concn (μg/ml)	Mean SI[b] RTS[c]	Mean SI[b] RPS[d]	P value[e]
R. typhi	D1	0.73	100	15.0 ± 1.4	9.8 ± 1.2	<0.05
			10	12.5 ± 1.1	8.4 ± 0.5	<0.02
	D3	5.8	100	43.0 ± 4.5	21.6 ± 2.3	<0.02
			10	23.0 ± 0.9	18.6 ± 2.1	>0.05
	D4	0.77	100	32.0 ± 2.3	19.4 ± 2.1	<0.01
			10	17.6 ± 2.1	11.4 ± 1.3	<0.05
	D8	2.4	100	17.5 ± 0.4	9.6 ± 0.3	<0.01
			10	14.8 ± 1.3	7.8 ± 0.7	<0.01
R. prowazekii	D2	0.51	100	4.7 ± 0.3	8.5 ± 0.8	<0.05
			10	4.2 ± 0.3	4.9 ± 0.6	>0.5
Exposure to both	O4	1.4	100	18.4 ± 1.5	10.4 ± 1.1	<0.01
			10	ND[f]	ND	
	O5	1.2	100	21.0 ± 2.3	12.7 ± 1.1	<0.02
			10	13.3 ± 0.9	8.0 ± 0.4	<0.4
	O6	1.4	100	11.1 ± 1.3	12.9 ± 1.4	>0.5
			10	ND	ND	

[a] Ratio: ELISA IgG titer against *R. typhi*/ELISA IgG titer against *R. prowazekii*; ELISA ratios for individual donors were calculated from serological data shown in Table 1.
[b] Mean SI ± standard error of the mean.
[c] Soluble fraction of *R. typhi*.
[d] Soluble fraction of *R. prowazekii*.
[e] The significance of the difference between mean SIs achieved with the soluble fractions of *R. typhi* and *R. prowazekii* was determined by Student's *t* test (33).
[f] ND, Not done.

34; Halle and Dasch, submitted for publication). Consequently, it is likely that rickettsial LPS is the major cause of the nonspecific proliferative responses observed with high concentrations of rickettsial antigens.

As in previous studies (8, 23) lymphocyte transformation to purified rickettsial fractions was an excellent indicator of prior exposure to typhus antigens. CMI, as measured by lymphocyte transformation, was demonstrated in all donors with histories of clinical disease, seven of eight occupationally exposed donors, and in all vaccinated individuals. These CMI-positive donors included two individuals infected 33 to 35 years previously who have experienced no further exposure to rickettsiae. Our ability to detect significant proliferative responses in individuals years after infection or vaccination indicated that rickettsial fractions contained antigens associated with persisting CMI in humans. The detection of cellular responses in three vaccinees 12 to 27 years after immunization was unexpected. Significant transformation responses have been detected in individuals after repeated immunizations with typhus or Rocky Mountain spotted fever vaccine. However, information on the persistence of this responsiveness has been somewhat limited. Coonrod and Shepard (8) detected significant responses to *R. typhi* and *R. prowazekii* antigens 1 to 1.5 years postimmunization with killed epidemic typhus vaccine, whereas Oster et al. (23) were not able to demonstrate responsiveness to *R. rickettsii* antigens in vaccinated individuals not currently working with this organism (mean years since last vaccination = 10.4 ± 4.4). The physical state of the antigens used in this study may explain our ability to demonstrate significant proliferative responses in these individuals. The soluble fraction generally elicited stronger proliferative responses than either whole cell or membrane preparations possibly because of more efficient processing by macrophages (1, 5, 29).

Transformation responses to rickettsial fractions compared favorably with the highly sensitive ELISA technique as an indicator of previous exposure. Significant responses to these fractions tended to correlate with both the presence and the level of anti-rickettsial antibodies. Previous investigators have noted a similar relationship between lymphocyte transformation and complement fixation and microagglutination titers to typhus and spotted fever group rickettsiae (8, 23). The fact that persisting cellular responses were detected in several individuals with little or no ELISA antibodies suggests that lymphocyte transformation may be a more sensitive indicator of previous *R. typhi* and *R. prowazekii* infection.

Dasch et al. (submitted for publication) have clearly shown that the soluble fractions of Renografin-purified typhus group rickettsiae contain species-specific antigens. This was also demonstrated in these studies, since individuals with documented *R. typhi* or *R. prowazekii* infections reacted more strongly with the homologous soluble fraction than with heterologous preparations. In two individuals, specificity was more evident in transformation results than in their serologies. The specificity of the transformation response to other bacterial, viral, and synthetic antigens has been documented by several investigators (6, 14, 18, 27), but specificity was not noted by Coonrod and Shepard (8) using rickettsial antigens prepared by ether extraction of infected yolk sacs. Other investigators have frequently encountered difficulty in demonstrating species-specific activity in antigens prepared by this method (10, 11). Our results suggest that Renografin-purified rickettsiae are a more satisfactory source of antigens for studies investigating the specificity of the immune response to these organisms. Whether more highly purified species-specific antigens from *R. typhi* and *R. prowazekii* are of greater value than crude soluble fractions in evaluating this specificity by lymphocyte transformation is under investigation.

Cell-transfer experiments in guinea pigs and mice (21, 30) and circumstantial evidence in humans (35) indicate that the cellular response is important in immunity to rickettsial infections. The results of these studies indicate that rickettsial antigens, present in cell-free extracts of disrupted purified *R. typhi* and *R. prowazekii*, are associated with both persisting humoral and cellular immunity in humans and may have potential as future candidate vaccines.

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