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PRELIMINARY CHARACTERIZATION OF INFLAMMATORY INFILTRATES IN RESPONSE TO *RICKETTSIA PROWAZEKII* REINFECTION IN MAN: IMMUNOHISTOLOGY

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Summary. - The local perivascular mononuclear cell inflammatory infiltrate (PVI) response to intradermal (ID) challenge with viable *R. prowazekii* was studied in a typhus-immune subject. An immunohistochemical method for specific mononuclear cell markers was used on skin punch biopsies taken 6, 24 and 48hr after challenge. By 24 to 48 hr, the histologic findings were consistent with a delayed--type hypersensitivity reaction (DTHR). Both $CD4^+$ and $CD8^+$ T lymphocytes dominated the PVI at 48h. $CD8^+$ cells entered the PVI more rapidly than $CD4^+$ cells. Local control of *R. prowazekii* challenge in an immune human subject was associated with recruitment into the PVI of T lymphocytes which are rich sources of γ-interferon, and $CD8^+$ T cells which are potentially cytotoxic for *R. typhi*-infected cells.

Key words: Rickettsia prowazekii; cell mediated immunity; delayed-type hypersensitivity; immunohistology

Introduction

Rickettsia prowazekii is an obligate intracellular bacterium which causes typhus, a severe febrile infectious disease of man. After primary infection, solid, long-term immunity to reinfection is established. The host immune response includes development of serum antibodies and evidence of cell-mediated immunity. Immunity to reinfection correlates with *in vivo* cutaneous delayed-type hypersensitivity reactions (DTHR) and *in vivo* lymphocyte proliferation after stimulation by *R. prowazekii* antigens (Bourgeois *et al.*, 1980; Wisseman *et al.*, 1967; Woodward, 1982).

Resistance to reinfection by rickettsiae is mediated predominantly by specific T lymphocyte cellular immune responses and to a lesser extent by immune serum or specific antibodies (Jerrells *et al.*, 1982; Murphy *et al.*, 1979). Guinea pig recipients of immune T cells demonstrated local control of rickett-

sial infection at the dermal challenge site, while recipients of *R. typhi* immune serum did not (Murphy *et al.*, 1979). *In vivo* and *in vitro* studies have strongly suggested a role for the T cell product γ-interferon in the intracellular killing of and resistance to infections caused by members of the genus *Rickettsia* (Li *et al.*, 1987; Turco and Winkler, 1983*a, b*; Wisseman and Wadell, 1983). Recently, *in vitro* cytotoxic T cell responses were shown to occur against *R. typhi* infected target cells, which suggests yet another potential mechanism by which rickettsiae-immune T cells may function *in vivo* (Carl *et al.*, 1987; Rollwagen *et al.*, 1986). In order to study the *in vivo* cellular components of this cell mediated immune response, we have utilized an immunohistochemical technique to characterize the mononuclear cell populations in a DTHR elicited by intradermal inoculation of viable virulent *R. prowazekii* into a typhus-immune human subject.

Materials and Methods

One of the authors (C.L.W.) has a long history of infection, laboratory exposure and immunity to *R. prowazekii*. Previous intradermal inoculation with *R. prowazekii* caused the development of a typical DTHR (Wisseman *et al.*, 1967). Informed consent was obtained, and the subject was judged to be healthy at the time of inoculation and skin biopsy.

R. prowazekii (Breinl) was plaque-purified and Renografin density gradient centrifugation purified. The rickettsial suspension was free of contaminating infectious agents. 10^4 viable *R. prowazekii* were diluted in phosphate buffered saline (PBS, pH 7.4), and inoculated intradermally into three locations on the volar surface of the left forearm.

At 6, 24, and 48 hours after inoculation, a 4 mm skin punch biopsy was obtained from each site. An additional 4 mm punch biopsy was obtained from normal skin to serve as control. The biopsies were divided immediately; one-half of each was fixed in 10 % neutral buffered formalin, and one-half was used for frozen sections. The formalin fixed tissues were processed by routine paraffin embedding for hematoxylin and eosin (H&E) stains and for *R. prowazekii* immunoperoxidase. The tissues for frozen section were embedded in OCT compound and snap-frozen. Frozen sections (4 microns) were cut on a cryostat and fixed in cold acetone (-20 °C) for 10 minutes. An avidin-biotin immunoperoxidase technique was used to stain mononuclear cells and rickettsiae (Dumler *et al.*, 1989, 1990). Monoclonal antibodies used included anti-CD4 (OKT4), anti-CD8 (OKT8), and anti-CD11b (OKM1) to recognize T helper/inducer, T cytotoxic/suppressor lymphocyte subsets, and human macrophages/monocytes, respectively (all from Ortho Diagnostic Systems, Raritan, NJ), and anti-CD19 (Leu12; Dickinson, Mountain View, CA) to recognize human B lymphocytes. Hyperimmune rabbit anti-*R. prowazekii* serum was prepared, the globulin fraction was purified, and the resulting product had a *R. prowazekii* IFA titer of $\geq$2000 and $\geq$51200 by the indirect immunoperoxidase technique.

Stained slides were examined by light microscopy. Five areas of PVI were identified on each slide. A digital image analyzer and data analysis software (Olympus Cue 2) were used, and the area of each PVI was determined. Stained mononuclear cells within these defined areas were counted for each antibody. Granulocytes in the PVI were excluded on the basis of nuclear morphology. Counts were normalized for area of PVI.

Results

Histologic examination showed an early (6>24 hr) perivascular neutrophilic infiltrate, accompanied by lymphocytes and fewer macrophages as seen on H&E stains. In some regions, early (6 h) acute vascular injury with leukocytoclasis and focal erythrocyte extravasation could be identified. At 24 hours the PVI was composed predominantly of lymphocytes and macrophages forming dense accumulations; focal vascular injury was seen. Scattered lymphocytes and macrophages were seen in the interstitial dermis. By 48 hours, a DTHR was present and histologic findings included dense PVI composed of lymphocytes and macrophages intimately associated with endothelial cells which were prominent and columnar in morphology. Many endothelial cells were swollen, had dispersed chromatin with nucleoli and cytoplasmic lucency. Mild perivascular and interstitial edema was present.

The mean area occupied by the PVI increased rapidly after 6 hours (20350 μm^2) post-inoculation, reached peak dimension at 24 hours (59857 μm^2), and decreased at 48 hours (36092 μm^2) as compared with control (13998 μm^2). The total cellular content per mm^2 of these PVI increased dramatically as expected, from 169 mononuclear cells per mm^2 in the control to 483 mononuclear cells per mm^2 in the 48 hour biopsy.

The results of mononuclear cell immunohistologic stains and cell quantitation are shown in Table 1. $CD4^+$ and $CD8^+$ T lymphocytes rapidly appeared in the PVI. Findings include an early increse in $CD8^+$ T cells at 6 hours and a marked late recruitment of $CD4^+$ T cells at 48 hours (Fig. 1). $CD19^+$ B lymphocytes comprised a consistently small pool of cells, and $CD11b^+$ macrophages maintained at a stable level throughout. The ratio of $CD4^+$ to $CD8^+$ cells at

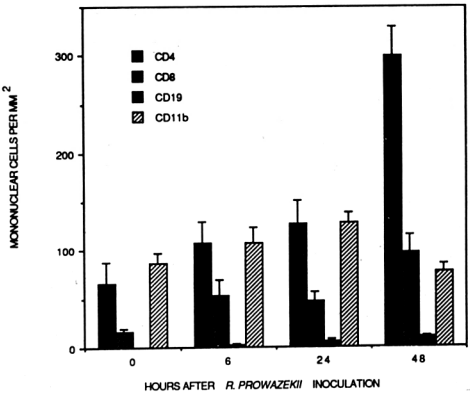

Fig. 1
Comparison of CD4, CD8, CD19, and CD11b mononuclear cell counts in skin biopsies of DTHR taken at various time-points after intradermal inoculation of live *R. prowazekii* into an immune human subject

Table 1. Immunohistologic quantitation of mononuclear cells in *R. prowazekii*-induced cutaneous DTHR perivascular inflammatory infiltrates. Results are expressed as mononuclear cells/mm^2 of perivascular inflammatory infiltrate ±s.e.m.

	Time after inoculation			
Antibody	0 hr*	6 hr	24 hr	48 hr
CD4 (OKT4)	66±22	108±21	127±24	299±30
CD8 (OKT8)	16±4	53±16	47±10	97±19
CD19 (Leu 12)	0	2±1	6±2	10±1
CD11b (OKM1)	87±10	108±16	128±11	77±9
CD4/CD8**	4.02	2.02	2.69	3.07

*uninoculated control biopsy
**results expressed as ratio

each time after inoculation is shown in Table 1. The changes in this ratio emphasize the early and late entry of $CD8^+$ and $CD4^+$ T lymphocytes respectively, into the PVI. No specific change in pattern or distribution was seen with regard to cell populations within PVI or interstitial regions as compared with the control.

Rickettsiae were not visualized at any timepoint throughout the series of biopsies.

Discussion

Rickettsia prowazekii, like other members of the genus and like other intracellular pathogens, presents an unique immunologic challenge to an infected host. The sequestered intracellular environment would seem to preclude effective immune recognition and elimination by circulating antibodies. The cellular immune system has proven more adept at controlling infection by such organisms and presents several possible mechanisms for elimination of these intracellular parasites. Various *in vivo* and *in vitro* tests of T lymphocyte mediated cellular immunity, including cutaneous DTHR and lymphocyte proliferation, are present and correlate with protection against reinfection by rickettsiae (Bourgeois *et al.,* 1980; Coonrod and Shepard, 1971; Jerrells and Osterman, 1982; Woodward, 1982). Such T cell responses effectively control rickettsial infection as has been shown in studies in which immune T lymphocytes were adoptively transferred into naive guinea pigs, which were then able to control infection at the site of dermal inoculation with *R. typhi* (Murphy *et*

al., 1979). Other studies have shown unchecked or poor control of rickettsial proliferation in athymic mice and T lymphocyte-depleted guinea pigs (Montenegro *et al.,* 1984; Walker and Henderson, 1978). Jerrells and Osterman (1982) studied DTHR in mice made immune to *R. tsutsugamushi* and showed correlation with protection to intraperitoneal challenge. DTHR was transferred to naive recipients by immune spleen cells, and abrogation of this transfer was effected by pretreatment of splenic immune cells with anti-Thy 1.2 alloantiserum.

In vivo immunohistologic evaluation of the lymphoid subsets in Taches Noires of human *R. conorii* infection revealed the prevalence of the T helper/inducer population in these early stages of immune response (Herrero-Herrero *et al.,* 1987). The data shown here confirm these results and reaffirm the importance of T cells *in vivo*. A rapid inflammatory cell response to intradermal inoculation of *R. prowazekii*, as shown here, would bring immune T lymphocytes directly to the site of early infection. The mechanism of resistance at the cellular level has not been established. Previous investigators have implicated activated macrophages (Nacy and Meltzer, 1982), and lymphokine products of immune T lymphocytes, especially γ-interferon (Li *et al.,* 1987; Turco and Winkler, 1983*a, b*; Wisseman and Waddell, 1983). Both $CD4^+$ and $CD8^+$ populations of T cells are known to produce γ-interferon (Mosmann and Coffman, 1989), and such cells were recruited in large numbers into the inflammatory lesions, in this immune subject.

Other studies of the immunity elicited by rickettsial infection have suggested roles for lymphokine-activated killer cells (Carl and Dasch, 1986*a*), antibody-dependent cellular cytotoxicity (Koster *et al.,* 1983), and cytotoxic T lymphocytes which recognize surface expressed rickettsial products in conjunction with class I major histocompatibility complex molecules in humans (Carl *et al.,* 1987; Rollwagen *et al.,* 1986). The early alterations in the ratio of increasing populations of CD4 T helper/inducer and CD8 T cytotoxic/suppressor cell infiltrating the inflammatory foci, as seen here, have not been noted previously in rickettsial infection. Recent data has suggested that protection against reinfection of immune mice by the facultative intracellular bacterium *Listeria monocytogenes* is mediated by the $CD8^+$ T cytotoxic subset, while other manifestations of DTHR are mediated by the $CD4^+$ T helper subset (Mielke *et al.,* 1988; 1989). It is tempting to speculate about the possible dichotomy of the contributions made by both T cell subsets in this DTHR elicited by *R. prowazekii.*

The results presented here cannot be conclusively interpreted; however, the *in vivo* findings are consistent with resuls of other authors concerning the importance of T cell mediated immunity in control of reinfection by rickettsiae in immune hosts. Such preliminary findings suggest the need for additional *in vivo* and *in vitro* studies to elucidate the contributions of distinctive $CD4^+$ and $CD8^+$ populations, and to determine whether they serve functionally separate

roles in cell mediated immune responses (such as DTHR) to rickettsial agents as has been shown with *L. monocytogenes.*

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