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# Cyclophosphamide-sensitive activity of suppressor T cells during treponemal infection

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## SUMMARY

When hamsters were infected with *Treponema pallidum* subspecies *endemicum*, the composition and activity of the cellular immune components were markedly altered compared to those of sham-infected controls. A population of suppressor T cells (Ts) developed that diminished the ability of the macrophage (M$\phi$) to perform C3b receptor-mediated ingestion (C3bMI) of erythrocytes coated with antibody and complement. Using cyclophosphamide (CY) we examined node and peritoneal cells to determine their role in regulating M$\phi$ activity during this infection. *In vitro* the node and peritoneal T cells from treponemal-infected/CY-treated animals showed considerably less suppressive activity than treponemal-infected/untreated T cells when co-cultured with M$\phi$ from infected animals. This response was greater with node T cells compared to peritoneal T cells. Moreover, a quantitative analysis of the mononuclear leucocyte populations from each of these regions showed that CY-treated/uninfected animals had a decreased percentage of node T cells. Despite this reduction of node T cells, peritoneal T-cell populations were only minimally reduced. However, treponemal-infected hamsters concomitantly treated with CY had a significant reduction in the T-cell percentages in both compartments. These results imply that, during this infection, most Ts generated in the node remain there although some are dispersed to supplementary regions. Thus, the development of a suppressor system that effects M$\phi$ function may be one way in which treponemes escape total elimination by the host.

## INTRODUCTION

Both the humoral (Turner & Hollander, 1957; Bishop & Miller, 1976; Blanco, Miller & Hanff, 1984) and cell-mediated (Maret, Basman & Folds, 1980; Bagasra, Kushner & Hashemi, 1985; Pavia & Niederbuhl, 1985) components of the host's immune system respond to *Treponema pallidum* infection. During these processes the macrophage (M$\phi$) can participate by presenting antigens (Bagasra & Damjanov, 1982), phagocytosing (Lukehart & Miller, 1978) and reacting to immune lymphocyte signals (Lukehart, 1982). However, investigations have suggested that suppression may play an important role during treponemal infection (Friedman & Turk, 1975; Baughn, Tung & Musher, 1980). Studies show that, during treponematoses, lymphocytes can either be directly rendered unresponsive (Pavia, Folds & Baseman, 1975, 1976) or may act to suppress select M$\phi$ behaviour (Tabor *et al.*, 1984). Either of these events could compromise the host's ability to completely eliminate this organism. To determine how treponemal infection influences the production and function of these suppressor T cells, we studied their effects on M$\phi$ activity by using cells from infected and cyclophosphamide (CY)-treated animals. The results showed that treponemal infection induced the production of a CY-sensitive suppressor T cell responsible for reducing M$\phi$ C3b receptor-mediated ingestion. The data imply that these suppressor T cells first appear in the node and subsequently disseminate producing similar effects on M$\phi$ from other regions. This activity alters the host response enough to facilitate the survival of the organism and contributes to the chronicity of the disease.

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## MATERIALS AND METHODS

### *Animals*

Inbred 10-week-old LSH/Ss Lak male hamsters (Charles River Breeding Laboratories Inc., Wilmington, MA) weighing 80–100 g were housed four per cage and given antibiotic-free food and water *ad libitum*. The animal quarters were maintained at 18°, which facilitates the development of cutaneous lesions (Hollander & Turner, 1954).

### *Organisms and infection hamsters*

*T. pallidum* subspecies *endemicum* are continuously maintained by passage in LSH hamsters in our laboratory (Tabor *et al.*, 1984). Three to four weeks post-intradermal infection, trepo-

neme-containing lymph nodes were surgically excised and treponemes were collected as described elsewhere (Tabor, Bagasra & Jacobs, 1986). Briefly, treponeme-containing suspensions were purified using Percoll gradient densities (Hanff *et al.*, 1984), which has been confirmed as a highly effective method for preparing these organisms (Hanff, Fernandez & Folds, 1986). Treponemes were resuspended in media and enumerated by dark field microscopy as previously described (Miller, 1976). Hamsters were intradermally infected at two sites in the shaved inguinal region by delivering $1 \times 10^5$ to $5 \times 10^5$ organisms suspended in 0·1 ml volume of media to each site. This consistently produces chronic lesions that persist for 6–9 months post-infection (Schell, 1983).

*Cyclophosphamide treatment*

Hamsters were intraperitoneally injected with a 0·5 ml volume of CY (Cytoxan, Bristol Meyers, Syracuse, NY) adjusted to yield a final concentration of 10 mg/kg. This dose has previously been shown to selectively reduce hamster suppressor T-cell populations (Bagasra & Tabor, 1986). Control animals received PBS. Treatment was administered weekly throughout the entire course of the experiment. In those hamster groups inoculated with treponemes, CY was given 3 days prior to infection and subsequently continued on a schedule of weekly injections.

*Cell harvest and macrophage preparation*

All cells were prepared as previously described (Tabor *et al.*, 1984, 1986). In brief, washed hamster peritoneal and lymph node cells suspended in RPMI containing 100 U/ml penicillin and 100 μg/ml streptomycin (media + p/s) were pooled within their respective groups. Trypan blue exclusion studies routinely performed showed 90–94% viability in these preparations. All cell concentrations were adjusted to yield $10^5$ macrophages when plated upon glass coverslips (Bellco, Vineland, NJ) in wells of tissue culture plates (Costar, Cambridge, MA). Total and differential cell counts were performed directly on the coverslips using Wright's-stained (Sigma Chemical Co., St Louis, MO) preparations and ocular grid analysis. After 1-hr incubation at 37°, 5% $CO_2$, individual coverslips were vigorously washed and the non-adherent cells collected were enriched for T cells. The adherent cells were recounted to standardize monolayers and identified as macrophages by morphology, neutral red uptake, and non-specific esterase staining (Yam, Cy & Crosby, 1971).

*Lymphocyte preparations*

Enrichment of all non-adherent populations for T cells was performed exactly as described elsewhere (Tabor *et al.*, 1986). Briefly, pooled hamster non-adherent cells suspended in media were fractionated by differential adherence to nylon-wool columns (Haddada, deVaux Saint Cyr, & Duthu, 1983). Washed effluent cells were panned in flasks coated with goat anti-hamster Ig antiserum (Cappel, Malvern, PA). The T-cell enriched populations were collected and their character verified by cytotoxic analysis using monoclonal anti-murine Thy 1.2 antibody (New England Nuclear, Boston, MA) and absorbed guinea-pig complement. Nylon-wool retained cells were recovered by immersing the nylon-wool packing in warm media and incubating for 30 min, at 37°, in 5% $CO_2$ with constant vigorous shaking. Subsequently the wool was teased apart and vigorously rinsed. All of these cell suspensions were pooled, washed twice, and enumerated. Representative samples from each of these groups were monitored for the expression of markers throughout the experiments. When select SIg ($^-$), Thy 1.2 ($^+$) groups were examined for their response to LPS using a mitogenic assay, they were unresponsive. Identification of B-cell characteristics was performed by immunofluorescense staining using FITC goat anti-hamster IgG (Cappel). Fluorescein-conjugated goat anti-mouse Ig (Cappel) served as negative controls. In all groups, cells were preincubated with excess mouse Fc pieces (Jackson Immuno Research, Avondale, PA) to prevent non-specific surface binding. Following incubation, at least 200 cells per slide from five random fields were examined from triplicate preparations. Background staining did not exceed 4%.

*Co-cultivation of macrophages with T lymphocytes*

Co-cultivation systems were prepared exactly as described elsewhere (Tabor *et al.*, 1986). In brief, fresh macrophage explants from normal or treponeme-infected hamsters were overlaid with T-cell suspensions from either (1) normal, (2) CY-treated, (3) treponemal-infected, or (4) CY-treated/treponemal-infected hamsters. Following a 48-hr incubation period all macrophage-containing coverslips were washed to remove non-adherent cells and assayed for activation by C3bMI. Macrophages cultured in media alone served as control groups. There was no significant loss of macrophages or death of T cells within the culture period. This was determined by periodic removal of representative samples and evaluation of the cell numbers by grid counts, esterase staining, and viability of all populations.

*C3b ingestion assay*

Macrophage activation was assessed by using the criteria of C3b receptor-mediated ingestion activity as originally described (Bianco, Griffin & Silverstein, 1975) with slight modifications (Tabor *et al.*, 1984). Briefly, washed sheep erythrocytes (E) were opsonized with IgM rabbit anti-E (Cordis, Miami, FL) and complement (C). Ingestion controls consisted of E opsonized with antibody alone (EA), and E alone to identify Fc or non-specific ingestion, respectively. Triplicate slides were examined microscopically under oil immersion.

The percentage of macrophages ingesting erythrocytes multiplied by the average number of erythrocytes ingested per 100 macrophages (ingestion index) was used to evaluate the extent of macrophage activation. The results were reported as the difference between the EAC ingestion index minus the background, which did not exceed 2% of the EAC.

*Statistical analysis*

Tests for statistical differences were performed using the Student's *t*-test. Values for the *t* statistic were calculated with data from experiments performed in triplicate using a minimum of eight animals per group.

## RESULTS

In both normal and treponemal-infected hamster nodes a predominance of lymphocytes and a relatively small percentage of macrophages were apparent. In the peritoneum the percentages of Mϕ and lymphocytes were quantatively equal (Table 1). Despite fulminant treponemal infection in the immediate inguinal region, the Mϕ:lymphocyte ratio remained unaltered in both compartments. There was, however, an increase in the

**Table 1.** A profile of hamster lymph node and peritoneal cell populations following treponemal infection and/or cyclophosphamide treatment*

| Tissue source† | % macrophages | % lymphocytes | % NWE‡ | Thy 1.2 (+)§ | % NWR¶ | % IgG(+)** |
|---|---|---|---|---|---|---|
| Control | | | | | | |
| Normal node | 10·4±3 | 89·2±10 | 65·4±5 | 95·0±2 | 21·4±3 | 95·5±3 |
| Normal peritoneum | 43·3±4 | 56·4±7 | 62·1±4 | 98·1±4 | 28·9±9 | 98·8±1 |
| Infected node | 5·7±2 | 94·3±2 | 31·0±2 | 96·3±2 | 54·1±2 | 94·2±3 |
| Infected peritoneum | 56·7±4 | 43·2±9 | 48·1±5 | 99·0±5 | 46·0±3 | 96·4±2 |
| Cyclophosphamide | | | | | | |
| Normal node | 13·5±3 | 86·3±4 | 41·5±3 | 96·5±3 | 26·9±3 | 96·1±3 |
| Normal peritoneum | 51·4±3 | 48·3±2 | 54·6±8 | 97·9±4 | 30·1±2 | 97·7±1 |
| Infected node | 8·2±2 | 91·6±3 | 18·1±4 | 96·8±2 | 57·5±4 | 92·4±3 |
| Infected peritoneum | 57·3±3 | 41·9±4 | 33·2±3 | 97·3±2 | 49·7±4 | 95·3±1 |

* Results were obtained using eight hamsters/group and reported as ±SEM.
† Cells were harvested from lymph nodes and peritoneum of normal and treponemal-infected animals from either control or cyclophosphamide-treated groups.
‡ Percentage of total cells eluted from nylon-wool (NWE) columns.
§ Percentage of NWE (eluted cells) as determined by cytotoxic analysis.
¶ Percentage of total cells retained on nylon-wool (NWR) columns.
** Percentage NWR positive for FITC goat anti-hamster IgG.

absolute cell numbers of each type, which reflected a significant cell-mediated response to the insult. The most apparent differences occurred within the lymphocyte group. A two-fold increase in B cells compared to T cells in both the nodes ($P < 0{\cdot}01$) and the peritoneum ($P < 0{\cdot}05$) consistantly developed post-infection, which produced a marked shift within the B to T cell ratio. Using cytotoxic and immunofluorescense analyses on *in vitro* cultures of fractionated lymphocytes, we confirmed that these two populations remained distinctly identifiable as either T or B cells. Neither class of lymphocytes released or concealed their surface markers during the time they were cultured for study. Using such periodic monitoring we confirmed that the ratios (B:T) expressed remained stable throughout the infection process. Although the percentage of uninfected node and peritoneal T cells were initially equal, treponemal infection induced a decrease in the percentage of T cells. However, this T-cell reduction was greater in the node than in the peritoneum. Concurrently, the percentage of node and peritoneal B cells was elevated, although the magnitude of the increase was much greater in the node population (Table 1). Following CY treatment, there was an appreciable reduction in the node T-cell population without a significant change in the B-cell population. The T-cell reduction became apparent when values for normal control nodes were compared with normal CY nodes ($P < 0{\cdot}01$). A similar response was observed when control nodes from treponemal-infected animals were compared with nodes from CY-treated/treponemal-infected animals ($P < 0{\cdot}05$). Conversely, no significant difference in the percentages of T cells was seen when peritoneal cells from normal controls and CY-treated animals were analysed ($P > 0{\cdot}05$). Moreover, a comparison between groups of peritoneal T cells showed that the percentage of these cells from CY-treated/treponemal-infected hamsters was significantly less than those from control/treponemal-infected animals ($P < 0{\cdot}01$). Although the infection induced an increase in the appearance of T cells, CY treatment readily diminished a discrete subset of this population. As the infection progressed, the node appeared to be a primary focus for CY-sensitive T cells. Subsequently, these cells became more easily ablated by the CY treatment. The dose of CY used significantly diminished a fraction of the T-cell populations in both naive and treponemal-infected nodes. However, with the same dose of CY, peritoneal T-cell populations from treponemal-infected animals were selectively reduced without any effect on the naive peritoneal populations. This implied that the normal peritoneum contained only small quantities of CY-sensitive T cells (Table 1). Although the B-cell quantities were minimally altered throughout the course of infection in both controls and CY-treated animals, in infected hamsters there was an increase in the percentage of B cells in both the node and peritoneum of control and CY-treated animals.

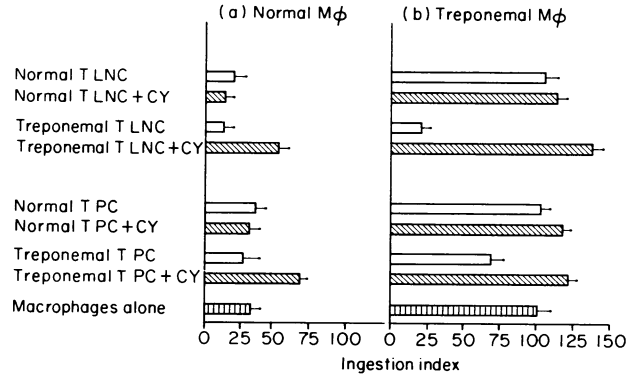


**Figure 1.** Lymph node macrophages from either normal animals (Normal Mϕ) or animals 3 weeks post-treponemal infection (Treponemal Mϕ) were co-cultured for 48 hr with either peritoneal (PC) or lymph node (LNC) T cells derived from either normal or cyclophosphamide-treated (+CY) animals with or without concurrent treponemal infection. Results are presented as the C3bMI ingestion index ±SEM of experiments performed in triplicate.

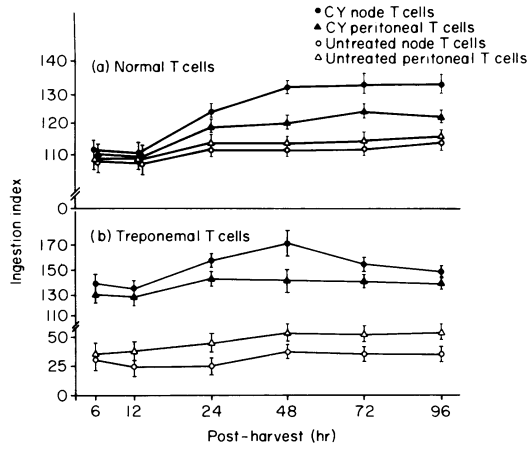


**Figure 2.** The effect of node or peritoneal T cells from cyclophosphamide (CY)-treated and/or treponemal-infected hamsters on the expression of treponemal node macrophage (IM$\phi$) activation. IM$\phi$ were co-cultured with T cells derived from either the peritoneum or the inguinal nodes of CY-treated/treponemal-infected or treponemal-infected hamsters alone. Controls consist of T cells from uninfected animals with or without CY treatment. At selected intervals post-incubation IM$\phi$ were removed from culture and assayed for activation. The results are presented as the C3bMI ingestion index of experiments performed in triplicate ± SEM.

*In vitro,* normal hamster node and peritoneal macrophages expressed only a very low level of C3bMI activity post-co-culture with naive homologous T cells (Fig. 1a). Conversely, when M$\phi$s from infected animals were co-cultured in the presence of these same naive T cells, their state of activation was markedly greater than that seen with normal M$\phi$s (Fig. 1b). A similar response was observed when these M$\phi$s were cultured alone (Fig. 1). However, incubating these M$\phi$s with T cells from treponemal-infected animals significantly reduced their activity (Fig. 1b). Normal M$\phi$ appeared to be uneffected directly by T cells from treponemal-infected animals (Fig. 1a). In parallel experiments when peritoneal T cells were substituted for node T cells the results were similar. The magnitude of inhibition produced by peritoneal T cells from infected animals was crucial. This response was significantly less than the effect that the node T cells alone ($P<0{\cdot}01$) had upon this M$\phi$ function. These results are consistant with our previous studies showing that T cells from treponemal-infected animals derived from these two separate compartments are functionally different (Tabor *et al.*, 1986).

When animals were treated with CY and concomitantly infected with treponemes, they demonstrated a significantly different pattern of activation (Fig. 1). The C3bMI responses from M$\phi$s post co-culture with T cells from CY-treated/treponemal-infected animals were markedly enhanced beyond the controls. Conversely, M$\phi$s co-cultured with T cells from CY-treated/uninfected animals did not differ from controls in their C3bMI response (Fig. 1). In parallel studies, when peritoneal T cells were substituted for node T cells similar results were produced. Again the most notable exception was that the magnitude of this activity was significantly less.

A kinetic analysis of this function demonstrated that maximum suppression occurred at 48-hr post-co-culture of M$\phi$s with T cells from treponemal-infected animals (Fig. 2b). The magnitude of this response, however, was considerably less when normal T cells were used (Fig. 2a). M$\phi$s incubated with these normal T cells generated at least a three-fold increase in activity at 6 hr without any significant reduction in function during subsequent time intervals. Conversely, when treponemal M$\phi$s were co-cultured with T cells from CY-treated/treponemal-infected animals, their response was substantially greater than that produced at 6 hr and 48 hr post-incubation with normal T cells from CY-treated animals ($P<0{\cdot}01$). Therefore, by using CY-selective sensitivity we ablated the suppressor T cells that developed during treponemal infection. However, the quantitative differences between treponemal M$\phi$ activity post co-culture with T cells from CY-treated/treponemal-infected animals and CY-treated/uninfected animals was significant at both 6 hr and 48 hr (Fig. 2). Those T cells from treponemal-infected animals that were insensitive to ablation by CY appeared to amplify the M$\phi$ response. However, when treponemal M$\phi$s were incubated with CY-treated/uninfected T cells, the activity developed much more slowly and the overall magnitude of their response was substantially less (Fig. 2).

Most significantly, the effect of node and peritoneal cells differed in their influence upon the M$\phi$. During infection, node T cells consistently elicited a greater response, whether inhibitory or stimulatory, than peritoneal T cells. The major exception to these results occurred when experiments were performed using normal untreated T cells. Using CY, we found that the differences between the compartments were amplified, with each region discretely varying in the percentage composition of CY-sensitive T cells (Table 1). Moreover, a larger population of T cells with suppressive character was localized within the node.

## DISCUSSION

Despite effective immune defences to treponemal infection, the organism often manages to evade total eradication (Baker-Zander & Sell, 1980; Pepose *et al.*, 1980; Sell *et al.*, 1980). One explanation may be that a transient state of immunosuppression is produced (Baughn, 1983; Musher *et al.*, 1975; Wright & Grimble, 1974). In new-born guinea-pigs, passive immunity to syphilis (Pavia, 1986b) may be assisted by the decreased ability of the pups to produce effective suppressor T cells. Moreover, thymectomized-irradiated bone marrow-reconstituted animals, which reputedly lack sufficient T cells (Pavia, 1986a), demonstrate an even greater resistance to treponemal infection. Other studies have shown that syphilitic T cells can suppress syphilitic macrophage activity following intradermal treponemal inoculation (Tabor *et al.*, 1984). Collectively, our results suggest that suppressor T-cell activity is promoted by treponemal infection. However, this response appears preferentially to involve regional node immunological processing since similar activity is not readily apparent when these organisms are directly introduced intraperitoneally (Tabor, Azadegan & Le Frock, 1985). The local preservation of such regional immunological differences is well documented for several infectious disease processes (Bach *et al.*, 1981; Godal, 1978).

More recently, we have demonstrated that syphilitic node T

cells can suppress syphilitic macrophages located both proximal and distal to the site of infection (Tabor *et al.*, 1986). Results from the current study extend those observations by showing that (1) the nodes from treponemal-infected hamsters contained the most predominant population of T cells with suppressive activity, and (2) selective CY elimination of these cells facilitated the restoration of a select M$\phi$ function.

In recent work it has been suggested that syphilitic infection occurs in phases, with different cell types gaining prominence as the disease progresses. These investigators found that the B cell becomes most predominant between the second and sixth weeks of infection (Bagasra *et al.*, 1985). Our results corroborate these observations. Additionally, studies also suggest that the increases in humoral activity may be associated with either a loss of suppressor T cells during this period or a dilution of their numbers which would render them functionally ineffective (Bagasra *et al.*, 1985). Our data would suggest that this marked increase in the B-cell population during treponemal infection may represent a dilution of the suppressor T-cell response.

Hamsters have at least two recognizable T-cell subpopulations (Witte, Stein-Streilein & Streilein, 1985), and the possibility for more has been strongly suggested (Witte & Streilein, 1986). Although studies have shown that the Thy 1.2 antigen is present on the surface of both hamster B and T cells (Witte & Streilein, 1983b), B cells may be distinguished from non-B cells by exploiting the presence of their surface immunoglobulins (SIg) (Witte & Streilein, 1983a). Using this information we designed experiments to enrich the cell preparations for T cells. Although this does not completely eliminate the possibility that NK cells, null cells, and other remaining unidentifiable cell types may be involved, it strongly implies that the response is attributable to the T-cell population. Moreover, recent evidence has demonstrated that the putative NK-cell activity in the LSH hamster is very low (Wright, Clark & Rawls, 1984), suggesting that its contribution to the observed results would be negligible.

The results presented here show that a CY-sensitive population is responsible for generating suppression of M$\phi$ C3bMI during treponemal infection. By using CY to dissect these groups, we found that only normal control node T-cell numbers decreased while normal control peritoneal T cells were negligibly affected. However, when cells from CY-treated/treponemal-infected populations were analysed, both the node and peritoneal cells were quantitatively diminished. Collectively, these data suggested that the number of available T cells within each compartment differs with respect to CY sensitivity. We speculate that when the host is infected, the development of suppressor T cells is accelerated. However, they are rapidly and selectively eliminated according to their CY sensitivity. The node appears to be most markedly affected since upon infection the majority of the suppressor T cells are both generated and sequestered within this region. Moreover, some of these cells may be dispersed to other areas. The mechanism by which the treponemal T cells actually inhibit M$\phi$ activity has not been determined. However, M$\phi$s are not irreversibly blocked or damaged since they can resume their activity when removed from the T-cell containing milieu. We therefore presume that they become activated prior to the emergence of a substantial suppressor T-cell population. Thus, the induction of C3bMI activity is not inhibited but it is the expression of this function that is restricted. Additional studies will be required to resolve the mechanisms responsible.

Significant advances have been made by examining the immune functions that develop during experimental syphilis. However, it remains unclear how the treponeme avoids total elimination from the host. These observations are important for probing the mechanisms that regulate some of the cell-mediated responses to treponemal infection. Moreover, they may assist in explaining how the treponeme can evade the immune defences of the host.

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