In Vitro Lymphocyte Response to *Treponema refringens* in Human Syphilis

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The response of lymphocytes from patients with syphilis and normal subjects was studied in vitro by using phytohemagglutinin (PHA), pokeweed mitogen (PWM), streptolysin O (SLO), and a preparation of *Treponema refringens*. Normal lymphocytes exhibited a dose-response curve to treponemes. Although lymphocytes from patients with primary and secondary syphilis responded normally to PHA and PWM, their response to SLO was suppressed and they failed to show significant stimulation by treponemes. Serum from syphilitic patients did not affect normal lymphocytes, and culturing lymphocytes from patients with syphilis in normal serum did not restore their responsiveness. Six to 10 weeks after syphilitic patients had been treated, the degree of stimulation by treponemes was the same as for normal subjects. These data give indirect support to the hypothesis that immunological suppression occurs during active infection with *T. pallidum*.

Several lines of evidence suggest that infection with *Treponema pallidum* stimulates a cell-mediated immune (CMI) response in the host. (i) Syphilis progresses through primary and secondary stages despite the presence of specific (*T. pallidum* immobilizing and fluorescent treponemal absorbed) and nonspecific (VDRL) antibodies (22). (ii) Cutaneous delayed hypersensitivity to treponemal antigens is associated with latency (3, 15). (iii) Migration of leukocytes from patients with tertiary syphilis is inhibited by treponemal antigens (7). (iv) Rabbits infected with *T. pallidum* resist challenge with *Listeria monocytogenes*; this resistance reaches its peak at the time that lesions are regressing (13). Progression of active syphilis might result from the failure to stimulate a CMI response or from suppression of the CMI response. In the present paper we describe studies that show that lymphocytes from syphilitic patients have a decreased lymphoblastic response to stimulation in vitro with *T. refringens*.

MATERIALS AND METHODS

**Selection of subjects.** Patients with clinical diagnoses of syphilis were identified at the Venereal Disease Clinic, City of Houston Public Health Laboratories. The diagnosis of primary syphilis was made if a chancre was present and *T. pallidum* was demonstrated by darkfield microscopy. Patients with secondary syphilis had a typical, generalized papulosquamous eruption; palmar and plantar lesions, condyloma lata, and/or alopecia were frequently present. All patients thus selected had a positive VDRL reaction.

Control subjects with negative VDRL reactions were matched by race, sex, age, and approximate socioeconomic status from premarital examinees at the City Health Laboratories and laboratory personnel or maintenance workers at the Houston Veterans Administration Hospital. Rights of privacy were assured to all subjects, and an informed consent was signed.

**Lymphocyte transformation.** Lymphocyte transformation was studied by using a previously described method (4). Briefly, blood was defibrinated with swirling with glass beads. Leukocytes were separated by dextran sedimentation, washed in Spinner modified minimal essential medium without glutamine (Difco), and resuspended to yield 5 x 10⁶ lymphocytes in 0.7 ml. Glutamine and neomycin were added to give 5.8 and 100 μg/ml, respectively. Serum (0.3 ml) from syphilitic or normal subjects and mitogens (see below) were then added, and the tubes were loosely capped and placed into a CO₂-enriched humid atmosphere at 37 C. One microliter of [3H]thymidine was added on the morning of the 5th day, and 2 h later the reaction was stopped by placing tubes into an ice bath. Lymphocytes were recovered by cold centrifugation and washed in saline at 4 C. Deoxyribonucleic acid (DNA) was precipitated by the addition of trichloroacetic acid, dissolved in Soluene-100 (Packard), and washed into glass scintillation vials with scintillation fluid (0.01% 1, 4-bis-(5-phenyloxazolyl)-benzene, 0.5% 2, 5-diphenyloxazole). The amount of [3H]thymidine that had been incorporated into DNA was counted in a Packard liquid scintillation spectrometer for 5 min.

**Antigens.** Phytohemagglutinin M (PHA; Difco), 0.05 ml; pokeweed mitogen (PWM; Gibco), 0.05 ml;...
and streptolysin O (SLO; Difco), 0.1 ml were used in lymphocyte cultures. Treponemal antigens were prepared as follows. T. refringens was grown in spiroplate broth (8) at 35 C for 5 days; the final concentration was 8 \times 10^7 per ml. These organisms were isolated by centrifugation at 4 C (10,000 \times g for 25 min), washed four times with saline, and resuspended to give 2 \times 10^8 per ml. Protein concentration was determined by the Biuret method, and dilutions were made in saline so that 0.05 ml contained amounts of treponemal protein (TP) ranging from 0.17 to 140 \mu g. These treponemal suspensions were divided into samples and stored at -70 C; one sample was used for each study. In our initial studies three other antigenic preparations were investigated: spiroplate broth in which treponemes had grown for 5 days; the first wash (presumably rich in cell envelope); and sonicated treponemes (Bio- sonik III, small probe, 50% output for 3 min; darkfield examination showed complete disruption). Each of these preparations had been dialyzed in tapwater at room temperature for 24 h and then overnight in saline at 4 C. The treponemal wash was concentrated by evaporation. Protein concentrations were determined, and a range of concentrations was studied similar to that for TP. Sonicated treponemes gave identical results to whole treponemes and were not used after the initial studies. Stimulation of normal lymphocytes by the first wash or by low concentrations of spiroplate broth was not detected; higher concentrations of the broth were suppressive. These preparations were not used in the studies reported in this paper.

**Statistical analysis.** All studies were carried out in triplicate. Values for each tube in counts per minute after subtraction for background were converted to log_{10} and averaged. The analysis of variance (14) was used. Because unequal numbers of subjects were present in each group, the means were weighted. Fischer's least significant difference test was used to examine pairs of means when a significant F ratio indicated reliable mean differences. The alpha level was set at 0.05 prior to the initiation of the experiment.

**RESULTS**

**Normal control subjects.** Lymphocytes from normal subjects incubated with their own serum incorporated \(^{3}H\) thymidine to give 2.146 ± 0.143 counts/min. Addition of PHA, PWM, and SLO increased these counts to 4.506 ± 0.015, 4.268 ± 0.059, and 4.358 ± 0.239 counts/min, respectively (Table 1). Addition of increasing concentrations of treponemes gave a dose-response curve with a peak increase of 23 times base-line values (3.505 ± 0.189) at 14 \mu g of TP per ml and suppression at 140 \mu g/ml (Fig. 1). The difference between \(^{3}H\) thymidine incorporation at rest and after exposure to treponemes was statistically significant (P < 0.001) for every concentration of TP between 0.17 and 70

### Table 1. Response of lymphocytes to mitogens in normal and syphilitic serum

<table>
<thead>
<tr>
<th>Lymphocytes</th>
<th>Serum</th>
<th>Conditions of lymphocyte culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unstimulated</td>
</tr>
<tr>
<td>Normal (12)*</td>
<td>Own</td>
<td>2.146 ± 0.143</td>
</tr>
<tr>
<td></td>
<td>Syphilitic</td>
<td>2.301 ± 0.249</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>1.956 ± 0.369</td>
</tr>
<tr>
<td>Primary syphilis (12)</td>
<td>Own</td>
<td>1.914 ± 0.269</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>2.094 ± 0.184</td>
</tr>
<tr>
<td>Secondary syphilis (8)</td>
<td>Own</td>
<td>2.037 ± 0.263</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>2.270 ± 0.234</td>
</tr>
<tr>
<td>Primary and secondary syphilis after treatment</td>
<td>Normal</td>
<td>2.177 ± 0.122</td>
</tr>
</tbody>
</table>

* Peak stimulation (14 \mu g of TP per ml).
* Number of subjects in each group is in parentheses.

**FIG. 1. Responses of lymphocytes from normal subjects (○), patients with primary (■) and secondary (▲) syphilis, and syphilitic patients 6 to 10 weeks after treatment (○) to T. refringens. Data are reported as mean log_{10} counts/min after correction for background. The variance is indicated by brackets. In each case data are shown for lymphocytes in the subject's own serum. The difference between normal and syphilitic lymphocytes is significant at every point from 0.17 to 70 \mu g of treponemal protein per ml (P < 0.001). The variance is not given for treated patients at 140 \mu g of TP, since only one patient was studied at this concentration.**
µg/ml. Nearly identical results were obtained for lymphocytes from normal subjects in the presence of serum from patients with primary or secondary syphilis (Table 1).

**Syphilitic patients.** Unstimulated lymphocytes from patients with primary or secondary syphilis incubated in their own serum incorporated the same amount of [³H]thymidine as did those from normal subjects, and the addition of PHA and PWM induced the same degree of lymphoblastic transformation (Fig. 1 and Table 1). Stimulation by SLO was significantly less for primary and secondary syphilic patients than for normal controls (3.713 ± 0.596 and 3.811 ± 0.797, respectively, compared to 4.358 ± 0.239; \( P < 0.05 \)). The uptake of [³H]thymidine by syphilitic lymphocytes was significantly less than that of normal lymphocytes at all concentrations of TP between 0.17 and 70 µg/ml (\( P < 0.001 \); Fig. 1). In fact, exposure to treponemes did not significantly increase thymidine uptake by syphilitic lymphocytes at any concentration of TP (\( P > 0.05 \)). Identical results were obtained when syphilitic lymphocytes were incubated in pooled normal serum (Table 1).

**Syphilitic patients after treatment.** These same studies were repeated 6 to 10 weeks after syphilitic patients had been treated with curative doses of penicillin (or tetracycline in one case of allergy to penicillin). Only nine of the syphilitic patients (five primary and four secondary) could be located for follow-up studies. Lymphocyte transformation during exposure to treponemes, SLO, PHA, and PWM was then identical to that of normal control subjects. There were no differences when the results for five treated primary and four treated secondary syphilic patients were compared, and the data for all nine patients are combined in Fig. 1 and Table 1.

**DISCUSSION**

Our studies show that exposure to *T. refringens* stimulates lymphocytes from normal subjects to undergo blastic transformation in vitro. The resulting dose–response curve is consistent with natural exposure to antigenically related treponemal antigen in vivo. Lymphocytes from patients with primary or secondary syphilis exhibited marked depression of this normal response. Six to 10 weeks after treatment, the response of lymphocytes from syphilitic patients to *T. refringens* had returned to normal.

*T. refringens* was used as the source of treponemal antigen for these studies. *T. pallidum*, the virulent organism that causes syphilis, has been cultivated only in vivo, and vigorous attempts to separate it from rabbit testicular material partially disrupt the cell envelope (16). In contrast, *T. refringens* can be grown in vitro and isolated by washing. This avirulent organism is known to share common antigens with other treponemes, including *T. pallidum* (6, 9, 11).

Suppression of lymphocyte reactivity to specific antigens from the causative organism has been described for disseminated tuberculosis (21), histoplasmosis (1), and cryptococcosis (5). In the case of miliary tuberculosis, decreased response of lymphocytes to tuberculin in vitro has been associated with decreased production of migration inhibiting factor and absence of cutaneous delayed hypersensitivity (21). This phenomenon is thought to result from desensitization of lymphocytes by antigen overload (2, 12, 19, 20); this same situation might well apply to syphilis and might explain the progression of syphilitic lesions in the presence of circulating immobilizing antibodies. We found the suppressed response to be a property of the lymphocytes themselves, although one preliminary study had suggested that serum from patients with syphilis suppressed the reaction of normal lymphocytes to PHA (10). Our data show that serum from syphilitic patients does not affect the stimulation of normal or syphilitic lymphocytes by any of the mitogens studied.

In syphilitic patients the lymphoblastic response to SLO was reduced, suggesting that a generalized suppression of the immune response to specific antigens might occur; however, these results barely achieved statistical significance. This suppression did not involve nonspecific mitogens such as PHA and PWM. These results are similar to those seen in disseminated cryptococcosis (5). Turner and Wright recently described lymphocyte depletion of paracortical areas in syphilitic lymph nodes (17), which would be consistent with selective or generalized impairment of thymus-dependent immunity.

If a CMI response brings about latency in syphilis (13, 18, 22) and if lymphocyte reactivity in vitro truly reflects cell-associated immune events, it should be possible to demonstrate an increased response to treponemal antigens in individuals with latent syphilis. Eleven patients with this diagnosis were studied. Lymphocyte response was generally found to be suppressed just as in patients with active infection. However, the diagnosis of latent syphilis was based upon the presence of positive VDRL and FTA-ABS tests in the absence of clinical manifestations. All of our patients had received some kind of antibiotic treatment in the recent past. From
an immunologic point of view they may have had partially suppressed active syphilis rather than the truly latent disease which develops from untreated secondary infection.

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LITERATURE CITED


