Studies of rabbit testes infected with *Treponema pallidum*

III Immunosuppressive activity of infiltrating mononuclear cells

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SUMMARY  When mononuclear cells infiltrating rabbit testes infected with *Treponema pallidum* were cocultured with autologous or homologous peripheral blood lymphocytes spontaneous stimulation and that induced by concanavalin A were suppressed. The inhibition was not due to the cytotoxic effect of the mononuclear cells or to their interference with the active site of concanavalin A (competitive inhibition). The suppressor activity was present in both T and non-T cells but was not affected by pretreatment of the mononuclear cells with indomethacin, a prostaglandin synthetase inhibitor. The suppressor activity may be intrinsic to the mononuclear cells or acquired by the cells in the testicular environment.

Introduction

*Treponema pallidum* infection is associated with an accumulation of mucoid material largely composed of hyaluronic acid and chondroitin sulphate. The material has been associated with virulence of *T pallidum* but as reported recently it does not seem to contain *T pallidum* antigen and is likely to be of host origin. Fitzgerald *et al* have reported that testicular fluid from syphilitic rabbits contains immunosuppressive substances which can reactivate treponemal lesions and inhibit the proliferative response of autologous rabbit lymphocytes to concanavalin A. We have observed similar inhibitory activity in the serum and testicular fluid from infected rabbits in experiments using phytohaemagglutinin. It is not clear what role these inhibitory substances play in the initial growth of *T pallidum* or in the host response at the site of infection. We have observed that *T pallidum* organisms which had been washed three times suppressed the deoxyribonucleic acid (DNA) synthesis of autologous rabbit peripheral blood lymphocytes in a direct lymphocyte transformation test. Supernatant of identical cells cultured for 48 hours, however, contained a significant amount of mitogenic factor which could only be detected by using it diluted in secondary cultures containing fresh autologous peripheral blood lymphocytes.

In this report we present some data on the immunological competence of the cells infiltrating the testes. The results indicate that more than one immunoregulatory mechanism may be implicated in the deficient clearance of the virulent treponemes and in delayed immunity in the infected host.

Material and methods

**PREPARATION OF ORCHITIC TESTICULAR CELLS**

Adult Nys (Flemish Giant) rabbits were inoculated intratesticularly with about 20 × 10⁶ *Treponema pallidum* (Nichols strain) per testis. Infected testes were removed aseptically at the peak of inflammation (days 9-12) and processed as described earlier in this series. The testicular cell suspension consisted of about 70-75% mononuclear leucocytes, 2-5% polymorphonuclear leucocytes, and 20-30% cells of testicular origin, including a few spermatozoas. The few red cells contaminating the testicular cell suspension were removed by lysis with cold hypotonic saline (0-2%) for 2 minutes, after which an equal volume of hypertonic saline (1·6%) was added to bring the solution to isotonicity. The yield of testicular cell suspension from each pair of testes varied, containing 3 × 10⁶ to 20 × 10⁶ cells, nearly all (>95%) of which were viable.
IDENTIFICATION OF MONONUCLEAR CELLS IN THE TESTICULAR CELL SUSPENSION

Fresh testicular cell suspension was treated with fluorescein isothiocyanate labelled bovine IgG anti rabbit thymocyte (previously absorbed with rabbit testicular cells) or with fluorescein labelled goat IgG F(ab')2 fragment anti rabbit immunoglobulins (Cappel Laboratories, Cochrantonville, Pennsylvania, United States), according to the procedure described.10 Macrophages in a testicular cell suspension depleted of testicular cells (Tcd-TCS) were examined by ingestion of latex particles10 and by histochemical staining of fixed smears of Tcd-TCS with alpha naphthyl acetate to show cytoplasmic non-specific esterases.11

PREPARATION OF TESTICULAR CELL SUSPENSION DEPLETED OF TESTICULAR CELLS AND T LYMPHOCYTES

The various cell suspensions were prepared as described earlier.9 For this experiment, however, the goat anti rabbit thymocyte serum was rendered specific for T cells by three additional absorptions with 10% rabbit macrophages and four with 10% bone marrow. This antiserum was cytotoxic for approximately 60% of peripheral blood lymphocytes, 85% of lymph node cells, and 65% of spleen cells.

LYMPHOCYTE TRANSFORMATION

Samples of peripheral blood lymphocytes or testicular cell suspension purified with Ficoll-Hypaque were resuspended in RPMI (Roswell Park Memorial Institute) 1640 medium containing antibiotics and 10% inactivated fetal calf serum. Triplicate samples of each suspension were incubated in microplates for 48 hours at 37°C in a 5% carbon dioxide incubator with 4 μg concanavalin A (con A) per culture or with phosphate buffered saline (PBS) as control. Six hours before the end of incubation tritiated thymidine (3H-TdR) were added. Uptake was measured in a liquid scintillation spectrophotometer, and the average counts per minute (cpm) of triplicate samples were estimated. The net cpm was obtained by subtracting that of non-stimulated from that of stimulated cultures.

EFFECT OF TESTICULAR CELL SUSPENSION ON MITOGENIC RESPONSE OF NORMAL RABBIT PERIPHERAL BLOOD LYMPHOCYTES

Triplicate 0·1 ml samples (0·2 × 10⁶ cells) of cryopreserved autologous peripheral blood lymphocytes (taken before infection) or fresh homologous peripheral blood lymphocytes (which we called responder cells (R)) were cocultured with 0·1 ml of irradiated (2000 rad) testicular cell suspension (TCSx) containing 0·2 × 10⁶ cells (which we called modulator cells (M)). Control cultures received similar numbers of irradiated peripheral blood lymphocytes (PBLx). The cultures were each incubated with 4 μg con A or with PBS. To minimise any competitive binding12 for the mitogen, it was added to the responder cells 30-40 minutes before the modulator cells were added. This timing had been established by preliminary experiments in which various concentrations of the polysaccharide methyl-a-D-mannoside (MAM), which competes for binding to con A, were ineffective if added to responder cells 30 minutes after the mitogen and left in the culture during the incubation period.

DNA synthesis was measured as described above. The net cpm for each set of cultures was determined, and the percentage of inhibition was calculated as follows:

\[
1 - \frac{\text{cpm (PBL + TCSx)}}{\text{cpm (PBL + PBLx)}} \times 100 = \% \text{ inhibition}
\]

To determine separately the effects of testicular or lymphoid cells, the testicular cell suspension was also examined after depletion of testicular cells, T lymphocytes, or both by complement mediated cytotoxicity.

INDOMETHACIN TREATMENT

Testicular cell suspension depleted of testicular cells (Tcd-TCS) was incubated for 1 hour at 37°C with a 10⁻⁴ to 10⁻⁶ mol/l solution of indomethacin (Sigma Chemical Co, St. Louis, Missouri, United States), which is an irreversible inhibitor of prostaglandin synthetase.13 A stock solution in 95% alcohol was freshly prepared and diluted with RPMI 1640 medium in a 1:100 ratio before use. After treatment with indomethacin the Tcd-TCS was well washed and irradiated for use in cultures as modulator cells. In other experiments indomethacin was present in the culture throughout the incubation period.

NYLON WOOL FRACTIONATION

To further identify cells with inhibitory activity, the testicular cell suspension depleted of testicular cells (Tcd-TCS) was subjected to nylon wool fractionation to separate adherent from non-adherent cells.14 To this end 10-20 × 10⁶ cells in 0·5 ml Eagle's minimal essential medium with 5% inactivated fetal calf serum was pipetted into a 5 ml syringe containing 0·3 g nylon wool (Associated Biomedical System, Buffalo, New York, United States) which had been washed and equilibrated in the same medium. After incubation for 45 minutes at 37°C the non-adherent cells were eluted from the column with 10 ml of warm medium. Adherent cells could be recovered by pressing and squeezing the nylon wool with forceps.
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several times while washing the column with medium.

Results

MITOGENIC RESPONSE OF TESTICULAR CELL SUSPENSION

The suspension prepared from cells extracted from orchitic testes consisted mostly of mononuclear leukocytes (70-75%). Their response to con A was therefore measured and compared with that of autologous peripheral blood lymphocytes. Results of paired testicular cell suspension and peripheral blood lymphocytes from 12 animals infected with *T. pallidum* bled and killed between days 9 and 11 were compared (fig 1). The testicular cell suspension response to con A was significantly (*p<0.005*) reduced, compared with that of the corresponding peripheral blood lymphocytes. In the testicular cell suspension the cpm ranged from 4020 to 11 000 (mean 1967) whereas in the peripheral blood lymphocytes it ranged from 7500 to 163 000 (mean 67 011). Although testicular cell suspension had fewer lymphocytes than did the peripheral blood lymphocytes, this difference could not explain the relatively low mitogenic response. We therefore considered the possibility that some of the cells infiltrating the testes may interfere with the proliferative response.

MODULATORY EFFECT OF TESTICULAR CELL SUSPENSION

To examine the above hypothesis, well washed irradiated testicular cell suspension was cocultured with cryopreserved autologous peripheral blood lymphocytes at three ratios of responder to modulator cells. Inhibition related to ratio was observed (fig 2), with the maximum (95% inhibition) at a ratio of 2:1, less (90% inhibition) at 4:1, and only slight (17% inhibition) at 20:1. Binding of the lectin to the lymphocytes seemed essentially complete within minutes of addition. Thus addition of 50-200 μg methyl-α-D-mannoside (MAM) to responder cells 30 minutes after the addition of con A was ineffective in blocking the binding or the mitogenic activity started by it (table I). Irradiated testicular cell suspension also affected the spontaneous blast transformation of autologous or homologous

![Fig 1](http://sti.bmj.com/)

**Fig 1** In vitro lymphocyte transformation. Comparison of responses to concanavalin A of testicular cell suspension (TCS) and autologous peripheral blood lymphocytes (PBL) from 12 rabbits infected with *T. pallidum.*

![Fig 2](http://sti.bmj.com/)

**Fig 2** Inhibition by irradiated testicular cell suspension (TCSx) of response to concanavalin A of cryopreserved peripheral blood lymphocytes (PBL) dependent on ratio of responder to modulator cells. Control used irradiated peripheral blood lymphocytes (PBLx).
lymphocytes although the degree of inhibition varied widely (9-89%). The inhibitory activity was similar whether autologous or fresh homologous peripheral blood lymphocytes were used. In most of the following experiments, therefore, fresh homologous lymphocytes were used as responder cells, and the ratio of responder to modulator cells was 4:1 unless otherwise indicated.

**EFFECT OF TESTICULAR CELL SUSPENSION FROM HEALTHY RABBIT TESTES**

As indicated earlier the testicular cell suspension consisted mostly of infiltrating mononuclear cells, with a small percentage of testicular cells. As substances with immunosuppressive activity have been found in mammalian seminal plasma and spermatozoa,\(^\text{16-18}\) it seemed feasible that the inhibitory activity of the testicular cell suspension originated from the contaminating testicular cells. To examine this possibility, testicular cell suspensions were prepared from healthy rabbit testes exactly as from infected testes, irradiated, and used as modulator cells. Results from three experiments (fig 3) showed inhibition related to ratios of responder to modulator cells, which, although considerable (71% inhibition) at ratios of 2:1 and still high (40% inhibition) at 4:1, was much lower than that exerted by the testicular cell suspension from orchitic tissue which contained no more than 30% testicular cells.

**MODULATORY EFFECT OF TESTICULAR CELL SUSPENSION DEPLETED OF TESTICULAR CELLS**

Testicular cells and spermatozoa may contribute to the inhibitory activity of the testicular cell suspension. Their low percentage at the peak of inflammation, however, and the finding in our previous studies\(^\text{9}\) that removal of testicular cells did not always enhance mitogenic stimulation in the remaining cells, led us to consider the possibility that both testicular cells and infiltrating mononuclear cells may contribute to the inhibitory activity of the testicular cell suspension. To test this hypothesis, irradiated testicular cell suspension depleted of testicular cells (TCD-TCSx) was used as a source of modulator cells. The elimination of testicular cells caused only a partial decrease of the inhibitory activity; substantial (58-93%) inhibition was still exerted by the testicular cell suspension depleted of testicular cells (TCD-TCSx) (table I).

**EFFECT OF TESTICULAR CELL SUSPENSION DEPLETED OF T CELLS AND TESTICULAR CELLS ON MITOGENIC RESPONSE OF HOMOLOGOUS PERIPHERAL BLOOD LYMPHOCYTES**

Since the testicular cell suspension depleted of testicular cells consisted mostly of T and B cells and...
TABLE II  Effect of TcS before and after treatment with antiserum to TC (TcD-TcS) on mitogenic response of normal rabbit homologous PBL (R cells)*

<table>
<thead>
<tr>
<th>Responder (R) cells</th>
<th>Modulator (M) cells</th>
<th>Con A</th>
<th>Mean (SD) cpm</th>
<th>Net cpm</th>
<th>Percentage inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBL</td>
<td>PBLx</td>
<td>No</td>
<td>1149 (18)</td>
<td>180477</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>181626 (9785)</td>
<td>180477</td>
<td></td>
</tr>
<tr>
<td>TcS</td>
<td>No</td>
<td>565 (63)</td>
<td>18570</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>19135 (13565)</td>
<td>18570</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>TcD-TcS</td>
<td>No</td>
<td>548 (95)</td>
<td>46949 (33601)</td>
<td>46401</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>46949 (33601)</td>
<td>46401</td>
<td>74</td>
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</table>

*Results of three representative experiments; TC = testicular cells; TcD-TcS = testicular cell suspension depleted of testicular cells; for meaning of other abbreviations see table I.

TABLE III  Effect of TcD-TcS before and after treatment with antiserum to rabbit T cells (Td-TcD-TcS) on mitogenic response of normal rabbit homologous PBL (R cells)*

<table>
<thead>
<tr>
<th>Responder (R) cells</th>
<th>Modulator (M) cells</th>
<th>Con A</th>
<th>Mean (SD) cpm</th>
<th>Net cpm</th>
<th>Percentage inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBL</td>
<td>PBLx</td>
<td>No</td>
<td>2793 (359)</td>
<td>222377</td>
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<td></td>
<td>Yes</td>
<td>225170 (4281)</td>
<td>222377</td>
<td></td>
</tr>
<tr>
<td>TcD-TcS</td>
<td>No</td>
<td>2626 (845)</td>
<td>54774</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>57401 (3647)</td>
<td>54774</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Td-TcD-TcS</td>
<td>No</td>
<td>1742 (67)</td>
<td>78822 (19354)</td>
<td>78080</td>
<td>65</td>
</tr>
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<td>Yes</td>
<td>78822 (19354)</td>
<td>78080</td>
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<td></td>
</tr>
</tbody>
</table>

*Results of three representative experiments; Td-TcD-TcS = testicular cell suspension depleted of T cells and testicular cells; for meaning of other abbreviations see tables I and II.

macrophages, the next step was the elimination of the T cells. The use of testicular cell suspension depleted of testicular cells and T cells (TcD-TcS) as modulator cells indicated that elimination of T lymphocytes lowered the inhibitory activity of the testicular cell suspension depleted of testicular cells but did not totally reverse it (table III).

Table IV shows that T cells were removed from the preparation. The response to the T cell mitogen con A was virtually suppressed after treatment with anti-serum and complement.

TABLE IV  Con A stimulation of TcD-TcS before and after treatment with goat anti-rabbit thymocyte serum (Ats)*

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Con A</th>
<th>Mean (SD) cpm</th>
<th>Net cpm</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>No</td>
<td>1614 (160)</td>
<td>69133</td>
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<td>70747 (12373)</td>
<td>69133</td>
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<td>Ats</td>
<td>No</td>
<td>1316 (114)</td>
<td>710</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>2026 (482)</td>
<td>710</td>
</tr>
</tbody>
</table>

*Results of two representative experiments; for meaning of abbreviations see tables I and II.

INDOMETHACIN TREATMENT OF MODULATOR CELLS

The substantial inhibition by testicular cell suspension depleted of both testicular cells and T lymphocytes suggested that the remaining cells, mostly macrophages and B cells, may also play some role in the observed inhibitory activity. It was technically difficult to remove either population from the testicular cell suspension depleted of testicular cells because of the low number of available cells and the similarity of their adherence properties. We attempted, however, to further characterise the nature or cellular origin of the suppressor factor(s) by using testicular cell suspension depleted of testicular cells. As prostaglandins are effective inhibitors of lectin induced DNA synthesis by T cells, and since macrophages are one of the main cell populations producing prostaglandins,10 we pretreated testicular cell suspension depleted of testicular cells with freshly prepared indomethacin before irradiating it and adding it to the responder cells. In other experiments indomethacin was added to the cultures. No significant change in the inhibitory activity after either treatment with indomethacin was observed (table V).

ACTIVITY OF CELLS ADHERENT TO NYLON WOOL

Nylon wool fractionation of testicular cell suspension depleted of testicular cells provided a non-adherent cell population comprising mostly T cells (about 92%) and a very small percentage of B cells (about 6%). The adherent population contained up to 89% non-specific esterase positive cells and approximately 4% T and 7% B cells. Examination of the unfractionated testicular cell suspension depleted of testicular cells (TcD-TcS) in parallel with the non-
adherent testicular cell suspension depleted of testicular cells (NA-TCd-TCS) as modulator cells indicated that inhibitory cells were present in both fractions (table VI).

Discussion

Our data show that infection of rabbit testes with virulent T pallidum leads to an infiltration with mononuclear cells which have a potent inhibitory activity on con A stimulation of autologous or homologous peripheral blood lymphocytes. This suppressor activity related to the ratio of responder to modulator cells and was not due to the cytotoxic effect of the mononuclear cells. Several reports have shown that healthy rabbit testes and those infected with T pallidum contain substances with immunosuppressive activity. Most of these substances are soluble factors which are contained in seminal plasma or testicular fluid and presumably carried by the testicular cells and spermatozoa.

In our studies testicular cell suspension obtained at the peak of inflammation contained no more than 30% cells of testicular origin, including spermatozoa, yet its immunosuppressive activity was much greater than that of similar preparations from healthy testes, which contained a higher proportion of spermatozoa. The small percentage of contaminating testicular cells could in most cases be almost totally removed by one or two cycles of complement mediated cytotoxicity, leaving a population of mostly mononuclear leucocytes. The use of irradiated testicular cell suspension depleted of testicular cells or subfractions of it as modulator cells indicated that the suppressor activity was attributable to more than one cell population. Eliminating T cells from the testicular cell suspension depleted of testicular cells thus only partially reversed the inhibitory effect while virtually suppressing the response to con A. Nylon wool fractionation, on the other hand, provided non-adherent and adherent cell populations which both had inhibitory activity.

We did not confirm the possibility that macrophages might play some role in the suppression through production of prostaglandin. Experiments with modulator cells pretreated with indomethacin or cultures containing indomethacin during the incubation period showed no reversal of the suppressor activity. Many other mechanisms of macrophage suppression not explored in these studies are not excluded by the above results, and a possibility that the infiltrating B cells have a role cannot be excluded. An alternative which should also be considered is that the suppressor activity of the testicular cell suspension depleted of testicular cells is not intrinsic to the cells but caused by substances from the testicular environment which are passively carried by the mononuclear cells.

The suppressive substances may originate from the testis itself or be produced during the inflammatory process and the local growth of treponemes. They seem to be tightly bound to the surface of the mononuclear cells as they were unaffected by repeated washes and the prolonged experimental treatments.
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Taking these conditions into account, two mechanisms of suppression can be postulated: competitive binding with the active site of the lectin, or a direct effect on T cell response to the mitogen. The first possibility seems improbable. To secure the binding of lectin to the peripheral blood lymphocytes thus minimising any competitive inhibition, all modulator cells were added at least 30 minutes after the addition of con A to the responder cells, and a ratio related inhibition of the mitogenic response was observed in cultures containing the modulator cells. At concentrations of 50-200 µg per culture, however, methyl-α-D-mannoside, the specific competitive inhibitor for con A, was unable to block the mitogenic response of responder cells.

The second mechanism, inhibition by direct interference with the T cell response, is more feasible. Prakash et al. have shown that a seminal plasma protein of molecular weight above 100 000 inhibits both the primary antibody response of normal spleen cells to thymus dependent antigens and the one way mixed lymphocyte reaction. In addition, this protein inhibited the spontaneous proliferative response of lymphocytes and that induced by con A. In earlier studies we found that fluid from testes infected with *T. pallidum* and a suspension of washed *T. pallidum* were equally inhibitory to DNA synthesis by normal rabbit homologous lymphocytes, which suggested that substances of treponemal or testicular origin present in the infected testes could be firmly attached to the treponemes. Similarly, the substances could firmly attach to cells, but we do not know whether the suppressor activity of the testicular cell suspension depleted of testicular cells originates from or relates to the mononuclear cells (lymphokines, monokines, and immune complexes) or is passively acquired by the cells in the testicular environment. Whatever its origin, the modulatory effect on the T cell proliferative response is undeniable. Its relevance to the slow clearance of treponemes from the site of infection and to the somehow deficient local production of IgG merits further investigation.

References

11. Anonymous. For cytologic demonstration of esterases, naphthol as-d chloracetate esterase and a-naphthyl acetate esterase in blood or bone marrow films and tissue imprints or section. St Louis: Missouri: Sigma Chemical Co, 1979; No. 90 A-1.