Circulating immune complexes in experimental syphilis and their relation to immunological response against *Treponema pallidum*

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1. SUMMARY

It was found that circulating immune complexes (CIC) were formed in rabbits at different times after infection with *Treponema pallidum*. The CIC which appeared at the beginning of the disease were short-lived (2–6 weeks) but those appearing later than 20 weeks after infection remained for 10–25 weeks. CIC contained both IgM and IgG classes of immunoglobulin. The antibodies present in CIC were found to be specific and nonspecific for *T. pallidum*. The presence of CIC led to a marked decline of treponemal antibodies in rabbit sera. The cell-mediated immune response measured by the macrophage migration inhibition (MMI) test at the beginning of the disease (up to 12 weeks) was not decreased. However, when syphilis lasted for more than 14 weeks and when CIC were formed mainly from IgG, a distinct decrease in the ability of lymphocytes to cause MMI was observed. These findings strongly suggest that IgG-complexes suppress the immunological responsiveness of lymphocytes against *T. pallidum* which in turn facilitates the multiplication of treponemes in the host.

2. INTRODUCTION

Recent data have indicated that a cell-mediated immune response is involved in protection against *Treponema pallidum* infection [1,2]; despite such a response, self-cure is exceptional. The question arises as to what is the reason for syphilis development in spite of the presence of immunologically competent cells in the host. Numerous explanations were suggested; one of the most probable mechanisms is a suppression of the cell-mediated immune response indicated by the lack of macrophage migration inhibition, (MMI) [1]. There was no agreement on the timing of, and reason for, this suppression in syphilis. Immune complexes are formed in different diseases and can stimulate or inhibit immunological response of lymphocytes [3], and they may play a similar role in syphilis. To shed some light on this problem it was decided
3. MATERIALS AND METHODS

3.1. Animals

Danish albino rabbits of both sexes (6 months old, 3 kg) were obtained from the Breeding Centre of this Institute. At the start of experiments they had negative VDRL and TPI reactions. Rabbits were given antibiotic-free, granulated food and water ad libitum. Twice a week the diet was supplemented with green forage.

3.2. Treponema pallidum

The virulent Nichols strain of *T. pallidum* was used throughout. Infection of rabbits was done by a single injection of 2.0 ml of treponemal suspension \(1.8-2 \times 10^7 \text{ ml}^{-1}\) intravenously.

3.3. Detection of circulating immune complexes

The assay for immune complexes (CIC) was done by three methods: (a) precipitation of sera with 3.5% (w/v) polyethylene glycol PEG-6000 Fluka AG and measurement of absorbance at 280 nm according to Bout et al. [4]. Briefly, to 3.0 ml of serum diluted 1/10 with 0.2 M borate buffer pH 8.4, 4.5 ml of PEG solution (5% (w/v) concentration in borate buffer) was added to obtain the final 3.5% (w/v) concentration of PEG. After 2 h incubation at 4°C the samples were centrifuged for 20 min at 4000 rpm. The sediments were washed 3 times with 3% PEG. One sediment prepared from the same serum was dissolved in distilled water equal in volume to the initial volume of serum (0.3 ml). The absorbance of this dissolved PEG-precipitate was determined with an aliquot of the aqueous immune complexes solution diluted 1/10 with 0.1 M NaOH (2.7 ml). The measurement was done in a Specord UV VIS Carl Zeiss Jena at 280 nm in a 1 cm cuvette. As a control we used: (1) 0.3 ml of distilled water and 2.7 ml of 0.1 N NaOH and, (2) PEG-sediment from normal rabbit sera dissolved in water and diluted to 1/10 with NaOH; (b) immunoelectrophoretic analysis of PEG-precipitates according to Świerczeńska et al. [5,6]. The second PEG-sediment of the same sera were dissolved in 0.1 ml of phosphate buffer (Sörensens) pH 7.2 and were tested in agar gel (1.5%) in immunoelectrophoresis according to Scheidegger [7], with goat antisera to rabbit immunoglobulin (Heitel, Vienna) or from the Institute of Sera and Vaccines (Prague), and monovalent goat antisera to rabbit IgG (Laboratory of Sera and Vaccines, Warsaw). The control serum was rabbit IgG isolated from pooled fresh rabbit sera by filtration on Sephadex G-200. The fraction containing IgG was concentrated by ultrafiltration to the initial volume of serum and divided into 0.1 ml amounts and stored at \(-70^\circ\text{C}\.\) Immediately before assay samples were thawed, diluted 1/40 and divided into two aliquots. One was used as nonaggregated and the other was aggregated by heating it at 63°C for 10 min according to Świerczeńska et al. [5]. The controls were PEG-precipitates from normal rabbit sera and fresh rabbit sera aggregated and not aggregated; and (c) platelet aggregation test (PA) described by Haeney [8] with slight modifications. The platelets were isolated, not as originally recommended by adding acid-citrate-dextrose to peripheral venous blood to obtain the platelet-rich supernate, but the blood was defibrinated and centrifuged on a gradient where two solutions of different density and viscosity were prepared according to Tellez and Rubinstein [9] with slight modifications. Uropolinum (Polfa) (natrium amidotrizoate, 1.6 g, N-methylglucaminum amidotrizoate, 10.4 g and sterile distilled water, 20 ml) was used instead of Hypaque M. To maintain reproducibility the platelets were freshly isolated from the same preselected rabbit donors and every serum was examined with platelets from two donors. The test was done in Terasaki plates (Falcon plastic). To 2.0 \(\mu\text{l}\) of serum diluted 1/20–1/640, an equal volume of the platelet suspension \((4 \times 10^8 \text{ ml}^{-1})\) was added. The mixture was incubated at 4°C overnight. As controls platelets were suspended in platelet buffer, normal rabbit serum or in aggregated IgG. The test was read in a phase contrast microscope (magnification \(\times 500\). Platelet aggregation at a serum dilution greater than 1/20 was recorded as positive IE contained.
3.4. The VDRL slide flocculation test

This test was performed as described in the Manual of Tests for Syphilis [10].

3.5. The TPI test

This test was carried out by the technique of Nelson and Diesendruck [11] modified by Metzger [12]. The TPI test was done with the addition of 200 μg of egg-white lysozyme (Worthington Biochemical Corporation) to the test mixtures per ml, to reduce the incubation time required for immobilization, from 18 to 6 h. The basal medium contained double-strength sodium thioglycolate, and gelatin to give a final concentration of 100 mg 100 ml⁻¹.

3.6. The capillary macrophage migration inhibition (MMI) test

The MMI test was performed by the technique introduced by George and Vaughan [13] and elaborated by David and co-workers [14] with slight modification. Lymphocytes were separated from peripheral blood of rabbits by the method of Tellez and Rubinstein [9].

Macrophages were derived from peritoneal exudate of normal guinea-pig, which were injected intraperitoneally four days before with 30 ml of sterile light paraffin oil.

An aliquot of macrophages and an aliquot of lymphocytes tested were counted in a haemocytometer chamber; the concentration adjusted to 40 000 cells per mm³ and 10 000 cells per mm³, respectively. The suspension of lymphocytes was incubated with antigen in RPMI-1640 medium supplemented with 5% heat-inactivated foetal calf serum for 48 h at 37 °C in an incubator with 5% CO₂.

The supernates from lymphocyte cultures were examined for the presence of MIF against freshly isolated guinea-pig macrophages. The antigen was prepared by the same method as for skin test, see below. A freshly prepared suspension of T. pallidum (in PBS), containing about 500 treponemes per microscopic field (× 400), was disrupted by ultrasounds. Each new part of the antigen was tested in at least three dilutions by the MMI test with lymphocytes from several normal and syphilitic rabbits. For routine use, the dilution which gave the greatest difference between migration areas of macrophages incubated with lymphocytes from normal rabbits and those from syphilitic rabbits was chosen. The antigen in 1.0 ml portions was stored at −70 °C until used.

3.7. Preparation of T. pallidum antigen and skin test for delayed hypersensitivity (SDH)

The preparation was done according to Podwińska [15]. Briefly, rabbits were given an intra-testicular inoculation of 1.8–2 × 10⁷ T. pallidum Nichols strain. On the day of inoculation and every second day thereafter, the rabbits were given hydrocortisone (Polfa, Poland; hydrocortisonum aceticum 0.125, lignocainum hydrochloricum 0.025, vehiculum et conserv. ad 5 cm³) intramuscularly in a dose of 6 mg kg⁻¹ body weight. 12 days after inoculation the treponemes were eluted from the testicular tissue with PBS, and purified by differential centrifugation, initially at 2000 rpm for 10 min to remove small tissue particles, red cells and spermatozoa. The supernate was centrifuged at 12000 rpm for 15 min to sediment the spirochaetes. The sediment was resuspended in PBS, and centrifuged at 1000 rpm for 10 min to remove treponemes coated with minute tissue debris. The supernate containing treponemes freed of contaminating material was adjusted with PBS to a density of about 500 treponemes per microscopic field at x 400. The treponeme suspension was stored at 4 °C for 40 h and sonicated (20 Kc, 3 × 1 min with pause 1–2 min, 100 W MES Ultrasonic Disintegrator) to retain the thermolabile protein antigen, divided into 1.0 ml aliquots and stored at −70 °C until used. The skin test was done by the intracutaneous injection of 0.1 ml of T. pallidum antigen on the clipped back of the rabbit. For the control 0.1 ml of PBS was injected. Reactions were read after 48 h and recorded as: 0, no reaction or reaction comparable with that at control site; +, patchy erythema; ++, homogenous erythema and oedema; ++++, homogenous erythema with oedema and induration; and ++++, very intensive homogenous erythema with oedema, induration and central necrosis.
4. RESULTS

The experiments were done with 33 rabbits infected with *T. pallidum* and with 10 normal rabbits. During the development of a syphilitic infection, as checked by VDRL and TPI tests, rabbits were examined for the presence of CIC every 2–4 weeks up to 25 weeks and subsequently every 6–10 weeks.

4.1. CIC in immunoelectrophoresis (IE)

The immune complexes (IC) present in the PEG-precipitates from syphilitic sera were seen in immunoelectrophoresis plates as a short round line near the origin, whereas control serum or immunoglobulin G (IgG) diluted to 1/40, precipitated near the trough containing antisera (Fig. 1); PEG-precipitates from normal rabbit sera gave negative results.

4.2. Determination of optical-density (OD) of PEG-precipitates

PEG-precipitates from sera containing IC, positive in IE, had higher absorbance values (0.20–0.85, mean 0.44 ± 0.12) than those from normal rabbit sera (0.05–0.11, mean 0.07 ± 0.04) or from syphilitic sera without IC (0.12–0.30, mean 0.27 ± 0.11). These differences were found to be statistically significant (*P* < 0.001).

3.8. Statistical analysis

The statistical analysis of data was done by the Student’s *t*-test.

![Fig. 1. CIC in immunoelectrophoresis. A constant current of 10 mA/117 mm plate was applied. Electrophoresis was done without cooling for 90 min.](image)

![Fig. 2. The percent of syphilitic rabbits containing CIC. * No. of examined rabbits.](image)
Table 1

Class of immunoglobulin in CIC

<table>
<thead>
<tr>
<th>Weeks after infection with CIC/total</th>
<th>No. of sera</th>
<th>No. of PEG-precipitates in immunoelectrophoresis with serum against:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ig</td>
</tr>
<tr>
<td>2-3</td>
<td>7/33</td>
<td>7/33</td>
</tr>
<tr>
<td>4-5</td>
<td>9/33</td>
<td>9/33</td>
</tr>
<tr>
<td>6-7</td>
<td>12/33</td>
<td>12/33</td>
</tr>
<tr>
<td>8-9</td>
<td>10/33</td>
<td>10/33</td>
</tr>
<tr>
<td>10-11</td>
<td>15/28</td>
<td>15/28</td>
</tr>
<tr>
<td>14-18</td>
<td>7/28</td>
<td>7/28</td>
</tr>
<tr>
<td>20-93</td>
<td>63/94</td>
<td>63/94</td>
</tr>
</tbody>
</table>

4.3. Platelet aggregation (PA) assay

When PEG-precipitates from syphilitic sera were negative in IE, but the absorbance values were higher than those with normal rabbits, the sera were additionally examined in the PA test.

All rabbit sera containing IC, as checked by IE, and three of six negative in IE were positive in the PA test. Three sera with high absorbance values (0.3) but without IC as indicated by IE and PA test had autolymphocytotoxins which are also precipitated with PEG.

4.4. Occurrence and persistence of CIC

The proportion of rabbits containing CIC, at defined times during a syphilitic infection, is shown in Fig. 2. In some rabbits CIC were detected as early as 3 weeks after infection. Between 5–12 weeks about 45% of the rabbits had immune complexes in their serum. From 14–24 weeks, the number of rabbits with CIC decreased and in the following weeks it increased again. The greatest number of rabbits with CIC was noted between 59–91 weeks after infection. The persistence of IC in the circulation depended on the time they were formed in the disease; 2–6 weeks at the beginning of the disease and longer, 10–25 weeks, when they appeared in syphilitic rabbits after more than 20 weeks.

4.5. Classes of immunoglobulin in CIC

CIC from most syphilitic rabbits, at 2–6 weeks, precipitated in IE with anti-immunoglobulin (Ig) serum but not with anti-IgG serum, which suggested that they contained another class of immunoglobulin, possibly IgM. IgG was present in PEG-precipitates from sera with high levels of TPI antibodies (data not shown). The presence of IgG-containing complexes increased after week 10 of infection and subsequently, all PEG-precipitates contained IgG (Table 1).

4.6. Antibodies in CIC

To determine whether immunoglobulins present in PEG-precipitates were antitreponemal antibodies, some of the precipitates with protein concentration 2.24–3.15 mg ml\(^{-1}\) were dissolved in Sörensens buffer pH 7.2 containing 0.15 M NaCl and were examined in the VDRL and TPI tests.

Table 2

Presence of antibodies in CIC examined by VDRL and TPI tests

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Weeks after infection</th>
<th>Antibodies in sera examined by tests:</th>
<th>Absorbance of PEG-precipitates at 280 nm</th>
<th>Presence of CIC</th>
<th>Antibodies in CIC examined by tests:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>VDRL/titre</td>
<td>TPI/%Tpi/titre</td>
<td></td>
<td>Presence of CIC</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>–</td>
<td>0</td>
<td>0.17</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>–</td>
<td>0</td>
<td>0.10</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>2–3</td>
<td>8</td>
<td>ND</td>
<td>0.36</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>2–3</td>
<td>8</td>
<td>ND</td>
<td>0.36</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>6–7</td>
<td>8</td>
<td>100</td>
<td>0.32</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>8–9</td>
<td>16</td>
<td>100</td>
<td>0.33</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>28</td>
<td>16</td>
<td>1000</td>
<td>0.4</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>28</td>
<td>–</td>
<td>900</td>
<td>0.32</td>
<td>+</td>
</tr>
</tbody>
</table>

ND = undiluted
Tpi = *Treponema pallidum* immobilized.
tests. The results obtained were compared with the titre of serum antibodies (Table 2). CIC from sera with high levels of antibodies were positive in both tests.

4.7. Correlation between the presence of CIC and immunological response

The levels of TPI and VDRL antibodies in syphilitic rabbit sera containing IC were lower
compared to the levels in sera without IC (Figs. 3 and 4).

Lymphocytes from some rabbits containing CIC during the initial weeks of a syphilitic infection showed a stronger capacity to inhibit macrophage migration than lymphocytes from rabbits without CIC (Fig. 5). This capacity increased to week 12, with an average value of 70% of inhibition. Between weeks 14–25 of the disease this capacity was almost abolished and in subsequent weeks it was maintained at a low level until the end of the observation period. On the contrary, lymphocytes from rabbits without CIC developed this capacity slowly. A similar inhibition of the cell-mediated immune response was observed when a skin test for delayed-type hypersensitivity against T. pallidum antigen was used (Fig. 6). In rabbits containing CIC the skin reaction was weak (1+ or 2+) and lasted only 2–3 days, while in rabbits without immune complexes these reactions were stronger (3+ and 4+) and were maintained for 4–6 days.

5. DISCUSSION

The results indicated that CIC were formed in rabbits infected with T. pallidum at different times after infection. Their persistence in sera depended on the time in the course of the disease at which they appeared; usually for 2–6 weeks at the beginning of disease, and for 12 weeks after 20 weeks of rabbit syphilis. Lack of CIC does not mean that they are completely removed from the host. Jorizzo et al. [16], found immune complexes in the damaged vessel wall and in the skin, which suggested that CIC could be translocated. Deposits of immune complexes were also found on the glomerular basement membrane in syphilitic nephropathy [17] and it was suggested that they take part in the pathogenesis of syphilis.

The immunoglobulin in the CIC was similar to the antibodies produced during a syphilitic infection [18]. It would seem to be IgM at the beginning of the disease and IgG later in rabbit syphilis.

The data presented here also indicate that antibodies in CIC are directed against specific antigens of T. pallidum and lipid antigens present not only in these bacteria, and which may be responsible for stimulating anti-cardiolipin antibody examined by the VDRL test [19]. The presence of specific TPI antibodies in immune complexes corresponds with the results of Engel and Diesel [20].

When CIC were present there was a distinct decrease of TPI and VDRL antibody in sera. A decrease in the VDRL antibody titre was previously observed during the course of syphilis in human beings and rabbits [21–23]. It was sug-
The relation between skin reaction and CIC content in rabbits

A

normal rabbits / control / DTH /--/ CIC /--/

B

syphilitic rabbits / DTH + / CIC +

C

syphilitic rabbits / DTH +++ / +++ / CIC /--/

D

syphilitic rabbits / DTH +++ / CIC /--/

Fig. 6. The relation between skin reaction and CIC content in rabbits. A. Normal rabbits without CIC and DTH negative. B. Syphilitic rabbits containing CIC, weak +ve DTH. C. Syphilitic rabbits without CIC and a stronger DTH + + / + + +. D. Syphilitic rabbits without CIC and a strong DTH + + + +.

Suggested that this decrease was connected with the presence of cold autolymphocytotoxins, which are directed mainly against B lymphocytes [22]. The reduced level of TPI antibodies specific for *T. pallidum* observed in this study, seems to suggest that both VDRL and TPI antibodies were removed from the circulation during immune complexes formation. Such complexes may precipitate or agglutinate in the circulation leaving diminished level of unbound antibodies ready to react with antigen. CIC can also induce the formation of anti-antibodies. Such anti-idiotypes will also interact with circulating antibody and neutralize its activity [24].

The CIC appear to influence the cell-mediated immune response; during the initial weeks of infection it was possible to elicit a cell-mediated immune response. Early in the disease the CIC were formed mainly from IgM which did not inhibit immunological response of lymphocytes [25]. The suppression of the cell-mediated immune response was observed 10–14 weeks after infection when IgG-containing complexes were formed.

IgG complexes are known to bind to Fcγ receptors on B lymphocytes and to inhibit differentiation of B cells into antibody secreting cells [25]. Inhibition of B cell antibody production by CIC in syphilis was suggested by Baughn et al. [27]. They found that lymphocytes from syphilitic rabbits containing CIC did not produce IgG plaques after immunization with sheep erythrocytes, a T-dependent antigen. The results reported here seem to indicate that IgG complexes can also inhibit lymphocyte mediated macrophage inhibition. MIF is produced by T and B cells [28]. The inhibition of MIF production after 20 weeks of the syphilitic infection suggest that both T and B cells are probably inhibited in the same way. The suppressing effect of IgG complexes on cells was also observed in our latest experiments (paper prepared for publication). T and B lymphocytes and macrophages, from rabbits containing CIC, were unable to produce antitreponemal lymphotoxin, a very important factor required to kill *T. pallidum* in vivo [2] and in vitro [26].

Cell-mediated immune responses contribute towards protection against *T. pallidum* [1,2]; suppression of these responses by CIC may facilitate
the survival of treponemes and thus enhance multiplication of the organism.

REFERENCES


