Influence of (+)-Cyanidanol-3 on the Leukocyte Migration Inhibition Test Carried Out in the Presence of Purified Protein Derivative and Hepatitis B Surface Antigen

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It has been shown that (+)-cyanidanol-3, therapeutically administered during the course of acute hepatitis B, is able to favor the elimination of hepatitis B surface antigen (HBsAg) from the blood. This observation suggests that (+)-cyanidanol-3 might stimulate the cell-mediated immune response, since it is known that this type of response is responsible for elimination of the virus. In the present study, the possible action of (+)-cyanidanol-3 on this type of immunity was investigated by adding the substance in vitro to leukocyte migration inhibition tests, performed with the antigens purified protein derivative (PPD) and HBsAg and with leukocytes from individuals sensitized to these antigens. In normal individuals sensitized to PPD, the addition of (+)-cyanidanol-3 amplified the inhibition of migration by 7.0% \((P < 0.05)\). In patients previously infected by hepatitis B virus and sensitized to HBsAg, the addition of (+)-cyanidanol-3 amplified the migration inhibition by 10.5% \((P < 0.05)\). A trend to a dose-response relation was observed with the antigen PPD. (+)-Cyanidanol-3 did not modify leukocyte migration in the absence of an antigen. (+)-Cyanidanol-3 therefore seems capable of amplifying the cell-mediated immune response as measured by the leukocyte migration inhibition test. It is thus possible that it favors the elimination of HBsAg in patients by this mechanism.

Important progress has recently been made in the understanding of immune mechanisms responsible for viral hepatitis B. Most authors agree that specific cellular immunity plays an essential role in liver cell cytology as well as in virus elimination (1, 7, 9, 12, 14–16, 27, 28) and that this immunity is deficient in asymptomatic carriers of hepatitis B surface antigen (HBsAg) (11, 18, 28). However, little progress has been made in the treatment of the disease. Attempts to improve the clinical course of chronic hepatitis B with antiviral substances (5, 21, 24), immunostimulants (19; R. G. Chadwick, S. Jain, H. C. Thomas, and S. Sherlock, Gut 18:A979, 1977), and immunosuppressives (9, 26) have given disappointing results.

(+)-Cyanidanol-3, a substance belonging to the group of flavonoids, has the property of preventing the liver cell necrosis induced in experimental animals by various toxic agents (17, 20, 22). In patients with acute hepatitis B, this drug has been shown to decrease bilirubinemia (3, 4), to reduce the severity of symptoms, and to increase the blood elimination of HBsAg (3). This last observation might be consistent with a possible stimulation of specific cellular immunity by (+)-cyanidanol-3, since this type of immunity is responsible for blood clearance of the virus (11, 12, 14, 16, 18).

The cellular immune response to a given antigen can be demonstrated by an in vitro test, the leukocyte migration inhibition test (LMIT). Leukocyte migration can be inhibited by a lymphokine liberated by lymphocytes in the presence of the antigen, to which they have been previously sensitized. A variant of this test, in which leukocytes migrate under an agarose layer (6), is especially suitable for the study of an antigen available only in small amounts, like purified HBsAg (13). The LMIT has allowed the demonstration of a response to this antigen in nearly all cases of acute hepatitis B after recovery (16, 18, 28), in about one-half of the cases of chronic persistent hepatitis (14, 15, 23), and more rarely in chronic active hepatitis and post-hepatitis cirrhosis (2, 10, 14). This response is lacking in asymptomatic carriers of HBsAg (11).

The aim of the present study was to investigate the possible action of (+)-cyanidanol-3 on an in vitro model of cellular immunity by using two different antigens: tuberculin purified protein derivative (PPD) and HBsAg.

MATERIALS AND METHODS

Leukocytes. Leukocytes were isolated from the blood of the following individuals: (i) 15 normal blood donors, aged 23 to 73 and (ii) 12 patients, aged 24 to 87, all HBsAg positive by counterimmunoelectropho-
resis or radioimmunoassay and sensitized to one of the antigens of hepatitis B virus (HBV) infection; these patients were at different stages of hepatitis B. In addition, a second group of normal individuals was used as controls in the experiments using HBsAg; in these individuals, previous contact with HBV was excluded by the absence of anticore antibody (Corab; Abbott Laboratories, North Chicago, Ill.).

LMIT. The LMIT under agarose, as performed in our laboratory, is described in detail elsewhere (13). The only modification introduced was a reduction in the size of the wells and, accordingly, in the number of incubated cells, so that a larger number of variants could be carried out on the same population of cells. Like others (25), we observed that a micromethod of the LMIT was reproducible. Briefly, the procedure was the following. A total of 5 × 10⁶ leukocytes, separated by dextran sedimentation, were distributed in a number of tubes corresponding to the number of variants to be tested. After centrifugation, the supernatants were discarded and the cells were suspended in 10 μl of bicarbonate medium, with or without the antigen or (+)-cyanidanol-3. After 30 min of incubation, 1 μl of cell suspension (5 × 10⁶ cells) was introduced into each of the 1.5-mm wells punched in an agarose layer. The suspension of each tube was sufficient for eight wells. The wells had been previously punched in the agarose layer of two petri dishes (30-mm diameter) into which 1.5 ml of agarose had been poured. The preparation was covered by an oil layer and incubated for 20 h at 37°C in a CO₂ atmosphere.

Antigens. Preliminary experiments showed that the most reproducible results with PPD were obtained with a concentration of 200 μg/ml and that those with HBsAg were obtained with a 1:16 dilution of a preparation titrating 1:2 in counterimmunoelctrophoresis. HBsAg was purified in our laboratory according to a technique described previously (16).

Experimental design. The leukocytes from the normal subjects were used to test the (+)-cyanidanol-3 alone and to test the effect of this substance on the leukocyte migration inhibition by PPD. In addition, leukocytes from normal donors in whom a previous infection by HBV could be ruled out were used to test the effect of this substance on leukocyte migration inhibition by HBsAg (nonsensitized controls). Cells from the patients infected by HBV were used to test the effect of (+)-cyanidanol-3 on the LMIT performed in the presence of HBsAg, as well as to test the influence of (+)-cyanidanol-3 alone. Different concentrations of (+)-cyanidanol-3 were used (from 5 to 1,000 μg/ml) in order to detect a possible dose-effect response as well as a possible toxic effect on the cells. The same population of cells was used to test simultaneously the different concentrations of (+)-cyanidanol-3.

Readings and calculations. The areas of migration were measured by planimetry. The mean surface area of the eight wells of each variant was calculated. In each experiment, a control migration without antigen or (+)-cyanidanol-3 was performed. The means of the migration surfaces in the presence of the antigen were expressed as a percentage of the average migration in the control without antigen (migration index [MI]). The individual MI values of one variant allowed the calculation of the mean MI.

In addition, the difference between the MI obtained in the presence of (+)-cyanidanol-3 and the MI obtained in its absence, i.e., with the antigen only, was calculated for each individual. All individual differences of one variant were then used to calculate the average difference for the whole group (expressed as the percentage difference from the control measurements). The paired Student t test and the Wilcoxon test were applied to these differences.

RESULTS

(+)-Cyanidanol-3, added alone in the absence of an antigen, did not inhibit leukocyte migration at concentrations of 5, 25, and 100 μg/ml: the MI values were close to 100% (Fig. 1). However, significant inhibition was obtained with a concentration of 1,000 μg/ml. Table 1 shows the means of the individual differences calculated between migration with and without (+)-cyanidanol-3 from the same experiment; significant results were obtained only at the higher (+)-cyanidanol-3 concentration of 1,000 μg/ml.

(+)-Cyanidanol-3 increased the inhibition of leukocyte migration due to PPD, and this effect increased with its concentration (Fig. 2). When the antigen alone was added to leukocyte suspensions from normal individuals, the mean MI was 80.5%; if (+)-cyanidanol-3 at concentrations of 5, 25, and 100 μg/ml was added, the mean MI of the same individuals decreased respectively to 76.7, 74.5, and 72.5%. The mean of the indi-

![FIG. 1. Effect of (+)-cyanidanol-3, added at different concentrations without antigen, on the migration of leukocytes from normal individuals. Each dot represents the MI of the leukocytes as one individual, that is, the surface of migration expressed as the percentage of the migration in a control preparation without (+)-cyanidanol-3. In each column, the average migration index is represented with ± 1 standard error.](http://iai.asm.org/ on May 22, 2021 by guest)
TABLE 1. Means of the differences (Δ) calculated between leukocyte migration obtained in the presence and absence of (+)-cyanidanol-3

<table>
<thead>
<tr>
<th>Donor group</th>
<th>n</th>
<th>(+)-Cyanidanol-3 (µg/ml)</th>
<th>Antigen</th>
<th>Δ</th>
<th>Standard error</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>10</td>
<td>5</td>
<td>None</td>
<td>+2.5</td>
<td>3.0</td>
<td>NS*</td>
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<tr>
<td></td>
<td>14</td>
<td>25</td>
<td>None</td>
<td>-1.21</td>
<td>5.85</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>100</td>
<td>None</td>
<td>+6.5</td>
<td>5.66</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>1,000</td>
<td>None</td>
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<tr>
<td></td>
<td>14</td>
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<td>3.53</td>
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<tr>
<td></td>
<td>14</td>
<td>25</td>
<td>PPD</td>
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<tr>
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<td>PPD</td>
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<td>&lt;0.05</td>
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<td>Patients infected with HBsAg</td>
<td>12</td>
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<td>HBsAg</td>
<td>-10.58</td>
<td>4.48</td>
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<td></td>
<td>8</td>
<td>100</td>
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<td>9.79</td>
<td>NS</td>
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<tr>
<td></td>
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<td>25</td>
<td>HBsAg</td>
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<td>3.97</td>
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<tr>
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<td>100</td>
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<td>-7.18</td>
<td>4.13</td>
<td>NS</td>
</tr>
<tr>
<td>Normal (previous HB infection excluded)</td>
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<td>0</td>
<td>HBsAg</td>
<td>+5.00</td>
<td>4.51</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>25</td>
<td>HBsAg</td>
<td>+1.18</td>
<td>3.03</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Expressed as the percentage difference from the control migration.

** NS, Not significant.

Leukocyte migration inhibition by PPD was increased in 9 of the 14 normal individuals when (+)-cyanidanol-3 was added at a concentration of 25 µg/ml (Fig. 3). With a concentration of 100 µg/ml, the inhibition by PPD was increased in 12 of these 14 individuals, whereas it was increased in only 3 with the lowest concentration, 5 µg/ml.

FIG. 2. Effects of (+)-cyanidanol-3 at different concentrations on the inhibition by the antigen PPD of the migration of leukocytes from normal individuals. Each dot represents the surface of migration expressed as the percentage of the migration in a control preparation without the antigen.

FIG. 3. Leukocyte migration inhibition in the presence of the antigen PPD: comparison, for each individual, of the results obtained in the absence and in the presence of (+)-cyanidanol-3 at a concentration of 25 µg/ml.
Purified HBsAg produced an inhibition of leukocyte migration in the majority of the patients sensitized to this antigen: the mean MI was 86.7\% (Fig. 4). (+)-cyanidanol-3 at a concentration of 25 \( \mu g/ml \) significantly increased this inhibition, but did not do so at a concentration of 100 \( \mu g/ml \). The mean of the individual differences (Table 1) was -10.58\% at a concentration of 25 \( \mu g/ml \), but only -1.63\% at 100 \( \mu g/ml \). (+)-cyanidanol-3 added alone (in the absence of HBsAg) to the leukocytes from the same infected individuals had no significant effect (Fig. 4). HBsAg and (+)-cyanidanol-3 at concentrations of 25 and 100 \( \mu g/ml \) did not modify significantly the migration of leukocytes from normal subjects without markers of a previous HBV infection (Table 1).

The inhibition of leukocyte migration by HBsAg was increased in 10 of the 12 patients when (+)-cyanidanol-3 at 25 \( \mu g/ml \) was added (Fig. 5). However, at the higher concentration of 100 \( \mu g/ml \), this effect could not be shown. The calculation of P by the Wilcoxon test gave results similar to those obtained by the paired Student t test.

**DISCUSSION**

(+)-cyanidanol-3, when added at concentrations of 5, 25, and 100 \( \mu g/ml \) to a leukocyte suspension, had no influence on the migration of leukocytes under an agarose layer, provided it was added alone, in the absence of an antigen. The concentration of 1,000 \( \mu g/ml \), however, was inhibitory in itself; this concentration is probably cytotoxic and was therefore not used further in experiments carried out with an antigen (Fig. 1).

(+)-cyanidanol-3, at a concentration of 25 \( \mu g/ml \), amplified significantly the response of leukocytes incubated in the presence of an antigen to which they had been previously sensitized. This effect was observed in both antigenic systems tested (Fig. 3 and 5). Therefore, it seems that this effect is not specific for one antigen. In the first of these systems (that is, PPD tested on homogeneous population of healthy subjects), the potentiating effect of (+)-cyanidanol-3 was more pronounced with 100 \( \mu g/ml \) and less marked with 5 \( \mu g/ml \). This dose-related effect was, however, not detectable when using HBsAg and patients infected by this antigen. In this latter system, no increase in the mean MI was observed at a concentration of 100 \( \mu g/ml \). This might be explained by the more inhomogeneous character of the group of patients infected with HBsAg: these patients were suffering from different forms of hepatitis B or were at different stages of the disease.

On the whole, the dispersion of the individual results obtained by the LMIT was large (Fig. 1, 2, and 4). This explains why the differences calculated between the average MI values were not significant and why only a trend toward a dose-response relation could be shown. On the
contrary, the pairs of results with and without (+)-cyanidanol-3 (Fig. 3 and 5) showed significant differences when the paired Student t test was applied (Table 1).

(+)-Cyanidanol-3 had no significant effect on the response of leukocytes incubated in the presence of an antigen to which they had been previously sensitized (Table 1). This could mean that the substance interferes only with a specific immune process.

It has been demonstrated that LMIT positivity in the presence of HBsAg is closely related to the elimination of HBV and to the production of the liver lesions (11, 12, 14-16, 28), and it is accepted that this test reflects a functional state of the T lymphocytes sensitized to a given antigen. Under certain conditions and at certain concentrations, (+)-cyanidanol-3 seems to amplify the results of the LMIT obtained in the presence of an antigen and, thus, seems capable of stimulating a specific cell-mediated immune response in vitro. These results may explain those of the study of Blum et al. (3), in which HBsAg was eliminated in a larger proportion of patients with acute viral hepatitis B treated by (+)-cyanidanol-3 than in the control group.

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