Effect of corticosteroids on mouse hepatitis virus infection

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Corticosteroid administration has been shown to have a deleterious effect in various viral infections in experimental animals. High mortality rates have occurred in animals but in most studies pharmacological doses of corticosteroids were used and were given before the viral infections (Shwartzman, 1950; Kilbourne and Horsfall, 1951). Starr and Pollard (1958) and Bang and Warwick (1960) found that mice which were normally resistant to mouse virus hepatitis became more susceptible if they were treated with corticosteroids before virus inoculation. Manso, Friend, and Wróblewski (1959), Vella and Starr (1965), and Hirano and Ruebner (1966) showed that the severity of infection with mouse hepatitis virus was increased in susceptible animals when corticosteroid therapy preceded or accompanied virus inoculation. In contrast to these animal studies, Katz, Velasco, Klinger, and Alessandri (1962) claimed that massive doses of corticosteroids were of benefit in the management of patients with fulminating viral hepatitis.

The administration of murine hepatitis strains of virus (MHVS) to mice produces a disease which evolves slowly, is associated with abundant inflammation, and leads to a spectrum of morphological lesions which closely simulate human viral hepatitis (Jones and Cohen, 1962). In view of these effects of MHVS infection we considered it of interest to determine the results of corticosteroid administration on the course of infection with this virus. The disease process was followed by changes in plasma enzymes, liver histology, and mortality.

These studies have shown that corticosteroids have an adverse effect on mouse hepatitis virus infection and that this response is associated with an increase of virus particles in the liver. Of significance was the finding that the steroid effect was dependent on the time at which the hormone was administered.

MATERIAL AND METHODS

MICE Swiss-Webster male weanling mice (21 days), weighing 8 to 10 g and free from Eperythrozoon coccoides, were used. A standard diet was fed and the animals were fasted overnight before bleeding or sacrifice. In all experiments 20 to 30 mice were bled at each point in time unless otherwise stated. A similar number of animals were used for mortality experiments.

INOCULUM The virus inoculum consisted of 10% homogenerate of liver infected with two strains of mouse hepatitis virus (MHVS and MHV-A59) in phosphate buffered saline, pH 7.4. Normal liver inocula consisted of 10% homogenerate of normal liver in the same buffered saline. Numerous aliquots of both inocula were made and stored at -40 °C. Whenever virus or normal liver inoculum was injected, a fresh aliquot was thawed and used for injection. In each experiment mice received either 0.1 ml of virus or 0.1 ml of normal liver inoculum intraperitoneally. Titration of the virus inoculum was carried out at the time of its preparation; it was shown that 0.16 ml of inoculum was equivalent to 1 LD50. This inoculum was used in all experiments.

CORTICOSTEROID PREPARATIONS Hydrocortisone (Cortef) or a long-acting preparation of methyl prednisolone (Depo-Medrol) was given intramuscularly. The steroid preparations were diluted with the appropriate vehicle so that animals received 0.1 ml of steroid solution regardless of the dose employed. Whenever steroids were used the control animals received 0.1 ml of vehicle.

The dose of hydrocortisone varied from 0.05 to 2.0 mg daily. Methyl prednisolone was given as a single injection; the dose was either 0.1 mg (approximately equal to 0.05 mg of hydrocortisone daily for eight days) or 4.0 mg (approximately equal to 2.0 mg of hydrocortisone daily for eight days).

COLLECTION OF BLOOD Plasma for enzyme estimation was obtained by pooling blood from two to four mice. The blood was obtained by retro-orbital puncture using a capillary tube. It was then placed in a heparinized tube and centrifuged at 3,000 g for 30 minutes at 2 °C. Enzyme estimations were performed on the same day or plasma was frozen for subsequent estimation.

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BIOCHEMICAL ASSAY β-Glucuronidase activity was measured by the method of Fishman, Springer, and Brunetti (1948) using 0.2 ml of phenolphthalein glucuronidate (0.01 M) as a substrate, 0.1 ml of plasma, and 0.7 ml of 0.1 M acetate buffer, pH 5. The mixture was incubated for 18 hours at 37°C and the phenolphthalein measured colorimetrically at 540 mμ. One unit of activity was equivalent to the release of 1 μg of phenolphthalein during the period of incubation. Plasma glutamic pyruvic transaminase (GPT) was measured according to the Sigma modification of Frankel's method and expressed as Sigma-Frankel units (1965).

HISTOPATHOLOGICAL STUDIES Liver tissue obtained after sacrifice of the animals was fixed in formalin and embedded in paraffin. Sections were stained with haematoxylin and eosin.

VIRUS TITRATION Livers obtained from six animals were excised, pooled, and homogenized in phosphate-buffered saline. Serial dilutions were made from 10⁻¹ to 10⁻⁷. One-tenth ml of each dilution was injected into 15 to 20 mice and mortality recorded for a period of eight days after the virus inoculation. On the basis of the mortality data the LD₅₀ was calculated according to the method of Reed and Muench (1938).

STATISTICAL TESTS The difference between means was analysed by the t-test (Hill, 1961) and P < 0.05 was accepted as significant.

SCHEDULE OF VIRUS AND STEROID ADMINISTRATION In one series of experiments hydrocortisone was given before virus inoculation. All animals received 2 mg of hydrocortisone daily for three days and were inoculated with virus on the second day. The animals were bled and sacrificed two days after virus inoculation.

The effect of steroids after virus inoculation (MHVS) was studied as follows: hydrocortisone (either 0.5 or 2.0 mg/day) was given on the day of viral inoculation or on the first, third, fifth, seventh, or ninth day after virus inoculation; steroids were given for two days only and the animals were bled and sacrificed on the third day.

Since initial experiments indicated that most of the animals died within eight days after inoculation with hepatitis virus, we elected to give a single dose of a long-acting preparation, methyl prednisolone (which obviated daily injections) and then noted the effect over the subsequent eight days. In this study the steroid was given either before, on the day of virus inoculation, or three, five, seven, and nine days after virus inoculation.

RESULTS

PLASMA ENZYMES Plasma glutamic pyruvic transaminase and β-glucuronidase levels in mice inoculated with MHVS virus are shown in Figure 1. No elevation was evident until the third day after virus inoculation. Levels were maximal on the fifth day but then fell rapidly. After seven days plasma enzyme levels were only slightly higher than those found in control animals given normal liver homogenate. No alteration in plasma enzyme levels was observed in normal mice when hydrocortisone was injected in a dose of 2 mg per day for three days.

In Table I are shown enzyme levels in animals given hydrocortisone for three days and in which the first injection was given a day before virus (MHVS or MHV-A59) inoculation. Whereas animals given virus alone showed no rise in enzyme levels during the two days following virus inoculations, hydrocortisone-treated animals showed a significant elevation during this period (P < 0.01). Hydrocortisone alone produced no significant alteration in plasma enzymes (Table I).

That the plasma enzyme level alterations were dependent on the dose of hydrocortisone administered before virus inoculation is shown in Figure 2.
FIG. 2. Effect of dose of hydrocortisone pretreatment on plasma enzymes (mean ± SE) in murine virus hepatitis. The schedule is shown in the footnote to Table I.

FIG. 3. Effect of the time of hydrocortisone administration on plasma enzymes of mice inoculated with MHVS virus.

TABLE I

EFFECT OF HYDROCORTISONE (2 MG/DAY) ON PLASMA ENZYMES 48 HOURS AFTER MHV-A59 AND MHVS VIRUS INFECTION

<table>
<thead>
<tr>
<th>Virus</th>
<th>− Hydrocortisone</th>
<th>+ Hydrocortisone</th>
<th>− Hydrocortisone</th>
<th>+ Hydrocortisone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>48.0±10.0</td>
<td>41.7±6.0</td>
<td>103.2±17.0</td>
<td>81.5±5.6</td>
</tr>
<tr>
<td>MHVS</td>
<td>46.0±34.9</td>
<td>273.4±479.1</td>
<td>94.3±7.6</td>
<td>711.6±112.6</td>
</tr>
<tr>
<td>MHV-A59</td>
<td>52.9±7.6</td>
<td>4168.6±352.2</td>
<td>123.1±12.8</td>
<td>403.8±41.8</td>
</tr>
</tbody>
</table>

*Hydrocortisone was administered for three days, the first injection being given a day before virus inoculation. Animals were sacrificed two days after virus inoculation. Control animals received only the vehicle in which the steroids were dissolved.

Results expressed as mean ± SE

It will be noted that as the dose of hydrocortisone was increased there was also a rise in the plasma enzymes. Pretreatment with hydrocortisone with as little as 0.05 mg per day increased the plasma enzyme levels above those found with virus inoculation alone (P < 0.05).

The importance of the time of hydrocortisone administration in relation to the time or duration of viral infection (MHVS) is shown in Figure 3. A significant rise (P < 0.01) in plasma enzymes was noted when hydrocortisone (2 mg day for two days) was either administered at the time of virus inoculation or at days 1 and 3 after virus inoculation. When hydrocortisone administration was delayed until five days after virus inoculation, there was still some increase in plasma enzymes over mice receiving virus alone but this difference was not statistically significant. However, when hydrocortisone treatment was delayed until seven or nine days after virus inoculation there was no difference in plasma enzymes between the steroid-treated and control groups. Results comparable to those shown in Fig. 3 were noted when 0.05 mg of steroid was given.

LIVER HISTOPATHOLOGY In normal mice the administration of hydrocortisone (2 mg/day) for three days did not result in significant histological changes in the liver except for some increase in glycogen deposition.

Mice infected with virus (MHVS) and pretreated with hydrocortisone according to the schedule shown in Table I differed from untreated infected mice in that severe hepatic lesions were noted as early as 48 hours; in those inoculated with virus alone severe lesions were usually noted at 72 hours. Thus at 48 hours about 52% of the animals receiving steroid plus virus had severe lesions while almost no severe lesions were noted in the group inoculated with virus alone. Similar effects were also seen in mice inoculated with the MHV-A59 virus plus hydrocortisone.

A typical example of the severe hepatic lesion seen
Effect of corticosteroids on mouse hepatitis virus infection

FIG. 4. Microscopic appearance of liver in hydrocortisone-pretreated animals 48 hours after virus inoculation. Note the severe necrosis. Hydrocortisone (2 mg/day) had been given for three days, the first injection being given a day before virus inoculation. Haematoxylin and eosin. × 250.

48 hours after virus inoculation in the hydrocortisone-pretreated group is shown in Figure 4. This lesion is in striking contrast to the liver histology at 48 hours in mice receiving only virus (Fig. 5). When hydrocortisone treatment was delayed until seven days after virus inoculation, no difference in the incidence of severe hepatic lesions could be observed (approximately 9% in each group).

Mortality rate. Figure 6 shows the effect of pretreatment with a single dose of methyl prednisolone (0·1 or 4·0 mg) one day before virus inoculation. Animals treated with virus alone showed a mortality rate of 38% when followed for 20 days. However, virus-inoculated animals pretreated with methyl prednisolone died earlier and had a higher mortality rate. Infected animals pretreated with 4 mg methyl prednisolone showed a mortality rate of 100% over the 20-day period and those pretreated with 0·1 mg methyl prednisolone showed a 70% mortality. No further increase in mortality was observed after eight days of virus inoculation.

In another experiment observations were made on the effect of time of administration of methyl prednisolone in relation to virus inoculation on the mortality over the subsequent eight-day period. As seen in Table II the deleterious effect on mortality rate was obvious when methyl prednisolone was either injected simultaneously with, or three and five days after, virus inoculation. However, no such effect on mortality was seen when steroid was administered seven or nine days after virus inoculation. The deleterious effect on mortality was greater when higher doses of steroids were administered.

Virus content of liver. Virus titrations in liver
FIG. 5. Microscopic appearance of liver two days after virus inoculation. Note the absence of the severe necrosis noted in Figure 4. There is focal inflammation. Haematoxylin and eosin. × 250.

<table>
<thead>
<tr>
<th>Time of Steroid Inoculation (days after virus inoculation)</th>
<th>Eight-day Period of Observation</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Virus Alone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1 mg</td>
</tr>
<tr>
<td>0</td>
<td>0-8</td>
<td>38</td>
</tr>
<tr>
<td>3</td>
<td>3-11</td>
<td>38</td>
</tr>
<tr>
<td>5</td>
<td>5-13</td>
<td>28</td>
</tr>
<tr>
<td>7</td>
<td>7-15</td>
<td>14</td>
</tr>
<tr>
<td>9</td>
<td>9-17</td>
<td>10</td>
</tr>
</tbody>
</table>

*Mortality is the cumulative mortality during the eight-day period of observation.

*Animals received a single injection of methyl prednisolone.

Table II shows the effect of methylprednisolone on mortality of mice inoculated with MHVS virus. The table indicates that there is a significant decrease in mortality for animals receiving methylprednisolone compared to those receiving virus alone.

Table III shows virus titration expressed as LD₅₀ of livers from mice two and nine days after MHVS inoculation. Data are from mice receiving steroid plus virus and those inoculated with virus only. It is evident that virus was demonstrable in the livers of animals two days after inoculation (LD₅₀ = 10⁻¹⁻²²). However, infected animals which received hydrocortisone for three days (the first injections being given a day before virus inoculations) showed a marked increase in the virus content of their livers. When compared with non-steroid-treated animals, steroid-treated animals showed a roughly 50-fold (LD₅₀ = 10⁻⁰⁻¹⁰) and 1,000-fold (LD₅₀ = 10⁻⁷⁻¹⁰) increase in virus in the liver, using 0.05 and 2 mg hydrocortisone, respectively.

Tissue were carried out in order to determine if there was any correlation between hepatic virus content and the degree of plasma enzyme elevation, severity of hepatic lesions, and mortality rate.
FIG. 6. Effect of methyl prednisolone pretreatment on mortality after MHVS virus infection. Animals received a single injection of methyl prednisolone the day before virus inoculation.

**TABLE III**

<table>
<thead>
<tr>
<th>Hydrocortisone (mg/day)</th>
<th>Virus Titre (log dilution LLD_{50}/0.1 g of liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day Two¹</td>
</tr>
<tr>
<td>None</td>
<td>-1.52</td>
</tr>
<tr>
<td>0.05</td>
<td>-3.10</td>
</tr>
<tr>
<td>2.00</td>
<td>-5.10</td>
</tr>
</tbody>
</table>

¹Animals received hydrocortisone for three days, the first injections being given a day before virus inoculation. Animals were sacrificed on the second day after virus inoculation.
²Animals were inoculated with virus and seven days later received hydrocortisone for two days, animals were sacrificed on day nine.

**DISCUSSION**

These studies have demonstrated the deleterious effect of corticosteroids in mice infected with mouse-hepatitis virus. This adverse effect was evidenced by high plasma enzyme levels, severe hepatic lesions, and a higher mortality rate. It is noteworthy that these effects occurred only when corticosteroids were started before or during the first five days after virus inoculation but not when steroid administration was delayed until seven or nine days after infection. These results are consistent with previous observations of a high mortality (Shwartzman, 1950; Kilbourne and Horsfall, 1951; Smith, Murphy, and Mirick, 1951; Vella and Starr, 1965) and elevated plasma transaminase levels (Manso et al, 1959) in experimental animals given large doses of corticosteroids before inoculation with different viruses. It was of interest that in the present study a deleterious effect could be demonstrated with lower amounts of steroids than was noted in other reports.

It should be emphasized that plasma for enzyme estimations was obtained from animals at the end of a two- or three-day period of steroid therapy. When hydrocortisone was administered early in the course of infection many animals died before blood was withdrawn. It is likely that the animals which died had high plasma enzyme levels and therefore the effect of hydrocortisone therapy on plasma enzymes was probably underestimated. This might explain the failure to show a significant effect on plasma enzyme levels in animals which first received hydrocortisone five days after virus inoculation even though the mortality rate in animals receiving methyl prednisolone five days after virus inoculation was higher than in controls.

The finding of an increase in plasma β-glucuronidase is of interest since this is primarily a lysosomal enzyme with the greatest concentrations in the liver (Rutenburg and Seligman, 1953). Although these studies were not designed to investigate the role of lysosomal injury in the pathogenesis of liver cell necrosis, the observation of an increase in plasma β-glucuronidase is consistent with the suggestion that lysosomal injury may play a role in the pathogenesis of liver cell necrosis (Allison and Sandelin, 1963; Datta, Jones, and Isselbacher, 1967). The present observations parallel those of Pineda, Goldbarg, Banks, and Rutenberg (1959) who found a rise in both of these plasma enzymes in patients with acute viral hepatitis. Allison and Mallucci (1965) have suggested that lysosomal enzymes may contribute to the uncoating of virus following attachment to the host cell surface. In the present experiments no studies were carried out to examine this concept.

Hirano and Ruebner (1966) showed that corticosteroids, if administered immediately before MHVS inoculation, had a deleterious effect as evidenced by a higher mortality rate and severe hepatic lesions. However, they noted no consistent difference in the concentration of virus in the liver of corticosteroid-treated and untreated animals and no explanation for the adverse effect of corticosteroids was offered. In the present experiments virus titration studies demonstrated a marked increase in the yield of virus from the livers of infected mice when they were pretreated with corticosteroids and the amount of this increase was related to the amount of steroid administered. Smith et al (1951) found a similar increase in the yield of pneumonia virus of mice from the lungs when mice were treated with corticosteroids before virus inoculation; a similar effect
was shown by Kilbourne (1957) in chick embryos infected with influenza virus B. The mechanism for this increase in virus content is not clear but it is evident that corticosteroid administration produces this effect only in the early stages of infection.

Reticuloendothelial cells are thought to be the major cells for the initial removal of mouse hepatitis virus from the blood as well as the site of early virus replication (Mims, 1964). These viruses have been shown to replicate in vitro in macrophages obtained from susceptible strains of mice (Bang and Warwick, 1960). Recently fluorescent antibody (Boss and Jones, 1963) and electron microscopic studies (Sabesin, Datta, Maglio, and Isselbacher, 1967) have shown that changes in the Kupffer cells precede alterations in the hepatic parenchymal cell in mouse hepatitis virus infection and that virus particles can be demonstrated in Kupffer cells at an early stage. These findings suggest that virus may first be taken up by macrophages, subsequently replicated there, and that this is followed by secondary infection of nearby hepatic parenchymal cells (Mims, 1964).

Recent studies in our laboratory with a macrophage culture system have suggested one possible mechanism for the hydrocortisone effect in most mouse viral hepatitis (Sabesin et al., 1967). It has been noted that when hydrocortisone is added to a macrophage culture 24 hours before or two hours after inoculation with virus in vitro, severe cytopathogenic effects occur. These changes are accompanied by a marked increase in the virus titre. No such effect is demonstrable when hydrocortisone is added 24 hours after virus inoculation. Thus it appears possible that the effects in vivo of corticosteroids in enhancing the severity of the mouse viral hepatitis lesion are related to a direct action of the steroids on the reticuloendothelial cells of the liver. It should be mentioned that Smart and Kilbourne (1966), on the basis of studies on the kinetics of interferon synthesis, suggested that the deleterious effects of steroids in their system might, at least in some strains, be mediated by a suppression of interferon synthesis. In the present studies, no experiments on interferon synthesis were carried out.

It is impossible to conclude from the present studies whether the observed effects of corticosteroids in mice infected with mouse hepatitis virus have any counterpart in man infected with one of the human hepatitis viruses. There is in fact no adequate evidence that corticosteroids can alter the degree of liver necrosis, increase the rate of healing, or improve immunological mechanisms in patients with hepatitis (Sherlock, 1968). One group of workers (Katz et al., 1962) have claimed beneficial effects from the use of large doses of cortisone in cases of fulminant viral hepatitis, but the value of corticosteroid therapy in this condition is a subject of controversy (Sherlock, 1968). It is nevertheless of interest that in the present study the deleterious effects of corticosteroids were demonstrated with two different strains of mouse hepatitis virus (MHVS and MHV-A59), and with low as well as high doses.

SUMMARY

A study was undertaken to observe the effect of corticosteroids on mouse virus hepatitis. A deleterious effect was noted when corticosteroids were administered before or early in the course of the viral hepatitis. This effect was reflected by increases in plasma glutamic pyruvic transaminase and \( \beta \)-glucuronidase, by severe histopathological changes in the liver, and by a higher mortality rate. Similar results were obtained with two different strains of murine virus hepatitis (MHVS and MHV-A59). The deleterious effects were most pronounced with high doses of corticosteroids but could be demonstrated with as little as 0·05 mg of hydrocortisone per day or 0·1 mg of methyl prednisolone. When corticosteroid treatment was delayed until seven or nine days after virus inoculation, no deleterious effect was observed.

Infected animals pretreated with hydrocortisone showed from 50-fold to a more than 1,000-fold increase of virus in the liver compared with untreated infected animals. It is probable that the increase of virus in the liver is an important factor in the increased severity of mouse hepatitis virus infection in animals treated with corticosteroids.

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REFERENCES


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