Effects of Immunosuppression on Coxsackie B-3 Virus Infection in Mice, and Passive Protection by Circulating Antibody

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(Accepted 19 January 1973)

SUMMARY
Young adult CBA mice developed lethal infections with Coxsackie B-3 virus when immunosuppressed with cyclophosphamide. In the immunosuppressed mice high titres of virus and severe lesions were found in target organs, including the heart and pancreas, as well as persistent viraemia. Immunosuppressed animals showed transient production of IgM but no IgG virus neutralizing antibody; levels of interferon in peripheral blood were higher than in controls. Immunosuppressed mice could be passively protected by administration of serum antibody after infection. The results suggest that circulating antibody plays a critical role in limiting infection of young adult mice by Coxsackie B-3 virus.

INTRODUCTION
Intraperitoneal inoculation of adult mice with Coxsackie B-3 (CoxB-3) virus leads to a mild infection. Although some virus replication occurs in the heart, pancreas, brain and other organs, animals quickly recover. Increased susceptibility of adult mice to CoxB-3 virus has been reported after pretreatment with cortisone (Kilbourne & Horsfall, 1951) or when mice were forced to swim during the course of the infection (Gatmaitan, Chanson & Lerner, 1970). Virus persistence and increased severity of lesions have also been demonstrated in mice with post-weaning undernutrition (Woodruff & Kilbourne, 1970). The undernourished mice had atrophic lymphoid tissues and deficiencies in the production of specific antiviral neutralizing antibodies and interferon. From these and adoptive transfer studies Woodruff & Woodruff (1971) have postulated a major role for cell-mediated immunity in protection against Coxsackie virus infection. The relative importance of circulating antibody, interferon and cell-mediated immunity in defence against Coxsackie viruses has not been ascertained. We have therefore examined the results of immunosuppression with cyclophosphamide and passive protection with antibody on CoxB-3 infection of adult mice.

METHODS
Mice. Inbred CBA mice from the breeding colony of the Clinical Research Centre were used.

Virus. Coxsackie B-3 (Nancy strain, CoxB-3) passaged in primary rhesus monkey kidney tissue culture, was obtained from the Central Public Health Laboratory, Colindale. Virus stocks were propagated in newborn mice brains; 10% (w/v) mouse brain suspensions (MBS) were stored at −70 °C.

Virus titrations. Organs were made up as 10% (w/v) suspensions in phosphate-buffered
saline. The suspension is taken as $10^{-1}$ dilution in the titration, so that titres are expressed in p.f.u./g of solid tissue. Bloods were collected into heparin and assayed for virus. The remaining blood was centrifuged and plasma was stored at 0°C for antibody and interferon assays. Monolayers of Vero cells grown in WHO haemagglutinating trays were inoculated with 0.1 ml of serial 10-fold dilutions of the appropriate organ suspensions or blood. Following adsorption for 1 h at 37°C, infected monolayers were overlaid with 0.5 ml of carboxymethylcellulose (CMC) in L-15 medium (Russell, 1962), and further incubated at 37°C. Three days later plates were rinsed once in saline and stained with crystal violet. Virus titres are expressed as p.f.u./ml.

Cyclophosphamide (Endoxana). This drug was purchased from Ward Blenkinsop & Co. Ltd., London, as dry powder and stored at room temperature. Each vial was rehydrated with 10 ml of sterile distilled water immediately before use and further diluted in saline to provide the desired total dose. Young adult mice weighing 15 to 20 g were given a single intraperitoneal (i.p.) injection of 6 mg/o.6 ml of the drug (Turk & Poulter, 1972).

Histology. Organs were fixed in formol saline and stained with haematoxylin and eosin.

Antibody titrations. Neutralizing antibodies were measured in Vero cell monolayers according to the micro-culture technique of De Madrid & Porterfield (1969). Antibody titres are expressed as the highest serum dilution that neutralized 50% of the virus inoculum.

Interferon assays. Interferon titrations were carried out by plaque count reduction of vesicular stomatitis virus (VSV) in L-cells. The interferon titre was taken as the highest dilution giving a 50% reduction of the number of VSV plaques as compared with the number of plaques in the controls. Mouse brain interferon with a known titre of 1:2000 i.u./ml was used as a positive control in all assays. No effort was made to inactivate infective virus from plasma samples before interferon titration since this virus did not replicate in L-cells and did not interfere with plaque formation by VSV (Rytel, 1969).

Preparation of anti CoxB-3 mouse serum. Adult mice were inoculated i.p. with $3 \times 10^6$ p.f.u. of virus, and were challenged 7 days later with the same dose of virus. Seven days after the second virus inoculation mice were bled and serum was collected. In some experiments mice were bled 7 days after virus inoculation. Titres of antisera obtained, as measured by a standard virus neutralization assay, were 1:80 for immune antiserum and 1:320 for hyperimmune antiserum.

RESULTS

Increased severity of CoxB-3 virus infection in adult mice treated with cyclophosphamide

Groups of 6- to 7-week-old CBA mice were infected i.p. with $10^6$ p.f.u. of CoxB-3 virus. These groups received also an i.p. injection of 6 mg of cyclophosphamide at different times before or after virus, and times of deaths were recorded. As shown in Fig. 1 the most marked effect of the drug was achieved when it was injected 72 h after virus, when 100% of the mice were dead by day 7. No deaths occurred when mice were infected with virus only, or treated with cyclophosphamide alone.

Effects of cyclophosphamide on the morbidity and mortality of CoxB-3 infection in adult CBA mice

A group of 6- to 7-week-old CBA mice was infected i.p. with $10^6$ p.f.u. of CoxB-3 virus, followed 72 h later by an i.p. injection of 6 mg of cyclophosphamide. A control group of mice was infected with virus only.

From the first day after virus infection two mice in each group were killed daily. Organs were prepared for histological examination by fixation in formol saline. Virus titrations
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Fig. 1. Effects of cyclophosphamide on CoxB-3 virus infection of adult mice. All mice were infected i.p. with $10^5$ p.f.u. of virus on day 0 and received a single dose of 6 mg cyclophosphamide i.p. at different times before or after virus. ——, CoxB-3 only (control); ••••, cyclophosphamide (cy) 6 h before virus; ••••, cy 6 h after virus; ——, cy 24 h after virus; ——, cy 48 h after virus; ——, cy 72 h after virus. Each group consisted of 10 mice.

Fig. 2. Virus titres in hearts of infected mice on various days after inoculation. Each point represents an average for two mice. ••••, intact mice infected with CoxB-3 virus; •—••, mice infected with CoxB-3 virus followed by cyclophosphamide 72 h later.
Fig. 3. Section of heart muscle from an intact mouse, taken 7 days after CoxB-3 virus infection (×250).

Fig. 4. Section of heart muscle from a cyclophosphamide-treated mouse, taken 7 days after CoxB-3 infection (×250).
Fig. 5. Virus titres in organs of infected mice on various days after inoculation. Each point represents the average for two mice. —, mice infected with CoxB-3 virus; •—•, mice infected with CoxB-3 virus followed by cyclophosphamide 72 h later. I, pancreas; II, liver; III, spleen; IV, brain.

were performed on 10% suspensions of brain, liver, pancreas, heart, spleen and blood. Interferon and antibody assays were performed on plasma as described earlier. Times of death of mice from each group were recorded, survivors being followed for an additional 6 months. No deaths occurred in control groups whereas the mortality was 100% in virus-infected and cyclophosphamide-treated mice.

**Virus in the heart**

The course of infection in the heart of both groups of mice is shown in Fig. 2. Virus could not be isolated from the hearts of mice in the control group at any time after infection. In cyclophosphamide-treated mice infective virus was first detected on the fifth day after virus infection, with titres increasing progressively until the death of animals. Grey-white lesions were observed macroscopically in cyclophosphamide-treated mice from the seventh day after infection until death. On microscopic examination foci of myocardial fibre necrosis with little mononuclear infiltration were found in both control and cyclophosphamide-treated mice. In control mice lesions disappeared by the seventh day following infection, whereas in cyclophosphamide-treated mice myocarditis became progressively more severe (Figs. 3, 4).

**Virus in the pancreas**

Virus titres in the pancreas of both groups are shown in Fig. 5, I. High levels of virus were present in both groups on the first day after infection and slowly dropped thereafter. By the
Fig. 6. Section of pancreas of a normal adult mouse (× 250).

Fig. 7. Section of pancreas from a mouse 4 days after Coxsackie B-3 virus infection, showing destruction of acinar cells (× 250).
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Fig. 8. Virus and antibody titres in peripheral blood of infected mice at various times after inoculation of virus, with or without cyclophosphamide (cy) 72 h later. Each point represents an average for two mice. ■—■, virus in blood of intact infected mice; •—•, virus in blood of infected, cy-treated mice; ○—○, antibody titres in infected, intact mice; ●—●, antibody titres in infected, cy-treated mice.

Tenth day no virus could be detected in either group. In the pancreas, necrosis of the acinar cells was observed in mice of both groups from the third day after virus infection. However, recovery followed in the control group whereas pancreatitis became progressively more severe in the cyclophosphamide-treated group, and led to complete necrosis of the organ. These changes did not occur in mice treated with cyclophosphamide only. On histological examination complete destruction of pancreatic acinar cells was observed whereas the islets and duct system remained intact. The infiltrate was predominantly of macrophages (Figs. 6, 7).

Virus in the liver

Virus titres in livers are shown in Fig. 5, II. Moderate titres were detected in control mice on the third and fourth day after infection. By the fifth day virus disappeared completely in this group. In the cyclophosphamide-treated animals virus persisted throughout the experiment. On histological examination mild focal necrosis was seen in both groups only at the early stages of the infection.

Virus in the spleen

Virus titres in the spleen are shown in Fig. 5, III. Peak titres of virus were detected in both groups on the third or fourth day after infection. By the seventh day virus had disappeared from spleens of control mice whereas it persisted in the cyclophosphamide-treated animals throughout the experiment. No microscopical lesions were seen other than changes typical of cyclophosphamide treatment in uninfected animals (Turk & Poulter, 1972).
Virus replication to moderately high titres in the brain was detected in both groups on the third day after infection (Fig. 5, IV). In control animals virus titres gradually dropped and by day 7 no virus could be isolated. In cyclophosphamide-treated mice moderate virus titres persisted until 9 days after infection and later disappeared. On histological examination mild meningitis and focal necrosis was seen at the beginning of the infection.

Viraemia and antibody response

Virus and antibody titres in the bloods of mice from both groups are shown in Fig. 8. In the control group viraemia was relatively short, so that by day 5 virus was cleared from the blood, whereas in cyclophosphamide-treated mice virus persisted in the blood indefinitely.

Neutralizing antibodies could be detected from the second day after infection and gradually increased. In cyclophosphamide-treated animals the early immune response was similar to that in control mice. However, 24 h after administration of the drug antibody titres began to fall, and by day 8 after infection treated mice did not have detectable levels of neutralizing antibodies. The antibody found transiently in cyclophosphamide-treated animals was sensitive to dithiothreitol and had the properties of IgM antibody. No IgG antibody against the virus was demonstrable at any time in immunosuppressed animals.

Blood interferon

Blood interferon levels in control and cyclophosphamide-treated mice are presented in Fig. 9. Soon after infection the interferon levels were similar in both groups. However, after 7 days interferon levels were higher in cyclophosphamide-treated mice, which perhaps correlates with the higher viraemia in these mice.

Effect of cyclophosphamide on intracerebral (i.c.) infection with CoxB-3 virus

Five-week-old CBA mice were inoculated i.c. with 1000 p.f.u. (0.03 ml) of virus followed 72 h later by i.p. injection of 6 mg of cyclophosphamide. A control group of mice was infected with virus only. Survivors from each group were killed 10 days after virus infection.
Organisms were prepared for histological examination by fixation in formol saline. Virus titrations were performed on 10% suspensions of organs as described earlier. No deaths occurred in the control group whereas the mortality was 100% in virus-infected and cyclophosphamide-treated mice. Virus (1 × 10^3 p.f.u.) was isolated from the heart of cyclophosphamide-treated mice, whereas all other organs were negative. Furthermore no virus was isolated from organs of mice which received virus alone.

On histological examination of the cyclophosphamide-treated virus-infected mice, many foci of myocardial necrosis were found in the hearts. In other organs, generalized necrosis was observed in the pancreas and mild meningitis in the brain. In mice that had received virus alone, one small focus of myocardial fibre necrosis was found in the heart and several small foci of mononuclear infiltration in the brain. No histopathological changes could be observed in the pancreas of these mice.

**Effects of thymectomy on resistance against CoxB-3 infection**

Twenty-two CBA mice were neonatally thymectomized, and inoculated i.p. with 10^5 p.f.u. of CoxB-3 virus 6 weeks later. As shown in Table I no death occurred in the thymectomized or intact mice of the same age which were infected with virus (controls). Furthermore levels of neutralizing antibodies in thymectomized mice were the same as in control mice.

**Effects of passive immunization on morbidity and mortality of CoxB-3 virus-infected and cyclophosphamide-treated mice**

Four groups of CBA mice 6 to 7 weeks old were infected i.p. with 10^5 p.f.u. of CoxB-3 virus. An i.p. injection of 6 mg of cyclophosphamide was given 72 h later. Groups then received: (1) a single i.p. inoculation of 0.4 ml of undiluted immune (titre 1:80) anti-CoxB-3 virus mouse serum 6 h after cyclophosphamide injection, (2) a single i.p. inoculation of the
Fig. 11. Effects of serum transfer on virus titres in heart of infected and cyclophosphamide-treated mice. Each point represents an average for two mice. ••••, mice infected with virus; — — , mice infected with virus followed by cyclophosphamide 72 h later; — — , mice infected with virus followed by cyclophosphamide 72 h later and anti-CoxB-3 serum 24 h and 15 days after cyclophosphamide.

Table 1. Effects of neonatal thymectomy on CoxB-3 virus infection in adult mice

<table>
<thead>
<tr>
<th>Virus</th>
<th>Treatment</th>
<th>Number of mice</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoxB-3</td>
<td>None</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>CoxB-3</td>
<td>Thymectomy</td>
<td>25</td>
<td>0</td>
</tr>
</tbody>
</table>

Six-week-old CBA mice were inoculated i.p. with 10³ p.f.u. of CoxB-3 virus.

Table 2. Neutralizing antibody levels in CoxB-3 virus-infected cyclophosphamide-treated mice following passive immunization

<table>
<thead>
<tr>
<th>Antibody titres</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoxB-3 virus</td>
</tr>
<tr>
<td>only (I)</td>
</tr>
<tr>
<td>CoxB-3 + cyclophosphamide (II)</td>
</tr>
<tr>
<td>CoxB-3 + cyclophosphamide + passive antibodies* (III)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day after CoxB-3 virus infection</th>
<th>CoxB-3 virus only (I)</th>
<th>CoxB-3 + cyclophosphamide (II)</th>
<th>CoxB-3 + cyclophosphamide + passive antibodies* (III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>1:160</td>
<td>1:80</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>1:160</td>
<td>1:20</td>
<td>1:160</td>
</tr>
<tr>
<td>6</td>
<td>1:160</td>
<td>1:20</td>
<td>1:80</td>
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<td>7</td>
<td>1:160</td>
<td>1:20</td>
<td>1:80</td>
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<td>8</td>
<td>1:160</td>
<td>&lt;1:10</td>
<td>1:20</td>
</tr>
<tr>
<td>9</td>
<td>1:160</td>
<td>&lt;1:10</td>
<td>1:20</td>
</tr>
</tbody>
</table>

(I) Three groups of 6- to 7-week-old mice were inoculated i.p. with 10⁵ p.f.u. of CoxB-3 virus.
(II) As for I followed 72 h later with 6 mg (i.p.) of cyclophosphamide.
(III) As for II followed by administration of 0.4 ml (i.p.) of anti-CoxB-3 serum 24 h after cyclophosphamide.
* Neutralizing antibodies were measured in Vero cell monolayers. Antibody titres are expressed as the highest dilution that neutralized 50% of the virus inoculum.
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same dose of antiserum 24 h after cyclophosphamide injection, (3) two inoculations of 0.4 ml of hyperimmune anti-CoxB-3 virus serum (1:320) 24 h and 15 days after cyclophosphamide injection, (4) no further treatment.

As shown in Fig. 10, prolonged protection of CoxB-3 virus-infected cyclophosphamide-treated mice from fatal infection was obtained only when relatively high-titre antibodies were administered twice. Some protection was achieved by injection of antibody 24 h after cyclophosphamide injection. No protection but delayed death was observed in animals which received a single injection of antibody 6 h after cyclophosphamide treatment.

Virus titres in organs of fully protected mice were much lower than in virus-infected immunosuppressed animals (Fig. 11). By the tenth day no virus could be isolated from hearts of protected mice, whereas 10^6 p.f.u. of virus were isolated from unprotected mice. In all other organs – pancreas, liver, spleen, brain and blood – virus titres were similar to those in mice infected with CoxB-3 virus without immunosuppression. On histological examination in the pancreas of protected mice, necrosis with no cellular reaction was observed, but on the eleventh day after infection normal acinar cells reappeared. The antibody level in the blood was high on the fifth day after virus infection, which is probably due to presence in the circulation of administered antibody (Table 2).

DISCUSSION

In the present study we have demonstrated that susceptibility of adult mice to intraperitoneal infection with CoxB-3 virus can be greatly increased by administration of a single dose of cyclophosphamide. All virus-infected mice died after injection of the drug, whereas no deaths occurred in mice infected with virus alone. Mortality of the immunosuppressed mice was associated with increased virus replication and pathological changes in target organs – notably the heart and pancreas – and persistent viraemia. The brain was not affected even when virus was inoculated intracerebrally. Mortality of these mice was a result of virus replication in heart and pancreas.

In intact mice clearance of virus from the blood was correlated with the appearance of circulating antibodies. Similar results were obtained with encephalomyocarditis virus by Murphy & Glasgow (1968).

In our experiments with CoxB-3 virus the effect of the drug depended on the time of administration: when this was 72 h after virus, all animals were dead by 7 days, and mortality was delayed when the drug was given at other times (Fig. 1). Cole et al. (1971) also found that the administration of cyclophosphamide to mice 72 h after they were infected with LCM virus was most effective; 85% of the adult mice became permanent virus carriers. Neutralizing antibodies were detectable in the bloods of intact and drug-treated infected mice 72 h after virus infection. Antibody in virus-infected, cyclophosphamide-treated mice was exclusively of the IgM class, and the level dropped soon after injection of the drug. There is some evidence that the switch mechanism which changes IgM to IgG antibody production is highly sensitive to cyclophosphamide (Makinodan, Santos & Quinn, 1970). In our system this change probably occurs about 72 h after virus inoculation. IgM (19 S) antibodies were found in virus-infected mice before cyclophosphamide was given. Following administration of cyclophosphamide 72 h after virus inoculation, neutralizing antibody levels dropped and no IgG (7 S) antibodies were detected at any time. On the other hand, in mice infected with virus alone IgM and IgG antibodies were found from 72 h onwards. The destructive effects of cyclophosphamide on rapidly proliferating and differentiating immunocompetent cells following antigenic stimulation would explain this result (Santos, 1967; Petrov et al. 1971). A similar prolonged depression of capacity to form antibody against
dengue virus after cyclophosphamide treatment has been described by Weiner, Cole & Nathanson (1971). The absence of circulating anti-virus antibody in the mice infected with CoxB-3 virus could account for the persistence of viraemia and an increased seeding of the target organs. The importance of circulating antibody in preventing spread to and extensive replication in target organs was demonstrated by the efficacy of passive protection with antibody. Soon after inoculation of antibody into immunosuppressed mice, virus titres in blood dropped to undetectable levels. Furthermore, in heart and pancreas – organs in which virus replication has already taken place and produced some pathological changes – virus replication was terminated and recovery commenced. A possible mechanism by which circulating antibody may contribute to recovery is described in the accompanying paper. Other suggested mechanisms include: (a) prevention of virus adsorption to cells (Svehag, 1968); (b) lysis of virus (Berry & Almeida, 1968); (c) altered intracellular handling of virus-antibody complexes (Silverstein, 1970), and (d) lysis of virus infected cells (Brier, Wahlenberg & Notkins, 1971; Smith et al. 1972). The relative importance of these and possibly other mechanisms in antibody-mediated recovery from enterovirus infections requires further evaluation.

Interferon levels were higher in cyclophosphamide-treated than in intact mice, which may be related to the presence of more virus in blood of immunosuppressed animals. Similar results were obtained with mice infected with encephalomyocarditis virus following whole-body irradiation (Murphy & Glasgow, 1968) or when mice were infected with CoxB-3 virus and treated with cortisone (Rytel, 1969). It is therefore unlikely that interferon alone can terminate a systemic virus infection, although collaboration of interferon and antibody cannot be excluded.

Histopathological studies of the virus-infected organs showed necrosis of heart muscle fibres with macrophages present in large numbers, together with some polymorphs. In the pancreas acini were destroyed but islets and duct systems remained intact. The inflammatory infiltrate consisted also predominantly of macrophages.

Woodruff & Woodruff (1971) have reported that the severity of CoxB-3 infection of undernourished mice can be reversed by transfer of immune lymphoid cells, but that survival in these mice is poorly correlated with levels of neutralizing antibody in circulating blood. They conclude that the acquisition of lymphocyte-mediated defence mechanisms is essential for recovery from primary virus infection. Our results do not support the view that classical cell-mediated immunity plays a major role in recovery. Neonatally thymectomized mice do not show increased susceptibility or a depressed antibody response to Coxsackie virus infections, in contrast to their increased susceptibility to other viruses such as herpes simplex (Mori et al. 1967; Allison, 1972a). Evidence is presented in the accompanying paper (Rager-Zisman & Allison, 1973) that antibody collaborates with non-immunocompetent host cells in recovery from Coxsackie-B virus. The host cells with this capacity may function poorly in undernourished mice, and transfer of immune lymphoid cells may restore this deficiency as well as supplying lymphocytes that can rapidly synthesize antibody in virus-infected recipients.

The results of our experiments now reported, as well as those previously published (Zisman, Hirsch & Allison, 1970; Zisman, Wheelock & Allison, 1971) and observations on human patients with various immuno-deficiency syndromes (Allison, 1972b), strongly suggest that in certain virus infections, such as those with enteroviruses and togaviruses, circulating antibody, acting in collaboration with non-immunocompetent host cells, plays the major defensive role, whereas with other viruses, such as herpesviruses and poxviruses, cell-mediated immunity is also of great importance in host cell protection.
We wish to thank Miss Mary Purdy for excellent technical assistance.

REFERENCES


(Received 27 September 1972)