MECHANISM OF RECOVERY FROM SYSTEMIC VACCINIA VIRUS INFECTION

I. THE EFFECTS OF CYCLOPHOSPHAMIDE

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The recovery of an animal from a primary viral infection has been extensively studied, but the relative importance of the several immune factors in this process has not been established. In recent years increased emphasis has been placed on the importance of cellular immunity and the relative lack of importance of humoral antibody in this recovery process (1, 2). This impression is partially based on the observation that patients thought to have primarily defects in cellular immunity are much more susceptible to certain viral infections, particularly vaccinia virus infections (2, 3). In addition it has recently been shown that administration of anti-thymocyte or anti-lymphocyte sera will significantly increase the mortality of mice infected with a number of viruses, including vaccinia virus (4-7).

A series of investigations was designed to study the role of cellular immunity, humoral antibody, and interferon in recovery from primary systemic vaccinia virus infection in mice. Vaccinia virus was chosen because it appears to be generally regarded as a viral infection in which there is a critical role for cellular immunity in the recovery process, both in man and in experimental animals (3, 5). It was planned to eventually use a number of different immunosuppressive agents, in the hope that different deletions of the components of the immune system would result in patterns of enhanced infection in proportion to the importance of the immune factors. In the first of the studies experiments were performed using cyclophosphamide (Cytoxan) as an immunosuppressive agent; the results suggest an essential role for humoral antibody, but not for cellular immunity, in recovery from primary vaccinia virus infection in the mouse.

Materials and Methods

Animals.—6-wk old NIH Swiss male mice and 6-wk old C57/BL6 male mice were obtained from the National Institutes of Health colonies.

Vaccinia Virus Strain.—Neurovaccinia virus was obtained from Dr. W. A. Casel, Emory University, Atlanta. Vaccinia virus was grown on the chick embryo chorioallantoic membrane; the pool titered $10^{6.0} \text{ ID}_{50}/0.05 \text{ ml}$ when inoculated intracutaneously (i.c.) into weanling Swiss male mice and $10^{5}$ plaque-forming units (PFU)/ml on vero cells.

1 Abbreviations used in this paper: ATS, anti-thymocyte serum; EMC, encephalomyo-
**Vaccinia Virus Assay.**—Individual mice were bled by the orbital technique, and serial 10-fold dilutions of each serum in Eagle's medium with 2% fetal calf serum (FCS) were assayed for vaccinia virus content by plaque titration on vero cells.

**Neutralizing Antibody Tests.**—Individual mouse sera were assayed for anti-vaccinia virus antibody by a plaque reduction method. Samples of vaccinia virus containing 50 PFU/0.2 cc were incubated with 3-fold dilutions of serum in Eagle's medium with 2% FCS at 37°C for 2 hr; each mixture was then plaque assayed on vero cells. A control sample of vaccinia virus incubated with normal mouse serum was included in each experiment.

**Lymphocytic Choriomeningitis Virus.**—Lymphocytic choriomeningitis virus (LCM) strain CA1371 was obtained from Dr. W. Rowe of the NIH. It had been grown in mouse brains; a 10% w/v suspension was made just before use, using Eagle's medium with 10% FCS. Further dilutions were also made in Eagle's medium with 10% FCS.

**Interferon Assays.**—At various time intervals after injection of virus, five mice from each group were bled and their blood pooled. The blood was centrifuged at 1500 rpm for 20 min and the serum was then stored at -20°C in a mechanical freezer. The serum interferon titers were determined as the reciprocal of the highest dilution of serum which inhibited the hemagglutinin yield of GD-7 virus during a single growth cycle in mouse L cells by 0.5 log2. Titers were adjusted in accordance with the titer of a laboratory reference interferon which was titrated in each assay. The international reference mouse serum interferon titered 10^{1.5} units/ml.

**Hyperimmune Anti-Vaccinia Virus Antibody.**—2-kg female rabbits were injected subcutaneously (s.c.) with 10⁶ PFU of vaccinia virus, and 2 wk later they received a similar s.c. injection. 1 wk after this they received a third s.c. injection of virus, and a week later they were bled by intracardiac puncture. The blood was allowed to clot overnight at 0°C and sera were then separated by centrifugation at 1500 rpm for 25 min.

**Immune Lymphocytes.**—The method described by Fred and Smith was used to prepare immune lymphocytes (8). 2 wk after intravenous (i.v.) injection of 10⁵ PFU of vaccinia virus, mice were sacrificed and their spleens collected. At this time the mice were immune as indicated by serum anti-vaccinia antibody titers of about 1:1000. Mice immunized to LCM virus received three weekly intraperitoneal (i.p.) injections of 10⁵ i.e. LCM of this virus. Their spleens were collected 1 wk after the third i.p. injection. Suspensions of individual spleen cells were prepared and the viability of the cells was determined by trypan blue exclusion. Each recipient mouse was injected slowly i.v. with 10⁶ viable cells in 0.5 cc of phosphate-buffered saline (PBS) on the day indicated. Some control mice received just 0.5 cc of PBS while others received no injection.

**Cyclophosphamide.**—Cyclophosphamide (Cytoxan) was obtained from Mead Johnson and Co., Evansville, Ind., and a solution in phosphate-buffered saline with a final concentration of 20 mg/ml was prepared just before use.

**Experimental Design**

All mice were injected i.v. with 10⁵ PFU of vaccinia virus in 0.1 cc of Eagle's medium with 2% fetal calf serum on day 0. 24 hr later one group of mice was injected i.p. with 150 mg/kg body weight of Cytoxan in 0.3 cc of PBS. A control group of mice received only 0.3 cc of PBS i.p. Each day after injection of virus three mice from each group were bled individually by the orbital method and the titer of viremia determined; three other mice from each group were also bled and the titer of serum anti-vaccinia antibody determined. The sera from five mice from both Cytoxan-treated and control groups were pooled within groups each day and assayed for interferon. After these initial studies replacement with vaccinia immune sera or immune lymphocytes was performed on different days after injection of virus. All mice were held for at least 28 days before an experiment was terminated.
RESULTS

Effect of Cytoxan on Mortality from Primary Vaccinia Virus Infection.—The results presented in Fig. 1 demonstrate that Cytoxan treatment markedly increased the mortality of vaccinia virus-infected Swiss male mice. Mice which received just vaccinia virus had 10% mortality, while Cytoxan-treated mice had 94% mortality. Cytoxan treatment alone did not kill any mice.

Effect of Cytoxan on Viremia.—The results presented in Fig. 2 demonstrate that viremia in Cytoxan-treated Swiss mice paralleled that of nonimmunosuppressed control mice for the 1st 3 days after infection. However, viremia in Cytoxan-treated mice did not decline on the 4th day after infection, but rather continued to rise and remained high until the death of the mice on the 7th or 8th day after infection. These results suggest that Cytoxan interfered with some host defense mechanism(s) which became effective on about the 4th day after infection and resulted in a termination of viremia.

Effect of Cytoxan on Serum Interferon Levels.—Mice injected with vaccinia virus had serum interferon level of 80 units/ml 8 hr after virus infection, but no interferon was detected in the sera of either Cytoxan-treated or control mice on any day after this. Interferon levels in organs were not studied.

Effect of Cytoxan on Serum-Neutralizing Antibody.—Cytoxan-treated mice formed no neutralizing antibody to vaccinia virus (with a single exception), while all control Swiss mice studied had significant levels of antibody by the 4th day after infection (Fig. 3). Identical results were obtained in C57/BL6 mice except that only one of three control mice had antibody on day 4; three of three had antibody on day 5 after infection and on each subsequent day. Cytoxan-
treated mice again did not form any detectable neutralizing antibody. These results suggest that suppression of neutralizing antibody formation might be the mechanism by which Cytoxan potentiated vaccinia virus infection. To test this

![Graph showing viremia in vaccinia virus infection in Swiss mice.](image)

**Fig. 2.** Effect of Cytoxan on viremia in vaccinia virus infection in Swiss mice.

![Graph showing formation of neutralizing antibody to vaccinia virus.](image)

**Fig. 3.** Effect of Cytoxan on formation of neutralizing antibody to vaccinia virus.

possibility vaccinia-neutralizing antibody was administered passively to Cytoxan-treated mice at various times after vaccinia infection.

*Effect of Passive Antibody Administration in Cytoxan-Treated, Vaccinia Virus-Infected Mice.*—Before using passively transferred antibody it was necessary to demonstrate that the levels of serum antibody obtained by the transfer were physiologic. Four C57/BL6 mice were injected i.p. with 0.4 cc of undiluted
vaccinia immune sera and bled at various time intervals. 6 hr and 3 days after injection of vaccinia immune sera these mice had serum antibody titers of between 30 and 70. This is similar to the serum antibody titers of nonimmunosuppressed mice 4 or 5 days after primary infection (Fig. 3).

Table I summarizes four experiments using C57/BL6 mice in which antibody was passively transferred on various days after virus infection. In each experiment there were 40 mice in the group receiving just vaccinia virus and Cytoxan, and 20 mice in each of the other groups. The results of these four experiments are combined in Fig. 4, which shows the time-course of mortality in the different groups. It is apparent that passive administration of physiologic amounts of antibody on days 4 and 5, when similar levels of antibody are present in non-immunosuppressed controls, largely reversed the enhancement of this infection by Cytoxan. Administration of antibody as late as the 7th day after infection still reduced final mortality by about 50%. Similar results were obtained when Swiss mice were used instead of C57/BL6 mice. These results thus strongly suggest that formation of neutralizing antibody is a critical host defense mechanism in this infection.

Effect of Cytoxan on Cellular Immunity.—Because of the unavailability of a reliable test for cellular immunity to vaccinia virus in mice, it was not possible to determine the effect of Cytoxan on this parameter of host defense. A single dose of Cytoxan has been reported to protect mice against acute disease after i.c. injection with LCM virus (9); this acute LCM disease is thought to be a cell-mediated immunopathological disease (9–11).
Fig. 5 shows the protective effect of a single dose of 150 mg/kg of Cytoxan i.p. 2 or 3 days after i.c. injection of Swiss male mice with $10^5$ i.c. $LD_{50}$ LCM virus in 0.05 cc. There were 15 mice in the group receiving just LCM virus, and 10 mice in each of the Cytoxan-treated groups. Similar results were obtained with C57/BL6 male mice. Thus, the dose of Cytoxan used in these experiments suppressed the cell-mediated immune response of mice to LCM virus.

Effect of Transfer of Immune Lymphocytes on LCM Virus Infection in Cytoxan-
**Treated Mice.**—Cytoxan has been shown to suppress cellular immunity in a variety of systems (12), and this was confirmed under the present conditions by its effect on experimental infection with LCM virus. To determine if correction of this defect would influence the outcome of this infection, immune lymphocytes were transferred to Cytoxan-treated C57/BL6 mice. Before this could be done, it was necessary to demonstrate that the transferred immune lymphocytes were capable of manifesting an immune response. Male C57/BL6 mice were injected i.c. with \(10^8\) LD\(_{50}\) of LCM virus; 3 days later they received a single dose of 150 mg/kg of Cytoxan i.p. As already noted, mice receiving Cytoxan were protected against acute LCM disease. 7 days after receiving Cytoxan, one group of 15 mice were injected i.v. with \(10^8\) spleen cells from LCM immune mice; another group of 15 mice received just PBS i.v. All 15 mice receiving the immune lymphocytes developed acute LCM disease and were dead by 9 days after injection of the cells; 14 of the 15 mice injected with PBS were alive and appeared well 3 wk after receiving the PBS. These results indicate that the transferred immune spleen cells were capable of mounting a potent immune response.

**Effect of Transfer of Immune Lymphocytes on Vaccinia Virus Injection in Cytoxan-Treated Mice.**—In each experiment on various days after vaccinia virus infection 15 mice were injected slowly i.v. with 0.5 ml of PBS containing \(10^8\) viable spleen cells from vaccinia immune mice. Fig. 6 summarizes three experiments performed in this manner. Injection of immune lymphocytes on day 3 after infection gave minimal protection, about the same effect as that of antibody transferred on day 7. Transfer of immune cells later than the 3rd day after infection had no significant effect on final mortality (Fig. 6). Four of the surviving mice injected with immune cells on day 3 after infection were bled on the 12th day after infection and their sera tested for antibody to vaccinia virus. All four sera had neutralizing antibody to vaccinia virus ranging from \(10^2\) to \(10^4\) units/0.2 cc. This finding raises the possibility that these cells might have exerted their antiviral effect by making neutralizing antibody. These results indicate that even large numbers of immune lymphocytes are far less effective at reversing the effect of Cytoxan on vaccinia virus infection than passive antibody administration.

**Pathology.**—On days 3, 5, and 7 after injection of vaccinia virus, three mice from each of the experimental groups were sacrificed; the brain, heart, lungs, liver, spleen, and kidneys of each mouse were removed, fixed in B-5 solution (formal sublimate), sectioned, and stained with hematoxylin and eosin. On day 3 after infection vaccinia-infected mice, both Cytoxan-treated and control, had focal areas of mild interstitial pneumonia and rare areas of focal hepatic necrosis. On day 5 after infection both groups demonstrated areas of focal necrosis with some polymorphonuclear cell infiltration in the lungs and the liver. There was no definite difference in the type or severity of these histopathological lesions in these two experimental groups on either day 3 or day 5 after infection.
By day 7, however, a clear difference between the two groups was apparent. Two of the three nonimmunosuppressed mice had no pathological lesions noted in any of the organs examined; the third mouse had only very rare areas of focal necrosis in the lungs and liver. All three mice receiving Cytoxan, on the other hand, had moderately severe bronchopneumonia and multiple areas of hepatic necrosis with many inflammatory cells. Two of three Cytoxan-treated mice also had areas of necrosis with inflammation in the myocardium at the base of the heart and in the walls of the aortic and pulmonary arteries; these same two mice also had moderate polymorphonuclear cell infiltration of their spleens. None of the mice had pathological lesions in the kidneys.

**DISCUSSION**

The present results indicate that administration of Cytoxan, in amounts sufficient to suppress humoral and cellular immunity, markedly potentiated primary systemic vaccinia virus infection in mice. Cytoxan-treated mice did not form detectable neutralizing antibody to vaccinia virus, and had a prolonged and more severe viremia than nonimmunosuppressed control mice. Passive transfer of physiologic amounts of neutralizing antibody late in the course of
infection, at a time when nonimmunosuppressed mice had similar levels of serum antibody, largely reversed the enhancing effect of Cytoxan on vaccinia virus infection. Passive transfer of 100 million immune spleen cells on day 3 after infection was partially protective but was ineffective thereafter in reversing this effect of Cytoxan on vaccinia infection, and mice receiving these cells did appear to make some antibody.

These results suggest that the formation of neutralizing antibody in the non-immunosuppressed mouse resulted in a termination of viremia, and that in this manner antibody played a critical role in limiting the primary vaccinia virus infection. The more severe viremia in immunosuppressed mice apparently resulted in more extensive dissemination of vaccinia virus to the target organs, particularly the lungs and liver. Our results do not exclude the possibility that antibody also played some direct role in recovery of infected target organs. Because of a lack of an adequate, direct test for cellular immunity to vaccinia virus in the mouse, it is more difficult to reach a firm conclusion concerning the importance of this component of the immune response in the recovery process. However indirect evidence indicates that Cytoxan did effectively suppress cellular immunity under the present experimental conditions. Cytoxan in the dose used has been shown to suppress cellular immunity in a variety of experimental systems (12). Cytoxan has been highly effective in preventing rejection of immunologically foreign lymphocytes by mice, even when the donor and recipient differ across the H-2 locus (13). A single dose of Cytoxan protected mice against acute LCM disease (9), which is thought to be a cell-mediated immunopathological disease (10, 11). We have confirmed, under the conditions of the present experiments, the effect of Cytoxan on experimental infection with LCM virus. Perhaps most impressive is the ability of a single dose of Cytoxan to inhibit development of immunity to a bacteria, *Listeria monocytogenes*, in mice (14); in this system Mackaness and his colleagues have demonstrated that cellular immunity and not antibody is critical for recovery (15). These studies would suggest that Cytoxan probably had some effect on cellular immunity to vaccinia virus in our experiments. Whatever degree of suppression of cellular immunity to vaccinia virus was obtained with Cytoxan did not appear to be enough to explain the potentiation of the infection, since the effect of Cytoxan was almost entirely reversed by antibody administration. Additional support for the suggestion that cellular immunity may not play a decisive role in recovery from primary vaccinia virus infection in mice comes from the observation in our laboratory that neonatally thymectomized mice, which have a very significant defect in cellular immunity, recover normally from this infection (16).

In recent years it has been demonstrated that an immune response is critical in recovery from a number of experimental, primary viral infections (4–7, 17–21). There are a number of possible mechanisms by which the immune response might contribute to recovery during a primary virus infection. Neutralizing antibody could control viremia, as appeared to be the case in our experiments,
and thus prevent further virus dissemination; alternately antibody could directly promote recovery of the infected target organs. Likewise cellular immunity could play an important role in a primary virus infection by either helping to control viremia or by assisting directly in the recovery process at the level of the target organ. Obviously these possible roles for the immune response are not mutually exclusive.

Further clarification of specific roles for the components of the immune response comes from consideration of selected reports from the literature. A number of studies have demonstrated that immunosuppression may potentiate a primary virus infection and that this potentiation may be accompanied by a suppression of neutralizing antibody formation and by a more severe viremia (17-21). In most of these studies it is likely that cellular immunity was also suppressed to some extent, and specific replacement with antibody or immune cells has generally not been performed. However, in an excellent series of studies, Murphy and Glasgow showed that either X-irradiation or a combination of cyclophosphamide and thioguanine dramatically potentiated primary systemic encephalomyocarditis (EMC) virus infection in mice and that formation of neutralizing antibody to EMC virus was suppressed by both of these methods of immunosuppression (22, 23). They also demonstrated that passive transfer of anti-EMC antibody could protect immunosuppressed mice if administered early enough in the course of infection (23). The authors concluded that formation of neutralizing antibody was critical during the viremic phase of primary EMC virus infection in mice. Cellular immunity was not measured and transfer of immune cells was not performed. These results with EMC virus are similar to our results with vaccinia virus. It would thus appear that in certain experimental virus infections neutralizing antibody plays a critical role by controlling viremia and preventing further virus dissemination.

It is more difficult to find strong evidence to support a possible role for antibody in directly promoting recovery of an infected target organ, and the studies already referred to provide no direct information on this point. In general, passive transfer of even large amounts of antibody has not exerted a protective effect when the virus has been introduced directly into an organ such as the brain (24). There are, however, at least two reports of instances in which transfer of antibody may have enhanced the recovery process (25, 26). In addition, immunosuppression has generally not potentiated primary virus infection when the virus is introduced directly into the target organ (4, 6, 23, 27). Exceptions to this general observation sometimes occur when experimental respiratory and intracranial viral infections are studied. Mice immunosuppressed with Cytoxan and infected intranasally with Sendai virus develop higher titers of virus in the lung and have more extensive histologic lesions than nonimmunosuppressed control mice (19). In experiments in which intranasal infection of mice with Sendai virus was potentiated by immunosuppression, serum antibody titers were found to be suppressed (19). This does not prove, however, that this suppression of antibody formation resulted in the increased severity of this infec-
tion. More recently Cytoxan-treated mice have been shown to be much more susceptible to i.c. infection with certain arboviruses (18). Antibody response to these arboviruses (18) was again suppressed in Cytoxan-treated mice, but it is quite likely that cellular immunity was also suppressed. These studies with arboviruses and Sendai virus do indicate that in certain experimental nonviremic infections an immune response is necessary for control of virus growth in the target organs. They do not, however, allow a definite statement as to which component of the immune response, antibody or cellular immunity, is critical in this process. The mechanisms by which antibody might promote target organ recovery could include prevention of virus adsorption to cells (28), lysis of virus (29), altered intracellular handling of virus-antibody complexes (30), and lysis of virus-infected cells (31).

There is little evidence to support an important role for cellular immunity in controlling viremia in experimental virus infections. Neonatally thymectomized mice and anti-thymocyte serum (ATS)-treated mice have been shown to be more susceptible to certain virus infections, including herpes simplex, vaccinia, and reovirus (5–7, 32). These two methods of immunosuppression have sometimes been regarded as specific methods of suppression of cellular immunity; this is clearly not a valid conclusion, because they have also been demonstrated to suppress antibody formation to a variety of antigens (33, 34). In our laboratory ATS-treated mice have also been demonstrated to be more susceptible to intravenous infection with vaccinia virus; this enhancement was accompanied by a suppression of antibody formation and a more severe viremia. Furthermore, replacement with physiologic amounts of antibody essentially completely reversed the enhancement of this infection by ATS. Another study in ATS-treated monkeys demonstrated enhancement of monkeypox infection which was accompanied by a prolonged and more severe viremia (35). In this study, the appearance of neutralizing antibody was also delayed. In a recent careful study by Blanden a single dose of ATS was sufficient to potentiate ectromelia infection in mice (36). Mice treated with ATS had higher titers of virus in the blood, liver, and spleen, but not in the footpad injection site, well before antibody could be detected in the serum. Transfer of immune lymphocytes markedly reduced the titers of ectromelia in the spleen and liver; transfer of hyperimmune serum had a definite, but less dramatic effect (37). In this study suppression of cellular immunity appeared to be the major mechanism by which ATS potentiated ectromelia infection in the spleen and liver, but not in the footpad. The increase in virus titer occurred at the same time in the blood and the target organs, and so it is difficult to determine if the ATS acted by suppressing cell-mediated immune mechanisms first at the target organ level, or if a cell-mediated immune mechanism for directly controlling viremia was also suppressed. Although not highly likely it is still possible that the ATS also acted to delay the first appearance of low levels of antibody which can effectively control viremia since undiluted serum was not used for antibody assays.

Cytoxan is a widely used immunosuppressive and antitumor agent in man; it
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has recently been demonstrated to be effective in suppressing graft rejection in renal transplant patients (38), and it is quite possible that it will be used more extensively in the future in this group of patients. Fatal varicella infection has been reported in a child on Cytoxan therapy (39). Clearly such infections should be watched for carefully in patients receiving Cytoxan.

SUMMARY

Administration of Cytoxan in doses capable of inhibiting both humoral and cellular immunity markedly potentiated primary systemic vaccinia virus infection in mice. Immunosuppressed mice did not form neutralizing antibody to vaccinia virus and had a prolonged and more severe viremia than nonimmunosuppressed control mice. Passive transfer of physiologic amounts of neutralizing antibody late in the course of infection, at a time when nonimmunosuppressed mice had similar levels of serum antibody, largely reversed the effect of Cytoxan on vaccinia virus infection. Transfer of 100 million immune spleen cells was much less effective than antibody in reversing the effect of Cytoxan on vaccinia virus infection, and mice receiving these cells did make some antibody. Serum interferon levels were not affected by Cytoxan. The results suggest an essential role for humoral antibody, but not for cellular immunity, in recovery from primary vaccinia virus infection in the mouse.

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