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CYTOMEGALOVIRUS-INDUCED IMMUNE SUPPRESSION

I. HUMORAL IMMUNITY

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SUMMARY

Murine cytomegalovirus (CMV) infects cells of the lymphoreticular system and causes alteration in immune function. Four to 6 days after infection, histological changes are observed in the spleen, consisting of intranuclear inclusions around germinal centres and destruction of germinal centres at higher virus inocula. Gradual healing takes place after this period. CMV also causes suppression of immune function, the maximal suppression also occurring in the 1st week after infection. Both the primary and secondary immune responses to sheep red blood cells are suppressed. The degree of immune suppression is directly related to virus inocula. When mice are first vaccinated with attenuated CMV, infection with virulent CMV results in an immune response greater than that observed in control mice.

INTRODUCTION

Many virus infections significantly affect immune function. The murine leukaemia viruses, lymphocytic choriomeningitis virus, M–P virus, and Junin virus inhibit humoral immunity (Notkins, Mergenhagen & Howard, 1970). Lactic dehydrogenase and Venezuelan equine encephalitis viruses, on the other hand, lead to an augmented antibody response to human gamma-globulin (Notkins *et al.*, 1966; Howard *et al.*, 1969). Viruses may also lead to altered serum immunoglobulin concentrations, to depressed cellular immunity, and to depressed and enhanced phagocytosis (Notkins *et al.*, 1970).

Several human viruses may also lead to altered immune function (Notkins *et al.*, 1970). Cytomegalovirus (CMV) infection is associated with abnormal serum globulins and depressed cellular immunity (Kantor *et al.*, 1970). In renal transplant recipients, CMV infection may lead to bacterial superinfection and transplant rejection (unpublished observations). Murine CMV causes suppression of the primary immune response to sheep red blood cells (Osborn, Blazkovec & Walker, 1968). The following experiments were undertaken to delineate the effect of CMV infection on immune function.

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MATERIALS AND METHODS

Animals

Swiss–Webster mice were obtained from Thorp Industries, Incorporated, White Bear Lake, Minnesota. Unless otherwise mentioned, female mice were used and were 4 weeks old at the time of infection with CMV.

Virus

Wild-type murine CMV was kindly provided by Dr June E. Osborn, Madison, Wisconsin. The virus was prepared from mouse submaxillary gland 21 days after infection. Mice were sacrificed by cervical dislocation and the submaxillary glands were removed sterilely and weighed. Cells were disrupted by grinding the tissue with a mortar and pestle using fine sand. A 10 per cent suspension (w/v) was prepared in medium 199, 5 per cent foetal calf serum, and 10 per cent dimethylsulphoxide. The mixture was centrifuged at 200 *g* for 10 min to remove cellular debris. The suspension was then aliquoted into small vials and stored at −70°C until used. Virus was assayed by the microplaque method as previously described (Osborn & Walker, 1970). The LD_{50} is approximately 5×10^6 plaque-forming units (PFU). All mice were infected intraperitoneally with 2×10^3 to 2×10^6 PFU.

Tissue culture passaged CMV was obtained from the American Type Tissue Collection, Bethesda, Maryland. A pool was prepared by growing the virus in mouse embryo fibroblasts. Culture medium was collected 6 days after infection and was made into a 10 per cent solution with dimethylsulphoxide. It was aliquoted into small vials, stored at −70°C, and assayed. The same pool was used in these experiments and had a titre of 8×10^7 PFU/ml.

Histological studies

Organs (spleen, thymus, lymph nodes, lung, liver, kidney, and muscle) were obtained following CMV infection. Samples were taken 2, 4, 6, 8, 10 and 14 days after infection and weekly thereafter. They were fixed in 10 per cent formalin, embedded in paraffin, sectioned, and stained with Haematoxylin and Eosin.

Carbon clearance

The rate of clearance of colloidal carbon was determined by the method of Biozzi *et al.* (Biozzi, Benacerraf & Halpern, 1953). Ten milligrams of colloidal carbon (Pelikan, Gunther, Wagner, Incorporated)/100 g of body weight was injected intravenously. Twenty microlitre blood samples were obtained from the same mouse from the retro-orbital venous plexus before and at 1, 4, 7 and 12 min after carbon clearance and diluted in 1·0 ml water containing 0·1 mg sodium carbonate. The optical density was read at 650 nm, and clearance rates (k) were determined from the slope of the line obtained by plotting the optical density against time on a semi-log scale.

Sheep erythrocytes

Sheep blood was obtained in Alsever's solution and stored at 4°C until used. Prior to use, the erythocytes were washed three times in phosphate-buffered saline. For immunization, mice were administered 0·2 ml of a 25 per cent ($7{\cdot}5 \times 10^7$ red cells) suspension of sheep red blood cells (SRBC) intraperitoneally (i.p.).

Haemagglutinin assay

Blood was collected from the mice by cardiac puncture. The serum was separated and decomplemented by heating at 56°C for 30 min. Serial two-fold dilutions of mouse sera in Amos and Peacock buffer were prepared in U-bottom microtitre plates (Linbro Chemical Company, New Haven, Connecticut). An equal volume (0·25 ml) of 1 per cent washed SRBC was added to each dilution and to control wells. The plates were incubated at room temperature for 2 hr and the titre was read as the last well showing agglutination.

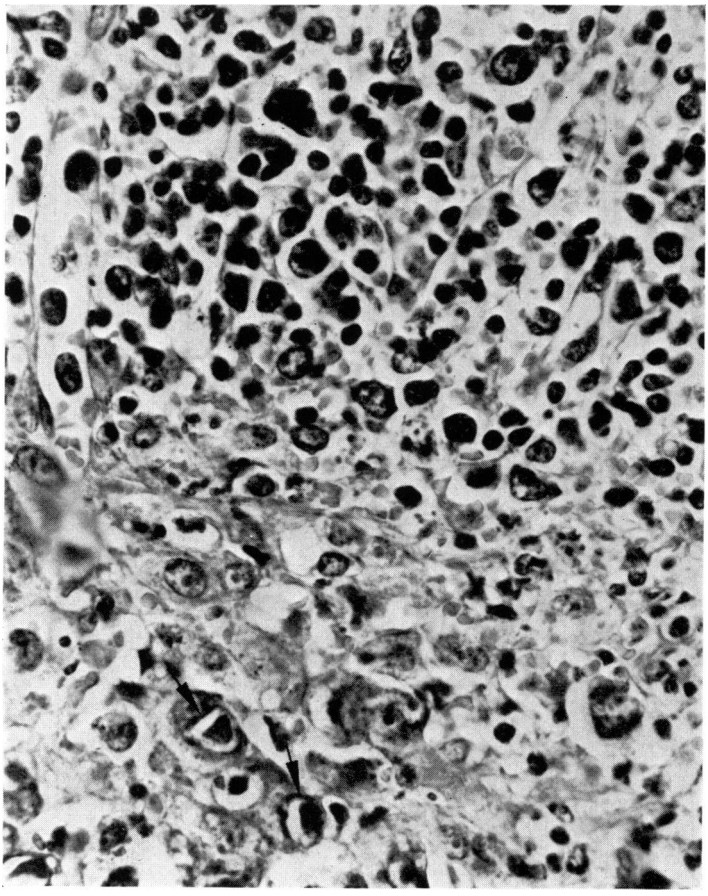

FIG. 1. Spleen from a mouse infected 6 days previously with 2×10^5 PFU CMV. Numerous intranuclear inclusions (arrow) can be seen in the space surrounding the intact germinal centre. (Magnification × 80.)

Haemolysin assay

Serial two-fold dilutions of decomplemented mouse sera were prepared in phosphate-buffered saline in U-bottom microtitre plates. An equal volume of one per cent SRBC and rabbit complement (0·25 ml) (1 : 2 dilution of rabbit serum) was then added to each well. The plates were incubated at 37°C for 30 min and the titre of each sample was read as the last well showing haemolysis.

RESULTS

Histological changes in lymphoid tissues following CMV infection

Characteristic intranuclear inclusions can be seen in many organs following CMV infection including spleen, lymph node, liver, salivary gland, kidney, and lung. Notably, in liver an acute inflammatory reaction can be observed around these inclusion-bearing cells. The spleen is the main lymphoid organ affected. When the mouse is infected with 5×10^4–2×10^5 PFU, CMV intranuclear inclusions can be seen surrounding germinal centres (Fig. 1).

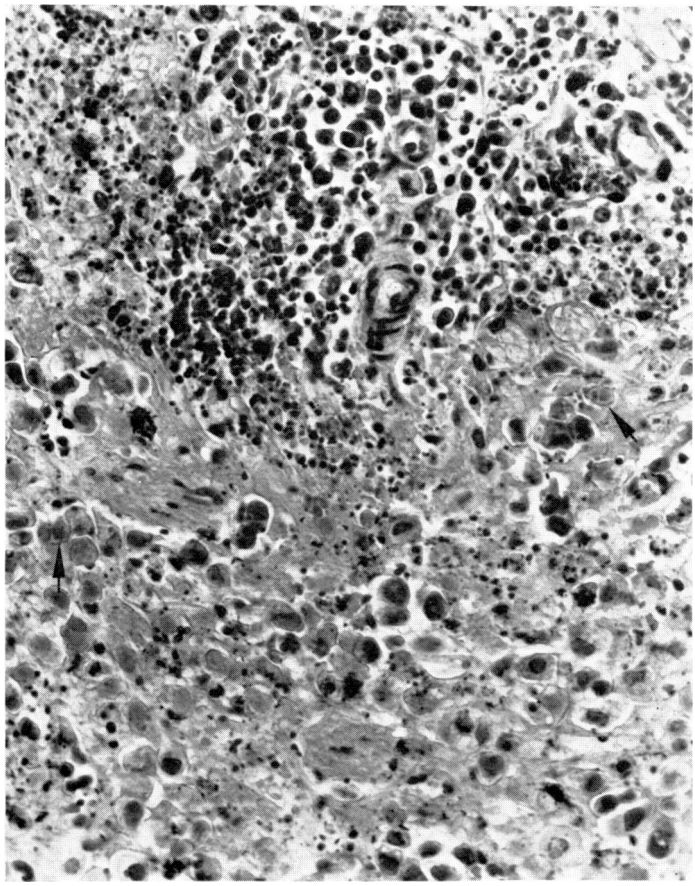

FIG. 2. Spleen from a mouse infected 6 days previously with 10^6 PFU CMV. There is destruction of the cellular elements of the germinal centre, and nuclear debris can be observed. Intranuclear inclusions (arrow) are not as readily observable as in Fig. 1 because of cellular destruction. (Magnification $\times 80$.)

These findings are most striking 4–6 days following infection. After this period, the intranuclear inclusions gradually disappear from the spleen, although virus can still be cultured from the organ. At higher virus inocula (10^6 PFU), the germinal centres undergo destruction (Fig. 2). There is gradual healing after the 1st week so that by 3 weeks after infection, the germinal centres have been reconstituted.

Intranuclear inclusions can also be identified in lymph nodes after CMV infection although germinal centre destruction does not occur. No histological changes can be identified in the thymus.

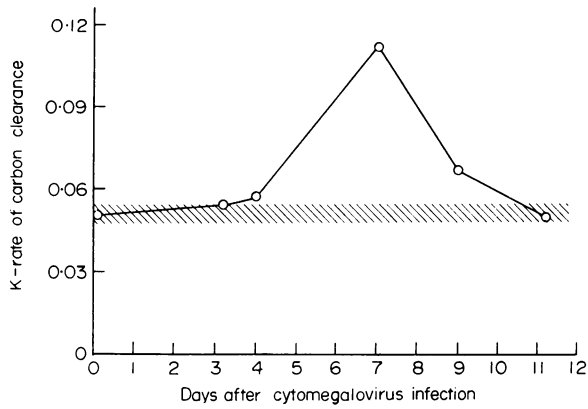

FIG. 3. Rate of carbon clearance in mice infected with 2×10^5 PFU CMV. The shaded area indicates the average ± 1 standard deviation for uninfected control animals. (○) The daily average carbon clearances for infected mice.

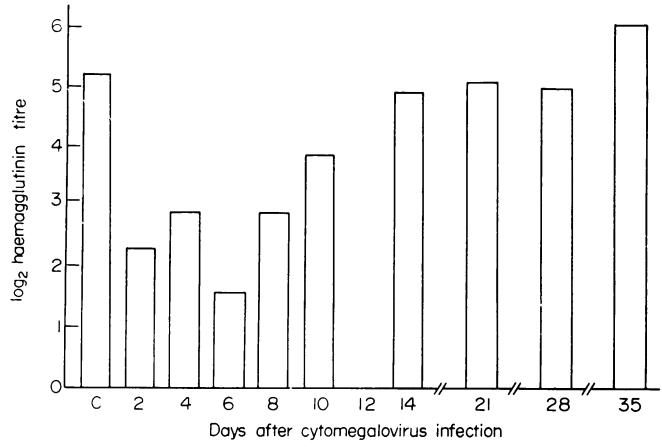

FIG. 4. Haemagglutinin antibody titre to sheep red blood cells in mice infected with 2×10^5 PFU CMV. Animals were immunized with SRBC 2–35 days after infection. C = uninfected control animals.

Carbon clearance

Processing of antigen by macrophages is thought to play an important role in the immune response. Therefore, clearance of colloidal carbon was measured in Swiss–Webster mice at various times after CMV infection. Mice infected with CMV 7 and 9 days prior to testing had significantly increased rates of carbon clearance compared to uninfected controls (Fig. 3). By 11 days after infection, clearance rates had returned to normal.

Primary response to SRBC in CMV-infected mice

Groups of eight to ten Swiss–Webster mice were infected with 2×10^5 PFU CMV at various times before immunization with SRBC. Control mice were injected with 0·2 ml medium 199 with 5 per cent foetal calf serum prior to immunization. Animals were bled 8 days after immunization. A greater than four-fold difference in antibody titre between control and experimental groups was considered to be significant. Mice infected with CMV 2–8 days prior to immunization with SRBC have significantly depressed antibody responses compared with uninfected control mice (Fig. 4). Whereas control animals had a mean $\log_2$ titre of 5·2, the mean $\log_2$ titre was 1·6 when mice were immunized 6 days after infection. The immune response to SRBC was still suppressed in mice immunized 10 days after CMV infection, although not significantly. If animals were immunized 14 or more days after infection, there was no suppression of the immune response to SRBC. Heat-inactivated virus (56°C for 30 min) does not lead to immune suppression. Tissue culture passaged CMV also does not cause immunosuppression (mean $\log_2$ haemagglutinin titre of 7·2 *versus* 6·1 for controls).

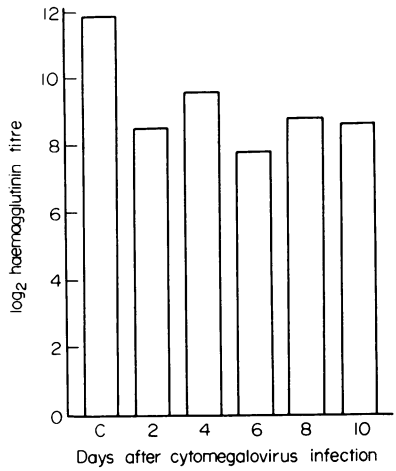

FIG. 5. Secondary haemagglutinin antibody response to sheep red blood cells in mice infected with 2×10^5 PFU CMV. Animals were infected 8 days after immunization with SRBC. A secondary immunization was administered 2–10 days after infection. C = uninfected control animals.

Secondary response to SRBC in CMV-infected mice

Groups of eight to ten 4-week-old Swiss–Webster mice were immunized with SRBC. Eight days later, they were infected with 2×10^5 PFU CMV. Control mice were not infected. Groups were given a secondary immunization from 2 to 10 days after infection. Control mice were given a booster injection of SRBC 8 days after the primary immunization or did not receive a secondary immunization.

CMV infection also inhibited the secondary haemagglutinin response to SRBC (Fig. 5). Whereas control mice given a secondary immunization had a mean $\log_2$ titre of 11·8, the titre was decreased to a mean $\log_2$ titre of 7·9 if animals received a secondary booster 6 days after infection. The secondary antibody response to SRBC was also significantly

decreased in animals given a booster immunization at 2, 8 and 10 days after CMV infection. The secondary response in animals infected 6 days before a booster immunization was not significantly greater than animals given a primary response alone (mean $\log_2$ titre of 6·2).

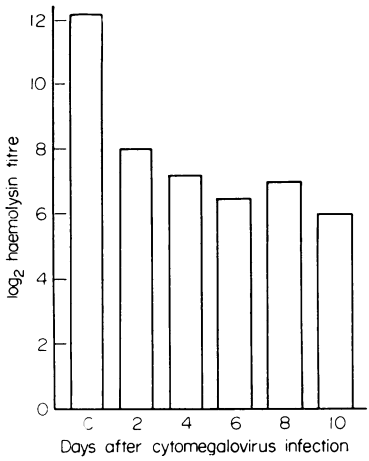

FIG. 6. Secondary haemolysin antibody response to sheep red blood cells in mice infected with 2×10^5 PFU CMV. Animals were infected 8 days after immunization with SRBC. A secondary immunization was administered 2–10 days after infection. C = uninfected control animals.

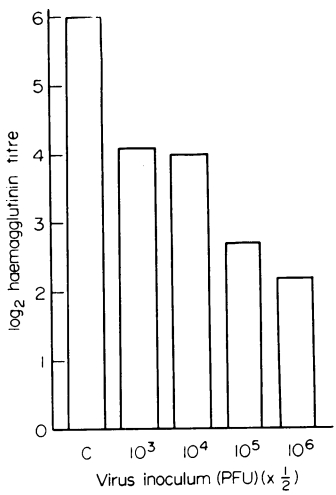

FIG. 7. Effect of virus inoculum on antibody response to sheep red blood cells. Mice were immunized with SRBC 6 days after infection with various doses of CMV. C = uninfected control animals.

The secondary haemolysin titre was also significantly suppressed in animals infected 2–10 days before a booster immunization. The mean $\log_2$ secondary haemolysin titre was 6·4 in animals infected 6 days prior to a booster injection of SRBC compared to 12·1 in uninfected animals (Fig. 6). Mice given a primary immunization only had a mean $\log_2$ titre of 5·9.

Effect of virus inoculum on the primary immune response to SRBC

In order to determine the effect of virus inoculum on the immune response, groups of female Swiss–Webster mice were infected with from 2×10^3 to 2×10^6 PFU CMV. They were immunized with SRBC 6 days later and were bled 8 days after that. The immunosuppression caused by CMV infection is related initial virus inoculum (Fig. 7). Control mice had a mean $\log_2$ haemagglutinin titre of 6·0. This titre was significantly reduced in mice infected with 2×10^4 PFU CMV (mean $\log_2$ titre of 4·0). The haemagglutinin titre was further reduced to 2·7 and 2·2 in mice infected with 2×10^5 and 2×10^6 PFU CMV, respectively. Thus, the immune suppression is related to initial CMV inoculum.

Effect of prior vaccination with tissue culture passaged CMV on the immunosuppressive effect of wild-type CMV

CMV is rapidly attenuated in tissue culture and loses its ability to grow in lymphoid tissue and to cause immune suppression (Osborn & Walker, 1970). The following experiment was undertaken to determine whether prior vaccination with tissue culture-passaged CMV could protect mice from the immunosuppressive effects of challenge with wild-type CMV. Two-week-old male and female Swiss–Webster mice were infected with 10^4 PFU tissue

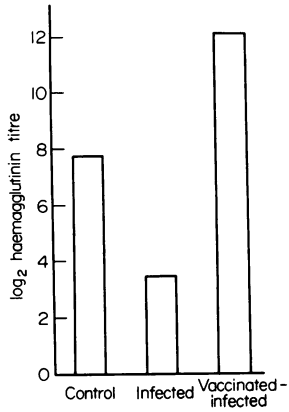

FIG. 8. Effect of vaccination with tissue culture-passaged CMV on immunosuppression by wild-type CMV. See text for experimental design.

culture-passaged CMV. This group and another previously uninfected groups of mice were infected with 2×10^5 PFU CMV when 4 weeks old. These two groups and a previously uninfected control group challenged with SRBC 6 days later and were bled 8 days after that. Haemagglutinin titres were then determined. Again, mice infected with wild-type CMV had a suppressed immune response to SRBC (Fig. 8). Uninfected control animals had a mean $\log_2$ agglutinin titre of 7·8 compared to 3·5 for infected mice. Prior vaccination with tissue culture passaged CMV not only protected against the immunosuppressive effects of wild-type CMV, it led to greatly augmented antibody titres. The previously vaccinated group had a mean $\log_2$ titre of 12·1. Thus, wild-type CMV infection in previously vaccinated mice seems to act as an immunological adjuvant instead of an immunosuppressive.

DISCUSSION

Murine cytomegalovirus infects cells of the lymphoid and reticuloendothelial systems. Histological changes occur as early as 3 days after intraperitoneal infection and are maximum 6–7 days later. At low (10^5 PFU) doses of CMV, intranuclear inclusions can be seen around germinal centres, but there is minimal destruction of lymphoid cells. At higher (10^6 PFU) doses, destruction of lymphoid elements also occurs. Following the acute phase of infection, gradual healing takes place. The concentration of virus in these tissues is greatest during this acute period when histological changes occur (Osborn & Walker, 1970). This acute phase of maximal virus concentration and histological change also corresponds to the time of greatest immunosuppression. Both the primary and secondary immune responses are suppressed to the maximal degree 4–6 days after CMV infection.

In addition, human CMV, like mouse CMV, can infect cells of the lymphoid and reticuloendothelial systems. An atypical mononucleosis-like illness can occur in the post-perfusion syndrome following cardiopulmonary bypass or after the transfusion of fresh blood. Atypical lymphocytes can be identified in the peripheral blood smear. Human CMV infection has also been associated with transient immunological abnormalities during acute infection (Kantor *et al.*, 1970). They identified immunological abnormalities in nine out of ten patients with post-perfusion CMV infection. Antinuclear antibody, rheumatoid factor, cold agglutinins, and cryoglobulins were found in the serums of these patients. Two also developed positive direct Coomb's tests; three had transient haemolytic anaemia; and one had a transient glomerulitis. No immunological changes were noted in 127 infants with congenital CMV infection (Montgomery *et al.*, 1973), however, the finding of immunological abnormalities in patients with post-perfusion CMV infection but not in infants with congenital CMV infection may be analogous to our finding impaired immune function only in acutely infected animals.

The mechanism whereby CMV infection causes immunosuppression is not fully known. Since maximal suppression of humoral immunity occurs at the time of maximal virus concentration, antigenic competition of viral antigens inhibiting antibody to another antigen has been proposed as a possible mechanism (Osborn, Blazkovec & Walker, 1968). However, CMV-induced immunosuppression occurs before the time the virus is present in any great amount.

A more likely possibility, it seems to us, is the infection of potential antibody-producing cells by virus with the result that cellular metabolic activity is directed toward production of virus rather than antibody. Virus may be present in lymphoid cells in a form not detectable by tissue culture assays. Furthermore, attenuated virus, which does not produce immunosuppression, does not grow well in lymphoid organs (Osborn & Walker, 1970). Many other viruses that directly infect cells of the lymphoid system are also capable of causing suppression of the immune response, and it may be that all viruses that infect lymphoid cells can be shown to alter immune responsiveness if properly studied. Some viruses, of course, cause increased immune responsiveness (Notkins, Mergenhagen & Howard, 1970). Most viruses that cause increased immune suppression—the murine leukaemia viruses—may also cause immune suppression by directing lymphoid cell processes toward other than antibody production, i.e. neoplasia. Even though defeats in humoral immunity can be seen prior to neoplasia, cellular processes in that direction may already be under way.

An interesting but somewhat paradoxical finding was obtained when animals were

vaccinated with attenuated CMV prior to inoculation with the virulent virus. One might have expected that, if protective, the vaccination with attenuated virus would have led to a normal immune response after immunization with SRBC. Instead, however, an augmented immune response was observed. How CMV causes increased immune responsiveness when given after vaccination is unknown at present. This adjuvant like effect of virulent CMV, when given after prior vaccination with attenuated CMV, may be due to non-specific proliferation of T or B cells caused by reinfection with virus or to increased efficiency of helper T cells. (Armerding & Katz, 1974). The mechanism may be similar to the increased secondary response to 2,4-dinitrophenol keyhole limpet haemocyanin (DNP–KLH) observed in mice undergoing a graft-*versus*-host reaction (Osborn & Katz, 1972; Katz & Osborn, 1972). These investigators were able to obtain a greater secondary response to DNP–KLH in the animals underwent a graft-*versus*-host reaction after the initial immunization. This allogenic effect did not occur if the graft-*versus*-host reaction was elicited before the primina immunization of DNP–KLH.

A similar type of mechanism may occur with CMV. CMV-primed cells may undergo cell division in response to the secondary injection of CMV and may elicit a factor which increases the immune response to SRBC or enables these cells to serve as helper cells more efficiently.

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