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Multiple autoantibodies following cytomegalovirus infection: virus distribution and specificity of autoantibodies

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SUMMARY

Multiple autoantibodies were found in the sera of BALB/c, C57BL/10 and C3H mice following mouse cytomegalovirus (MCMV) infection. The complex pattern of intra-organ, intratissue and intracellular reactivity observed by immunoperoxidase histochemistry suggested that many autoantibodies of varying specificities were elicited. This evidence from immunoperoxidase histochemistry was confirmed by immunoblot, where autoantibodies binding to polypeptides of a variety of sizes and tissues of origin were observed. In addition to these central findings, the tissue and organ distribution of MCMV in three mouse strains of differing genetic resistance were described. No correlation was found between the distribution of virus and the tissue and organ specificity of the autoantibodies produced following infection. Inflammatory responses accompanied MCMV infection in a number of tissues. In BALB/c mice, myocarditis and salivary gland inflammation were evident at Day 56 post-infection in the absence of MCMV, but in the presence of autoantibodies to cardiac muscle and salivary duct epithelium. This model for virus-induced autoimmunity can be applied to studies of the relationship between virus infection, autoimmunity and disease.

INTRODUCTION

Autoimmunity often accompanies virus infection in man, and viruses are increasingly implicated in the aetiology of autoimmune diseases. Both the role of autoimmune responses during virus infection and the role of viruses in the induction of autoimmunity are poorly understood. During virus infection, autoimmune reactions could exacerbate tissue damage caused by virus or by virus-directed immune responses, as with coxsackie B virus and acute perimyocarditis (Maisch, 1984), and hepatitis B virus and acute hepatitis (Mondelli & Eddleston, 1984). Autoimmune tissue damage could continue with chronic virus infection or if the homeostatic mechanisms responsible for self-tolerance were disturbed by virus, as with hepatitis B virus chronic hepatitis (Mondelli & Eddleston, 1984) and Epstein–Barr virus-associated systemic lupus erythematosus (Smith & Steinberg, 1983). Viruses have also been implicated in the aetiology of human diseases of unknown aetiology but with a major autoimmune component. Such associations in man frequently rely upon the circumstantial evidence of antecedent viral illness and viral seroepidemiology. For example, autoimmune responses to peripheral nerve proteins in acute inflammatory polyneuropathy (Guillain–Barre syndrome) have been linked with preceding infection with cytomegalovirus, Epstein–Barr, coxsackie, echo, influenza, varicella, measles and mumps viruses (Weiner & Hauser, 1982).

Animal models of virus-induced autoimmunity can provide direct evidence of the linkage of certain viruses with autoimmune disease and provide an opportunity to study the mechanisms involved in virus-induced autoimmunity. There are few animal models of virus-induced autoimmunity. They include autoimmune polyendocrinopathy associated with reovirus type 1 infection in mice (Onodera *et al.*, 1981), coronavirus-induced subacute demyelinating encephalomyelitis in rats (Watanabe, Wege & Ter Meulen, 1983), autoantibody to liver-specific lipoprotein (LSP) following acute hepatitis induced by mouse cytomegalovirus (MCMV) (Bartholomaeus *et al.*, 1983; Bartholomaeus, O'Donoghue & Reed, 1984) and autoantibodies to cardiac muscle following mouse coxsackie B virus myocarditis (Alvarez *et al.*, 1987). Here we report a marked loss of self-tolerance during MCMV infection of mice, with autoantibody produced to a variety of organs and tissues. The relationship between organ distribution of MCMV in infected mice and organ specificity of autoantibodies was investigated, and no correlation found. Autoantibodies in the sera of MCMV-infected mice reacted predominantly with different polypeptides within each tissue.

Abbreviations: C3H, C3H/HeJ; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; H&E, haematoxylin and eosin; i.p., intraperitoneal; LD_{50}, minimum virus dose lethal for 50% of animals; LSP, liver-specific lipoprotein; MCMV, mouse cytomegalovirus; MW, molecular weight; PFU, plaque-forming units; PI, post-infection; SDS, sodium dodecyl sulphate.

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MATERIALS AND METHODS

Mice

Specific pathogen-free inbred female BALB/c, C57BL/10 and C3H/HeJ (C3H) mice of 8 weeks of age were supplied by the Animal Resources Centre, Murdoch.

Virus

MCMV (Smith strain) was maintained by passage in weanling female BALB/c mice and stored in the gas phase of liquid nitrogen, as described elsewhere (Allan & Shellam, 1984). The minimum virus dose that killed 50% of the animals inoculated (LD_{50}) was determined in adult BALB/c mice as described by Chalmer, Mackenzie & Stanley (1977). In comparison with BALB/c mice, C57BL/10 mice are two to four times more resistant to lethal infection and C3H mice 24–28 times more resistant [Grundy (Chalmer) Mackenzie & Stanley, 1981; Allan & Shellam, 1984]. Where required, MCMV was inactivated by exposure to ultraviolet (UV) light at 800 $\mu W/cm^2$ for 4 min.

Experimental design

BALB/c, C57BL/10 and C3H mice were infected with 1×10^4 plaque-forming units (PFU) of MCMV (0·3 LD_{50} for BALB/c mice) by intraperitoneal (i.p.) injection. At Days 0, 2, 3, 5, 7, 10, 14, 21, 28 and 56 PI, eight animals were killed; blood was collected by cardiac puncture for serum separation and organs and tissues were collected and fixed for 15 hr in Bouin's Fluid. After fixation the tissues were transferred to 70% ethanol before processing to composite paraffin blocks. The tissues assessed were liver, spleen, salivary gland, adrenal, ovary, pancreas, kidney, cardiac muscle, peripheral nerve, brain, striated muscle, lung, thymus, thyroid, lymph node (mesenteric), stomach, duodenum and ileum. Sections were stained with haematoxylin and eosin (H&E) for histopathological studies or used for immunoperoxidase histochemistry. Control mice of each strain were injected i.p. with the equivalent of 1×10^4 PFU of UV light-inactivated MCMV and similarly treated.

Antiserum to MCMV

Hyperimmune antiserum to MCMV was prepared in BALB/c mice by an initial i.p. injection of 0·02 LD_{50} of MCMV followed by i.p. injections of 0·3 LD_{50} of MCMV at Days 14 and 21, and the serum collected on Day 31. Organs taken from 10 of fifty hyperimmunized mice at Day 31 PI were examined for MCMV by immunoperoxidase histochemistry; no virus was found.

Immunoperoxidase histochemistry

Dewaxed sections were blocked with 0·3% hydrogen peroxide in methanol for 30 min at room temperature and treated with 20% normal swine serum (CSL, Melbourne) diluted in Tris-buffered saline (Sigma, St Louis, MO) (TBS; 20 mM Tris, 500 mM NaCl, pH 7·6) for 20 min at room temperature. The sections were then incubated with mouse sera diluted 1/20 in TBS with 10% normal swine serum for 30 min at room temperature. After incubation with primary antibody the slides were washed twice in TBS for 3 min before the addition of peroxidase-conjugated rabbit antibody to mouse immunoglobulins (Dakopatts, Glostrup, Denmark) for 20 min at room temperature. After washing twice in TBS, the slides were incubated with freshly prepared diaminobenzidine substrate (Sigma) (DAB; 6 mg DAB in 10 ml TBS containing 0·01% H_2O_2, filtered through Whatman No. 1 paper before use) for 8 min at room temperature. Slides were washed for 5 min in TBS, stained with haematoxylin, dehydrated and mounted. DAB-positive reaction products appeared brown, while cellular morphology and nuclei stained blue.

Preparation of tissue homogenate supernatants

Normal C57BL/10 mice were killed by cervical dislocation. Striated muscle, cardiac muscle, kidney, liver, brain and thymus were excised and washed in sucrose buffer (Ajax Chem., Sydney) at 4° (0·25 M sucrose, 0·05 M Tris, 0·1 M NaCl, 0·50 mM disodium EDTA, pH 8·0). Forty percent (v/v) homogenates were prepared in sucrose buffer using a Waring blender (Thomas Co., PA) followed by a Braun pestle homogenizer (FRG). Homogenates were then centrifuged at 105,000 *g* for 60 min at 4°, and the supernatants stored at −80°.

Polyacrylamide gel electrophoresis and immunoblot

Tissue homogenate supernatants at 1 mg/ml were dialysed for 18 hr against 10 mM Tris-HCl buffer containing 1 mM EDTA, 1% sodium dodecyl sulphate (SDS; especially pure, BDH, Poole, Dorset, U.K.), 40 mM dithiothreitol and 10 M urea (Bio-Rad Ltd, Richmond, CA) (pH 8·0) at room temperature with stirring. The samples were heated for 5 min in a boiling water bath, centrifuged at 11,500 *g* for 10 min and applied to 10% polyacrylamide gel with a 3% polyacrylamide stacking gel. Four identical gels were run; one gel was stained with Coomassie Blue; polypeptides from the remaining three gels were transferred to nitrocellulose and immunoblot was performed after the method of Dao (1985). After blocking of the nitrocellulose membranes in a solution of 10% dry skim milk powder with 0·01% anti-foam A emulsion (Sigma, St Louis, MO) in phosphate-buffered saline (PBS, pH 7·2) at room temperature for 1 hr, mouse serum diluted to 1/25 was applied to the nitrocellulose membranes overnight at room temperature with gentle agitation. The nitrocellulose membranes were briefly rinsed in distilled water and washed three times for 10 min in PBS with 0·05% Tween 20. Alkaline phosphatase-conjugated goat antisera to mouse IgG and IgM (Tago Inc., Burlingame, CA) at a dilution of 1/1125 was then applied for 1 hr at room temperature with gentle agitation. The nitrocellulose membranes were again washed before immersion in substrate solution (0·25 mg/ml O-dianisidine tetrazotized and 0·25 mg/ml beta-naphthyl acid phosphate in 0·06 M sodium tetraborate buffer, pH 9·7, containing 1·2 mg/ml $MgSO_4.7H_2O$) for 15 min at room temperature to give a pink reaction product.

Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) was performed using a modification of the method previously reported from this laboratory (Swanson *et al.*, 1985). Tissue homogenate supernatants were adjusted to a concentration of 10 μg/ml before adsorption onto polystyrene microtitre plates (EIA plates, Flow Laboratories Inc., Mclean, VA) for 1 hr at 37° and overnight at 4°. The plates were washed, incubated with mouse sera diluted 1/25 for 1 hr at 37°, washed again and incubated for 1 hr at 37° with alkaline phosphatase-labelled goat antiserum to either mouse IgG and IgM or the individual G or M isotypes (Tago Inc.) at a dilution of 1/1500. After further washing, the plates were incubated with the chromogenic substrate *p*-nitrophenyl phosphate (1 mg/ml) for 1 hr before measurement of absorbance at 405 nm.

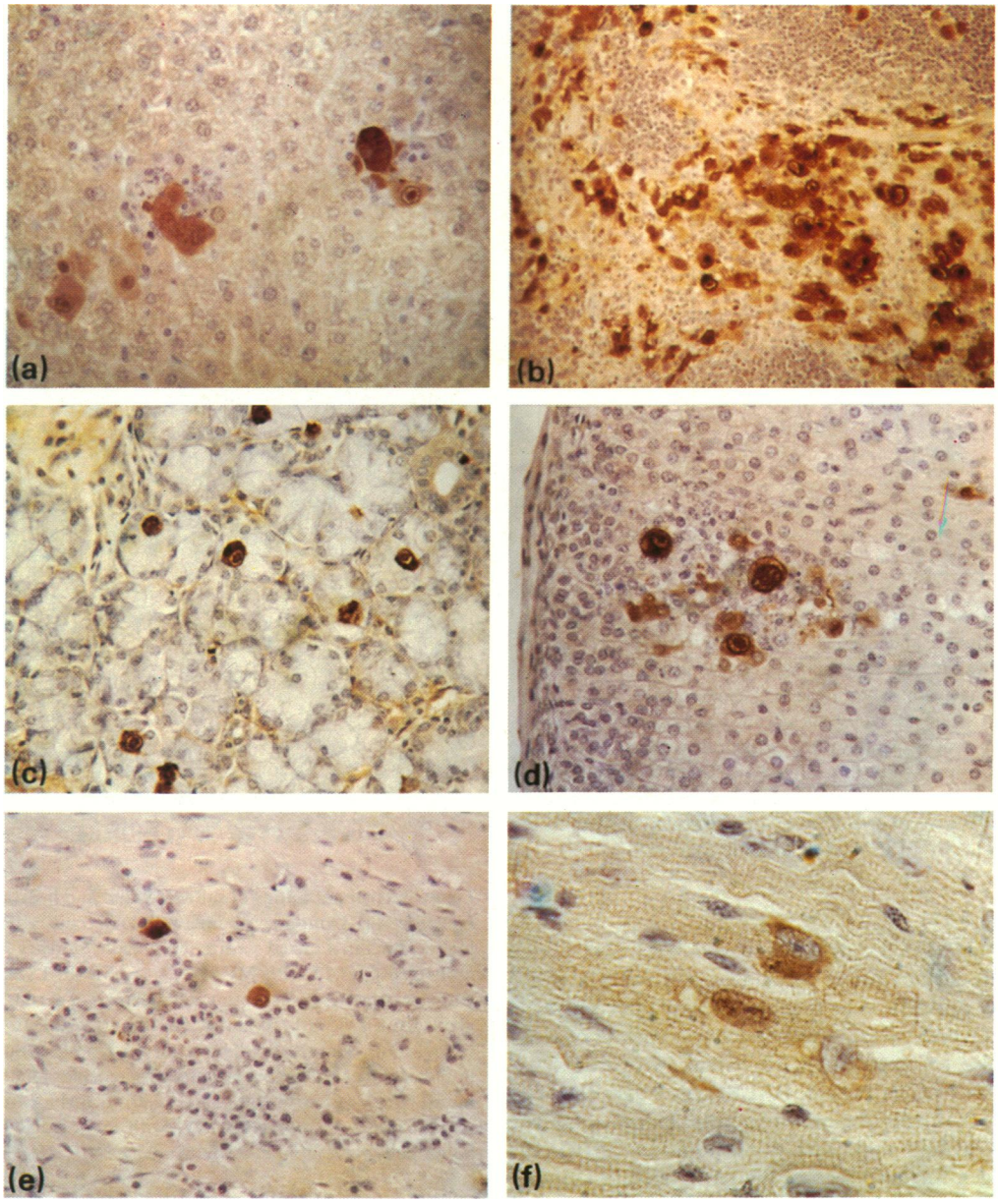

Figure 1. MCMV antigens detected in infected cells by immunoperoxidase histochemistry using MCMV hyperimmune mouse serum. All sections were counterstained with haematoxylin. (a) Six MCMV-infected hepatocytes shown in BALB/c mouse liver, 2 days PI with MCMV. One hepatocyte shows a characteristic intranuclear viral inclusion, a densely stained area surrounded by a clear zone between it and the nuclear membrane; two central hepatocytes show immunoperoxidase staining without intranuclear viral inclusions, adjacent to these cells is a mixed inflammatory infiltrate (magnification × 400). (b) Virus-infected reticular cells in the red pulp and lymphoid follicles of BALB/c spleen 3 days PI (× 250). (c) MCMV-infected acinar cells of mucous alveoli within salivary gland of BALB/c mouse 7 days PI (× 400). (d) Virus-infected adrenal cortical cells with accompanying necrosis and inflammation in a BALB/c mouse, Day 5 PI (× 400). (e) Two virus-infected cells in cardiac muscle with associated myocarditis in a BALB/c mouse 7 days PI (× 400). (f) Early nuclear and perinuclear MCMV antigens in cardiac myocytes and vacuolation of infected myocyte, 5 days PI in a BALB/c mouse (× 1000).

RESULTS

Virus distribution and histopathology

Immunoperoxidase histochemistry employing hyperimmune anti-MCMV serum readily detected cells expressing viral antigen. Immunoperoxidase-positive cells were identified as virus-infected cells by the concurrence of characteristic intranuclear viral inclusions and, in some instances, evidence of cytomegala (Fig. 1a–e). The intensity of expression of cytoplasmic and nuclear immunoperoxidase reaction reflected antigen expression from early infection to cell necrosis (Fig. 1a–f). Serum from normal, uninfected BALB/c mice applied to the same MCMV-infected tissue sections did not result in immunoperoxidase staining of cells with intranuclear inclusions. Hyperimmune anti-MCMV sera applied to tissue sections from eight

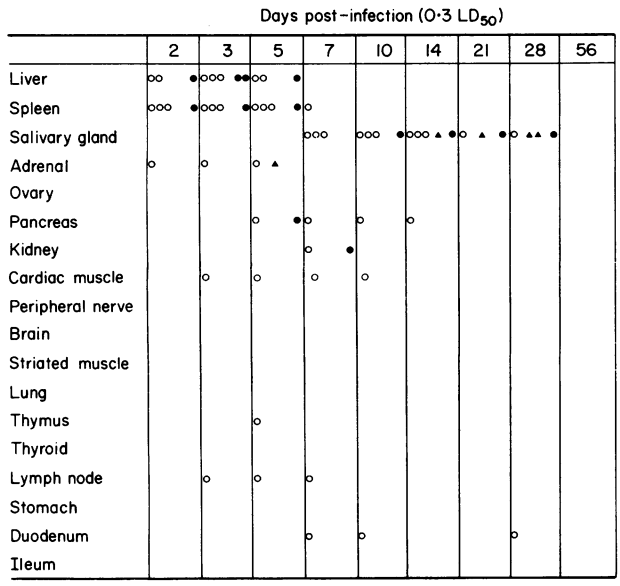

Figure 2. Distribution of MCMV in BALB/c (○), C57BL/10 (●) and C3H (▲) mouse tissues and organs detected by immunoperoxidase histochemistry using MCMV hyperimmune mouse serum. Each assessment was based upon examination of each organ and tissue taken from eight mice of each strain at each time point. The absence of a point indicates the absence of detectable viral antigen in all mice; one point indicates a mean of up to four virus-infected cells per low power field (× 10); two points indicates a mean 4·1–12 virus-infected cells per field; three points indicates a mean of > 12 virus-infected cells per field. Mice were infected with 1×10^4 PFU MCMV which is equivalent to 0·3 LD_{50} for BALB/c.

normal, uninfected BALB/c, C57BL/10 and C3H mice (pre-infection, Day 0) did not detect peroxidase-positive cells. The principal organs infected were the liver, spleen and salivary glands (Fig. 2). Overall, most virus antigen was seen in organs from BALB/c mice, followed by C57BL/10 mice, with little virus antigen detectable in C3H tissues. BALB/c mice also showed virus antigen in the adrenal gland, pancreas, kidney, cardiac muscle, thymus, lymph node and duodenum. In C57BL/10 mice, virus antigen was also seen in the pancreas and kidney, and in C3H mice only in salivary gland and adrenal.

MCMV infection of the liver in BALB/c and C57BL/10 mice elicited a focal hepatitis with aggregates of between 10 and 30 lymphocytes, Kuppfer cells and granulocytes surrounding some but not all infected hepatocytes (Fig. 1a). By Day 7 PI the focal hepatitis had subsided in both mouse strains. The spleen was a major focus of virus infection in both BALB/c and C57BL/10 mice (Fig. 2). At Day 2 PI, perifollicular reticular cells and others spread throughout the red pulp harboured MCMV; on Days 3 and 5 PI, reticular cells and other large mononuclear cells in the lymphoid follicles were also infected (Fig. 1b). Marked necrosis of the red pulp was observed in BALB/c mice on Days 3 and 5 PI. The serous and mucous cells in salivary gland were a major site of infection in all three strains of mice (Fig. 1c), and infection was accompanied by an inflammatory response from Days 10 through to 28. At Day 56 PI lymphocytic inflammation in the absence of virus was observed in salivary gland tissues of each mouse strain, juxtaposed to intra- and interlobular ducts and surrounding necrotic and fragmented acini (Fig. 3a and b).

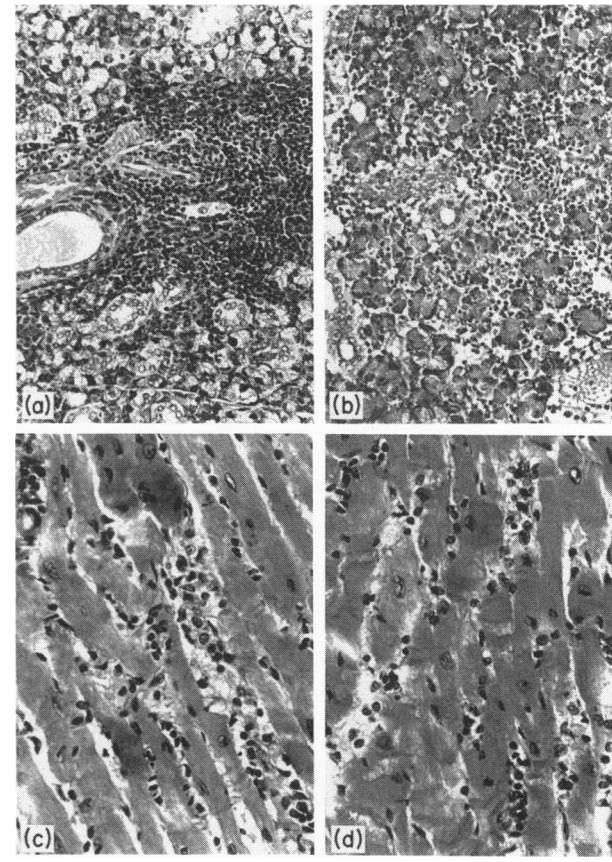

Figure 3. Histopathology of salivary gland and heart following MCMV infection. (a) Lymphocytic inflammatory focus adjacent to an interlobular duct in a mixed serous and mucous salivary gland from a C3H mouse 56 days PI with MCMV (× 162·5). (b) Lymphocytic infiltrate with accompanying disruption and necrosis of serous alveoli in salivary gland from a C57BL/10 mouse 56 days PI with MCMV (× 162·5). (c) Myocarditis characterized by a mixed lymphocytic and plasmacytic infiltrate, myocardial fibre loss and hypereosinophilic fibres in a BALB/c mouse 7 days PI with MCMV (× 260). (d) Myocarditis in a BALB/c mouse 56 days PI with MCMV, with myocardial fibre necrosis and accompanying lymphocytic infiltrate (× 260). H&E stained sections.

MCMV infection of adrenal cortical cells of BALB/c mice on Days 2, 3 and 5 PI was accompanied by a mixed lymphocytic, granulocytic and histiocytic infliltrate (Fig. 1d), whereas in C3H mice at Day 5, infection was accompanied by minimal inflammatory response and necrosis. MCMV infection of pancreatic acinar cells of BALB/c mice on Days 5, 7, 10 and 14 PI and of C57BL/10 mice on Day 5 PI and infection of the kidney glomeruli in BALB/c mice at Day 7 PI was not accompanied by either necrosis or inflammation. MCMV infection of the kidney tubule cells of C57BL/10 mice was accompanied by minimal lymphocytic infiltrate. MCMV was observed in BALB/c mice in thymus reticular cells on Day 5 PI, in lymph node reticular cells on Days 3, 5 and 7 and in cells of the Brunner glands of the duodenum on Days 7, 10 and 28 PI, without accompanying inflammation or necrosis.

MCMV infection of cardiac muscle on Days 3, 5, 7 and 10 PI in BALB/c mice was accompanied by myocarditis in each of eight mice on Days 7 and 10, with necrosis of myocardial fibres

and an infiltrate composed predominantly of mononuclear cells (Fig. 1e, Fig. 3c). MCMV infection of cardiac myocytes was established by the demonstration of early viral antigens prior to loss of cellular morphology (Fig. 1f). Virus was detected in five mice on Day 7, and three mice on Day 10 PI. Myocarditis was not evident on Days 14, 21 and 28 PI, but reappeared on Day 56 with lymphocytic infiltrate and necrosis of individual myocytes (Fig. 3d).

Specificity of autoantibodies

Autoantibodies in the sera of BALB/c, C57BL/10 and C3H mice following MCMV infection were assessed by immunoperoxidase histochemistry using composite sections of normal mouse tissues and by ELISA and immunoblot using 105,000 *g* supernatant of normal mouse tissue homogenates. Control mice of each mouse strain injected with UV-inactivated MCMV or an equivalent amount of normal BALB/c salivary gland homogenate did not produce autoantibodies detectable by immunoperoxidase histochemistry, ELISA or immunoblot (data not shown). No autoantibody activity was detected in normal BALB/c, C57BL/10 or C3H mouse serum, although immunoglobulin-positive lymphocytes and plasma cells in lymphoid tissues, lung and gastrointestinal tract were stained. Sera from MCMV-infected mice collected on Days 7 through to 56 PI showed strong reactivity with multiple normal (C57BL/10) mouse tissues and organs by immunoperoxidase histochemistry (Fig. 4a–h, Fig. 5). Autoantibodies reacting with normal mouse tissue were not detected on Days 2, 3 and 5 PI; the autoantibodies appeared on Day 7 and were still detectable by immunoperoxidase histochemistry at Day 56. In each mouse strain, serum autoantibodies were found to all 18 tissues or organs at some stage following MCMV infection, the intensity of autoantibody reactivity by this method appeared greatest at Day 10 PI. Sera from BALB/c mice reacted more strongly with peripheral nerve than did either C57BL/10 or C3H sera, and C3H sera reacted more strongly with lymphoid tissue than did either BALB/c or C57BL/10 sera.

A complex pattern of intra-organ, intratissue and intracellular autoantibody reactivity was observed. For example, within the salivary gland, autoantibodies reacted with parasympathetic nerve ganglia, cells lining intra- and interlobular ducts, and with the basket cells of the acini, but not with mucous and serous alveolar cells (Fig. 4e). Similarly, autoantibodies reacting with the pancreas bound to cells of the pancreatic acini and all cells in the islets of Langerhans, but most intensely with the alpha cells within the islets of Langerhans (Fig. 4f). In the kidney, autoantibodies reacted with cells lining the tubules but not with cells of the glomeruli. Autoantibodies reacted with the cell membrane, nucleus and cytoplasm of all adrenal cells, but showed greater reactivity with adrenal cortical cells than with adrenal medullary cells (Fig. 4g). Anti-nuclear antibodies were seen in the sera of approximately half of the mice of each strain from Day 7 through 56 PI (Fig. 4h). There was considerable heterogeneity of anti-nuclear antibody reactivity; for example, autoantibodies reacted with the nuclei of granular cells in the brain but not with the nuclei of astrocytes. Where autoantibody reactivity following MCMV infection was demonstrated against the liver, spleen, ovary, cardiac muscle, peripheral nerve, striated muscle, lung, thymus, thyroid, lymph node, stomach, duodenum and ileum, all cell types within these tissues and organs were involved.

The ELISA determined both the kinetics of autoantibody responses and the relative reactivity of autoantibodies with different tissue preparations. The ELISA data showed greater autoantibody reactivity in BALB/c mice (Fig. 6a) than in C57BL/10 (Fig. 6b) and C3H (Fig. 6c) mice with peak autoantibody responses to most protein preparations at Day 7 PI. The exceptions were autoantibodies to striated muscle and brain in BALB/c mice (Fig. 6a) and brain in C57BL/10 mice (Fig. 6b), which peaked at Day 10 PI. The ELISA data also showed considerable variation in the level of the autoantibody response to each protein preparation. ELISA employing second antibodies to either IgG or IgM established that autoantibodies of both isotypes were present throughout the response (data not shown).

The polypeptide specificities of autoantibodies were assessed by immunoblot. Four individual sera of each mouse strain on Days 10 and 21 PI were reacted by immunoblot against 105,000 *g* supernatants of homogenized striated muscle, cardiac muscle, kidney, liver and brain. A minimum of 20 polypeptides of molecular weight (MW) from in excess of 200,000 to approximately 20,000 were discernible within each protein preparation (Fig. 7, some bands have been lost in photographic reproduction). Individual mouse sera contained autoantibodies that were reacting predominantly with different polypeptides in each organ preparation. A typical result is shown in Fig. 7, where serum from one individual BALB/c mouse 10 days PI with MCMV reacted with nine polypeptides in brain, 12 in liver, nine in kidney, seven in cardiac muscle and six in striated muscle protein preparations. Autoreactivity with a polypeptide common to all five preparations was seen with only one polypeptide of approximately 43,000 MW. This serum also showed reactivity with two polypeptides of greater than 200,000 MW and approximately 68,000 MW common to brain, liver and kidney. An irregular pattern of antibody activity to a polypeptide of approximately 48,000 MW in liver, kidney, cardiac muscle and striated muscle was seen consistently and may reflect incomplete denaturation of a protein. Strong reactivities were also seen with a 110,000 MW polypeptide in brain, with 35,000, 36,000 and 138,000 MW polypeptides in liver, an 88,000 MW polypeptide in cardiac muscle and a 38,000 MW polypeptide in striated muscle (Fig. 7). The response pattern varied between individual mice sampled on the same day PI. When the pattern of responses observed for a particular mouse strain was compared with that for other mouse strains, there was no obvious strain-related contrasts. The only autoantibody specificities common to the 24 sera assessed from MCMV-infected mice were to a polypeptide of in excess of 200,000 MW in brain and a 38,000 MW polypeptide in striated muscle.

DISCUSSION

Multiple autoantibodies were found in the sera of BALB/c, C57BL/10 and C3H mice following MCMV infection. The complex pattern of intra-organ, intratissue and intracellular reactivity observed by immunoperoxidase histochemistry and confirmed by immunoblot suggested that many autoantibodies of varying specificities were elicited following MCMV infection. In addition to these central findings, the study has described comprehensively the tissue and organ distribution of MCMV

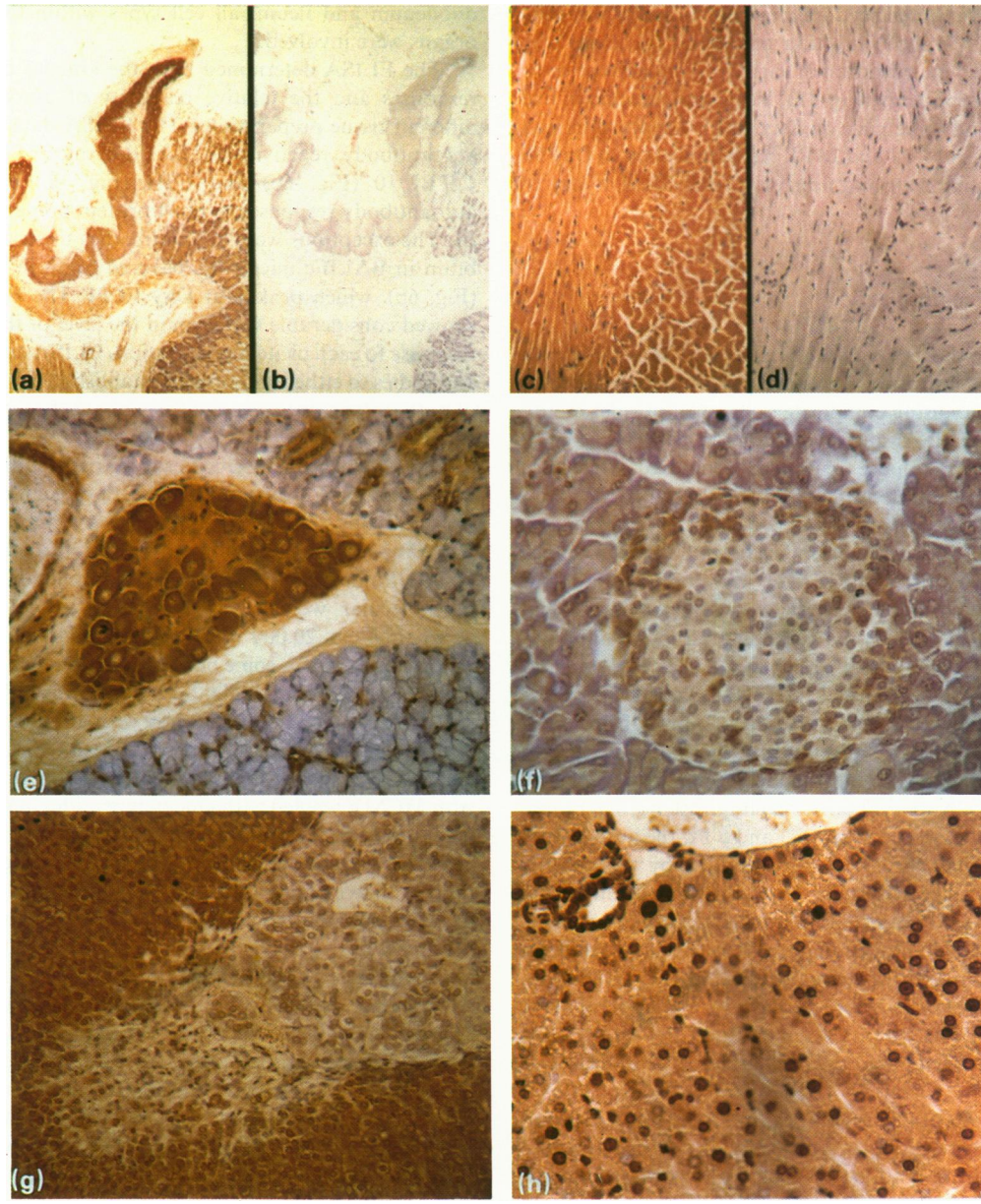

Figure 4. Autoantibodies to normal mouse tissues in the serum of mice infected with MCMV detected by immunoperoxidase histochemistry. (a) Esophagastric junction and serum from a C57BL/10 mouse 7 days PI with MCMV (×25). (b) Esophagastric junction and normal C57BL/10 serum (×25). (c) Cardiac muscle and serum from a C57BL/10 mouse 7 days PI (×100). (d) Cardiac muscle and normal C57BL/10 serum (×100). (e) Salivary gland showing autoantibody reactivity with a parasympathetic nerve ganglion, cells lining intra- and inter-lobular ducts and basket cells of alveoli in serum from a BALB/c mouse 10 days PI (×400). (f) Pancreas and serum from a BALB/c mouse 14 days PI with MCMV, showing marked reaction with the alpha cells of the islets of Langerhans (×400). (g) Adrenal showing the greater reactivity of autoantibodies in the serum of a C3H mouse 21 days PI with cortical cells than with medullary cells; anti-nuclear autoantibodies are also evident (×250). (h) Liver, showing anti-nuclear and anti-hepatocyte autoantibodies in the serum of a C57BL/10 mouse 21 days PI with MCMV (×400).

during sublethal infection of adult mice. No correlation was found between the distribution of virus and the tissue and organ specificity of the autoantibodies produced following infection.

Three strains of mice were selected for this study because of their varying resistance to lethal MCMV infection [Grundy (Chalmer) *et al.*, 1981; Allan & Shellam, 1984], which was reflected in the intensity of MCMV infection observed in this study. No previous report has described as extensive a pattern of virus distribution following sublethal MCMV infection of adult mice. In addition to the frequently reported sites of MCMV infection in the liver, spleen and salivary gland (reviewed in Osborn, 1982), MCMV infection of adult mice has been found to involve the adrenal (Mims & Gould, 1979), pancreas (Mims & Gould, 1978), kidney (Allan & Shellam, 1984) and lymph

Days post-infection (0·3 LD_{50})

	2	3	5	7	10	14	21	28	56
Liver				○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●
Spleen					○ ▲	○ ▲	○ ▲ ●	○ ▲ ●	○ ▲ ●
Salivary gland				○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●
Adrenal				○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●
Ovary				○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●
Pancreas				○ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●
Kidney				○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●
Cardiac muscle				○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●
Peripheral nerve				○ ●	○	○	○	○ ▲	○
Brain				○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲	○ ▲
Striated muscle				○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●
Lung				○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●
Thymus				○	○ ▲	○ ▲ ●	○ ▲ ●	▲	▲
Thyroid				○ ▲	○ ▲ ●	○ ▲ ●	○ ▲	○ ▲	
Lymph node					▲	○ ▲	○ ▲ ●	○ ▲	○
Stomach				○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●
Duodenum				○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●
Ileum				○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●	○ ▲ ●

Figure 5. Organ and tissue reactivity of autoantibodies detected by immunoperoxidase histochemistry following MCMV infection of BABL/c (○), C57BL/10 (●), and C3H mice (▲). Individual sera from eight mice of each strain at each time point were assessed and a positive result was determined if at least six sera showed reactivity with the tissue or organ. Mice were infected with 1×10^4 PFU MCMV which is equivalent to 0·3 LD_{50} for BALB/c.

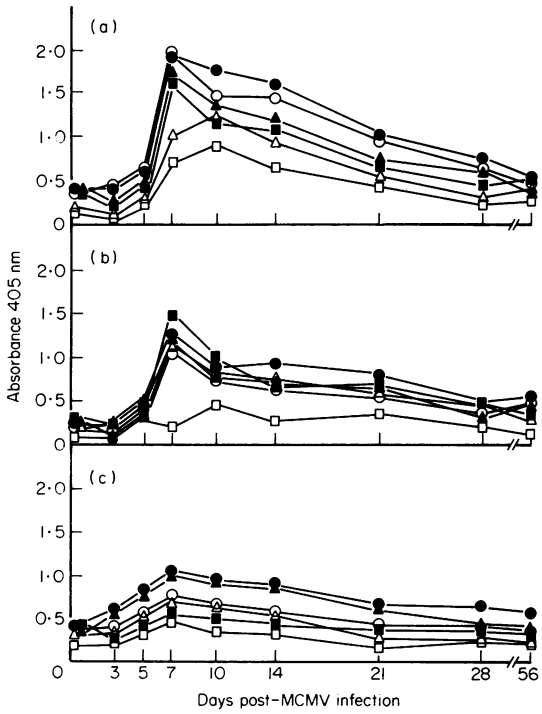

Figure 6. Autoantibody responses measured by ELISA to 105,000 *g* supernatant proteins of normal mouse tissue and organ homogenates. Eight individual BALB/c (a), C57BL/10 (b) and C3H (c) sera were tested for each time point and the mean absorbance is shown for reactivity against proteins from heart (○), liver (●), kidney (▲), striated muscle (△), thymus (■) and brain (□).

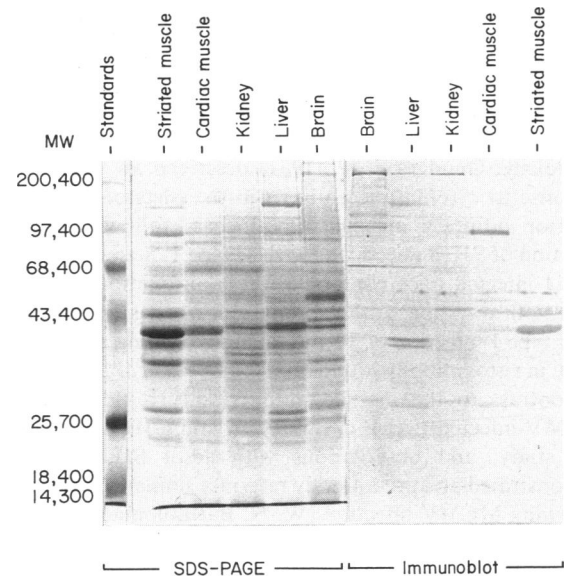

Figure 7. SDS–PAGE and immunoblot of 105,000 *g* supernatant proteins of normal mouse tissues and organs using serum from an individual BALB/c mouse 10 days PI with MCMV. SDS–PAGE of molecular weight standards and proteins is shown on the left and the nitrocellulose immunoblot developed with the MCMV-infected BALB/c mouse serum and alkaline phosphatase-labelled antisera to mouse immunoglobulins is shown on the right.

node (Howard & Najarian, 1974). In this study MCMV was also observed to infect cardiac muscle, thymus, and duodenum of BALB/c mice. It is possible that virus seen in the Brunners glands of the duodenum on Days 7, 10 and 28 in BALB/c mice was shed from the heavily infected salivary glands. MCMV infection of the heart and thymus (Mims & Gould, 1978, 1979) has been reported previously following neonatal infection of mice, but not following infection of adult mice.

Evidence of autoantibodies gained by immunoperoxidase histochemistry was not directly comparable with that derived by ELISA. Immunoperoxidase histochemistry is essentially a qualitative technique; by this technique the autoantibody response to most tissues was strongest at Day 10 and persisted through to Day 56 PI. In contrast, autoantibodies to 105,000 *g* supernatants of tissue homogenates measured by ELISA generally peaked at Day 7 PI and had diminished greatly by Day 56. Furthermore, by immunoperoxidase, each strain was observed to have autoantibody responses of similar intensity to several tissues (for example liver, adrenal, ovary, kidney, cardiac muscle). In contrast, by ELISA the autoantibody response of BALB/c mice was greater than that of C57BL/10 mice, and C3H mice demonstrated minimal autoantibody responses. Autoantibodies to organelles appeared to constitute a major component of the response detected by immunoperoxidase histochemistry. The cytosol prepared by 105,000 *g* centrifugation of organ homogenate was devoid of organelles except for a minor microsomal component (Lahav *et al.*, 1982). We have shown previously discordance in the kinetics of autoantibody responses following MCMV infection to a cytosol-derived antigen complex, LSP, by ELISA and to nuclear antigens by immunofluorescence (Bartholomaeus *et al.*, 1983). By immunoblot, autoantibodies binding to a variety of polypeptides of different sizes and different tissue origin were observed. The possibility

that these autoantibodies reacted with similar epitopes on different polypeptides is not excluded (Lane & Koprowski, 1982).

Autoantibody responses to such an extensive range of tissues and organs following virus infection have not been reported previously. Onodera *et al.* (1981) described an autoantibody response to cytoplasmic antigens in the islets of Langerhans, anterior pituitary and gastric mucosa, following neonatal infection of SJL/J mice with reovirus type 1. Sera from reovirus type 1-infected mice containing these autoantibodies did not react with intestine, adrenals, thyroid, ovaries or testes. Reovirus type 1 infection of 30–day-old mice (young adult) did not result in autoantibody production (Onodera *et al.*, 1981). This is in contrast to the marked autoantibody response of adult MCMV-infected BALB/c, C57BL/10 and C3H mice shown in this study, and observations with adult SJL/J mice that demonstrated an autoantibody response similar to these strains following MCMV infection (W. N. Bartholomaeus and H. L. O'Donoghue, unpublished results). A panel of monoclonal autoantibodies prepared from reovirus type 1-infected mice contained both organ-specific (Haspel *et al.*, 1983a) and multiple organ-reactive autoantibodies (Haspel *et al.*, 1983b), suggesting that the infection had broken tolerance to a wide variety of self-antigens. These workers subsequently showed that lymphocytes capable of making monoclonal autoantibodies reacting with multiple organs were a common feature of the normal B-cell repertoire (Prabhakar *et al.*, 1984); expression of autoimmune potential occurred after fusion with plasmacytoma cells. In another model of virus-induced autoimmunity, mice produced autoantibodies to heart and skeletal muscle within 15 days of infection with coxsackie B virus; some anti-kidney autoantibodies were detected at Day 45 PI, but no anti-liver autoantibodies were found (Wolfgram, Beisel & Rose, 1985). These workers claimed that myosin was one of the major autoantigens in coxsackie B virus-induced autoimmune myocarditis (Alvarez *et al.*, 1987).

In a previous report we described autoantibody production to LSP following MCMV hepatitis in adult C57BL/10 and BALB/c mice (Bartholomaeus *et al.*, 1983). LSP is a candidate target autoantigen in chronic active hepatitis (Bartholomaeus *et al.*, 1987). The LSP autoantibody response following MCMV infection in the mouse is T dependent (Bartholomaeus *et al.*, 1984). Anti-nuclear antibodies were also found following MCMV hepatitis in the mouse (Bartholomaeus *et al.*, 1983), consistent with an earlier report of anti-nuclear antibodies following latent infection with MCMV (Olding, Kingsbury & Oldstone, 1976). In clinical CMV infection in man, rheumatoid factors, anti-nuclear, anti-smooth muscle, and anti-erythrocyte autoantibodies have been demonstrated (Wager *et al.*, 1968; Kantor *et al.*, 1970; Andersen & Andersen, 1975). The present study suggests that the loss of self-tolerance following cytomegalovirus infection is considerably greater than described previously.

The mechanisms by which viruses induce autoimmunity are essentially unknown. A review of possible mechanisms (Hirsch & Proffitt, 1975) suggested release of sequestered antigens, virus-induced alteration of host cell membrane antigens, immunological cross-reactivity between viral and host antigens, T-cell bypass and alteration of immunocytes and/or immune regulator cells. More recently, idiotype networks (Cooke, Lydyard & Roitt, 1984) and alterations to the MHC microenvironment (Bottazzo *et al.*, 1986) have been advocated as further explanations for the emergence of autoantibodies following virus infection. CMV disturbs immunoregulation. CMV infection in man and mice produces a marked immunosuppression of both cellular and humoral immunity (Ho, 1984). It is paradoxical that despite reported immunosuppression, a potent autoantibody response occurs following MCMV infection. The production of autoantibodies following MCMV infection is more consistent with reports of enhancement of antibody responses (to T-dependent antigens) following MCMV infection (Tinghitella & Booss, 1979) and of polyclonal stimulation of immunoglobulin production in human lymphocytes by human CMV *in vitro* (Hutt-Fletcher, Balachandran & Elkins, 1983). Polyclonal B-cell activation *in vitro* by human CMV was found to occur with killed CMV as well as live virus (Hutt-Fletcher *et al.*, 1983). In this and an earlier study of autoantibody production following MCMV infection (Bartholomaeus *et al.*, 1983), injection of UV-inactivated MCMV was not found to induce autoantibody.

CMV has been implicated in the aetiology of three diseases with an autoimmune component in man: acute myocarditis (Maisch, 1984), acute inflammatory polyneuropathy (Weiner & Hauser, 1982) and Sjogrens syndrome (Shillitoe *et al.*, 1982). An association between a high incidence of maternal antibodies to cytomegalovirus and antibodies to fetal cardiac antigens has also been drawn in a study of congenital complete heart block (Taylor *et al.*, 1986). In relation to the possible aetiological association of CMV with myocarditis and Sjogrens syndrome in man, the MCMV-induced myocarditis with autoantibodies to cardiac muscle and the persistent salivary gland inflammation with autoantibodies to salivary duct epithelium reported here, are under further investigation in this laboratory. The model for loss of self-tolerance following virus infection described here is applicable to understanding further the aetiology and pathogenesis of autoimmune diseases and to unravelling the pathobiological puzzle surrounding the ubiquitous cytomegalovirus.

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REFERENCES

ALLAN J.E. & SHELLAM G.R. (1984) Genetic control of cytomegalovirus infection: virus titres in resistant and susceptible strains of mice. *Arch. Virol.* **81**, 139.

ALVAREZ F.L., NEU N., ROSE N.R., CRAIG S.W. & BEISEL K.W. (1987) Heart-specific autoantibodies induced by coxsackievirus B_3: identification of heart autoantigens. *Clin. Immunol. Immunopathol.* **43**, 29.

ANDERSEN P. & ANDERSEN H.K. (1975) Smooth-muscle antibodies and other tissue antibodies in cytomegalovirus infection. *Clin. exp. Immunol.* **22**, 22.

BARTHOLOMAEUS W.N., O'DONOGHUE H. & REED W.D. (1984) Thymus-dependence of autoantibody responses to liver specific lipoprotein in the mouse. *Clin. exp. Immunol.* **55**, 41.

BARTHOLOMAEUS W.N., SHELLAM G.R., ALLAN J.E., REED W.D. & JOSKE R.A. (1983) Autoantibodies to liver-specific lipoprotein follow-

ing hepatitis induced by mouse cytomegalovirus. *Clin. exp. Immunol.* **52,** 89.

BARTHOLOMAEUS W.N., SWANSON N.R., REED W.D., O'DONOGHUE H.L., FOTI D. & PAPADIMITRIOU J.M. (1987) The relationship between liver-specific lipoprotein and the hepatocyte plasma membrane. *Immunology*, **60,** 321.

BOTTAZZO G.F., TODD I., MIRAKIAN R., BELFIORE A. & PUJOL-BORRELL R. (1986) Organ-specific autoimmunity: a 1986 review. *Immunol. Rev.* **94,** 37.

CHALMER J.E., MACKENZIE J.S. & STANLEY N.F. (1977) Resistance to murine cytomegalovirus linked to the major histocompatibility complex of the mouse. *J. Gen. Virol.* **37,** 107.

COOKE A., LYDYARD P.M. & ROITT I.M. (1984) Autoimmunity and idiotypes. *Lancet*, **ii,** 723.

DAO M.L. (1985) An improved method of antigen detection on nitrocellulose: *in situ* staining of alkaline phosphatase conjugated antibody. *J. immunol. Meth.* **82,** 225.

GRUNDY (CHALMER) J.E., MACKENZIE J.S. & STANLEY N.F. (1981) Influence of H-2 and non-H-2 genes on resistance to murine cytomegalovirus infection. *Infect. Immun.* **32,** 277.

HASPEL M.V., ONODERA T., PRABHAKAR B.S., HORITA M., SUZUKI H. & NOTKINS A.L. (1983a) Virus-induced autoimmunity: monoclonal antibodies that react with endocrine tissues. *Science*, **220,** 304.

HASPEL M.V., ONODERA T., PRABHAKAR B.S., MCCLINTOCK P.R., ESSANI K., RAY U.R., YAGIHASHI S. & NOTKINS A.L. (1983b) Multiple organ-reactive monoclonal autoantibodies. *Nature (Lond.)*, **304,** 73.

HIRSCH M.S. & PROFFITT M.R. (1975) Autoimmunity in viral infections. In: *Viral Immunology and Immunopathology* (ed. A.L. Notkins), p. 419. Academic Press, New York.

HO M. (1984) Immunology of cytomegalovirus: immunosuppressive effects during infections. In: *CMV: Pathogenesis and Prevention of Human Infection* (eds S.A. Plotkin, S. Michelson, J.S. Pagano and F. Rapp), p. 131. Alan R. Liss, Inc., New York.

HOWARD R.J. & NAJARIAN J.S. (1974) Cytomegalovirus-induced immune suppression. I. Humoral immunity. *Clin. exp. Immunol.* **18,** 109.

HUTT-FLETCHER L., BALACHANDRAN N. & ELKINS M.H. (1983) B cell activation by cytomegalovirus. *J. exp. Med.* **158,** 2171.

KANTOR G.L., GOLDBERG L.S., JOHNSON L., DERECHIN M.M. & BARNETT E.V. (1970) Immunologic abnormalities induced by post-perfusion cytomegalovirus infection. *Ann. Int. Med.* **73,** 553.

LAHAV M., SCHOENFELD N., EPSTEIN O. & ATSMON A. (1982) A method for obtaining high recovery of purified subcellular fractions from rat liver homogenate. *Analyt. Biochem.* **121,** 114.

LANE D. & KOPROWSKI H. (1982) Molecular recognition and the future of monoclonal antibodies. *Nature (Lond.)*, **296,** 200.

MAISCH B. (1984) Cardiocytolysis by sera of patients suffering from acute perimyocarditis. In: *Viral Heart Disease*, p. 121. Springer-Verlag, Berlin.

MIMS C.A. & GOULD J. (1978) The role of macrophages in mice infected with murine cytomegalovirus. *J. Gen. Virol.* **41,** 143.

MIMS C.A. & GOULD J. (1979) Infection of salivary glands, kidneys, adrenals, ovaries and epithelia by murine cytomegalovirus. *J. Med. Microbiol.* **12,** 113.

MONDELLI M. & EDDLESTON A.L.W.F. (1984) Mechanisms of liver cell injury in acute and chronic hepatitis B. *Semin. Liver Dis.* **4,** 47.

OLDING L.B., KINGSBURY D.T. & OLDSTONE M.B.A. (1976) Pathogenesis of cytomegalovirus infection. Distribution of viral products, immune complexes and autoimmunity during latent murine infection. *J. gen. Virol.* **33,** 267.

ONODERA T., TONIOLO A., RAY U.R., JENSON A.B., KNAZEK R.A. & NOTKINS A.L. (1981) Virus-induced diabetes mellitus XX. Polyendocrinopathy and autoimmunity. *J. exp. Med.* **153,** 1457.

OSBORN J.E. (1982) Cytomegalovirus and other herpesviruses. In: *The Mouse in Biomedical Research* (eds H.L. Foster, J.D. Small and J.G. Fox), Vol. II, p 267, Academic Press.

PRABHAKAR B.S., SAEGUSA J., ONODERA T. & NOTKINS A.L. (1984) Lymphocytes capable of making monoclonal autoantibodies that react with multiple organs are a common feature of the normal B cell repertoire. *J. Immunol.* **133,** 2815.

SHILLITOE E.J., DANIELS T.E., WHITCHER J.P., STRAND C.V., TALAL N. & GREENSPAN J.S. (1982) Antibody to cytomegalovirus in patients with Sjogren's syndrome. *Arth. Rheum.* **25,** 260.

SMITH H.R. & STEINBERG A.D. (1983) Autoimmunity—A Perspective. *Ann. Rev. Immunol.* **1,** 175.

SWANSON N.R., BARTHOLOMAEUS W.N., REED W.D. & JOSKE R.A. (1985) An enzyme-linked immunosorbent assay for the detection of hepatocyte plasma membrane antibodies. *J. Immunol. Meth.* **85,** 203.

TAYLOR P.V., SCOTT J.S., GERLIS L.M., ESSCHER E. & SCOTT O. (1986) Maternal antibodies against fetal cardiac antigens in congenital complete heart block. *New Eng. J. Med.* **315,** 667.

TINGHITELLA T.J. & BOOSS J. (1979) Enhanced immune response late in primary cytomegalovirus infection of mice. *J. Immunol.* **122,** 2442.

WAGER O., RASANEN J.A., HAGMAN A. & KLEMOLA E. (1968) Mixed cryoimmunoglobulinemia in infectious mononucleosis and cytomegalovirus mononucleosis. *Int. Arch. Allergy*, **34,** 345.

WATANABE R., WEGE H. & TER MEULEN V. (1983) Adoptive transfer of EAE-like lesions from rats with coronavirus-induced demyelinating encephalomyelitis. *Nature (Lond.)*, **305,** 150.

WEINER H.L. & HAUSER, S.L. (1982) Neuroimmunology I: immunoregulation neurological disease. *Ann. Neurol.* **11,** 437.

WOLFGRAM, L.J., BEISEL, K.W. & ROSE, N.R. (1985) Heart-specific autoantibodies following murine coxsackie B3 myocarditis. *J. exp. Med.* **161,** 112.