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Lymphocytic Choriomeningitis Virus Can Persistently Infect Thyroid Epithelial Cells and Perturb Thyroid Hormone Production

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SUMMARY

Although viral infection has been suspected as the cause of some thyroid disorders, there has been limited data to support this contention seriously. Now we report the first evidence that lymphocytic choriomeningitis virus can persist in the thyroid gland, particularly thyroid epithelial cells in which thyroglobulin (Tg) the precursor of thyroid hormone, is synthesized. Concomitant with the infection of these cells is a significant reduction in Tg mRNA and in the level of circulating thyroid hormones. Another virus (lactate dehydrogenase virus) that causes persistent infection but does not replicate in the thyroid gland failed to alter levels of circulating thyroid hormones. These observations in an experimental model support the hypothesis that viruses may account for some thyroid disorders in man.

INTRODUCTION

Current knowledge about viruses and the diseases they cause is conditioned by the expectation of finding concurrent cellular destruction and/or inflammatory lesions at the site of virus replication. There are, however, numerous diseases of neuro-endocrine and immune dysfunction where there is no apparently definable aetiology or pathogenesis. Examples include diminished hormone synthesis, as in type I diabetes and thyroid disease. The involvement of viruses has often been speculated but even in the best documented cases not proven (Eylan *et al.*, 1957; Volpe *et al.*, 1967; Hung *et al.*, 1969; Ziring *et al.*, 1977).

Lymphocytic choriomeningitis virus (LCMV) is a relatively non-cytopathic arenavirus. It induces a persistent infection in its natural host, the mouse (for reviews, see Mims, 1970; Lehmann-Grube, 1971; Buchmeier *et al.*, 1980), due in large part to its ability to abort the LCMV-specific H-2-restricted cytotoxic T cell response which is required for clearance of virus-infected cells (Ahmed *et al.*, 1984). By escaping immunological surveillance and because of its non-cytopathic properties, the virus can replicate and viral materials continually accumulate in a variety of cells for which the virus is tropic throughout the life span of an animal (Southern *et al.*, 1984).

In the present study we have examined the replication of LCMV in the thyroid glands of BALB/c mice following neonatal infection and demonstrate an alteration in thyroid homeostasis as a result of persistent infection with LCMV but not with lactate dehydrogenase virus (LDV), an RNA virus not known to replicate in the thyroid gland (Notkins, 1965).

METHODS

Initiation of infection in mice. BALB/c mice were obtained from the breeding colony of the Research Institute of Scripps Clinic. Mice were inoculated either with 100 to 1000 p.f.u. of LCMV intracerebrally (within 18 h of birth) or with 10^5 ID₅₀ of LDV intraperitoneally when 4 weeks old. Details of inoculation, and assay for development of virus persistence have been reported previously for LCMV (Oldstone & Dixon, 1969; Buchmeier *et al.*, 1980; Oldstone *et al.*, 1982) and LDV (Notkins, 1965).

Virus. The origin, passage history (Dutko & Oldstone, 1983; Oldstone *et al.*, 1985) and recognition by monoclonal antibodies (Buchmeier *et al.*, 1981; Oldstone & Buchmeier, 1982) of LCMV Armstrong CA 1371 clone 53B (LCMV-ARM) has been reported. The virus was cloned and plaque-purified three times in Vero cells. Thereafter, stock virus was prepared after one passage in BHK cells (Dutko & Oldstone, 1983). LDV was kindly provided by Dr A. L. Notkins of the National Institute of Dental Research, Bethesda, Md, U.S.A.

Virus titration. Thyroids from individual mice were disrupted by Dounce homogenization in 10% culture medium (Dutko & Oldstone, 1983) and infectious virus was quantified by plaque assay on Vero cell monolayers.

Immunofluorescence. Thyroids were removed from individual mice, snap frozen in liquid nitrogen, and 4 μ m sections were cut and processed for immunofluorescence as reported previously (Oldstone & Dixon, 1969; Buchmeier *et al.*, 1981; Oldstone *et al.*, 1984a). Each tissue was stained with mouse monoclonal antibodies specific for either LCMV nucleoprotein (MAb 1-1-3) or LCMV glycoproteins (MAbs 2-11-10 and 9-7-9) kindly provided by Dr M. Buchmeier, Research Institute of Scripps Clinic. The specificities of these reagents have been described (Buchmeier *et al.*, 1981).

Autoantibody assays. Sera were tested for autoantibodies by indirect immunofluorescence on both unfixed frozen and Bouin's fluid-fixed paraffin-embedded sections of normal mouse thyroid as described previously (Prabhakar *et al.*, 1984). Autoantibodies to murine thyroglobulin were determined by ELISA assay (Romball & Weigle, 1987).

Histopathology. For light microscopy, tissues were removed, fixed in Bouin's solution and stained with haematoxylin and eosin. In other studies, mice were sacrificed by perfusion through the left ventricle with 2.5% glutaraldehyde in 0.1 M-phosphate buffer and processed for electron microscopy as described previously (Rodriguez *et al.*, 1985).

RNA dot blot hybridization. Individual thyroids were pooled separately from each of three persistently infected or uninfected control mice. RNA was extracted (Chirgwin *et al.*, 1979), denatured with formaldehyde, diluted fivefold with 15 \times SSC (where 1 \times SSC is 0.15 M-sodium chloride plus 0.015 M-sodium citrate) from 8 μ g stock and bound to nitrocellulose paper. The filter was hybridized with a 700 bp 32 P-labelled cDNA probe specific for the small segment of LCMV-ARM (Southern *et al.*, 1984). After autoradiography, the LCMV probe was removed by washing the filters under stringent conditions (90 $^{\circ}$ C in 0.1 \times SSC with 0.1% SDS) for 1 h. The same filter was then hybridized with a 32 P-labelled 3000 bp cDNA probe to rat Tg (prTg 39; Van Heuverswyn *et al.*, 1985) which cross-hybridizes with mouse Tg. The filter was then re-washed under stringent conditions and rehybridized with a 32 P-labelled 400 bp cDNA probe specific for 28S ribosomal RNA. The hybridization signal was quantified using a scanning densitometer (LKB Ultrascan XL).

Hormone analysis. Serum levels of tri-iodothyronine (T_3) and free thyroxine (FT_4) were determined from individual mice by solid-phase radioimmunoassay (Clinical Assays, Genentech).

Statistics. Students' *t*-test for unpaired comparisons was used.

RESULTS

To establish whether LCMV nucleic acid sequences are deposited and persist in thyroid tissue, total RNA was extracted from thyroids harvested from persistently infected and uninfected control mice 90, 180 and 360 days old. A nick-translated 700 bp 32 P-labelled cDNA probe, specific to the 5' region of the small RNA segment of LCMV-ARM hybridized specifically to RNA extracted from the persistently infected mice but not the controls, demonstrating LCMV nucleic acid sequences in these tissues (Fig. 1). By plaque infectivity assay, we detected 10^3 to 10^4 viral infectious units per gram of thyroid. The expression of LCMV nucleoprotein was localized principally to follicular epithelial cells of the thyroid (Fig. 2) in over

Fig. 2. Immunohistochemical and histopathological analysis of thyroid tissue taken from 90 day-old BALB/c mice persistently infected since birth with LCMV-ARM. (a) Cryostat-cut section (4 μ m) of thyroid gland stained with mouse monoclonal antibody 1-1-3 (specific to LCMV nucleoprotein) and fluorescein isothiocyanate-conjugated goat antibody to mouse IgG. Expression of LCMV nucleoprotein is localized to thyroid epithelial cells which secrete Tg, the precursor of thyroid hormone. Similar results were obtained in over 20 individual thyroids studied. (b) Haematoxylin- and eosin-stained sections of thyroid glands lacked cellular infiltration or necrosis despite persistent viral infection of the gland. However, follicles from virus-infected mice appeared larger than those of age- and sex-matched controls suggesting differences in synthetic activity. Similar results were observed in thyroid glands obtained for all 12 persistently infected mice studied. (c) Electron micrograph of a negatively stained thyroid gland showing LCMV virions above the apical surface of a thyrocyte. Release of virus is away from the colloidal border. (d) Enlargement of an area indicated in (c). Arrow points to LCMV virions. Bar markers represent (c) 500 nm and (d) 2 μ m.

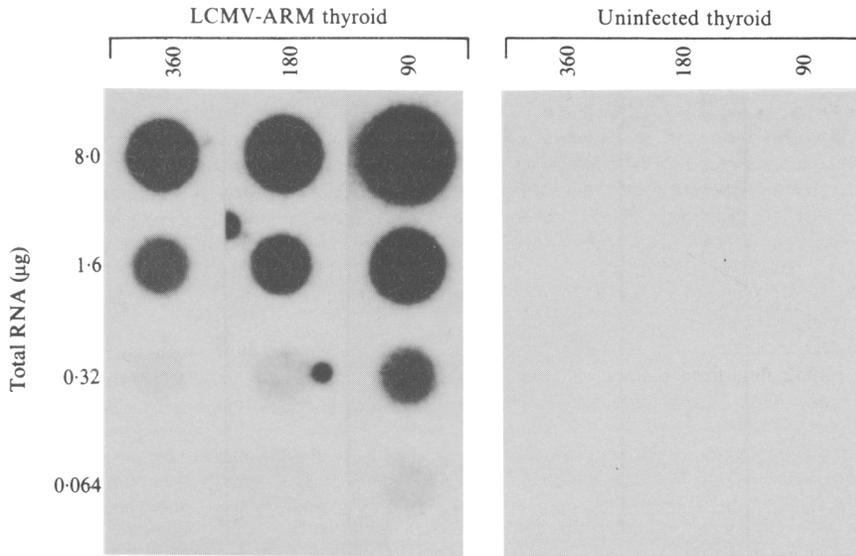
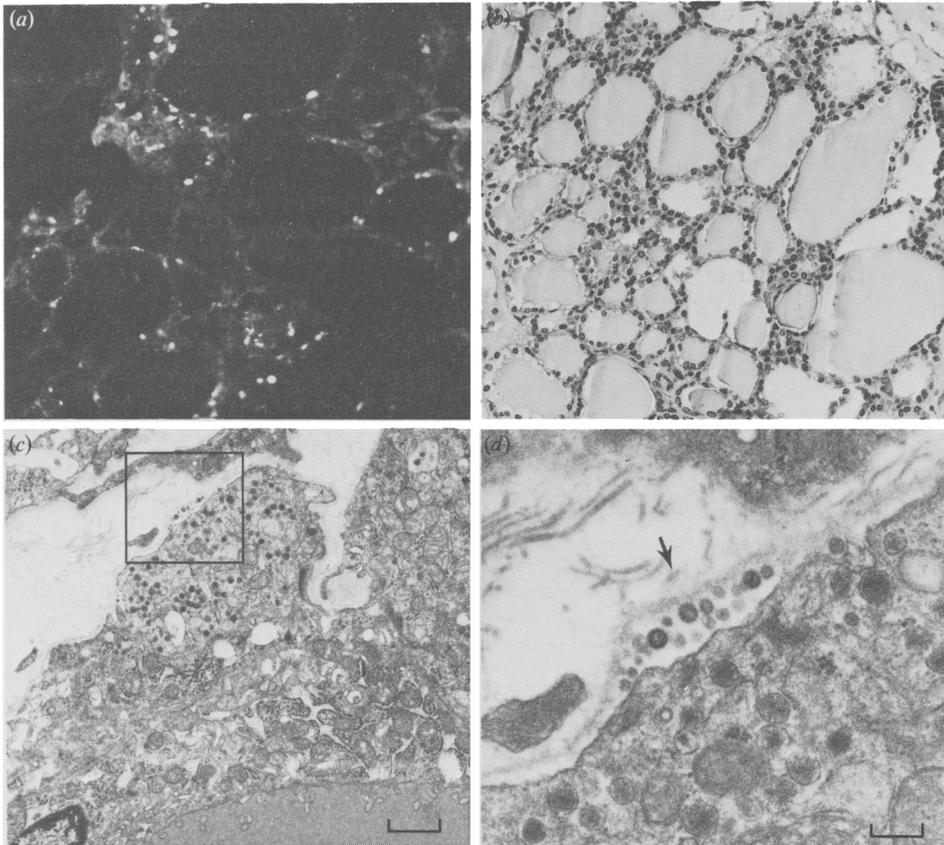


Fig. 1. Detection of LCMV-specific RNA in thyroids from persistently infected 90, 180 and 360 day-old BALB/c mice. These mice had been persistently infected since their inoculation within 18 h of birth with 100 p.f.u. of LCMV-ARM. RNA was extracted and probed for S RNA as described in Methods.



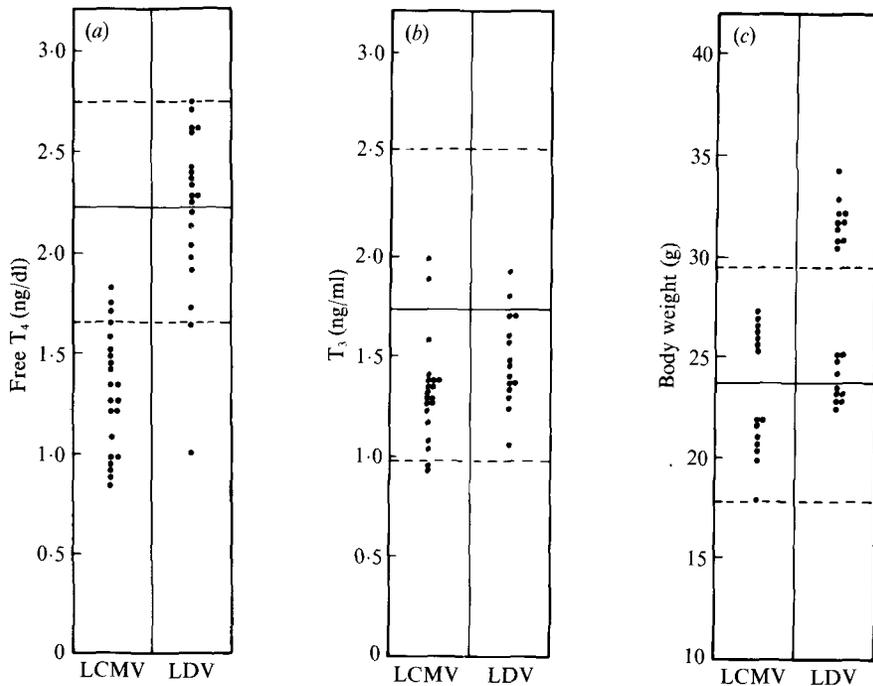


Fig. 3. Perturbation of thyroid free T_4 hormone in 90 day-old BALB/c mice persistently infected with LCMV since birth. Mice were injected intracerebrally with 100 p.f.u. LCMV-ARM within 18 h of birth or intraperitoneally with 10^5 ID₅₀ LDV at 4 weeks of age. (a) Free thyroxine (free T_4) and (b) triiodothyronine (T_3) measured by radioimmunoassay and (c) body weights for individual mice (●) are shown. The solid horizontal line indicates the mean, and the upper and lower broken lines 2 S.D. from the mean of 20 age- and sex-matched uninfected controls.

20 mice whose thyroids were tested with virus-specific monoclonal antibodies 90 days post-infection. However, no LCMV glycoprotein was detected in thyroids from the persistently infected mice at this time. Electron microscopy revealed that LCMV virions budded from follicular epithelial cells (Fig. 2), although the cells' morphology remained normal. Virus particles were released predominantly at locations away from the apical colloidal border of the follicle cell. Under a light microscope, neither necrosis nor inflammatory infiltration was visible in over 50 thyroids studied, indicating normal morphology of the persistently infected gland.

Because virus persisted in cells responsible for the synthesis of Tg, we measured levels of the circulating thyroid hormones T_3 and T_4 in persistently infected mice. There was a consistent decrease in the amounts of T_3 and free T_4 (the active form of T_4) in 15, 30, 90 and 180 day-old LCMV-infected mice compared to age- and sex-matched controls. Fig. 3 shows differences in free T_4 and T_3 levels at day 90. In LCMV-infected animals both free T_4 and T_3 were significantly decreased compared to control animals, $P < 0.001$ when $t = 10.78$ (39 degrees of freedom) and $t = 5.54$ (31 degrees of freedom) for free T_4 and T_3 respectively. This was not associated with a defect in dietary intake of mice persistently infected with LCMV and showing thyroid dysfunction. The body weights of LCMV-infected mice were within 1 S.D. of the mean weight of uninfected controls (Fig. 3). To demonstrate that this decrease in thyroid hormones was not caused by a non-specific physiological response to systemic infection, thyroid hormone levels were measured in mice persistently infected with LDV, a virus not known to replicate in the thyroid gland (Notkins, 1965). LDV persistently infected BALB/c mice did not develop thyroid hormone dysfunction since only 10% (2/20) had free T_4 levels 2 S.D. or more below the mean value of age-matched uninfected controls compared to 86% (19/22) of mice inoculated with LCMV-ARM (Fig. 3).

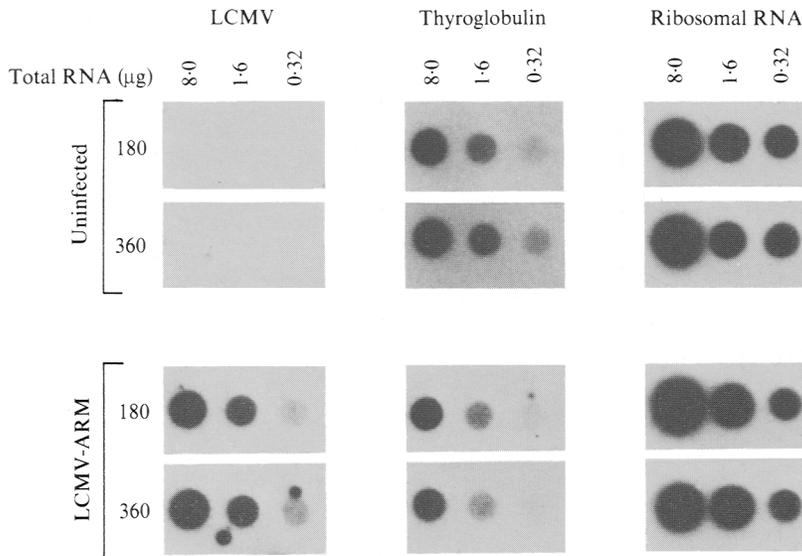


Fig. 4. Decreased level of Tg mRNA in adult BALB/c mice persistently infected since birth with LCMV. When 180 and 360 days old, mice were sacrificed; total RNA was extracted and probed as described in Methods.

To determine whether the abnormality in thyroid function during persistent infection with LCMV could be attributed to autoantibodies, we next analysed sera for binding to thyroid tissue sections and to purified murine Tg. In 10/10 sera from 90 day-old LCMV-infected mice we were unable to detect any binding either to cryostat thyroid sections or to Tg by ELISA.

Other studies showed that persistent infection with LCMV significantly lowered Tg mRNA levels. Total RNA extracted from the thyroids of infected and uninfected BALB/c mice at 180 and 360 days of age was analysed on nitrocellulose filters for hybridization with a cDNA probe to rat Tg. Comparison of the hybridization signal (Fig. 4) demonstrated that LCMV-infected mice had less cellular Tg mRNA than uninfected controls at both time points sampled. The dot blot in Fig. 4 was quantified by scanning densitometry. This demonstrated that there was a 2.17-fold reduction in the hybridization signal in 180 day old and 3.41-fold reduction in 360 day old LCMV persistently infected mice compared to age-matched controls after normalizing to the hybridization signal when the blots were rehybridized with a 400 bp probe to 28S ribosomal RNA (to correct for the amount of RNA on the filter). In other experiments a reduction in the amount of Tg RNA in LCMV-infected mice was similarly observed.

DISCUSSION

We have demonstrated for the first time that a virus can persist in the follicular epithelial cells of the thyroid gland and that infection is associated with abnormal levels of thyroid hormone in the circulation and abnormal levels of thyroglobulin mRNA in the thyroid.

In the thyroid, LCMV infection is restricted to follicular epithelial cells, the cells that secrete Tg. Viral antigen could be detected at least 180 days post-infection indicating that such cells can be continuously infected. Analysis of the viral proteins reveals that whereas nucleoprotein is being expressed, viral glycoprotein is not detectable. Limited expression of LCMV glycoproteins in the presence of abundant nucleoprotein has been observed previously in a variety of cells both *in vitro* (Welsh & Oldstone, 1977; Oldstone & Buchmeier, 1982) and *in vivo* (Oldstone *et al.*, 1982, 1984b; Oldstone & Buchmeier, 1982) and probably represents some

translational or post-translational modifications, since glycoprotein genes are expressed (Francis *et al.*, 1986). Persistent infection with LCMV is not accompanied by either marked cellular necrosis or inflammation, these being histological markers conventionally considered characteristic of virus infection. The lack of tissue necrosis despite active viral replication probably relates to the non-cytopathic nature of LCMV, the relative lack of LCMV-specific H-2-restricted cytotoxic T lymphocytes (CTL) (Ahmed *et al.*, 1984) and diminished expression of viral glycoproteins (Welsh & Oldstone, 1977; Oldstone & Buchmeier, 1982; Oldstone *et al.*, 1982; 1984*b*). Recent experiments using cloned CTL and LCMV glycoprotein genes expressed in vaccinia virus vectors indicate that the major epitope(s) of recognition with H-2^b mice are encoded in an H-2-restricted glycoprotein complex (Whitton *et al.*, 1986).

Diminished circulating levels of T₃ and free T₄, suggesting diminished synthesis, was associated with persistent LCMV infection. This is unlikely to be due to a non-specific physiological response to systemic infection because mice persistently infected with LDV fail to show disordered thyroid function (Fig. 3). Moreover, the alteration in thyroid homeostasis during LCMV infection was not accounted for by a defect in body weights, low iodine uptake or generation of autoantibodies.

Hence, we now demonstrate a novel way that viruses may interfere in the synthesis of thyroid hormones. This is accomplished by persistent infection of thyroglobulin-producing cells, which is associated with a significant reduction in Tg mRNA. We speculate that a defect at the level of Tg mRNA may account for the reduction in circulating thyroid hormone associated with LCMV replication in the thyroid.

Thyroid function is regulated by complex neuroendocrine feedback controls involving the secretion of thyrotropin by the anterior pituitary and of thyrotropin-releasing hormone by the hypothalamus (Ingbar, 1980). Transcription of the Tg gene is positively regulated by thyrotropin via a cAMP-dependent mechanism (Van Heuverswyn *et al.*, 1984). Thus, the alteration in thyroid homeostasis induced during persistent infection with LCMV may result either from primary thyroid failure, secondary to perturbation of the hypothalamic-pituitary axis or from a combination of pituitary and thyroid failure. Determining which of these is correct, as well as the transcription rate of the Tg gene, stability of the mRNA and the molecular basis by which a virus down-regulates a differentiated function are our current objectives. Finally, considering the many disorders of the thyroid and other endocrine glands for which no aetiology is known, it is worthwhile raising the issue that viruses may play a role in their pathogenesis.

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REFERENCES

- AHMED, R., SALMI, A., BUTLER, L. D., CHILLER, J. M. & OLDSTONE, M. B. A. (1984). Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice: role in suppression of cytotoxic T lymphocyte response and viral persistence. *Journal of Experimental Medicine* **60**, 521-540.
- BUCHMEIER, M. J., WELSH, R. M., DUTKO, F. J. & OLDSTONE, M. B. A. (1980). The virology and immunobiology of lymphocytic choriomeningitis virus infection. *Advances in Immunology* **30**, 275-331.
- BUCHMEIER, M. J., LEWICKI, H. A., TOMORI, O. & OLDSTONE, M. B. A. (1981). Monoclonal antibodies to lymphocytic choriomeningitis viruses: generation, characterization and cross-reactivity with other arenaviruses. *Virology* **113**, 73-85.
- CHIRGWIN, J. M., PRZYBYLA, A. E., MACDONALD, R. J. & RITTER, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294-5299.
- DUTKO, F. J. & OLDSTONE, M. B. A. (1983). Genomic and biological variation among commonly used lymphocytic choriomeningitis virus strains. *Journal of General Virology* **64**, 1689-1698.
- EYLAN, E., ZMUCKY, R. & SHEBA, C. (1957). Mumps thyroiditis and subacute thyroiditis: evidence of a causal association. *Lancet* **i**, 1062-1063.
- FRANCIS, S. J., SINGH, M. K., OLDSTONE, M. B. A. & SOUTHERN, P. J. (1986). Analysis of LCMV gene expression in acutely and persistently infected mice. *Medical Microbiology and Immunology* **175**(2-3), 105-108.

- HUNG, W. (1969). Mumps thyroiditis and hypothyroidism. *Journal of Pediatrics* **74**, 611–613.
- INGBAR, S. J. (1980). The thyroid gland. In *Williams Textbook of Endocrinology*, pp. 682–815. Edited by J.-W. Wilson & D. W. Foster. Philadelphia: W. B. Saunders.
- LEHMANN-GRUBE, F. (1971). *Lymphocytic Choriomeningitis Virus. Virology Monographs*, vol. 10. Wien: Springer-Verlag.
- MIMS, C. A. (1970). Observations on mice infected congenitally or neonatally with lymphocytic choriomeningitis (LCM) virus. *Archiv für die gesamte Virusforschung* **30**, 67–74.
- NOTKINS, A. L. (1965). Lactate dehydrogenase virus. *Bacteriological Reviews* **29**, 143–160.
- OLDSTONE, M. B. A. & BUCHMEIER, M. J. (1982). Restricted expression of viral glycoprotein in cells of persistently infected mice. *Nature, London* **300**, 360–362.
- OLDSTONE, M. B. A. & DIXON, F. J. (1969). Pathogenesis of chronic disease associated with persistent lymphocytic choriomeningitis viral infection. I. Relationship of antibody production to disease in neonatally infected mice. *Journal of Experimental Medicine* **129**, 483–505.
- OLDSTONE, M. B. A., SINHA, Y. A., BLOUNT, P., TISHON, A., RODRIGUEZ, M., VON WEDEL, R. & LAMPERT, P. W. (1982). Virus-induced alterations in homeostasis: alterations in differentiated functions of infected cells *in vivo*. *Science* **218**, 1125–1127.
- OLDSTONE, M. B. A., RODRIGUEZ, M., DAUGHADAY, W. H. & LAMPERT, P. W. (1984*a*). Viral perturbation of endocrine function: disorder of cell function leading to disturbed homeostasis and disease. *Nature, London* **307**, 278–280.
- OLDSTONE, M. B. A., SOUTHERN, P., RODRIGUEZ, M. & LAMPERT, P. W. (1984*b*). Virus persists in beta cells of islets of Langerhans and is associated with chemical manifestations of diabetes. *Science* **224**, 1144–1443.
- OLDSTONE, M. B. A., AHMED, R., BUCHMEIER, M. J., BLOUNT, P. & TISHON, A. (1985). Perturbations of differentiated functions during viral infections *in vivo*. I. Relationship of lymphocytic choriomeningitis virus and host strains to growth hormone deficiency. *Virology* **142**, 158–174.
- PRABHAKAR, B. S., SAEQUA, 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. *Journal of Immunology* **133**, 2815–2817.
- RODRIGUEZ, M., GARRETT, R. S., RAITT, M., LAMPERT, P. W. & OLDSTONE, M. B. A. (1985). Virus persists in beta cells of islets of Langerhans and infection is associated with chemical manifestations of diabetes. II. Morphologic observations. *American Journal of Pathology* **121**, 497–504.
- ROMBALL, C. G. & WEIGLE, W. O. (1987). Transfer of experimental autoimmune thyroiditis with T cell clones. *Journal of Immunology* (in press).
- SOUTHERN, P. J., BLOUNT, P. & OLDSTONE, M. B. A. (1984). Analysis of persistent virus infections by *in situ* hybridization to whole mouse sections. *Nature, London* **312**, 555–558.
- VAN HEUVERSWYN, B., STREYDIO, C., BROCAS, H., REFETOFF, S., DUMONT, J. & VASSART, G. (1984). Thyrotropin controls transcription of the thyroglobulin gene. *Proceedings of the National Academy of Sciences, U.S.A.* **81**, 5941–5945.
- VAN HEUVERSWYN, B., LERICHE, A., VAN SANDE, J., DUMONT, J. E. & VASSART, G. (1985). Transcriptional control of thyroglobulin gene expression by cAMP. *FEBS Letters* **188**, 192–196.
- VOLPE, R., ROS, V. V. & EZRIN, C. (1967). Circulating viral and thyroid antibodies in subacute thyroiditis. *Journal of Clinical Endocrinology and Metabolism* **27**, 1275–1284.
- WELSH, R. M. & OLDSTONE, M. B. A. (1977). Inhibition of immunologic injury of cultured cells infected with lymphocytic choriomeningitis virus. Role of defective interfering virus in regulating viral antigenic expression. *Journal of Experimental Medicine* **145**, 1449–1468.
- WHITTON, J. L., SOUTHERN, P., TISHON, A. & OLDSTONE, M. B. A. (1986). LCMV glycoprotein expressed in vaccinia virus allows H-2 restricted LCMV-specific CTL recognition/lysis. *Federation Proceedings* **45**, 979.
- ZIRING, P. R., GALLO, G., FINEGOLD, M., BUIMOVICI-KLEIN, E. & OGRA, P. L. (1977). Chronic lymphocytic thyroiditis: identification of rubella virus antigen in the thyroid of a child with congenital rubella. *Journal of Pediatrics* **90**, 419–420.

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