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Virus infection induces redistribution and membrane localization of the nuclear antigen La (SS-B): a possible mechanism for autoimmunity

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SUMMARY

To investigate the possibility that anti-La (SS-B) antibodies in Sjögren's syndrome were induced by virus infection we studied the distribution of La in virus-infected human cell lines. Three monoclonal antibodies to La were used with monoclonal anti-Sm (derived from MRL/lpr lupus mice) and anti-rat immunoglobulin antibodies as controls. In uninfected cells La was predominantly in the nucleus. Twenty-four hours after infection of HEp-2 cells with adenovirus 2, the La and Sm antigens appeared to aggregate and accumulate in the periphery of the nucleus and, after 48 h, La was seen in the cytoplasm and cell membrane. No cytoplasmic or membrane expression of Sm was seen. Infection with adenovirus or cytomegalovirus caused a 2–13-fold increase in the concentration of La in three cell lines. Treatment of HE--2 cells with interferon-gamma (IFN-γ) and infection with Epstein–Barr virus and cytomegalovirus caused cytoplasmic, but no definite membrane expression of La. The appearance of La on the surface of virally infected epithelial cells together with IFN-γ induced class II expression could form the basis of a T cell dependent mechanism for anti-La autoantibody induction.

Keywords Sjögren's syndrome adenovirus nuclear antigens

INTRODUCTION

Patients with autoimmune disorders frequently have circulating antibodies to nucleic acids or nucleic acid binding proteins. In 50% of patients with Sjögren's syndrome these antibodies are directed against the ribonucleoprotein antigen, La, also known as SS-B (Mattioli & Reichlin, 1974; Alspaugh, Talal & Tan, 1976; Venables *et al.*, 1983a). La is a nuclear ribonucleoprotein which contains a 45–50 kD molecular weight polypeptide (Francoeur & Mathews, 1982; Habets *et al.*, 1983; Venables, Smith & Maini, 1983b). Although the presence of high levels of anti-La antibodies is a useful marker of Sjögren's syndrome (Venables *et al.*, 1989) the mechanism by which antibodies to these sequestered nuclear polypeptides arise is unknown.

Viruses have frequently been considered as trigger factors for autoimmune disorders (Philips, 1988). It has been postulated that infection may result in the exposure of the normally hidden cellular proteins either by damage to the cell or as a result of host–virus interactions. La in particular is of interest because of its known interactions with a number of viral RNAs including adenovirus and Epstein–Barr virus (EBV) RNAs (Lerner *et al.*, 1981). Another mechanism suggested by Botazzo *et al.* (1983) for the induction of thyroid autoimmunity, was that autoantibodies to thyroid tissue could be explained by interferon-gamma (IFN-γ) induced class II expression together with thyroid antigens in thyroid epithelial cells. The observation that epithelial class II expression is also a feature of inflamed salivary glands (Lindahl *et al.*, 1985; Fox *et al.*, 1986) suggests that similar mechanisms may be operational in Sjögren's syndrome but does not explain why the autoantibodies are directed against nuclear antigens.

One possible hypothesis is that conditions which promote class II expression also cause certain nuclear antigens to be expressed on the cell membrane. Some supporting evidence is provided by the observations of Le Feber *et al.* (1984) who showed that exposure of human keratinocytes to sub-lethal doses of u.v. light caused Ro (SS-A), nRNP and Sm to move from the nucleus to the cell surface. Although this study could be criticised on the grounds that polyclonal human sera were used, it is in accord with our previous findings (Smith *et al.*, 1985) with monoclonal anti-La antibodies. In that study we showed that cell transformation caused the movement of La antigen from the nuclear matrix to the nucleolus and cytoplasm, although we were unable to demonstrate La on the membrane.

In this study we used three monoclonal anti-La antibodies to investigate the effect of infection with different viruses and the

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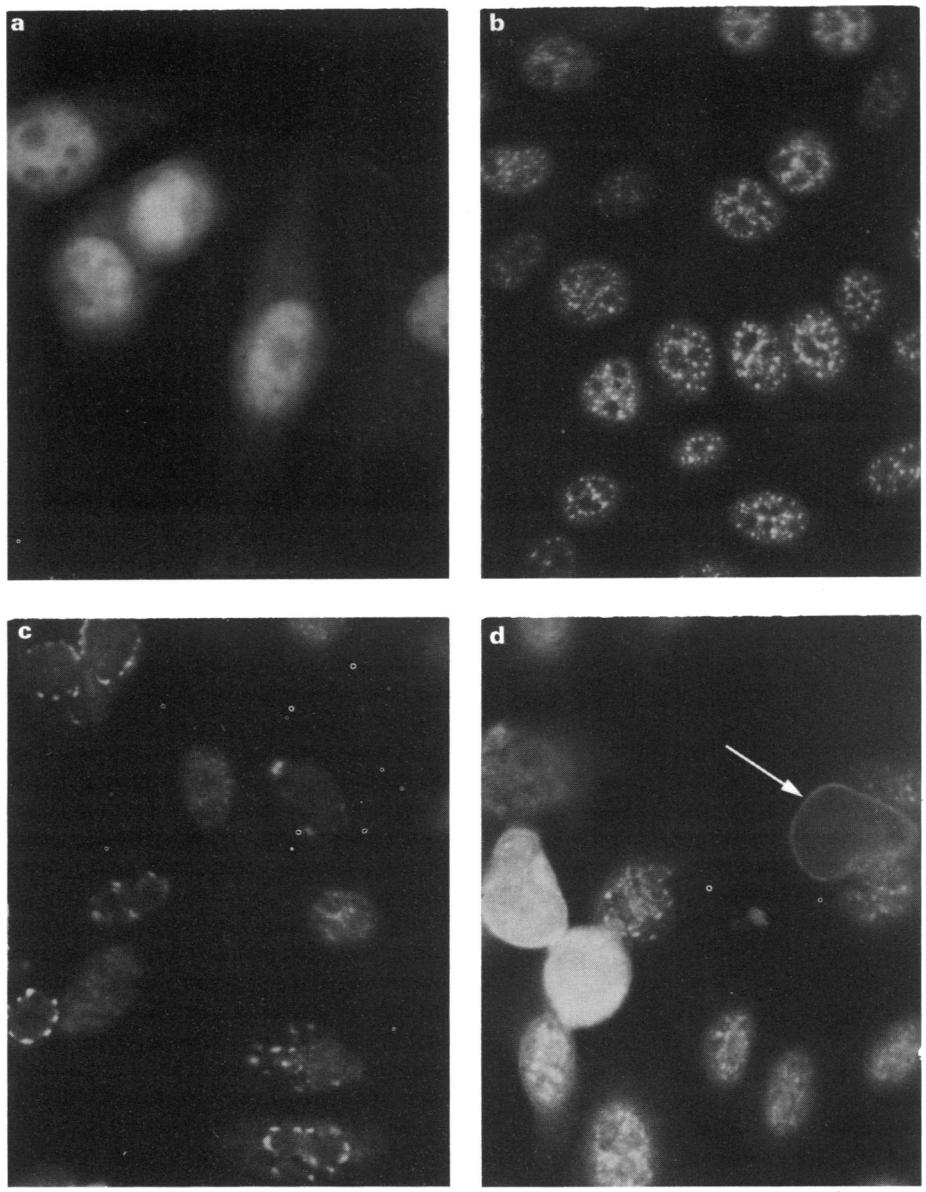

Fig. 1. Immunofluorescent microscopy of HEp-2 cells using SW-5 anti La monoclonal antibody. a Uninfected cells; b, 12 h after infection with adenovirus; c, 24 h after infection; d 48 h after infection. One of the cells (arrowed) shows fluorescence restricted to the cell membrane.

effect of IFN-γ on the cellular localization of La. By demonstrating La on the surface of adenovirus-infected epithelial cells, we are able to suggest a mechanism whereby virus infection of salivary glands could induce anti-La antibodies in Sjögren's syndrome.

MATERIALS AND METHODS

Cell culture

The cell lines HEp-2 and HeLa (both epithelial) and human embryo lung fibroblasts (HEL) were cultured in Eagle's minimum essential medium (MEM, GIBCO; Paisley, Scotland), supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal calf serum (FCS) (Flow laboratories; Rickmansworth, UK). EBV-infected transformed cell line WIL-2 was cultured in RPMI 1640 (Flow Laboratories) with supplements as described.

Indirect immunofluorescence

HEp-2 cells were cultured on multi-test slides (Flow Laboratories) in growth medium in 5% CO_2 at 37°C. When confluent monolayers were formed, the cells were inoculated with 0·1–0·3 plaque-forming units (PFU) per cell of adenovirus type 2. Samples were removed at 12, 24 and 48 h, the cell monolayers were washed once in phosphate-buffered saline (PBS) and fixed in acetone and methanol (1 : 1, v/v) on ice, air dried and stored at −20°C until used.

Thirty microlitres of ascitic fluid containing monoclonal antibodies directed against the antigen to be detected (diluted 1:100 in PBS) were placed on each well of the slide and

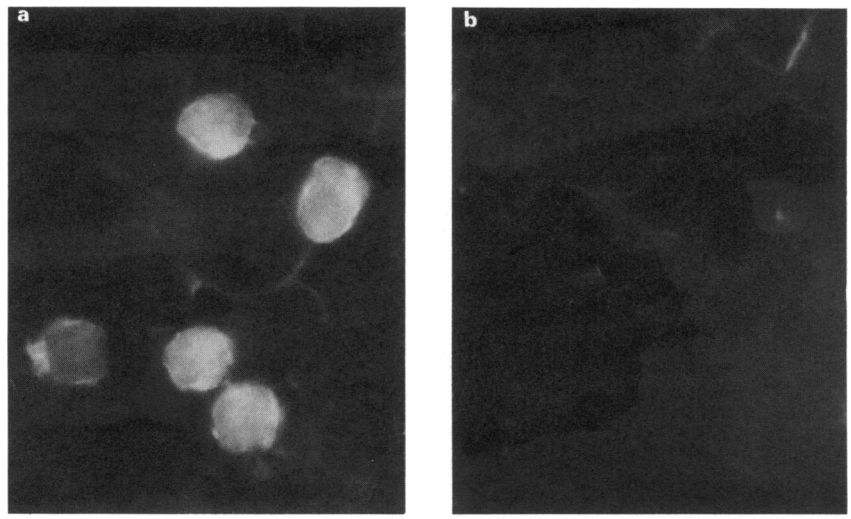

Fig. 2. Immunofluorescent microscopy of the surface of adenovirus-infected HEp-2 cells. Incubation steps with antibody and conjugate were performed on viable, unfixed cells. a, adenovirus-infected cells probed with anti-La monoclonal SW-5 showing surface fluorescence of 20% of the cells; b, adenovirus-infected cells probed with anti-Sm showing weak background fluorescence only.

Table 1. Effect of cell activation, virus infection and interferon-gamma (IFN-γ) treatment on La and DR expression

Cell line	Treatment	La expression	DR expression
HEp-2	Untreated	Speckled nuclear	Negative
HEp-2	Adenovirus	Cytoplasmic, membrane	Negative
HEp-2	IFN-γ (50 U/ml)	Speckled nuclear	Positive
HEp-2	IFN-γ (500 U/ml)	Nucleolar, cytoplasmic	Positive
HEL	CMV	Cytoplasmic	Negative
HeLa	Adenovirus	Cytoplasmic	Negative
WI-L2	EBV transformed (constituative)	Nucleolar, cytoplasmic	Positive

CMV, cytomegalovirus; EBV, Epstein–Barr virus.

incubated for 1 h at room temperature. We used three anti-La monoclonal antibodies SW1, SW3 and SW5 (Smith *et al.*, 1985); and F(ab′)$_2$ affinity-purified human anti-La (Horsfall, Brown & Maini, 1987); an anti-Sm monoclonal antibody, KSm2, developed in this laboratory (Williams, Smith & Maini, 1985a); an anti-class II monoclonal antibody, Tu 39 (prepared by Dr Zeigler, Tubingen, FRG); and an anti-adenovirus antibody (Seralab; Crawley Down, UK). An antibody to rat immunoglobulin, OX-12, prepared by Dr A. Williams, Oxford, was used as the negative control throughout. After washing for 20 min with two changes of PBS the slides were incubated with fluorescein-conjugated goat anti-mouse IgG (Sigma Chemical Company, Poole, UK) diluted 1:50 in PBS, for 30 min at room temperature. After a subsequent wash in PBS the slides were mounted in 90% glycerol/PBS containing *p*-phenylenediamine (0·1 mg/ml) and viewed with a Leitz Laborlux 12 fluorescent microscope with a × 50 water immersion lens.

Double staining for La and adenoviral antigens was carried out using biotin-labelled F(ab′)$_2$ affinity-purified human anti-La (Horsfall *et al.*, 1987) at 10 μg/ml mixed with a murine monoclonal anti-adenovirus antibody (Seralab) used at a final dilution of 1:100. After incubation and washing as above La antigen was identified with Texas red streptavidin (Amersham International, Amersham, UK) and adenoviral antigens with fluorescein-conjugated goat anti-mouse IgG, as described above.

The procedure for fluorescent staining of live HEp-2 cells was identical to that described above except that the cells were viable and were incubated with the monoclonal antibodies prior to fixation. Particular care was taken to ensure that the cells did not dry at any stage. Viability of the cells after incubation with conjugate was checked by 0·1% trypan blue exclusion. The cells were fixed with acetone/methanol before mounting.

IFN treatment

Uninfected HEp-2 cells cultured on multi-test slides were treated with recombinant human IFN-γ (generously donated by Professor M. Feldmann, Charing Cross Sunley Research Centre, London) at 0, 50, 125, 250, 500 and 1000 units/ml for 5 days. The slides were then fixed and processed as above.

Preparation of cell extracts

Confluent monolayers of HEp-2 and HeLa cells were inoculated with adenovirus type 2 and HEL cells with cytomegalovirus AD169 at 0·1–0·3 PFU/cell. A control batch of cells were mock-infected with uninfected cell supernatant. The cells were harvested daily by scraping into 0·5 ml of PBS followed by centrifugation. The cell pellets were weighed, and were suspended in PBS at a concentration of 400 mg of pellet/ml. The pellets were extracted by one cycle of freezing at −70°C and thawing at 37°C followed by sonication at maximum amplitude using Soniprep 150 (MSE, Crawley, UK) for 1 min. The cell debris was removed by centrifugation at 1000 ***g*** for 10 min. The supernatants were stored at −70°C until required.

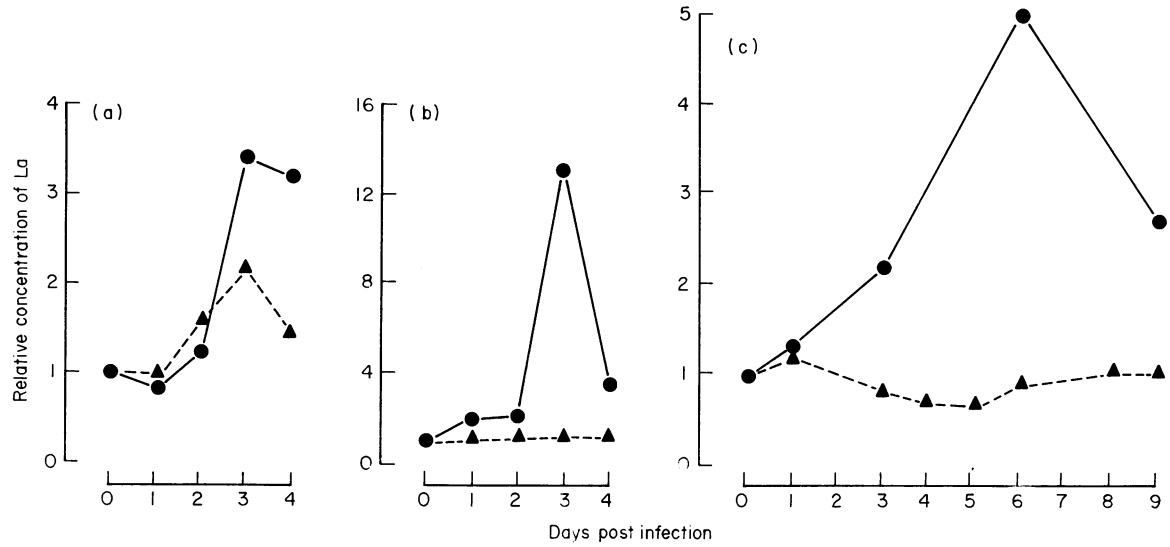

Fig. 3. Kinetics of the concentration of La in virus-infected human cells *in vitro*. a, adenovirus-infected HEp-2 cells; b, adenovirus-infected HeLa cells; c, CMV-infected HEL fibroblasts. ▲ Uninfected cells, and ● infected cells.

Quantification of La

Fifty microlitres of monoclonal anti-La antibodies (SW5, 1:100 dilution of ascitic fluid in PBS) were added to each well of 96-well micro-ELISA plates (Dynatech, Billingshurst, UK). After incubating for 1 h at 37°C, the plates were washed with PBS and blocked with 2% casein in PBS for 1 h at 37°C. After three further washes with PBS containing 0·1% Tween 20 (PBST), 50 μl of serial dilutions of the cell extracts were added to each well. In order to standardize the assay serial dilutions of a positive control antigen were included on each plate. This antigen was an affinity-purified preparation of La obtained from a commercially available acetone extract of rabbit thymus (Venebles *et al.*, 1983a).

After 1-h incubation at 37°C, the plates were washed three times with PBST and incubated at 37°C with 50 μl of 1:100 dilution of a human serum known to contain high levels of anti-La antibodies. They were then washed three times with PBST and 50 μl of a 1:1000 dilution of an alkaline phosphatase conjugated goat anti human immunoglobulin (Sigma) diluted in 0·5% casein in PBST were added to each well. After 1-h incubation at 37°C, the plates were washed six times with PBST and 50 μl of substrate were added. The substrate was 1 mg of *p*-nitrophenyl phosphate disodium/ml of 0·1 M glycine buffer, pH 10·4, containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$ (Sigma). Optical density was read after 1-h incubation at 37°C, at 405 nm on a Titertek Multiskan (Flow Laboratories). The results were expressed as a ratio of the concentration of La in each sample over that of the sample taken on day 0.

RESULTS

Intracellular localization of La antigen in HEp-2 cells

Immunofluorescence staining of HEp-2 cells with monoclonal antibodies to La revealed a predominantly speckled nuclear staining pattern. A similar pattern was observed with all three monoclonal anti-La antibodies (Fig. 1a). Twelve hours after infection with adenovirus type 2 the nuclear speckles appeared brighter and more discrete in all the cells (Fig. 1b). Twenty-four hours after infection La was still nuclear but the bright speckles appeared to aggregate in the region of the nuclear membrane in 20% of the cells (Fig. 1c), and 48 h after infection a mixture of patterns were observed; 80% cells still showed La in the nucleus while 20% showed the antigen in the cytoplasm (Fig. 1d). In a proportion of these cells it was possible to demonstrate staining of the cell membrane.

Membrane localization of La 48 hours after adenovirus infection was confirmed by incubating live cells with the antibodies (Fig. 2a). The positively stained cells were smaller and more rounded and lay on top of the monolayer of uninfected cells. The localization of the reaction to the cell surface was demonstrated by focusing up and down and by establishing that more than 95% of the cells were viable at the end of the staining procedure. By examining the cells consecutively with fluorescent (for La) and light microscopy (for trypan blue) we confirmed that the cells expressing surface La were viable. There was no evidence of surface staining when the monoclonal anti-Sm was used (Fig. 2b).

Using the monoclonal anti-adenovirus antibody, about 20% of the cells were shown to be infected. Double immunofluorescence on the fixed cells showed that the cells expressing La in peripheral nuclear speckles and in the cytoplasm, were also expressing adenovirus antigens, indicating that these changes were restricted to infected cells. Using the monoclonal antibody to Sm, similar changes in nuclear staining were observed after 24 h including the aggregation around the nuclear membrane in the infected cells. However, cytoplasmic or membrane staining was not seen with the anti-Sm antibody or with Ox-12 at any stage of virus infection.

Fibroblasts (HEL cells) infected with cytomegalovirus showed nucleolar and cytoplasmic staining of La but no membrane expression up to 10 days post-infection. WI-L2 cells which are inherently transformed with EBV also showed cytoplasmic staining of La as previously described (Smith *et al.*, 1985), although convincing membrane staining could not be demonstrated.

HEp-2 cells treated with IFN-γ (500 U/ml) showed nuclear and cytoplasmic staining of La but no membrane fluorescence was seen (Table 1).

The adenovirus-infected cells were negative for class II antigens, although HEp-2 cells could be induced to express HLA class II by treatment with 50 U/ml of IFN-γ. Higher doses of IFN-γ (500 U/ml) also resulted in the appearance of La in the nucleolus and cytoplasm. WI-L2 cells were class II positive without treatment and expressed cytoplasmic La.

Quantification of La in infected cells

Not only did the distribution of La alter with progression of viral infection but its concentration increased, too. Figure 3 demonstrates elevated levels of La in HEp-2 and HeLa cells infected with adenovirus and HEL cells infected with cytomegalovirus. It can be seen that compared with uninfected cultures, virally infected cells showed a 2–13-fold increase in the quantity of La.

DISCUSSION

We have shown that the La antigen, normally found in the cell nucleus, is induced to move to the cytoplasm and subsequently to the cell membrane following infection with adenovirus. The early changes to bright, discrete nuclear speckles occurred in all of the cells suggesting an effect of a soluble factor secreted by the cells. The later changes, the peripheral nuclear granules and cytoplasmic and surface staining were restricted to infected cells, suggesting a more direct effect of the virus itself.

The surface expression of La on adenovirus-infected cells was confirmed by incubating anti-La antibodies directly with living cells whose viability was shown by trypan blue exclusion. We also provided evidence that the antigen was La itself rather than a cross-reactive antigen, by showing that it reacted with three different monoclonal antibodies as well as with an affinity-purified human antibody. The identical reaction with the $F(ab')_2$ human anti-La antibody as well as the lack of staining with KSm2 and Ox-12 confirmed that the binding was not due to Fc receptors. Furthermore, the use of the antibody to Sm, which like La is another ribonucleoprotein, indicated that the phenomenon could not simply be explained by passive leakage of intracellular antigen onto the cell surface.

This report is the first to demonstrate La on the surface of virus infected cells. Previous studies have demonstrated other nuclear antigens on the cell surface, using 'monospecific' human sera. These include Ro, RNP, and Sm (Le Feber *et al.*, 1984) on u.v.-irradiated keratinocytes. In our study we used monoclonal antibodies because most human sera contain antibodies to adenoviral antigens which would have caused surface and cytoplasmic staining of infected cells.

Previous studies have shown that virus infection also has profound effects on the cellular distribution of ribonucleoprotein antigens. Martin *et al.* (1987) found that infecting HEp-2 cells with herpes simplex virus resulted in the nuclear staining pattern for Sm changing to more discrete speckles followed by the accumulation of bright speckles round the nuclear membrane. Their results are almost identical to our findings with both the anti-Sm and anti-La monoclonal antibodies.

Our results differ from those of Pizer *et al.* (1983) and Habets *et al.* (1983) who compared adenovirus infected and uninfected cells and found no change in the position of La. In these studies polyclonal sera were used and in one (Habets *et al.*, 1983) speckled cytoplasmic staining was seen which was attributed to anti-adenoviral antibodies. The most important difference in both these earlier studies was that the cells were examined at less than 24 h after infection and therefore adequate time may not have been allowed for the appearance of La in the cytoplasm. In our study the change to cytoplasmic staining was only seen 48 h after infection.

Infection with cytomegalovirus and EBV was associated with a movement of La to the cell cytoplasm, although with these viruses we were unable to demonstrate membrane staining. We have previously noted a change in La to a predominantly nucleolar and cytoplasmic distribution in activated cells (Smith *et al.*, 1985). Here we have extended these findings by showing that treatment with high doses of IFN-γ had the same effect.

We also showed that virus infection increases the quantity of the antigen in the cells. This is in agreement with the increased intensity of cytoplasmic staining that we observed in the adenovirus-infected cells. The observations also confirm those of Williams *et al.* (1985b) who found 15 times the quantity of La in EBV-infected continuous cell lines compared with normal peripheral blood mononuclear cells, and those of Bachmann *et al.* (1986) who found that herpes simplex virus infection of Vero cells caused a 10-fold increase in the concentration of La. Our results may also be explained by an alteration in the ability of La antigen to bind antibodies. Pizer *et al.* (1983) demonstrated that La from adenovirus-infected cells had additional phosphate groups and bound more strongly to specific antibodies. These findings, whether due to increased concentration or phosphorylation, are consistent with the active participation of La in the transcription of viral RNA, and, possibly, in its transport from nucleus to cytoplasm. We cannot, however, explain the mechanism by which the antigen appears on the surface.

Our study allows the proposal of a hypothesis for pathogenesis of autoimmunity in Sjögren's syndrome. Virus infection of the salivary epithelium leads to the expression of La antigen on the cell surface. This, together with IFN-γ-induced class II expression, could result in a cellular and humoral response directed not only against viral antigens but also to the La antigen itself. This response would cause both the generation of anti-La antibodies, and the inflammation characteristic of Sjögren's syndrome. It is possible that high levels of IFN-γ could also contribute to the surface expression of La, although in our study we were only able to demonstrate it in the cytoplasm.

All the essential elements of this response, including IFN-γ epithelial class II expression and infiltrates with CD4-positive T cells, have been detected in salivary gland biopsies from patients with Sjögren's syndrome (Fox *et al.*, 1986). Moreover, we have recently shown that a proportion of plasma cells in the glands secrete idiotypes present on anti-La antibodies (Horsfall *et al.*, 1988). This suggests that antigen expression, T cell response and production of anti-La antibodies can all take place within the confines of the gland. However, we have no evidence that adenovirus is the causative organism. In a recent study of EBV in salivary biopsies we found EBV DNA, early antigen and membrane antigen in salivary duct epithelium, but no expression of adenoviral proteins using the same monoclonal antibody as in this study (Veneables *et al.*, 1989). It is possible that surface expression of La following virus infection is a property of epithelial cells infected by a number of viruses and our results with adenovirus simply reflect its ability to infect

epithelial cells *in vitro*. Whatever the causative agent, our system has provided a new model for studying induction of autoimmunity in Sjögren's syndrome.

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