**In vivo** cell penetration and intracellular transport of anti-Sm and anti-La autoantibodies

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**Abstract**

Anti-nuclear autoantibodies (ANA) are the hallmark of systemic autoimmune diseases. Yet, the **in vivo** function of ANA remains controversial to a large extent due to the intracellular nature of their antigenic targets. It has been reported that a subset of autoantibodies can penetrate live cells and translocate into the subcellular compartments containing the corresponding antigens. The studies presented herein show that murine anti-Sm and anti-La monoclonal autoantibodies can also enter a variety of cell types from different animal species and that the cell penetration activity is not isotype-restricted. Interestingly, only mAb with cross-reactivity against double-stranded DNA did enter cells. Both these autoantibodies rapidly accumulate in the nucleus of viable cells but display different penetration kinetics. In co-localization experiments, monoclonal autoantibodies did not accumulate significantly within endocytic vesicles containing dextran, suggesting that they are internalized by mechanisms distinct from conventional receptor-mediated endocytosis. This report represents the first evidence that anti-La and anti-Sm autoantibodies are capable of entering live cells. Our observations support the notion that the phenomenon of intracellular autoantibodies may have a larger scope than previously reported and are consistent with a potential pathogenic role for ANA.

**Introduction**

Anti-nuclear antibodies (ANA) have been used to define systemic autoimmune diseases for decades, often with great specificity for the disease in question. As a result, ANA constitute important diagnostic tools for a number of diseases, e.g. systemic lupus erythematosus (anti-Sm and anti-double-stranded DNA antibodies), generalized systemic sclerosis (anti-DNA topoisomerase I antibodies) and localized systemic sclerosis (anti-centromere antibodies) (1–3).

A cardinal feature of ANA is that their antigenic targets usually carry out critical intracellular functions such as DNA and RNA processing and modification, protein synthesis, and cell cycle regulation (4–6). It is also of great significance that the recognized epitopes are highly conserved in evolution and localized in domains of functional importance. As a consequence, autoantibodies have been shown to be capable of inhibiting antigen function both **in vitro** and **in vivo**(7). At the same time, the very nature of the autoantigens raises a number of significant questions regarding the ability of autoantibodies to interfere with **in vivo** functions in a manner relevant to disease pathogenesis. Indeed, given the subcellular localization of the autoantigens and the systemic nature of the diseases in question, pathogenic autoantibodies should be able to enter live cells in multiple tissues, reach the appropriate intracellular compartment and interfere with cell function.

The ability of polyclonal IgG anti-ribonucleoprotein (RNP) autoantibodies isolated from autoimmune patients to penetrate live human peripheral blood mononuclear cells via Fc receptors was first proposed 20 years ago (8). Ever since, an increasing number of autoantibodies of different antigenic specificity have been added to the list of intracellular antibodies (9–19). Yet, it should be noted that only a subset of anti-DNA antibodies has been characterized in some detail for their ability to translocate inside the cell nucleus (16).

It should be noted, however, that other investigators have disputed this phenomenon and, indeed, it has been argued that nuclear localization of autoantibody represented a fixation artifact, with movement of Ig into cells during fixation (20). In order to clarify this issue, we have investigated the scope and
Intracellular autoantibodies

Fig. 1.

Fig. 2
nature of intracellular autoantibodies. Here we describe two murine monoclonal autoantibodies with anti-Sm (IgA) and anti-La (IgG) primary antigenic reactivity respectively, which are capable of penetrating live cells. This represents the first description of monoclonal autoantibodies of such specificity with in vivo nuclear localization ability. Our data indicate that the phenomenon of cellular penetration by autoantibodies has a larger scope than previously suspected and that ANA may indeed contribute to the pathogenesis of autoimmune diseases. The studies also contribute preliminary information regarding the utilization of distinct mechanisms of cell entry and/or intracellular transport of autoantibodies.

Methods

Antibodies origin and purification methods

Anti-La and anti-Smith monoclonal autoantibodies were kindly provided by Dr Stephen Clark (University of North Carolina at Chapel Hill). They were originally isolated from MRL/lpr mice and have been described elsewhere (21–23). Antibodies from cell culture supernatants were precipitated with 47% w/v ammonium sulfate and dialyzed in high salt buffer containing 3 M NaCl/10 mM Na borate (pH 8.9). Antibodies of IgA isotypes were affinity-purified using an anti-mouse κ agarose column (Zymed, San Francisco, CA), whereas IgG autoantibodies were purified using Gammabind beads (Pharmacia, Piscataway, NJ) according to the manufacturer’s instructions. All purifications were done under high salt conditions in order to achieve antigen dissociation. IgA and IgG isotype controls were purchased from Sigma (St Louis, MO).

Cell lines

Three cell lines previously shown to be susceptible to antibody penetration were selected for analysis. They included H35, a rat hepatoma cell line (16), PK15, a pig kidney cell line (24), and A431 (25), a human epidermoid carcinoma cell line, which were purchased from ATCC (Rockville, MD). Cells were grown and maintained in 10% FCS in DMEM supplemented with 1 mM l-glutamine, Na pyruvate and non-essential amino acids (Gibco/BRL, Gaithersburg, MD).

ANA assays

Target cells were cultured to near confluence as described above. Cultures with at least 95% cell viability were used for ANA determination. Cells were washed 3 times with PBS (pH 7.4) and fixed with cold acetone/methanol at −20°C for 10 min. After an additional 2×PBS washes, the fixed cells were blocked with 5% normal goat serum (Jackson Immuno-Research, West

![Fig. 3. Internalization kinetics of monoclonal autoantibodies in H35 cells.](image)

*Fig. 3.* Internalization kinetics of monoclonal autoantibodies in H35 cells. (a) Import kinetics of mAb21 in H35 cells was studied using flow cytometry. In vivo nuclear localization assays were performed as in Fig. 1. Intracellular accumulation of mAb21 was detected with Alexa 488-conjugated secondary antibody and measured as mean fluorescence intensity. mAb 21 was detectable inside the cells within minutes of incubation and reached plateau within 1 h. In contrast, neither intracellular mAb 2-12 nor an IgG2a isotype control were detected. (b) Kinetics of nuclear import of mAb SBS was determined by direct measurement of nuclear fluorescence using a CCD camera. Nuclear accumulation of SBS was maximal after 30 min of incubation with live cells. Consistent with multiple other experiments, the IgA isotype control was not detected in the nucleus.

![Fig. 1. ANA and in vivo nuclear localization activities of anti-Sm and anti-La murine monoclonal autoantibodies.](image)

*Fig. 1.* ANA and in vivo nuclear localization activities of anti-Sm and anti-La murine monoclonal autoantibodies. ANA activity of Alexa 488-conjugated mAb and control polyclonal IgA were tested by conventional indirect immunofluorescence (upper panel). mAb were incubated with acetone/methanol-fixed H35 cells. Bound antibody was then labeled with Alexa 488-conjugated goat anti-mouse IgG (H + L). All three mAb, 21, SBS and 2-12, generated distinct ANA patterns but the IgA isotype control was seen only in the cell membrane and in the cytosol. The right panel shows results obtained with the in vivo nuclear localization assay (lower panel). In this assay only mAb 21 and SBS were detected inside the nucleus. Images were obtained by conventional fluorescence microscopy (×40).

![Fig. 2. Nuclear localization of mAb as shown by confocal microscopy.](image)

*Fig. 2.* Nuclear localization of mAb as shown by confocal microscopy. In vivo nuclear localization experiments were performed as in Fig. 1 with mAb 21, SBS and 2-12 as well as with a polyclonal IgA control antibody. The cell nucleus was labeled with propidium iodide 0.5 µg/ml. Samples were scanned individually under FITC (left panel) and propidium iodide settings (middle panel) using a BioRad MRC 600 confocal microscope (×100). The merged images (right panel) show that only mAb 21 and SBS, but not 2-12 or IgA isotype control co-localize with propidium iodide-stained material within the cell nucleus. The IgA isotype antibody control can be observed in the cytosol, presumably due to pIgR-mediated endocytosis, but not within the nucleus.
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Fig. 4. Cell lineage susceptibility to autoantibody penetration. Penetration of mAb 21 and 5B5 was demonstrated using cell lines of different tissue origins derived from several animal species. Both monoclonal autoantibodies were able to penetrate a human epidermoid carcinoma cell line (A431) and a pig kidney cell line (PK15) as determined by *in vivo* nuclear localization assays performed as previously described for H35 cells. Appropriate antibody controls (mAb 2-12, IgA and IgG2a isotype controls) did not enter the cells (not shown). Images were taken using a conventional fluorescence microscope (×40).

Fig. 5A.
were then carried out in the cold-room at temperatures ranging
between 0 and 4°C. Cell viability was monitored by Trypan blue
exclusion and was consistently >90% at the time of fixation.
The amount of internalized antibodies was measured as mean
fluorescence intensity using an Elite flow cytometer (Coulter,
Fullerton, CA). Nuclear localization kinetics of mAb SBS was
determined by indirect immunofluorescence as described
above for in vivo penetration assays. Nuclear fluorescence was
then quantified from digital images obtained with a Sony DXC-
9000 3CCD camera (Tokyo, Japan) using the Image-Pro Plus
software (Media Cybernetics, Silver Spring, MD). Six fields
were counted from each sample in at least three independent
experiments

Fluid-phase endocytosis experiments
Intracellular transport of mAb was compared to trafficking of
Texas Red–dextran. To do so, H35 cells were incubated with
antibodies at 30 µg/ml and Texas Red–dextran (70,000 kDa) at
1 mg/ml (Molecular Probes) in 1% BSA/DMEM for 30 min at
37°C. After 3 washes with PBS, cells were fixed with cold acet-
one/methanol and intracellular mAb were detected with Alexa
488-conjugated goat anti-mouse IgG (H + L) as described for
the penetration assay. Samples were scanned using a Leica
confocal microscope (Heerbrugg, Switzerland) to obtain
images of Alexa 488 and Texas red labeling respectively. Image
processing was performed with Adobe Photoshop software
(San Jose, CA). Both granules containing only mAb, and those
containing dextran and mAb together were counted. Results
were expressed as the fraction of total mAb granules that also
contained dextran. A final score was generated from counting
a minimum of three fields from three independent experiments.

Antibody sequence analysis
The DNA sequences of the mAb have been previously reported
and were obtained from GenBank (accession nos X67195,
L08989, L08980, U26999, L09013, U18595, S71116). The sequences were imported into the EditSeq pro-
gram of the DNASTAR Lasergene software (Madison, WI) and
analyzed for VH and VL germline homology, and the presence
and pattern of somatic mutations using the Megalign program
of DNASTAR and the Blastn search program available through
the National Center for Biotechnology Information. Analysis
of the predicted protein sequences was performed using the
Protean program of DNASTAR.

Fig. 5. Co-localization of intracellular autoantibodies and endocytic
vesicles. (a) Texas Red–dextran (1 mg/ml) and the corresponding
mAb were co-incubated with live H35 cells at 37°C for 30 min.
Intracellular antibody was labeled with Alexa 488-conjugated
secondary antibody as before. Samples were scanned using a Leica
confocal microscope (×100) under the appropriate settings in order
to detect Texas Red (right panel) or Alexa 488 (middle panel).
Individual images of the same field were merged to detect co-
localization of mAb and dextran (right panel). As compared to
dextran-mediated IgA control, only a small percentage of vesicles
containing mAb 21 or SBS also contained dextran. Quantification of
dextran-mediated vesicles containing both mAb and dextran. (b) Results
from the experiments shown in (5) are expressed as the percentage
of Alexa 488+ vesicles also containing Texas Red.
Results

In vivo penetration of H35 cells by mouse monoclonal autoantibodies

In order to explore the prevalence of the intracellular autoantibody phenomenon and given that the preponderance of previous studies had focused on anti-DNA antibodies, we decided to test a panel of disease-associated monoclonal autoantibodies whose primary specificity was other than DNA. Moreover, we wanted to determine whether this phenomenon is restricted to any particular isotype. Thus, four anti-Sm and one anti-La mAb derived from autoimmune MRL/lpr mice were selected for our in vivo penetration assay. These mAb were previously characterized and have been described elsewhere (21–23). In selecting these autoantibodies we were mindful of the fact that anti-RNP autoantibodies often display significant cross-reactivity with single-stranded DNA and/or double-stranded DNA. Therefore, we chose to study antibodies with different degrees of cross-reactivity with DNA. Furthermore, it has been suggested that pathogenic anti-DNA antibodies may predominantly cross-react with Sm/RNP-associated polypeptides A and D (24). Accordingly, we selected anti-Sm antibodies with different degrees of reactivity against either the D or the B/ B' polypeptides of the Sm antigen, which represent the main antigenic targets of the anti-Sm response. Relevant characteristics of these mAb including in vitro ANA pattern and in vivo penetration ability are summarized in Table 1.

As expected, all mAb showed in vitro ANA activity. As shown in Fig. 1, among those five mAb, only two mAb, 21 (anti-La, IgG2a) and 5B5 (anti-Sm, IgA), were detected in the nucleus of live H35 cells. In order to ensure that nuclear localization of mAb was not due to leakage of cell and nuclear membranes, cell viability was monitored by Trypan blue or propidium iodide exclusion before fixation in all experiments (data not shown). The in vivo nuclear accumulation of mAb was not affected by different fixatives including cross-linking reagents such as freshly prepared 4% paraformaldehyde or methanol-free 3% formaldehyde (data not shown). Significant similarities could be observed between the ANA patterns and the in vivo staining profiles. Thus, mAb21 created a nuclear rim pattern in both assays while a consistent speckled pattern was seen with 5B5. In addition to the nuclear localization, granules containing mAb 21 were also observed in the cytoplasm of most H35 cells and somewhat finer ones with mAb 5B5. Large cytoplasmic granules were also observed with the IgA isotype control antibody, but in the absence of nuclear staining. This observation might be explained by endocytosis of IgA via the polymeric IgA receptor (pIgR) that mediates transcytosis in hepatocytes (26–29).

Taken together, these results strongly suggest that the cell entry and nuclear localization of the mAb is Fc receptor independent (as demonstrated by lack of activity of other mAb of the same isotype and of polyclonal IgG and IgA antibodies). Moreover, the consistently positive results obtained with mAb 21 and 5B5 under different fixation protocols along with the negative controls just discussed provide further proof that this phenomenon is not the result of fixation artifacts.

The staining profile obtained with mAb21 suggested that this antibody might simply bind to the nuclear membrane without actually entering the nucleus. This issue was resolved by using laser scanning confocal microscopy to show that both mAb 21 and 5B5 did indeed co-localize with propidium iodide-stained material within the cell nucleus (Fig. 2). It should be appreciated, however, that within the nucleus, there was only partial overlap between the two probes, consistent with the recognition by the mAb of nuclear antigens other than or in addition to double-stranded DNA. This is not surprising in light of their primary antigenic reactivity against the Sm and La proteins respectively.

In vivo penetration kinetics

Both mAb 21 and 5B5, penetrated live cells in a time-dependent manner. Internalization kinetics of mAb 21 was determined by flow cytometry. Cell aliquots sampled at each time point were also observed under fluorescent microscopy to confirm nuclear localization of the antibody. In turn, mAb 5B5 presented a different challenge based on its IgA isotype. As previously shown, IgA antibodies are internalized by H35 cells presumably through the plgR and, therefore, mere cell penetration as indicated by flow cytometry would not be indicative of nuclear localization. Accordingly, 5B5 kinetics was determined by direct measurement of nuclear fluorescence intensity from the digital images obtained by a CCD camera. As shown in Fig. 3, mAb 21 and 5B5 entered live cells in a very rapid manner, and could be detected in the cell nucleus within 15 min of incubation. Penetration of mAb 21 reached a plateau between 30 and 60 min, while maximal nuclear staining with mAb 5B5 was seen within 30 min. Furthermore, the maximum nuclear accumulation of mAb 21 was significantly higher than that of mAb 5B5 when both antibodies were assayed under fluorescent microscopy (data not shown). These results may reflect differences between both mAb in terms of efficiency in cell penetration.
phenomenon is Fc receptor independent and is most likely
receptors in the cell lines tested as well as the lack of internaliz-
elsewhere (20). Also of importance, both the absence of Fc
membrane and/or the nuclear envelope as it has been suggested
control antibody. The scattering pattern of the cytoplasmic IgA
and 18.7% for mAb 5B5 and mAb 21 respectively). In contrast,
of vesicles contained both mAb and dextran together (27.4
cytic pathway. As shown in Fig. 5, only a relatively small fraction
is the formation of circulating immune complexes with in
enon (32). The most widely accepted pathogenic mechanism
autoantibodies in the systemic autoimmune diseases are still
Discussion
The actual role and mechanism(s) of action of anti-nuclear
autoantibodies in the systemic autoimmune diseases are still
questionable 50 years after the discovery of the ANA phenom-
enon (32). The most widely accepted pathogenic mechanism
formation of circulating immune complexes with inflam-
matory properties and indeed such mechanism has been well
documented with anti-DNA antibodies. Given the intranuclear
nature of most of the autoantigens recognized, it seems obvious
that the ability of autoantibodies to enter subcellular compart-
ments would create additional and tantalizing opportunities for
these molecules to generate functional perturbation and tissue
damage. This report sheds light on this poorly understood
aspect of the autoimmune process. Our results demonstrate
that, in addition to DNA, other major nuclear autoantigens such
as the Sm and La RNP can also be the target of intracellular
autoantibodies. First, it should be emphasized that this property
was observed in only a subset of the autoantibodies tested and
that it was independent of the type of fixatives used, either
acetone or cross-linking agents including 4% paraformal-
dehyde. This result indicates that our observations are not the
result of fixation artifacts with disruption of the plasma mem-
brane and/or the nuclear envelope as it has been suggested
elsewhere (20). Also of importance, both the absence of Fc
receptors in the cell lines tested as well as the lack of internaliz-
ation of isotype control antibodies strongly suggest that this
phenomenon is Fc receptor independent and is most likely
mediated by the antigen binding site of the antibodies tested.
Being a liver epithelial cell line, however, H35 cells do express
the plgR that normally mediates endocytosis and transcytosis
of both dimeric IgA and pentameric IgM. Yet, as demonstrated
by using a control polyclonal IgA antibody, internalization of
IgA through the plgR does not result in nuclear translocation,
thus reaffirming the specificity of this phenomenon for the IgA
mAb with anti-Sm reactivity (5B5).

Several factors related to the antigenic reactivity of mAb 21
and 5B5 might be responsible for their intracellular activity.
First, anti-Sm and anti-La antibodies may display significant
cross-reactivity with single-stranded and/or double-stranded
DNA, and, therefore, it is plausible that such cross-reactivity
might be ultimately responsible for cell penetration. In our
sample, only autoantibodies with cross-reactivity with double-
stranded DNA but not single-stranded DNA were able to enter
cells (see Table 1) suggesting that double-stranded DNA
binding may be important for antibody penetration. Secondly,
anti-Sm antibodies may react with one or more of the multiple
polypeptides that form the snRNP complex. The mAb tested in
this study were chosen to represent a spectrum of anti-Sm
reactivity with special emphasis on the main antigenic targets,
i.e. the B/B’ and/or D polypeptides (21). While mAb 5B5 reacts
only with the Sm-D polypeptide, other anti-Sm-D mAb failed to
penetrate live cells, as did mAb 1-12 which binds to both Sm-
B/B’ and -D polypeptides. Hence, on a superficial analysis
it would seem that the global polypeptide reactivity of these
autoantibodies is not essential, or at least not sufficient, for cell
entry. It is possible, however, that the intracellular properties
could be dependent on fine epitope specificity in which case
the broader reactivity against the full-length polypeptides might
not be informative enough (detailed epitope mapping of the
anti-Sm mAb is currently underway). Along similar lines,
the fine specificity of anti-La antibodies might also represent an
important factor in their ability to translocate into the cell nu-
ucleus. The demonstration that the ability of anti-La antibodies
to recognize different La–RNP complexes is dependent on their
fine specificity would appear to support this notion (33,34).
Alternatively, intracellular autoantibodies might need to cross-
react with surface-exposed antigens as it has been suggested
for some anti-DNA antibodies that recognize myosin I (35).
Although our antibodies did not recognize myosin I as deter-
dined by conventional ELISA (results not shown), other
possible targets include laminin, a main component of basal
membranes that is recognized by some pathogenic anti-La
antibodies capable of inducing congenital heart block (36).
Another potential scenario contemplates the possibility that the
intracellular antigens themselves could be expressed in the
cell membrane, and, indeed, both La and the snRNP have been
shown to translocate to the cell surface under diverse stimuli
including UV radiation, estrogen stimulation, viral infections
and exposure to tumor necrosis factor-α (37–41).

Whether the antigen responsible for autoantibody penetra-
tion turns out to be the nominal antigen (Sm or La) or a cross-
reactive antigen(s) such as DNA, our results suggest that the
recognized target must be a widely expressed molecule since
several cell lines including rat liver cells, pig kidney cells and
human epidermoid carcinoma cells were susceptible to anti-
body penetration. This finding is consistent with the observa-
tions of Vlahakos et al., that the in vivo pathogenic potential of
a subset of anti-double-stranded DNA antibodies administered to normal mice correlated with their ability to accumulate in the cell nucleus in multiple tissues (16), and it supports the notion that anti-Sm and anti-La autoantibodies could mediate systemic autoimmune tissue damage.

The mechanism responsible for intracellular transport of mAb remains largely unexplored and may not correspond to the general pathways of protein kinesis. Our results represent a significant departure from the observations reported with the subset of anti-DNA autoantibodies that cross-react with myosin which are internalized by receptor-mediated endocytosis (35). Thus, in our case nuclear accumulation was faster with significant nuclear staining observed within minutes. This feature is reminiscent of the rapid cell entry and nuclear translocation observed with the HIV Tat protein. This translocation property has been assigned to a region of the Tat protein centered on a cluster of basic amino acids and recent data have demonstrated that chemical coupling of this Tat-derived peptide to several proteins allowed their internalization into several cell lines or tissues (42). As with our mAb, the rapid uptake of Tat peptides appears to be temperature independent, suggesting that the internalization process does not follow conventional endocytosis rules.

Multiple studies have shown that the presence of basic residues in the antibody hypervariable regions, mostly arginine residues in the HCDR3, may be responsible for anti-DNA reactivity (43) and that cationization of otherwise irrelevant antibodies also appears to induce cellular uptake (44). Furthermore, these stretches of basic residues may closely resemble conventional nuclear localization signals (NLS) such as the one used by the SV-40 large T antigen (45,46). Close inspection of the published hypervariable region sequences of mAb 21 and 5B5 did not reveal a typical NLS. However, some distinct features of potential significance can be found in the HCDR3 of these antibodies (Fig. 6). Thus, both HCDR3s display an RR doublet at the 5′ end where the second arginine appears to have been generated by junctional diversity and presumably selected by antigen. In the case of mAb 21 another amino acid with positive charge potential (histidine) is located one residue away from the RR doublet and contributes to create a significantly positive charge in the HCDR3 of this antibody. In contrast, these features were absent in the non-penetrating antibodies whose HCDR3 global charge was significantly less positive, as the result in some cases of a higher content of acidic residues which may promote binding to the basic snRNP polypeptides (47).

In summary, in this paper we demonstrate that autoantibodies whose primary antigenic specificity is directed against the major RNP targets of systemic autoimmune diseases such as systemic lupus erythematosus and Sjögren’s syndrome can rapidly traverse the plasma membrane of live cells of different tissue origin and translocate into the nucleus. Although cross-reactivity with double-stranded DNA may play a role, the actual requirements for this phenomenon remain to be determined, and, indeed, previous results by other investigators and our own studies show that anti-DNA reactivity per se is not sufficient for intracellular activity. The rapid rate of internalization, the lack of significant co-localization with endocytic vesicles and the apparent temperature independence observed in our studies strongly point towards a mechanism different from conventional receptor-mediated endocytosis. Experiments aimed at the elucidation of these issues are currently underway in our laboratory.

The observations presented herein hold significant promise for the study of important biological functions such as intracellular and nucleocytoplasmic transport of proteins as well as for our understanding of systemic autoimmunity. Furthermore, once the requirements for in vivo intracellular activity of (auto)-antibodies are established, this information should prove invaluable for the design of antibodies as intracellular therapeutics and drug-delivery systems.

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Abbreviations

ANA anti-nuclear antibody assay
NLS nuclear localization signal
pIgR polymeric IgA receptor
RNP ribonucleoprotein

References

Intracellular autoantibodies


