**In Vivo ANA Is a Fixation Artifact: Nucleosome-Complexed Antinucleosome Autoantibodies Bind to the Cell Surface and Are Internalized**


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**ABSTRACT**

It has been suggested that binding of anti-double-stranded DNA antibodies to cell surfaces, followed by internalization and nuclear binding (so-called in vivo ANA) is of pathophysiological significance for tissue damage in systemic lupus erythematosus. We have shown before that pathogenic antinuclear antibodies complexed to nucleosomal antigens can bind to heparan sulfate in the glomerular basement membrane in vivo. Because nucleosomes are also reported to bind to the cell surface, we hypothesized that in vivo ANA is a property of antinuclear antibodies bound to nucleosomal antigens. Therefore, we studied three antinucleosome monoclonal antibodies (mAb) that exhibit in vivo ANA as seen by immunofluorescence in mice inoculated intraperitoneally with the hybridoma producing the mAb. The same mAb complexed to nucleosomal antigens after intravenous injection into mice induced in vivo ANA, in contrast to purified noncomplexed mAb. To study this in more detail, we incubated complexed mAb with various cell lines and found binding to the cell surface and subsequent internalization into cytoplasmic vesicles. However, no binding to the nucleus was observed by immunoelectron microscopy (IEM) and confocal laser microscopy. Noncomplexed mAb did not bind to the cell surface. Next, from mice bearing the hybridomas producing the mAb intraperitoneally, a small part of the kidney was snap frozen in liquid N2, fixed with acetone, and studied in immunofluorescence, whereas the remaining part of the kidney was fixed in vivo by renal perfusion with a mixture of 0.01 M sodium periodate, 0.075 M lysine HCl, 0.0375 M Na2HPO4, and 2% paraformaldehyde (PPL) and studied in both immunofluorescence and IEM. In the acetone-fixed kidney sections obtained without in vivo fixation we again observed in vivo ANA. However, after in vivo PPL perfusion fixation, no nuclear binding was found. In IEM, localization in cytoplasmic vesicles was seen. In conclusion, antinucleosome antibodies complexed to nucleosomal antigens can bind to the cell surface and are transported into the cytoplasm, but do not bind to the nucleus. The reported nuclear localization of antinuclear antibodies is caused by a fixation artifact.

Key Words: Systemic lupus erythematosus, antinuclear antibodies, monoclonal antinucleosome antibodies in vivo ANA

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Systemic lupus erythematosus (SLE) is an autoimmune disease that affects many organs in the body. Antinuclear autoantibodies are the hallmark of the disease, especially anti-double-stranded DNA (dsDNA) antibodies (1). These anti-dsDNA antibodies are thought to be responsible for most of the tissue damage in this disease, although the mechanisms by which these lesions are produced in the various organs is still a matter of debate. It has been proposed that anti-dsDNA antibodies are able to bind to cell surface-bound DNA (2) or, alternatively, directly via cross-reactivity with cell surface proteins (3). This binding may lead to cell damage, for instance enhancement of apoptosis of rat mesangial cells (4). Others mentioned that cell surface-bound anti-dsDNA antibodies were internalized and that these antibodies were subsequently transferred to the nucleus leading to derangement of cell functions (5,6). However, one of the first authors who described the binding of anti-dsDNA antibodies to cell surface proteins (7) later discovered that this binding was caused by the intermediary action of nucleosomes or DNA/
histone complexes bound to the antibodies (8). Others found that nucleosomes or DNA/histone complexes could bind to cell surfaces, probably via a DNA receptor inducing cytokine production (9). At this moment, it is still unclear whether the binding of nucleosome material to cell surfaces, alone or complexed to antibodies, is a charge-related phenomenon or mediated via a specific receptor. In favor of a charge-dependent interaction is our finding that antinucleosome antibodies complexed to nucleosomal antigens are able to bind to the glomerular basement membrane (GBM) in vivo, through an interaction with the negatively charged heparan sulfate in the GBM (10).

These antinucleosome antibodies are antibodies that bind to the nucleosome, but not to its constituents, histones and DNA. These antibodies have been described in murine (11–14) and human SLE (15,16), but also in other diseases (17,18). Their relevance for SLE was reviewed recently (19). When inoculated in naive mice hybridomas producing the antinucleosome monoclonal antibody (mAb) that showed the above-described GBM binding, we invariably observed in vivo nuclear binding of antinuclear antibodies (in vivo ANA) in kidney sections by immunofluorescence (IF). This nuclear staining can be frequently observed in sections of kidneys from patients with SLE or lupus mice, and is sometimes explained as a smearing artifact occurring during sectioning of the tissue. Others state that this nuclear staining is the result of an active process in vivo (5,6). In this study we tested the hypothesis that in vivo ANA is produced by antibodies complexed to nucleosomal antigens. In this context the complexed autoantibody binds to the cell surface via nucleosomal material and, after binding, the complex is internalized. We also analyzed whether this internalization ultimately leads to nuclear binding.

**MATERIALS AND METHODS**

Preparation and Characterization of Pure Noncomplexed Antibodies and Antibodies Complexed to Nucleosomal Material

Three mAb (Clones 2, 32, and 34) derived from lupus mice were used. By purification on a protein A-Sepharose column under physiological conditions (Procedure I), high salt conditions (Procedure II), or DNase treatment followed by high salt conditions (and in the case of mAb 34, additional separation of histone-bound mAb on a DNA-cellulose column) (Procedure III), antibodies complexed to nucleosomal material (Procedures I and II) or pure noncomplexed antibodies (Procedure III) were obtained as described before (10).

Purified and complexed antibodies were tested in the Farr assay and in ELISA (anti-dsDNA, antihistone, and antinucleosome) as described (10). Composition and antigen reactivities of the antibody preparations used have been published (10) and are summarized in Table 1.

mAb 2 and 32 are antinucleosome mAb, which in purified form (purification Procedure III) only recognize the intact nucleosome and not its components, histones or DNA. In pure form (Procedure III) mAb 34 also recognizes histones, but its reactivity with the intact nucleosome is much stronger. All three mAb only show DNA reactivity when they are complexed to nucleosomal antigens (purification Procedures I and II).

**In Vivo Studies**

Hybridoma Inoculation. Hybridoma cells producing the mAb (5 × 10⁶ cells per mouse) were inoculated intraperitoneally in 8-week-old BALB/c mice (N = 3 per mAb), which were primed 8 days before with pristane. As controls three mice were inoculated with pristane alone and with the hybridoma cells producing the mAb.

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**TABLE 1. Composition of bound complexes (Panel A), reactivity in Farr assay and various ELISA (Panel B) of the different mAb preparations obtained after purification under physiological conditions (I), high salt conditions (II), or DNase treatment followed by high salt conditions (and, in the case of mAb 34, additional separation of histone-bound mAb on a DNA-cellulose column) (III) (Reproduced with permission from J Clin Invest 1994;94:568–577)**

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a Histone content as assessed on SDS-PAGE; the intensity of the histone staining was scored semiquantitatively by comparing the intensity of the staining with that of the heavy and light chain.

b Presence of DNA fragments (of predominately 120 base pair size) as assessed after labeling with [35]P.

c Percentage precipitation of labeled DNA per 12 µg of IgG; values ≤3% are regarded as negative.

d Titer/mg IgG.
doma-producing WT1, a mouse immunoglobulin (Ig) G2a antihuman CD7 mAb. Two days after development of ascites the animals were euthanized, ascites and blood was collected in EDTA vials and stored at −20°C, the kidneys were flushed with phosphate-buffered saline (PBS) and snap frozen in liquid N₂.

In an experimental addition only a small part of the kidney was snap-frozen in liquid N₂ after flushing with PBS and used for IF. The remainder of the kidney was perfused during 10 min with a mixture of 0.01 M sodium periodate, 0.075 M lysine HCl, 0.0375 M Na₂HPO₄, and 2% paraformaldehyde (PLP). This was done to obtain in vivo fixation. Thereafter, the kidney was cut into small pieces (5 × 5×1 mm) and immersed in PLP for another 3 h. A small part of tissue was used for IF, whereas the remaining pieces were used for immunoelectron microscopy (IEM). To this end they were washed in PBS for 30 min, cryoprotected by immersion in 80% wt/vol sucrose in PBS for 1 h, and snap frozen in liquid N₂.

**Intravenous Injection.** Complexed (Procedure I or II) and noncomplexed mAb (Procedure III), 1 mg Ig in 500 µL PBS, were injected intravenously in 6- to 8-week-old BALB/c mice. After 5 or 45 min kidneys were flushed with PBS, taken out, and snap frozen in liquid N₂. At 5 min three mice injected with noncomplexed and three mice injected with complexed mAb were studied (mAb 32 and 34). At 45 min three mice injected with noncomplexed mAb and seven mice injected with complexed mAb were studied (mAb 2, 32, and 34). In addition two mice were injected with a control IgG2a mAb (WT1).

**Immunohistology of Kidney Tissue**

Direct IF was performed on 2-µm cryostat sections. Sections were fixed for 5 min in cold acetone and air dried. Next, sections were incubated for 1 h at room temperature with Fab2 fluorescein isothiocyanate (FITC)-labeled sheep anti-mouse IgG (Cappel, Organon Teknika NV, Turnhout, Belgium) diluted 1:750 in PBS/1% wt/vol BSA, and 4% normal rat serum. After washing, the sections were embedded in Agar (BDH Ltd., Poole, England) and examined with a Zeiss fluorescence microscope.

Double labeling with 4,6-diamidino-2-phenylindole (DAPI, Sigma, St Louis, MO) was performed by diluting DAPI (1 µg/mL) in Agar in 1% BSA. For IEM, 20-µm sections were incubated with a peroxidase-labeled rabbit antitoxin Ig diluted 1:20 (Dakopatt, Copenhagen, Denmark) and washed three times with PBS. Subsequently, the sections were rehydrated in diaminobenzidine medium (0.05% diaminobenzidine wt/vol in Tris) for 10 to 15 min, followed by diaminobenzidine medium with addition of H₂O₂ to a final concentration of 0.05% vol/vol. The sections were washed in distilled water, postfixed in 0.1 M phosphate-buffered 1% wt/vol OsO₄ for 30 min at room temperature, dehydrated, and embedded in Epon 812. Thin sections were prepared on a LKB ultratome and examined unstained in an electron microscope (Jeol 1200 EX2, Jeol Europe BV).

**Binding of Complexed and Noncomplexed mAb to Surfaces of Cells in Culture**

For these experiments three cell lines were used. CEM and PEER are human T cell lines, that are Fc receptor negative. HL60 is a human myeloid cell line, which bears Fc receptors I and II. Cells were cultured in RPMI 1640, dutch modification (ICN, Costa Mesa, CA), supplemented with 2 mM glutamine and 1 mM pyruvate containing gentamycin (40 µg/mL) and 5% (vol/vol) fetal calf serum (FCS) (Life Technologies, Gaithersburg, MD). Only cultures less than 3 weeks old with a viability of >95%, as assessed with trypan blue, were used. Cells in suspension (100 µL, 5 × 10⁶ cells/mL in PBS/1%BSA) were incubated with complexed mAb, noncomplexed mAb, or an irrelevant mouse IgG2a mAb (100 µL, 200 µg/mL in PBS) for 30 min at 4°C. After washing twice with PBS/1%BSA they were incubated with FITC-conjugated Fab₂ sheep antimouse IgG (100 µL, diluted 1:300 in PBS/1%BSA) (Cappel, Organon Teknika) for 30 min at 4°C. After washing the cells twice again with PBS/1%BSA the fluorescence intensity was determined with a Fluorescence Activated Cell Sorter (Coulter Electronics Ltd., Luton, UK).

To analyze whether charged molecules could influence the cellular binding, complexed antibodies, mAb WT1 (mouse IgG2a antihuman CD7) and mAb 1210 (mouse IgG2a antihuman class II) were incubated with PEER cells in the presence of heparin 3 µg/mL, and subsequently analyzed by Fluorescence Activated Cell Sorter as described above.

**Internalization and Ultrastructural Intracellular Localization of Bound mAb**

PEER cells in suspension (1300 µL, 5 × 10⁶ cells/mL) were incubated with pure noncomplexed or complexed mAb for 30 min, 2, 5, or 18 h in RPMI containing 5% FCS at 37°C. After incubation the cells were fixed in 2% paraformaldehyde (PF)/0.1 M phosphate buffer (PB), pH 7.4, for 1 h at room temperature. The cell suspension was divided in two parts. One part was used for IF and the other part of the suspension for IEM. In addition a pulse-chase experiment was performed. Complexed mAb was incubated for 30 min at 4°C with cells (1300 µL, 5 × 10⁶ cells/mL) in RPMI containing 5% FCS at 37°C. After incubation the cells were fixed in 2% paraformaldehyde (PF)/0.1 M phosphate buffer (PB), pH 7.4, for 1 h at room temperature. The cell suspension was divided in two parts. One part was used for IF and the other part of the suspension for IEM. In addition a pulse-chase experiment was performed. Complexed mAb was incubated for 30 min at 4°C with cells (1300 µL, 5 × 10⁶ cells/mL) in RPMI containing 10% FCS. Cells were washed three times in PBS containing 1% BSA to remove unbound mAb. Next cells were incubated in RPMI containing 10% FCS at 37°C and studied by confocal laser microscopy at different time points (5 min, 30 min, 2.5, and 18 h).

**Immunofluorescence.** The cells were spun down in O.C.T. Tissue-Tek compound (Miles, Elkhart, IN) and snap frozen in liquid N₂. Six-micrometer sections were cut in a cryostat (Micron) at −20°C, picked up on silan-coated slides at −20°C, and air dried. After a brief rinse in PBS/0.05% Tween (PBST), the sections were rinsed with three changes of PBS. They were incubated for 1 h in a moist chamber at room temperature with goat antitoxin FITC (Cappel, Organon Teknika) diluted 1:80 in PBST. Then the slides were rinsed with three changes of PBST for 10 min each and mounted with Mowiol (Hoechst, Frankfurt am Main, Germany). The sections were viewed with a Zeiss fluorescence microscope.

**Immunoelectron Microscopy.** The cells were spun down in 10% gelatin/0.1 M PB, pH 7.4, and postfixed in 2% PF/0.1 M PB overnight at 4°C. The next day they were cut in small blocks, infiltrated with 2.3 M sucrose, placed on copper stubs, and snap frozen in liquid N₂. Ultrathin cryosections (80 nm) were cut with a cryoultramicrotome (Leitz Ultracut-FCS), picked up with 2.3 M sucrose, and placed on formvar-coated copper grids. After blocking the nonspecific protein binding with PBS/0.15% glycine/0.1% BSA/0.1% gelatin, pH 7.4 (PBSG), for 30 min the sections were incubated overnight at 4°C in a moist chamber with rabbit-antimouse IgG (Cappel) 1:500 PBSG. The next day the grids were rinsed twice with PBS and postfixed with 1% osmium tetroxide solution for 1 h at room temperature. The sections were then dehydrated and embedded in Epon 812. Thin sections were prepared on a LKB ultratome and examined unstained in an electron microscope (Jeol 1200 EX2, Jeol Europe BV).
three times with PBSG and incubated with protein-A gold 10 nm/1% BSA/PBS for 1 h at room temperature. After rinsing again three times in 0.1 M PBS and 3 times in milli-H2O the grids were placed on drops of a methylcellulose/uranylacetate (9:1) solution for 10 min on ice and picked up with stainless steel loops. Excess methylcellulose was removed by filter paper and the grids were air dried. The sections were viewed with a JEOL 1010 electron microscope.

Confocal Laser Microscopy. After fixing with 1% PF/0.1 M PB the cells were spread on silan-coated slides and air dried. The cells were permeabilized with 0.5% saponine/0.1 M PB for 30 min and incubated as described for the immunofluorescence technique. After mounting with Mowiol/2.5% NaN3 for 30 mm and incubated as described for the immunofluorescence technique. After mounting with Mowiol!2.5% NaN3, the cells were viewed with the confocal laser microscope (MRC 600, Biorad, Hemel Hempstead Herts, UK).

RESULTS

In Vivo ANA after Intraperitoneal Hybridoma Inoculation in Mice

BALB/c mice (N = 3 per clone) were injected intraperitoneal 1 with 5 × 10⁶ hybridoma cells. In ascites and blood samples from mice inoculated with antinucleosome-producing hybridoma cells anti-HS and anti-DNA reactivity was detected in contrast to control mice carrying the hybridoma producing the antihuman CD7 mAb WT1 (data not shown). Previously, we had shown that these antinucleosome mAbs only display anti-HS or anti-DNA reactivity if they are complexed to nucleosomes (10). In kidney sections of mice bearing either of the three hybridomas intraperitoneally a consistent in vivo ANA was observed in IF (Figure 1). DAPI double labeling revealed that all nuclei were ANA positive (data not shown). As we have shown previously with in vivo renal perfusion of nucleosome-complexed antinucleosomal mAb (10), we observed also glomerular binding in this experimental setup (Figure 1). Mice inoculated with pristane alone or the control mAb WT1 showed no nuclear binding and only limited glomerular mesangial deposition of IgG2a (data not shown).

In Vivo ANA of Complexed and Noncomplexed Antibodies after Intravenous Injection

To evaluate whether this nuclear binding was, as we hypothesized, due to antibodies complexed to nucleosomes, we injected complexed antibodies intravenously in mice. At 5 min again binding to glomerular capillary loops and some in vivo ANA was found by IF (Figure 2A), whereas after intravenous injection of pure noncomplexed mAb, no glomerular binding and a very faint in vivo ANA was observed (Figure 2B). When kidneys were studied 45 min after intravenous injection of complexed antinucleosomal mAbs the antibodies were found in the cell nuclei by IF, whereas some glomerular binding remained (Figure 2C). Findings in all three tested antinucleosome mAbs were essentially the same, except for complexed mAb 2, which did not show glomerular binding but only in vivo ANA after 45 min. Double labeling with DAPI revealed that the nuclei of all cells were positive. When purified noncomplexed mAbs were injected, only a very faint staining of nuclei, as shown in Figure 2B, was observed after 45 min. So, nuclear binding was a property of nucleosome-complexed antibodies. Injection of control mAb WT1 only led to some mesangial binding, neither nuclear nor glomerular binding was observed.

Binding of Complexed and Noncomplexed mAb to Surfaces of Cultured Cells.

To analyze the course of events leading to in vivo ANA, we turned to binding studies with well defined cell lines. Noncomplexed antinucleosome mAb showed hardly any binding to the cell surface of CEM, PEER, or HL60 cells (Table 2). However, when the antibodies were complexed to nucleosomes a marked binding was observed. In most cases binding of complexed antibodies containing less DNA (obtained by Procedure II) was higher than that of complexes of the same mAb, obtained by Procedure I, containing considerably more DNA. Cell surface binding was seen with all three cell lines studied, irrespective of the presence of Fc receptors, because CEM and PEER are Fc receptor negative. Fluorescence intensity observed after incubation of control IgG2a mAb in the same concentration did not exceed background levels (Table 2) except for mAb WT1, which was positive on CEM and PEER cells, because these cells express the CD7 antigen recognized by WT1.

To analyze whether charged molecules are important for the cellular binding, complexed antibodies were incubated with PEER cells in the presence of heparin. Heparin blocked this binding, whereas the binding of the positive control (mAb WT1, antihuman CD7), or the negative control (mAb 1210, antihuman class II) was not affected (Table 3).
Internalization after Binding of Complexed Antinucleosome mAb to Cultured Cells

To study whether cell surface binding of antibodies complexed to nucleosomes leads to nuclear binding, complexed mAb were incubated with cells. Internalization of antibodies was found after 2 h, increasing with longer incubation times (5 and 18 h). Antibodies were localized in cytoplasmic vesicles (Figure 3). In the pulse-chase experiment after initial binding to the cell surface (Figure 4A), the antibody rapidly appeared in the cytoplasm (Figure 4B). After 18 h of incubation capping of residual mAb was observed. In none of these experiments, repeated several times, nuclear binding of internalized mAb in living cells was seen.

In both sets of experiments viability of the cells was not affected by internalization of mAb as assessed by Fluorescence Activated Cell Sorter analysis.

Effects of Fixation on Localization of mAb in Vivo

Because, in the previous experiments, where no nuclear binding was observed, fixation was carried out with paraformaldehyde, we questioned whether the in vivo ANA was related to the fixation technique. To this end we analyzed in a separate experiment the effect of the different fixation procedures carried out on the same kidney. After intraperitoneal administration of the hybridoma in mice, kidney tissue was fixed in parallel either by freezing in liquid N2, subsequent cutting of sections and acetone fixation, or by initial in vivo fixation with PLP and subsequent processing of tissue. After cryopreservation and fixation with acetone in IF in vivo ANA was observed identical with that shown in Figure 1. After fixation of the remaining part of the same kidney by perfusion with and immersion in PLP, no nuclear binding was found in this material by IF (data not shown), whereas by IEM the mAb were localized in cytoplasmic vesicles (Figure 5), comparable with the localization found in the in vitro experiments with living cells with confocal laser microscopy.

DISCUSSION

In vivo ANA (in vivo binding of autoantibodies to the cell nucleus) as examined by IF has been described by numerous authors, in tissue sections of both patients with SLE (20–22) or other autoimmune diseases (23–25). Recently, it was reported that certain anti-dsDNA mAb injected into experimental animals reproducibly showed intranuclear localization, whereas others were deposited extracellularly within glomeruli and/or blood vessels (5). This intranuclear localization of anti-dsDNA mAb was a time- and ATP-dependent phenomenon. Before internalization binding to a cell surface receptor was demonstrated (6). It was purified mAb are injected at 5 (B: original magnification, ×400) and 45 min no glomerular binding and very faint in vivo ANA are observed.
TABLE 2. Binding of complexed and noncomplexed mAb to cell surfaces of CEM, PEER, and HL60 cells

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\(^a\) The binding is expressed as mean fluorescence intensity. The baseline negative control without mAb is set at 1, and all other measurements are adjusted accordingly. Data are derived from a representative experiment.

\(^b\) For description of purification procedures see Table I.

\(^c\) Histone content as assessed on SDS-PAGE.

\(^d\) Presence of DNA fragments as assessed after labeling with \(^32\)P (see text).

\(^e\) WT1 is an IgG2a mouse mAb toward human CD7, which is expressed on CEM and PEER cells, but not on HL60.

\(^f\) 1210 is an IgG2a mouse mAb against human HLA class II, which is not expressed on CEM and PEER. ND, not determined.

TABLE 3. Binding of complexed mAb to cell surfaces of PEER cells with and without heparin

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\(^g\) WT1, antihuman CD7 (present on PEER cells).

\(^h\) 1210, antihuman HLA class II (not present on PEER cells).

claimed that this in vivo nuclear binding had major pathological consequences like decreased cell proliferation, increased protein synthesis (26), glomerular hypercellularity and proteinuria (5). We also observed nuclear binding when hybridomas producing antinucleosome mAb were inoculated intraperitoneally in mice. Because in the ascites of these mice nuclear material is released from dying hybridoma cells, immune complexes are formed in vivo (27), which was confirmed by the finding of anti-HS and anti-dsDNA reactivity in the ascites and the circulation of these mice. So, this in vivo ANA might be a feature of antinucleosome antibodies complexed to nuclear antigens. Indeed, when we injected complexed antinucleosome antibodies intravenously into mice, strong in vivo ANA was observed after 45 min. After 5 min GBM binding was found which was similar to what we observed after renal perfusion of these complexed antibodies (10). We found that nuclear binding of mAb was not restricted to the kidney, but was also found in two other organs that we analyzed (liver and skin). In theory, the differences we found between complexed and noncomplexed mAb could be attributed to differences in clearance kinetics. This is unlikely, however.
Mechanism of in vivo ANA

Figure 4. Incubation of antibodies (mAb 32) complexed to nucleosomal material with lymphoid cells. By confocal laser microscopy in a pulse-chase experiment after initial binding to the cell surface (A: original magnification, x1500), the antibody rapidly appears in the cytoplasm (insert; B: original magnification, x3000). Most importantly these studies did not reveal any nuclear binding in living cells.

because the complexed antibodies are cleared more rapidly from the circulation via Fc and complement receptors than noncomplexed antibodies. So, if a difference in clearance will affect the results, it would rather decrease the nuclear appearance of complexed antibodies.

Our observations provide evidence that in vivo ANA is a feature of antibodies bound to nucleosomal material. Some of the discrepancies in the literature on this subject might therefore be explained by impurities of the antibody preparations containing variable amounts of nucleosomal antigens. This nucleosomal material can easily be copurified with antinuclear antibodies (10) and be missed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) when only Coomassie staining is used. Before in vivo ANA can develop, autoantibodies have to bind to the cell surface and have to be transported through the cytoplasm to the nucleus. Binding of anti-dsDNA mAb to the cell surface has been described before (28) and was attributed to the binding to the so-called "lupus associated membrane protein" (7). Soon, it was shown that this binding was not a direct cross-reactivity of the mAb with lupus associated membrane protein, but that it occurred via histones and DNA (8,29). These findings were challenged by a more recent paper describing again that anti-dsDNA mAb could directly bind to cell surfaces (3). However, one of the mAb utilized in this latter study (PME 77) was used by Jacob et al. (8) to identify that binding occurred via histones and DNA. In line with our observations (10), PME 77 shows only weak reactivity with DNA and strong reactivity with nucleosomes (8). So this mAb resembles the antinucleosome mAb used in this study.

We found only very weak binding of pure noncomplexed mAb to cell surfaces whereas the complexed antibodies bound considerably stronger. For our experiments we only used fresh cell cultures with a viability of >95%. This is important because cells in culture can be a rich source for nucleosomal material
released in the supernatant which may mediate subsequent binding of purified antibodies. Using cell cultures with a poor viability, we found considerable binding of purified noncomplexed mAb (data not shown).

The binding of antibodies to cell surfaces *via* nucleosomes is in line with findings of others, showing binding of nucleosomes to cell surfaces, maybe *via* a DNA receptor (9). It is also possible that binding of complexed antibodies is a charge-related phenomenon. This view is supported by our finding that complexed antibodies harboring less DNA bind better to the negatively charged cell surface and by the fact that heparin, which is strongly negatively charged, could block the binding completely. Recently, we postulated that it is possible that binding of purified antibodies noncomplexed antibodies is prevented by acetone fixation does not prevent migration of antibodies. This view is supported by our finding that complexed antibodies are able to bind to the GBM (30,31). Also for this binding, we postulated that it is due to charge. The positively charged histones in the nucleosome are thought to interact with the negatively charged heparan sulfate in the GBM.

When we incubated complexed antibodies with cells we found cytoplasmic localization of mAb, but did not observe any nuclear binding despite 18 h of incubation. Therefore, we returned to the *in vitro* experiments and found that in the same kidney nuclear binding can be detected when the tissue is processed conventionally for IF, whereas no nuclear localization is found when in *vitro* PLP fixation is used. By IEM, localization in cytoplasmic vesicles was observed, resembling the picture found with the confocal laser microscopy in the *in vitro* experiments. This suggests that when tissue is processed for conventional IF the procedure leads to disruption of intracellular structures, which allows antibodies present within the cytoplasm to diffuse and to bind to the cell nucleus. Apparently this occurs after cutting of the sections before the acetone fixation. Another possibility is that acetone fixation does not prevent migration of antibodies after the fixation procedure. This nuclear localization of mAb is prevented by PLP fixation. In line with this observation is that in previous studies describing the in *vitro* ANA conventional IF techniques were used, with acetone fixation (20,23,32,33). Of course, *in vitro* perfusion fixation with PLP is not feasible for human biopsies. We did not find reports about *in vitro* ANA after PLP immersion fixation. Therefore, whether in *vitro* ANA as found in SLE patients is due to *in vitro* binding of autoantibodies to the nucleus or to a fixation-related artifact, is not clear yet, although our studies suggest that the latter explanation is true.

A similar phenomenon has been described for the perinuclear binding of antineutrophil cytoplasmic autoantibodies (p-ANCA) due to antibodies directed against myeloperoxidase (34). Nevertheless, one has to be cautious to extrapolate findings from *in vitro* analysis of human cell lines to binding observed *in vitro* in mouse kidney cells. Very interestingly, however, it was recently reported that nucleosomes are able to bind to the cell surface of mouse mesangial cells *in vitro* (35), providing a link between the two experimental setups we describe.

Furthermore, one should realize that most previous studies analyzing nuclear penetration of autoantibodies used anti-dsDNA (6,36) and not antinucleosome antibodies. Therefore, it can not be excluded that the discrepancy between our and these studies is due to the difference in antibody specificity. Nonetheless, our study raises serious doubts whether *in vitro* ANA exists. It is claimed that *in vitro* nuclear binding of autoantibodies can lead to tissue damage (5). The fact that *in vitro* ANA is often encountered in noninflamed tissue (33) may be an argument that in these instances nuclear binding is a consequence of the fixation process.

Although our results indicate that *in vitro* ANA is an artifact, we want to stress that the phenomenon of *in vitro* ANA is a characteristic of pathogenic nephritogenic antinuclear autoantibodies complexed to nucleosomal antigens. The same complexed autoantibodies are able to bind to the GBM *in vivo* and activate complement (10) and are related to onset (37) and exacerbations (38) of nephritis in SLE patients. A similar suggestion has been put forward by Ohnishi and colleagues, who suggested that pathogenic anti-dsDNA antibodies are capable both of binding to nuclei *in vivo* and to renal basement membranes via DNA/histone (39). Furthermore, cellular binding or intracytoplasmic uptake of these complexed autoantibodies may be responsible for the derangement of cellular functions as observed by others like interference with apoptosis (4,19), cellular proliferation (5,26), protein synthesis (5), or proteinuria (26). Finally, our studies indicate that the analysis of *in vitro* ANA is only valid if the tissue is fixed properly.

NOTE ADDED IN PROOF

During the revision of this article, an article was published that described the same observation, namely, endocytosis of nucleosome-antinucleosome complexes by Green monkey fibroblasts. This endocytosis was mediated by putative DNA (180 kd) and nucleosome (50 kd) receptors (Eur J Immunol 1996; 26:472–486).

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REFERENCES

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