Penetration of Autoantibodies Into Living Cells, 2000

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Abstract

The formerly prevalent concept that intact autoantibodies could not penetrate into viable cells has been defeated by a large amount of experimental findings and clinical observations that indicate otherwise. The penetration of autoantibodies into living cells seems to participate in the pathogenesis of diverse autoimmune diseases, but it may also play a physiological role in healthy individuals. Although the fine mechanisms of the phenomenom remain to be elucidated, the potential use of penetrating autoantibodies as vectors to deliver molecules into cells, with diverse therapeutic purposes, has gained growing interest during the last few years.

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Not until the first definitive description that a human immunoglobin G autoantibody to nuclear ribonucleoprotein could enter into viable human lymphocytes and react with its antigen within the nucleus [1], a long-standing dogma held that antibodies could react with their respective antigens exclusively in the extracellular compartment. This idea seemed abstruse by itself, for it limited the setting of the humoral immune response to less than one-third of the total body water space. Although autoantibodies directed to intracellular antigens, found in the serum of patients with different autoimmune diseases, were considered as important and useful diagnostic tools, they were never attributed a direct pathogenetic role other than their participation in immune complex-mediated injury. Should immune complex disease be the only potential immunopathogenic mechanism for autoantibodies, one would expect all autoimmune diseases to be non-organ specific and bear a very limited clinical spectrum.

Following the initial demonstration that autoantibodies could enter into cells, a growing number of papers dealing with the penetration of many other antibodies into a large number of animal and human cells have confirmed the phenomenon and provided evidence that the interactions of autoantibodies with intracellular antigens may affect intracellular functions which, in turn, might explain physiopathological and clinical features of autoimmune diseases. A review of such reports was presented at the 4th Dresden Symposium on Autoantibodies held in Dresden in October 1998 [2].

The aim of this paper is to comment on selected recent publications describing antibodies that have been shown to penetrate into living cells, their possible physiopathologic role, and their potential use as therapeutic agents to deliver proteins, drugs, radiation or genes into the cell.

Effects of antibody penetration

It had already been shown that anti-U1snRNP antibodies of the IgG class can penetrate into subsets of human T lymphocytes, induce an arrest of the cycle in the G0/G1 phases, and ultimately trigger active cell death [3,4]. Antibodies to double stranded DNA that penetrate into human lymphocytes induce both – an abnormal activation pathway, as determined by the expression of several activation antigens, as well as apoptosis of a large fraction of penetrated cells [5,6]. Antibodies directed to dsDNA are also capable of causing podocyte fusion after penetrating glomerular renal cells [7], and anti-ribosomal P-protein antibodies result in decreased synthesis of apolipoprotein B and cholesterol accumulation after penetrating hepatocytes [8].

Penetration of autoantibodies into neural cells has been documented in several instances. Antibodies to the Hu nuclear antigen, present in the sera of some patients with small cell lung cancer, have been shown to penetrate in central nervous system cells and possibly participate in the pathophysiology of the paraneoplastic neuropathy of such patients [2]; antibodies to recoverin, a 23 kDa retinal protein, can penetrate photoreceptor and bipolar cells of the retina and induce apoptosis which, in turn, might explain the retinal cell damage and visual loss, without evidence of local inflammatory phenomena, observed in patients with these antibodies [9]; and antibodies to dsDNA have also been shown to penetrate into rat primary cortical neurons, either alone or serving as carriers for other proteins [10]. Antibodies to hsp27, which are found in patients with glaucoma, have been shown to penetrate into human retinal neuronal cells and induce their active death, most likely by inactivating the ability of hsp27 to stabilize actin cytoskeleton [11], thus suggesting a pathogenetic role of these antibodies. Patients with demyelinating IgM monoclonal neuropathy bear serum antibodies to myelin-associated-glycoprotein and to sulfated glucuronosyl glycolipids, which are capable of penetrating into the myelinated fibers and endoneurial space [12,13]. Furthermore, it has been suggested that autoantibodies to

Ig = immunoglobulin
dsDNA = double stranded DNA

several nervous system intracellular antigens, elicited by cell damage caused by environmental chemicals, may play a key role in the progression of neurodegenerative diseases [14].

Several findings support the fact that antibody penetration takes place *in vivo* as well. These include, among others: the finding of intranuclear immunoglobulins in viable epidermal cells and lymphocytes from patients with mixed connective tissue disease with high titers of serum antibodies to RNP; proteinuria following penetration of glomerular cells by anti-dsDNA antibodies in rodents; localization of anti-Ro/SSa antibodies in heart cells of neonates with complete heart block born to women with high serum titers of such antibodies [2]; diminution of the cytosolic-mitochondrial phosphorylation potential of ATP in myocardial cells after penetration of antibodies to the ADP/ATP carrier [15]; localization of anti-nucleolar antibodies in nucleoli of kidney and liver cells in the mercury-induced systemic disease of mice of the H-2s genotype [16]; and fetal loss or underdevelopment in pregnant mice injected with different human antinuclear antibodies [Garza-Elizondo et al., manuscript in preparation].

Perhaps the most interesting effect of antibody penetration is the induction of active cell death. Firstly, it appears that the induction of apoptosis by penetrating antibodies results from different, though not mutually exclusive mechanisms; and additionally, the pathophysiological role of active cell death induced by antibody penetration appears to be manifold. Regarding the underlying mechanisms, it has been proved that human lymphocytes express exhuberant amounts of CD95/Fas after penetration of anti-dsDNA antibodies and mitogen-driven activation [4,6], strongly suggesting that the Fas/Fas ligand interaction is involved; however, when resting lymhocytes are exposed to exactly the same anti-dsDNA antibodies, the cells undergo apoptosis without expression of cell surface CD95 [17]. Antibodies to hsp27 have been shown to induce apoptosis of penetrated cells apparently through the interference of such antibodies with the capability of hsp27 to stabilize the cytoskeleton structure [11], and intracellular antibodies to cistein aspartate protease-3 (caspase-3), the "executioner" of programmed cell death, have been shown to induce self-activation of caspase-3 moieties, which results in irreversible cell death [18]. These findings are consonant with the idea that once inside the cells, autoantibodies could directly trigger other pro-apoptotic pathways depending on their antigen specificity [2]. Preliminary experimental data from our laboratories suggest that some penetrating apoptogenic anti-dsDNA antibodies might recognize specific nucleotide sequences in DNA [Garcés-Eisele and Ruiz-Argüelles, manuscript in preparation]; hence, the direct interaction of autoantibodies with DNA itself may not be ruled out as an additional pro-apoptotic mechanism.

The physiological or pathogenetic role of apoptosis induced by penetration of autoantibodies also appears to be manifold. As mentioned above, active death of cells of the nervous system caused by penetration of antibodies to the Hu antigen, recoverin, hsp27, MAG, SGGLs and others, contributes to the immunopathogenesis and clinical features of the diseases where these antibodies are present. Similarly, antibodies to the Ro/SSa antigen and to the ADP/ATP carrier that penetrate into heart cells participate as late effectors of the pathophysiology of heart block and myocarditis, respectively. However, autoantibodies that are capable of inducing apoptosis may also be pathogenetic at an earlier stage through triggering autoimmune phenomena. Although it is generally accepted that the production of autoantibodies is not antigen driven, recent data suggest that in some cases, as for instance in the autoimmune response to the ribosomal P-protein [19], the activation of autoreactive clones might be elicited by autoantigens. In this context, it has been postulated that apoptosis might represent a mechanism of self-antigen presentation, since it is known that apoptotic blebs, as residuals of the programmed cell death, contain clusters of antigens that have been implicated in the generation of autoimmune phenomena [20]. In fact, immunization of normal mice with large amounts of apoptotic bodies leads to the production of hypergammaglobulinemia as well as antibodies to DNA and cardiolipin [21]. Furthermore, defective phagocytosis of apoptotic cells has been proposed as an additional mechanism to explain the induction of autoantibodies in systemic autoimmune disease [22].

We have advanced the possibility that apoptosis due to penetration of naturally occurring anti-dsDNA antibodies might indeed have a physiological role in the edition of the immune repertoire, through the elimination of autoreactive cells [3]. This possibility is supported by the fact that mitogen-activated immature lymphoid cells are more prone to penetration and apoptosis than their mature counterparts [4], and by the observation that the simultaneous injection of penetrating anti-dsDNA antibodies and small peptidic antigens into irradiated Balb/c mice renders them unresponsive to booster injections of the same, but not to unrelated antigens [Ruiz-Argüelles et al, manuscript in preparation]. These findings suggest that when immature cells become activated, either by antigens or by unspecific mitogens, they become liable to penetration of anti-dsDNA antibodies and commit apoptosis. In the physiological setting, it is possible that when fetal autoreactive lymphoid cells become exposed to and activated by autoantigens, they might be eliminated by penetrating naturally occurring anti-dsDNA antibodies. This mechanism might contribute to maintaining self-tolerance and by no means excludes the previously proposed ones.

Molecular basis of antibody penetration

The amino acidic sequence of several anti-dsDNA antibodies that are capable of entering cells has been established. Studies performed in antibodies derived from MRL-lpr/lpr mice show the presence of nuclear localization-like motifs in their heavy chain CDR3 regions and, although no consensus sequence has

MAG = myelin-associated glycoprotein
SGGLs = sulfated glucoronolyl glycolipids

been found, 3/3 nuclear localizing antibodies share a tertiary structure that is not present in non-nuclear localizing anti-dsDNA antibodies [2]. Similarly, penetrating anti-dsDNA monoclonal antibodies derived from naive NZB x NZW_{F1} mice display a large homology at the amino acidic sequences of their heavy chain CDR2 and CDR3 regions, despite the participation of a variety of germline VH gene families [2]. Synthetic peptides containing the CDR2 and CDR3 sequences of anti-dsDNA antibodies that are capable of translocating into the cell nucleus are themselves capable of entering into viable cells and translocating to the nucleus, either alone or as vectors to internalize other molecules. However, similar peptides constructed to mimic the sequence of non-penetrating antibodies are unable to enter the cell and translocate to the nucleus [23]. When comparing the amino acidic sequences of peptides derived from penetrating and non-penetrating anti-dsDNA antibodies, it can be presumed that the sequences involved in dsDNA recognition are located in the CDR2 region, while those enabling the antibody to enter cells and translocate to the nucleus reside in both the CDR2 and CDR3 regions. All three antibodies whose CDR2/CDR3 regions are shown below were derived from NZB x NZW_{F1} mice and exhibit anti-dsDNA reactivity, however antibodies F4.1 and J20.8 are capable of transgressing the cell membrane, localizing in the nucleus and inducing active cell death, while antibody F14.6 is not.

VAYYISRGGVSTYYSDTVKGRFTRQKYNKRA F4.1

AYISRGGGIFYYQDISKGRFTREKYGKRGMDY J20.8

AISRGGVYSYYLDVKRTARATWDWFAY F14.6

The analysis of VH gene families and cross-reactivity patterns of these antibodies indicate that they correspond to germline gene-encoded products not affected by mutational or recombinational events leading to specificity maturation [24]. Therefore it seems that naturally occurring, rather than pathogenetic anti-dsDNA antibodies, display the highest ability to penetrate into living cells, reach the nucleus and trigger apoptosis, thus stressing the importance of the possible physiological role of antibody penetration in immunobiology.

The mechanisms by which antibodies transgress the cell membrane, travel through the cytoplasm and reach their antigens, either in the nucleus or in other organelles, are still very obscure. Many of the proposed pathways involve the expression of the specific or a cross-reactive antigen at the cell surface level, while others implicate receptors for the Fc fragments of the antibody molecules [1,2,25,26]. Concerning the trans-cytoplasmic transit of antibodies, a large variety of mechanisms have been proposed and, although they are not mutually exclusive, no general agreement has been reached. While some investigators have reported that antibodies are internalized in clathrin-associated vesicles and later released by pH or hypotonic lysis of the pinosome [2,27,28], others have not confirmed the participation of pinocytic vesicles in the phenomenon, but instead have proposed different mechanisms of free transit of antibodies through the cytoplasmic space with the aid of other molecules, such as myosin 1 acting as a chaperone or anti-Ro/La autoantibodies as facilitators of nuclear import of other autoantibodies [24,29,30]. We ourselves have observed that only some anti-dsDNA antibody molecules co-localize with clathrin as assessed by immunoelectronmicroscopy, while others are found in the cytoplasm in no association with clathrin [Martinez-Villalpando et al., unpublished observations]. Whether this finding represents different stages of the trans-cytoplasmic transit of anti-dsDNA antibodies to the nucleus, or indicates that there might be several pathways for antibodies to traverse the cytoplasmic compartment, still remains to be determined.

Preliminary data from our own experience suggest that some carriers of the ABC pump family, or similar molecular carriers, might be involved in antibody internalization and transport, since several calcium-channel blockers provoke intracellular accumulation of anti-dsDNA antibodies. Interestingly, although Ca++ mobilization into the cell is a very early event in antibody penetration, internalization does not seem to be dependent on such a phenomenon [Ruiz-Argüelles et al., unpublished observations].

Potential therapeutic applications

More than two years ago it was shown that some penetrating anti-dsDNA antibodies, their Fab fragments and small peptides containing their CDR2/CDR3 heavy chain regions, were able to translocate covalently linked haptens or macromolecules into the cell nucelus [2,23]. At the same time, it was proved that a single heavy chain antibody, derived from another anti-dsDNA antibody, termed 3E10, obtained by transfection of COS-7 cells with a cDNA constructed by splice overlap polymerase chain reaction, was also able to transport large molecular weight antibody-enzyme complexes into the nuclei of intact cells [2,31]. Since then, the potential use of antibodies as vectors to translocate other molecules into cells with therapeutic purposes has rapidly expanded.

Although the direct cytotoxic effect of antibodies to tumor-associated antigens (e.g., carcinoembrionic antigen) could by itself contribute to the control of tumor growth [32,33], and thus explain the better clinical outcome of patients affected by colon cancer with high serum titers of anti-CEA antibodies [34], most therapeutic approaches are intended to use antibodies as carriers to deliver enzymes, drugs, radiation or genes into the cell.

An antibody-enzyme delivery system has been built by covalently coupling the anti-dsDNA monoclonal antibody 3E10 with catalase. The antibody conjugate has been demonstrated to localize in the cytoplasm and nucleus of rat cortical neurons and retain catalase activity. Cells thus transfected become resistant to hydrogen peroxide, in contrast to their non-transfected counterparts that are highly sensitive to peroxide. The system

CEA = carcinoembryonic antigen

might provide an approach for selective reconstitution therapy in hereditary enzyme deficiencies [10].

A most interesting example of a drug delivery system is that of the conjugate of an anti-HER2 mAb and geldanamycin. HER2 is a membrane receptor that is abundantly expressed in invasive breast carcinoma cells, and commercially available antibodies to HER2 have been used in the past with quite poor response rates. HER2 expression/activity can also be inhibited by the antibiotic geldanamycin, however this drug is not widely used clinically because of its severe toxic effects. A conjugate of an internalizing anti-HER2 mAb with geldanamycin has been constructed and proved to inhibit HER2 expression and tumor proliferation dramatically. A control conjugate built with a non-penetrating anti-HER2 mAb did not exert such effects, indicating that penetration of the conjugate is essential. Analysis of individual cells showed that cells into which the conjugate penetrates are the ones that exhibit reduced levels of HER2 [35,36].

Hydrophilic sensitizers appear to be most suitable for photodynamic therapy of tumors, but since they cannot readily transgress the cell membrane their effectiveness is rather poor. Coupling of the hydrophilic porphyrin derivative TrisMPyP-PhiCO_2H to the internalizing mAbs U36 and 425 yielded effective *in vitro* phototoxic agents, while conjugates constructed with the same sensitizers and the non-penetrating mAb E48 did not. *In vivo* biodistribution studies in tumor-bearing nude mice have shown that conjugates with sensitizer:-mAb molar ratios from 1:1 to 3:1 are selectively concentrated in the tumors, suggesting that hydrophilic photosensitizers might have therapeutic use when targeted into malignant cells by internalizing antibodies [37].

The potential use of anti-CEA antibodies in cancer immunotherapy has led to the bioengineering of several molecules that differ in molecular size, affinity, valency, specificity and tumor-penetrating capabilities [38]. Some of these antibodies have been labelled with isotopes to be used in cell-directed radiotherapy. Analysis of the anti-tumor efficacy of the internalizing mAb CO17-1A labelled with Auger electron emitters such as ^{125}I or $^{(111)}$In have shown that such conjugates display a higher *in vivo* antitumor activity than those prepared from non-internalizing anti-CEA antibodies or those constructed with conventional beta emitters (^{131}I or ^{90}Y). Besides the better therapeutic results of the Auger electron emitters, they have proved to be less toxic to the bone marrow. It is believed that Auger electron emitters display less toxicity because of the short path length of their low energy electrons, which can reach the nuclear DNA only when the antibody is internalized. Selective delivery of such radiometals by penetrating anti-CEA antibodies into tumor cells, while not into hemopoietic stem cells, seems to be a very promising approach [39].

Genetically engineered single chain anti-CEA antibodies have been successfully used in radioimmunoguided surgery: the radiolabeled antibody (^{125}I-MFE-23-his) is injected intravenously in cancer-bearing patients before they undergo surgery, and a hand-held gamma-detecting probe is used to locate the tumor and define tumor limits in the operative field. The rapid blood clearance and good tumor penetration of single chain antibodies offer potential advantages over larger antibody molecules that had been used for radioimmunoguided surgery in the past. Additionally, the short necessary interval between injection and operation, the absence of significant side effects and the relatively simple production of the antibody make radiolabeled MFE-23-his suitable for this type of procedure [40].

Concerning gene delivery, it has been shown that two monoclonal anti-dsDNA antibodies derived from naive NZB x NZW_{F1} mice, F4.1 and J20.8 (*vide supra*), when conjugated to polylysine tails can transfer genes in the presence of serum. A peptide of 30 amino acidic residues, corresponding to the joined heavy chain CDR2 and CDR3 regions of mAb F4.1 and carrying 19 lysine residues at its aminoterminal end, has been found to be an efficient vector for the transfection of the luciferase gene into different cell lines. These findings indicate that the addition of a polylysine tail is able to turn a spontaneously cell-penetrating peptide into a potent plasmid vector [23,41].

Another example of succesful *in vitro* gene transfer is the ability of mAb G250, directed to a tumor-associated antigen termed G250TAA which is present in most human renal cell carcinoma cells lines, to internalize a plasmid cDNA encoding for mouse interleukin-2. Human carcinoma cells that are penetrated by such conjugate become sustained producers of mouse IL-2 during 5–15 days, while cells exposed to the unconjugated plasmid DNA, which is not internalized, fail to produce the cytokine. The data suggest that internalization of mAb G250 could become the basis of gene transfer to renal carcinoma cells as a novel cancer gene therapy approach [42].

Recently published efforts to facilitate the rapid identification and selection of either murine monoclonal or tumor-specific human antibodies from phage libraries, which are capable of penetrating into living cells and hence useful for targeting intracellular antigens with therapeutic purposes, strongly suggest that a wide and new variety of applications of internalizing antibodies can be expected in the near future [43].

Concluding remarks

Antibody penetration into living cells, initially described as a novel mechanism of immunologically mediated damage, seems to have many other pathogenetic and physiological effects as well. Several clinical manifestations of autoimmune diseases may be attributed to selective cell damage due to antibody penetration. Morevover, antibody penetration could be respon-

mAb = monoclonal antibodies

IL = interleukin

sible for the induction of apoptosis of certain cells whose residuals, under specific circunstances, might participate in the induction of autoimmune phenomena.

The fact that naturally occurring anti-dsDNA antibodies may penetrate activated immature lymphoid cells, and result in their active cell death, supports the notion that antibody penetration into living cells may play a role in the maintenance of self-tolerance, by participating in the edition of the immune repertoire.

The capacity of several autoantibodies to enter into living cells and react with their respective intracellular antigens, skeptically questioned for many years, is now envisioned as an exploitable therapeutic resource. Antibody molecules or bioengineered derivatives have been succesfully used to deliver enzymes, drugs, isotopes and genes into the cellular compartment in order to selectively target cells bearing certain antigens without harming neighboring tissues.

It is foreseeable that in the years to come we will have unraveled the mechanisms that antibodies use to transgress the cell membranes and transit through the cytoplasm. We will have a better understanding of the physiological and pathogenetic roles of the phenomenon, and will be able to implement new therapeutic approaches taking advantage of antibody penetration for the benefit of human patients.

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