ABSTRACT
An hypothesis is presented in which the natural physiologic process of apoptosis may sometimes be linked to cell penetration by autoantibody. Apoptotic blebs as residuals of the programmed cell death process have recently been demonstrated to contain clusters of disparate autoantigens which have previously been implicated in the generation of autoimmune reactions. In parallel, there now exists a mounting body of evidence that certain autoantibody molecules are capable of living cell penetration and even nuclear membrane ingress. We suggest that these two processes, apoptosis and cell penetration by autoantibody, may be associated with one another in certain circumstances.

Hypothesis
Apoptosis and cell penetration by autoantibody may represent naturally linked events in the same cycle. A series of recent observations by many different workers have suggested that the cellular debris which are presented on the residual outpouchings of the apoptotic cell could account for the process of diverse intracellular antigens being presented to the immune system and subsequently stimulating the production of autoimmunity. In parallel it has also been suggested that certain autoantibodies are somehow capable of penetrating living cells or even subcellular barriers such as nuclear membranes and, in some cases, of initiating cell death and apoptosis. One of the problems in attempting to analyze whether indeed such processes of apoptosis and cell penetration by antibody are actually different stages of the same linked process is that currently our insight into which comes first - antibody cell penetration or apoptosis - is not yet clear. This review will attempt to provide information currently available and then let the reader decide for himself. Specific mechanisms actually involved in the generation of what is looked on as an autoimmune response have been examined by a number of workers and are the subject of several major hypotheses concerning how autoantibodies are produced and how such antibodies escape the usual presumed scrutiny and discipline of tolerance mechanisms (1-4). Recent reports by Casciola-Rosen, Anhalt and Rosen (5) have presented an interesting hypothesis which perhaps can explain how a number of diverse molecules such as DNA, histones, phospholipids and various cytoplasmic antigens cluster together extended on the cell surface during the terminal phases of the complex process of apoptosis (6-9). The basic hypothesis, simply stated, is that seemingly diverse and apparently unrelated structures such as DNA and phospholipid moieties suddenly find themselves as closely packed bedfellows in the cell surface during the final phase of apoptotic regurgitations outward to the cell margins before complete dissolution of the dying cell. Such an hypothesis may provide a convenient and rational explanation for the simultaneous generation of an extremely diverse immune response and ultimately autoantibodies to antigens on DNA, phospholipid moieties, histones, components of nucleosomes and other former intracellular structures during the course of active systemic lupus erythematosus (SLE). One of the major theoretical problems involved in accepting the “apoptotic belch” of cell contents as a universal truth to help explain immune system antigenic presentation of such diverse intracellular autologous structures as DNA, phospholipids and histones is that it may be difficult to document similar antigenic presentation of all antigens which react with lupus or other disease-related autoantibodies - including various conformational determinants on IgG (in the case of rheumatoid factors), thyroglobulin, thyroid gland cell peroxidase, erythrocyte membrane antigens (including the I, i system) and autoantibodies reacting with platelet components. There are a number of autoantigens which thus far are unaccounted for, or at least have not yet been definitely identified within the apoptotic ladder of DNA fragments or associated with the complex process during which various apoptosis-related cleaving enzymes called caspases appear to be very active in breaking down cell constituents. Even through apoptosis provides a unifying concept for how diverse structures within the cell may present themselves to the immune sys-
tem, it still does not adequately explain how, in parallel, such a process can account for loss of tolerance to these many self components.

Cell penetration by antibody - the other process which could initiate apoptosis

An inverse, contrasting process to apoptosis may also eventually be linked to the intracellular events leading up to apoptosis or modifying its cycle in some as yet undetected way. The actual spectrum of signals, which normally initiates apoptosis, is still being defined. One conceivably could relate to cell penetration by whole antibody molecules. The first report that whole antibody molecules were actually capable of directly entering living cells was published more than 20 years ago by Alarcón-Segovia et al. in 1978 (10). Subsequently this group along with a number of other workers made similar confirmatory observations, which have accelerated and become more abundant over the subsequent twenty years (11-29). Our own laboratory with the work of Okudaira (18) showed that monoclonal anti-DNA antibody could enter living cells and in concurrent studies that polyclonal antibodies from SLE patients could enter lymphocytes. Recently this subject was the focus of a special “conference within a conference” at the Fifth International Conference on Systemic Lupus Erythematosus held at Cancún on April 20-24, 1998 (30).

The phenomenon of whole antibody molecules actually penetrating not only the outer membranes of cells but their cytoplasmic interstices and eventually their nuclear membranes is now coming into its own - having been confirmed and extended by a number of other groups (19-29). However, apparently there are still a substantial number of workers who regard this subject with disdain or consider it fringe science - somewhat akin to acupuncture, melatonin, or the megavitamin hysteria. A number of additional new bits of information have recently been added to the antibody cell penetration story. Initially, Alarcón-Segovia and coworkers felt that the cell entry must be accomplished by engagement of antibody IgG molecules by cell surface Fc-receptors (10). However, Yanase, Madaio and coworkers have recently defined a distinct cell surface myosin-I molecule as being directly involved with cell membrane penetration and ultimate delivery into the living cell (28). Moreover, the same phenomenon of cell penetration has now also been described and studied by Reichlin and coworkers (25, 29), particularly as associated with anti-DNA antibody with anti-Sm(D) cross reactivity (31). Initial observations of intracellular events by this latter group appear to indicate that intracytoplasmic residence of the internalized IgG antibody coincides with the rather abrupt cessation of protein synthesis and yet the cells do not perish. Some 24 to 48 hours later, whole antibody molecules inside the cell have disappeared or are undetectable and protein synthesis recommences. One could wonder at this point whether cell penetration by antibody could initiate a sort of general cellular dyspepsia, culminating in a final apoptotic event? As a matter of record, it is fascinating to note that more than two decades ago Alarcón-Segovia and coworkers reported that anti-ribonucleoprotein IgG was capable of penetrating Ty lymphocytes and thereby abrogating their presumed suppressor function (12).

Several other examples of cell penetration by antibodies of other unique specificities have also now been described. One of the most interesting are the reports by Adamus and coworkers of retinal rod cells being entered and ultimately dying after penetration by IgG antibody to recoverin, the retinal protein implicated in cancer-associated retinopathy (CAR) (34, 35). In this example, antibody with anti-recoverin specificity from a human patient with small cell lung carcinoma and CAR was instilled into the ocular posterior chamber of an experimental animal. Serial studies of the events thereafter indicated that the IgG anti-recoverin antibody entered the retinal rod cells and subsequently was associated with the apoptotic death of these same cells with permanent blindness of the eye thereafter (36). Clear evidence for apoptosis of rod cell nuclear DNA was evident in this experimental model. This finding represents the first demonstration that specific antibody cell penetration can induce apoptosis of the target cells directly. A similar disease mechanism is presumed to be operative in naturally occurring CAR in the case of some patients with small cell carcinoma. Thus, in this particular dramatic example, the autoantibody with anti-recoverin specificity was directly responsible for apoptosis and target cell death.

There are also several other fascinating new observations related to antibody cell penetration. The first is that there appear to be unique or distinctive V-region antibody primary amino acid sequence motifs, which may actually regulate or be important for the ability of the antibody to cross the outer cell membrane. Madaio and coworkers studied the primary amino acid sequences of the V-region complementarity-determining regions (CDRs) in a panel of monoclonal anti-DNA antibodies demonstrated to be capable of cell penetration and found that a basic sequence motifwithin the heavy chain complementarity region (CDR3) of VSRKRPRPRA was somehow linked to this biological activity (37, 38). This observation was of great interest since it indicated that portions of the anti-DNA antibody intimately involved in making up the antibody combining site, namely the CDR3 of the heavy chain, were directly involved in intracellular localization. Along the same lines Buttin and coworkers from the Pasteur Institute reported that small peptides synthesized from the third complementarity-determining region of the V-region heavy chain sequence of cell penetrating monoclonal anti-DNA antibodies were themselves capable of cell and eventual nuclear membrane penetration (39). However, contrary to the anti-recoverin story, cell penetration by anti-DNA antibodies or peptides from the latter was not uniformly followed by cell death or apoptosis.

This entire concept has now also been ingeniously adapted by Weisbart and coworkers (40) as a possible method to transport molecules of interest inside the cell and, of course, eventually to the nuclear chromatin. These workers demonstrated that a very large molecule such as alkaline phosphatase (245 Kd) linked to the recombinant Fv portion of 3D10,
a mouse monoclonal anti-DNA known to be capable of cell penetration (27), was transported piggy-back with the monoclonal IgG antibody across the nuclear membrane and still stayed attached to the antibody to which it had been linked. Since the antibody or even the recombinant Fv portion of the antibody can deliver molecules directly to the chromatin structures, this mechanism also conceivably might provide an alternative pathway for either gene therapy or even chemotherapeutic molecules directed at tumors.

The investigative work in the general area of cell penetration by various autoantibodies is now only in its infancy. It seems possible that other connective tissue disorders associated with autoantibody production such as C-ANCA in Wegener’s granulomatosis may also somehow be involved in similar phenomena. In Wegener’s granulomatosis, Csernok and coworkers demonstrated an overexpression of the autoantigen proteinase-3 (PR-3) on the cell surface of neutrophils (PMNL) when PMNL from Wegener’s patients were compared to healthy controls (41). In this study, intracytoplasmic IgG antibodies were observed in PMNL from Wegener’s patients. Along a similar line, Gilligan and coworkers (42) showed that apoptosis of unprimed PMNL may also be associated with the translocation of cytoplasmic granules to the cell surface and alignment just beneath the intact cell membrane. By FACS analysis, they demonstrated reactivity of anti-PR3 positive sera with apoptotic but not with viable PMNL. Although these observations do not directly support cell penetration by anti-PR3 antibody, they do emphasize that apoptotic cells may be involved in the antigen presentation of neutrophil granule antigenic constituents to the immune system.

One of the most critical aspects of apoptosis and autoantigen presentation to the immune system certainly relates to how self antigens exposed within cell apoptotic blebs can escape built-in indigenous tolerance. One interesting mechanism recently suggested by Utz and Anderson (43) is that a host of self components may be cleaved by caspases or cysteine proteases with aspartic acid substrate specificity and that novel epitopes uncovered by such proteolytic cleavage might thereby be exposed. In such cases, no tolerance to these previously buried determinants would be present and an immune response would follow. In addition, the very process of apoptosis may be associated with the phosphorylation of various proteins such as serine arginine-rich splicing factors known to be contained in certain autoantigens such as the U1 snRNP autoantigen complex. Moreover, phosphorylation has also been shown to affect the immunogenicity of some proteins and these alterations as a product of apoptosis could produce new antigens to which tolerance has never been established.

Thus various post-translational protein modifications engendered by the apoptotic process could bypass tolerance and initiate an autoimmune response. Additional evidence for post-translational modifications of various autologous proteins has also recently been presented by Schellekens and coworkers (44), who demonstrated that autoantibodies in the sera of patients with rheumatoid arthritis (RA) reacted with synthetic peptides containing the unusual amino acid, citrulline, with a post-translationally modified arginine residue. Moreover, affinity purified antibodies were positive in the immunofluorescence-based anti-perinuclear factor assay.

The presence of autoantibodies in RA sera which react with citrulline-containing epitopes might suggest that deimination of arginine residues to produce citrulline could represent a post-translational alteration inducing a neoantigenic determinant capable of stimulating an immune response. The enzyme which modified arginine to citrulline is peptidylarginine, which is normally expressed in a wide range of tissues and cell types (45, 46). However, activation of these enzymes capable of the deimination of arginines to citrulline has not been specifically demonstrated to be linked to apoptosis and more work on this subject needs to be completed.

The apoptotic process itself has been demonstrated to be dysregulated or abnormal primarily in SLE, as evidenced by elevated levels of soluble Fas in some SLE patients’ sera as well as increased expression of bel-2 (47). Abnormal apoptosis in SLE could contribute to tolerance of T cells to self antigens since it could allow the persistence of auto-reactive T cells (48). It is not yet clear whether cell penetration by autoantibodies such as anti-DNA could also somehow distort the normal apoptotic process.

Thus one may be able to begin to look at the process of immune processing and generation of autoantigens by cells as a product of apoptosis and possibly also linked to cell penetration by autoantibodies. Which process comes first and which initiates which is still not clear because cell penetration by antibody is not always followed by cell death. Clearly one could envisage a scenario in which the presence in plasma of autoantibodies of various specificities and bearing various key sequence motifs essential to cell penetration in their VH CDRs capable of cell membrane and subsequent nuclear membrane penetration, could enter the cell and shortly thereafter initiate various degrees of cellular indigestion or what a rheumatologist might term “intracellular fibromyalgia”. In some susceptible cell populations this could then lead to the induction of apoptosis through the activation of caspases and other enzymes, leading eventually to apoptotic extrusion of a number of diverse autoantigens and presentation of the latter to antigen presenting cells of the immune system. The various steps involved in apoptosis are illustrated in Figure 1.

How much of the basic engine driving autoimmune reactivity could be relegated to such an apoptosis-antibody cell penetration concept now remains to be determined. Moreover, it raises another interesting question as to whether cell penetration by autoantibody could actually represent a natural function driving the apoptotic process. In this respect it has long been apparent that there exist in normal mice and in many normal humans a number of so-called natural autoantibodies (50, 51). These have been extensively documented and studied in detail by Avreamas, Coutinho and coworkers (51, 52). Could it be that this general pool or spectrum of natural autoantibodies might in some circumstances also be capable of initiating cell death.
Fig. 1. Schema proposed by Korsmeyer (52) to indicate various checkpoints, key elements and phases, which are involved in apoptosis. The pro-apoptotic BAX pro-death family member is known to have the capability of forming ion conductive pores. In situations where cell penetration by antibody somehow might activate BAX, the inevitable progression to cell death follows. Exactly how cell penetration by antibody could initiate apoptosis is not known but intranuclear penetration by antibody could possibly activate the endonuclease implicated in cell death (point of no return), or interfere with DNA repair pathways and initiate the death cascade. Activation of the caspase enzymatic systems is shown to the lower right. An alternative pathway to the point of no return is shown at the lower left.

as a natural phenomenon? Presently, of course, there is no direct experimental data to support such a notion. Much remains to be done and re-examined in this general area and we all look forward to a final balanced synthetic assessment.

References
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HYPOTHESIS


