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Annexin V Delays Apoptosis While Exerting an External Constraint Preventing the Release of CD4⁺ and PrPc⁺ Membrane Particles in a Human T Lymphocyte Model¹

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Phosphatidylserine exposure in the exoplasmic leaflet of the plasma membrane is one of the early hallmarks of cells undergoing apoptosis. The shedding of membrane particles carrying Ags testifying to their tissue origin is another characteristic feature. Annexin V, a protein of as yet unknown specific physiologic function, presents a high Ca²⁺-dependent affinity for phosphatidylserine and forms two-dimensional arrays at the membrane surface. In this study, we report the delaying action of annexin V on apoptosis in the CEM human T cell line expressing CD4 and the normal cellular prion protein (PrPc), two Ags of particular relevance to cell degeneration and with different attachments to the membrane. The effect of annexin V was additive to that of z-Val-Ala-Asp-fluoromethyl ketone, a potent caspase inhibitor. Annexin V significantly reduced the degree of proteolytic activation of caspase-3, and totally blocked the release of CD4⁺ and PrPc⁺ membrane particles. z-Val-Ala-Asp-fluoromethyl ketone was a more powerful antagonist of caspase-3 processing, but prevented the shedding of CD4⁺ vesicles only partially and had no effect on that of PrPc⁺ ones. These results suggest that an external membrane constraint, such as that exerted by annexin V, has important consequences on the course of programmed cell death and on the dissemination of particular Ags. In vivo, annexin V had a significant protective effect against spleen weight loss in mice treated by an alkylating agent previously shown to induce lymphocyte apoptosis. The Journal of Immunology, 1999, 162: 5712–5718.

Programmed cell death, apoptosis, is a fundamental process involved in the maintenance of homeostasis in multicellular organisms (1, 2). Early transverse redistribution of plasma membrane phosphatidylserine is one of the well-documented hallmarks of cells undergoing apoptosis (3, 4) and has been shown to depend on caspase-3 (CPP32) protease activity (5, 6). In nonstimulated cells, this lipid is almost entirely sequestered in the cytoplasmic leaflet (7, 8). The membrane remodeling occurring in stimulated cells includes the shedding of particles carrying Ags and exposed phosphatidylserine in the exoplasmic leaflet (9, 10). Once accessible, this anionic aminophospholipid enables the assembly of the enzyme complexes of the blood coagulation cascade and becomes a determinant for phagocyte recognition of cells and derived membrane fragments to be rapidly cleared (3, 11–13).

Owing to a strong affinity for phosphatidylserine (14), annexin V is now widely used for probing cell stimulation or death (4, 10, 15–17). Depending on local Ca²⁺ conditions and on the proportion of available phosphatidylserine, annexin V has been shown to form two-dimensional arrays on membranes (18–20) in an essentially interfacial interaction (21, 22). This probably explains its ability to counteract plasma membrane vesiculation when present during the whole course of platelet activation, but it does not prevent phosphatidylserine transmembrane migration (23). Although it is abundantly present at the surface of cells fulfilling a barrier function such as trophoblasts or placental endothelial cells, where it could exert an anticoagulant potential (24, 25), its genuine physiologic role remains to be unequivocally established (26).

The above considerations prompted us to assess the effect of annexin V on the execution of induced cell death programs in human CEM T cells. This cell line was selected because of its ability to express CD4 and cellular prion protein (PrPc) (27), two constitutive membrane Ags of particular significance with respective participation in the onset of cell degeneration in AIDS (2, 28) and prion diseases (29). In addition, CD4 is an integral membrane protein while PrPc is linked to the lipid bilayer by a glycosylphosphatidylinositol (GPI) anchor (29, 30). Annexin V was indeed observed to interfere in apoptosis with a direct impact on membrane features, and an indirect modulating effect on the cytoplasmatic caspase activation cascade, with possible repercussions in vivo.

Materials and Methods

Reagents

Cell culture reagents were obtained from Bioproducts (Gagny, France). FCS was obtained from Life Technologies (Paisley, U.K.). Actinomycin D, etoposide (VP-16), propidium iodide, type I-A RNase A, FITC, and human serum albumin were obtained from Sigma (St. Louis, MO). HN-2 was obtained from Laboratoires Delagranges (Paris, France). Natural annexin V was kindly provided by Dr. Jean-Marie Freyssinet, Institut d’Hématologie et d’Immunologie, Faculté de Médecine, Université Louis Pasteur, Strasbourg, France; ¹ Institut National de la Santé et de la Recherche Médicale, Unité 143, Hôpital de Bicêtre, Le Kremlin-Bicêtre; and ² Laboratoire de Biochimie, Hôpital Lariboisière, Paris, France.

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Abbreviations used in this paper: PrPc, cellular prion protein; GPI, glycosylphosphatidylinositol; VP-16, etoposide; z-VAD.fmk, z-Val-Ala-Asp-fluoromethyl ketone; DEVD-CHO, Asp-Glu-Val-Ala-Asp-aldehyde.

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was purified from human placenta, characterized according to a previous report (21), and conjugated with FITC (annexin VFITC) as already described (16). Recombinant annexin V from Bender MedSystems (Vienna, Austria) was also used in preliminary experiments and yielded identical results as the natural counterpart. mAb to CPP32 (caspase-3) was purchased from Transduction Laboratories (Lexington, KY). Biotinylated mAb to CD4, goat anti-mouse IgG, HRP-conjugated secondary Ab, and irrelevant biotinylated IgG1 were obtained from Leinco Technologies (Ballwin, MO). Biotinylated mAb to human PrPc (3F4) was obtained from Senetek (Maryland Heights, MO). z-Val-Ala-Asp-fluoromethyl ketone (z-VAD.fmk) was obtained from Calbiochem (La Jolla, CA). Human blood coagulation factors were the same as those used in a recent study reported by our group (10). The caspase-3 cellular activity assay kit was obtained from Biomol Research Laboratories (Plymouth Meeting, PA).

Cell culture and induction of apoptosis

Human lymphoid CEM T cells were cultured in X-vivo 15 medium (Bio-products, Gagny, France) under standard conditions. Human HL-60 promyelocytic leukemia cells (CCL-240; American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS. Cell counts were determined using a hemocytometer. The cell viability was checked by trypan blue exclusion. Cells were seeded at 5 × 10³ cells/ml in the presence or absence of actinomycin D (0.3 µg/ml) or VP-16 (5 µM) for 18 h. In some experiments, cells and released microparticles were examined separately after centrifugation at 12,000 × g for 30 s.

Animals and treatments by HN-2 and annexin V

Male BALB/c (Institut Français de la Fièvre Aftuse Centre de Recherche et d’Élevage des Oncins, Les Arbresles, France), 6 wk old, were used in these experiments. They were housed under conventional conditions and provided with standard feed and water. HN-2 was diluted in sterile 0.15 M NaCl shortly before use and injected i.p. at 6 mg/kg. Annexin V (50 µg) was also diluted in sterile 0.15 M NaCl and injected i.v. immediately after HN-2 injection. At 24 h following the injection, mice were sacrificed and their spleen were aseptically excised, weighed, and put in cold RPMI 1640. Single-cell suspensions were prepared by teasing the spleens with the plunger of a 2-ml syringe. Red cells were eliminated by incubating the cells for 1 min at 4°C in a solution referred to as ACK (NH₄Cl 0.83%, 10 mM Tris buffer, pH 7.4). After two washing steps, the pellets were resuspended in RPMI 1640. Cell number and viability were determined by counting trypan blue excluding cells using a Neubauer hemocytometer.

Flow cytometry analysis

The dilution or suspension buffer for flow cytometry experiments was HBSS. Phosphatidylserine exposure was evidenced using annexin VFITC added at a final concentration of 140 nM. Incubation at room temperature was allowed to proceed for 10 min in the dark before data acquisition. Samples were analyzed using a FACSscan flow cytometer (Becton Dickinson, San Jose, CA). The sheath fluid was Isoton II balanced electrolyte solution (Coulter, Krefeld, Germany). Suspensions to be analyzed contained 5 × 10⁵ cells/ml. Data acquisition, 10,000 events in each case, and analysis were conducted using CELLQuest software (Becton Dickinson). The forward light scatter setting was E01 when cells and microparticles were analyzed and E00 when cells only were analyzed.

Determination of hypodiploid DNA

After the different treatments, cells were harvested and numbered. Concomitant with the determination of 5 × 10⁶ cells/ml of 70% ethidium bromide (EB) in H₂O₂ and fixation was allowed to proceed during at least 1 h at 4°C. Cells were washed once in HBSS before resuspension in a solution containing type I-A RNase A (0.5 mg/ml) in HBSS and were incubated for 10 min at 37°C. Propidium iodide was then added at a final concentration of 0.1 mg/ml. Samples were allowed to stand another 15 min in the dark at room temperature before flow cytometry analysis.

Antigenic capture, characterization of released membrane particles, and prothrombinase assay

Particles were captured from the supernatant, after centrifugation of the cell suspension at 600 × g for 1 min, using 5 µM actinomycin D during 6 h with 8 µM VP-16 or with 0.3 µg/ml actinomycin D (not shown).

The only difference was that to remove annexin V from bound microparticles, three washing steps were performed in the presence of EDTA (0.5 mM in TBS, consisting of 50 mM Tris buffer, pH 7.5, containing 120 mM NaCl and 1.7 mM KCl), followed by three washing steps in the presence of CaCl₂ (1 mM in TBS) before prothrombinase assay. Phosphatidylserine was the rate-limiting parameter of the reaction. The assay is sensitive enough to allow the detection of minute amounts of generated thrombin corresponding to a minimum value of 125 pM catalytic phosphatidylserine. Background values obtained with insolubilized irrelevant IgGs were subtracted from those measured with mAbs. Linear absorbance changes were recorded at 405 nm using a microtitration plate reader equipped with a kinetics software. Results were expressed as nanomolar phosphatidylserine equivalent by reference to a standard curve constructed by using liposomes of defined composition and of known concentration. The liposomes containing 33% phosphatidylserine and 67% phosphatidylcholine (mol/mol) were prepared and observed by electron microscopy according to Pigault et al. (20). Background values obtained in the absence of anti-apoptotic mAbs never exceeded 0.5 nM phosphatidylserine equivalent, even in samples with the highest particle content, and were subtracted from all the data presented in this study.

Western blotting

After incubation with apoptosis-inducing agent, cells were sedimented, washed once with HBSS and solubilized in lysis buffer consisting of 50 mM Tris buffer containing 8 mM MgCl₂, 5 mM EGTA, 0.5 mM EDTA, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 10 µg/ml aprotinin, 1 mM PMSF, 250 mM NaCl, and 1% (v/v) Triton X-100, adjusted to pH 7.5. Samples containing 15–20 µg protein (Peterson’s protein assay kit; Sigma) were separated on 10% SDS-PAGE (31). Separated proteins were then blotted onto Protran nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Blots were probed with mAb to CPP32 and developed with the appropriate HRP-conjugated secondary Ab. Bound Abs were detected by chemiluminescence (Pierce, Rockford, IL).

Caspace-3 activity assays

A caspase-3 cellular activity enzyme assay kit was used according to the manufacturer’s instructions adapted to the use of CEM T cells. Briefly, incubation of a nonapoptosis-inducing agent, cells were sedimented, washed once with HBSS, and resuspended to 20 × 10⁶ cells/ml in ice-cold lysis buffer containing 0.03% 3-(3-cholamidopropyl)dimethylammonio) 1-propanesulfonic acid. After lysis, 20 µl of each cell extract was added to the assay buffer, incubated for 10 min at 37°C before the addition of 10 µl DEVD-p-nitroanilide (the final volume of each reaction was 100 µl). Linear absorbance changes were recorded at 405 nm using a microtitration plate reader equipped with a kinetics software. No endogenous inhibitor could be detected in cell extracts incubated with known amounts of purified caspase-3. Furthermore, in each sample, it was verified that the measured caspase-3 activity was totally inhibited by Asp-Glu-Val-Asp-aldehyde (DEVD-CHO), a potent caspase-3 reversible inhibitor.

Results

Induction of apoptosis and membrane alterations in CEM T and HL-60 cells

Two agents, VP-16 and actinomycin D, inhibitors of topoisomerase II and DNA-primed RNA polymerase, respectively, were used to induce apoptosis in CEM T and HL-60 cells. The CEM T cells were treated with 5 µM VP-16 or with 0.3 µg/ml actinomycin D during 18 h. Under these conditions, the content of hypodiploid DNA in CEM T cells was 31 ± 2% (mean ± SEM) after treatment with either VP-16 (n = 17) or actinomycin D (n = 13). Similar results were obtained with HL-60 cells treated during 6 h with 8 µM VP-16 or with 0.3 µg/ml actinomycin D (not shown).

Phosphatidylserine externalization by apoptotic cells was revealed by flow cytometry using annexin VFITC as a probe. A large majority of untreated cells (~95%) showed a low affinity for annexin VFITC (mean fluorescence intensity = 5 ± 1 arbitrary units, n = 5); the remaining ~5%, corresponding to basal cell death, had a mean fluorescence intensity in the range indicated below for treated cells. After treatment with VP-16 or actinomycin D, two cell populations were clearly evidenced, one with a low labeling similar to that of untreated cells and corresponding to the normal...
proportion of hypodiploid DNA as described in Materials and Methods.

Values are mean ± SEM of five independent studies performed likewise.

Growing population, the other one highly labeled (mean fluorescence intensity = 516 ± 55 arbitrary units for VP-16-treated CEM T cells, n = 5; and 421 ± 50 arbitrary units for actinomycin D-treated CEM T cells, n = 5) and mainly containing apoptotic cells (4). Other characteristic features of apoptosis, including cleavage of DNA into nucleosomal multiples of ~200 bp visualized as a ladder (not shown), dramatic plasma membrane blebbing (as assessed by optical microscopy; not shown), and collapse of cells into numerous vesicles (as observed by flow cytometry; Table I) were observed in treated cells. When examined by flow cytometry, released particles had light scatter parameters comparable to those of liposomes of mean diameter of 0.15 μm with extremes at 0.03 and 0.3 μm (20). All these observations confirmed the drastic changes of the plasma membrane organization that are hallmarks of apoptotic cells. The results obtained with actinomycin D as well as with HL-60 cells (not shown) were comparable with those shown for VP-16-treated CEM T cells.

Modulation of apoptosis and inhibition of the release of particles by annexin V.

To examine the possible effect of annexin V on the apoptotic process, VP-16 or actinomycin D-treated cells were maintained in the presence or absence of annexin V during the whole course of the 18-h apoptosis-inducing treatment. Annexin V counteracted apoptosis induced by 5 μM VP-16 in a dose-dependent manner with a plateau, corresponding to 40–45% inhibition at 18 h, at about 30 μg/ml annexin V (~850 nM) (Fig. 1). The binding of annexin V to phosphatidylserine is a relatively rapid reaction—about 20 min is sufficient to reach a plateau (15). Hence, it is not a rate-limiting step with respect to the apoptotic process, which requires several hours. However, the addition of annexin V after the first 2 h of treatment resulted in a progressive loss of effect. Comparable results were obtained with actinomycin D and with HL-60 cells, but at 6 h in this latter case. To verify the binding of annexin V, the apoptosis-delaying effect of annexin VFTTC was also tested at 20 and 30 μg/ml under the same conditions; identical results were obtained with a binding of annexin VFTTC to apoptotic cells reflected by a mean fluorescence intensity in the range of 300 arbitrary units. In the presence of annexin V, the release of particles was highly to totally reduced in the population of VP-16-treated cells as observed by photonic microscopy and by flow cytometry (Table I). The events recorded in the microparticle gate were considerably reduced in the presence of annexin V, and those recorded in the cell gate increased accordingly.

As previously reported (10), released particles bearing exposed phosphatidylinerine and specific Ags can constitute a valuable parameter for the estimation of the degree of cell apoptosis. The presence of membrane Ags associated with the shed particles allowed their capture by insolubilized corresponding Abs. According to this, the release of apoptotic microparticles from VP-16-treated CEM T cells was assessed using anti-CD4 or anti-PrPc mAbs. This method yielded an accurate evaluation of the respective effects of the proapoptotic agent VP-16 and annexin V on human membrane vesiculation, which confirmed the above results. As already observed with stimulated monocytic cells (10, 32), the increase of an Ag on microparticles was correlated with the loss of this Ag at the cell surface. VP-16 treatment resulted in a 35-fold increase of the shedding of CD4+ particles, while that of PrPc+ particles was 11-fold as indicated in Table II. At 20 μg/ml (~560 nM), annexin V totally inhibited the release of apoptotic particles bearing either of CD4 or PrPc Ag, and at half this concentration (10 μg/ml) the inhibition reached 90% (not shown).

Effect of annexin V and caspase inhibitor on CEM T cell apoptosis.

The permeable peptidic agent z-VAD.fmk is an irreversible inhibitor of several members of the caspase family of proteases, which become activated during the apoptotic cascade. It prevents DNA digestion, intracellular acidification, increased membrane permeability, and cell shrinkage (33, 34). When present during the 18 h proapoptotic treatment, z-VAD.fmk inhibited the generation of hypodiploid DNA normally induced by 5 μM VP-16 (Fig. 2) or 0.3 μg/ml actinomycin D (not shown) in CEM T cells. As shown in Fig. 2, the inhibitory effect of z-VAD.fmk measured by propidium iodide assay was dose-dependent and reached ~100% at 100 μM. As indicated in Table II, at 10 μM this inhibitor had a limited effect on the shedding of CD4+ particles and no effect at all on that
of PrP$^{\text{sc}}$ ones. DEVD-CHO, another caspase inhibitor more specifically directed to caspases 8 and 3, elicited a reduction of $\sim$50% in apoptotic cell population at a concentration of 50 nM, but had no effect on the release of membrane particles. Annexin V enhanced the inhibitory effect of z-VAD.fmk on VP-16-induced apoptosis as shown in Fig. 2. Thus the external effect of annexin V is additive to that of an intracellular inhibitor of apoptosis. This finding was verified by DNA ladder analysis (not shown).

Status of caspase-3 during the inhibition of apoptosis by annexin V

Caspase-3 is a member of the caspase family of proteases (35) for which the processing to the active form is inhibited by z-VAD.fmk. Because this peptide is a strong inhibitor of apoptosis-associated phosphatidylserine externalization (5), it was assessed whether annexin V also interfered in the caspase cascade. Caspase-3 underwent proteolytic processing during VP-16 treatment of CEM T cells, which was clearly reduced in the presence of 30 $\mu$g/ml ($\sim$850 nM) annexin V, as qualitatively shown in Fig. 3. As expected, z-VAD.fmk was efficiently protective against caspase-3 proteolysis. This processing was concomitant with an increased caspase-3 activity in VP-16-treated CEM T cells, and with the reduction of this activity of about 50% and 80% in the presence of annexin V and z-VAD.fmk, respectively (Table III).

**FIGURE 2.** Additive effects of z-VAD.fmk and annexin V on CEM T cell apoptosis. Cells were treated during 18 h with 5 $\mu$M VP-16, in the presence of z-VAD.fmk at different concentrations and the indicated concentrations of annexin V. The degree of apoptosis was estimated from the proportion of hypodiploid DNA as described in Materials and Methods. Values are mean ± SEM of five independent determinations.

**FIGURE 3.** Effect of annexin V on the processing of caspase-3 (CPP32). CEM T cells were treated during 18 h with 5 $\mu$M VP-16 in the presence of 30 $\mu$g/ml annexin V ($\sim$850 nM) or 10 $\mu$M z-VAD.fmk, and lysates (containing 16 $\mu$g protein) were submitted to SDS-PAGE and Western blotting as described in Materials and Methods. Caspase-3 underwent proteolytic processing from the 36-kDa pro-form during CEM T cells apoptosis, which was inhibited by annexin V as well as by z-VAD.fmk.

**Table III.** Effect of annexin V on the generation of caspase-3 activity

<table>
<thead>
<tr>
<th>Caspase-3 Activity (pmol p-nitroaniline/min×10^{6} cells)</th>
<th>Control</th>
<th>VP-16 (5 $\mu$M)</th>
<th>VP-16 (5 $\mu$M) + annexin V</th>
<th>VP-16 (5 $\mu$M) + z-VAD.fmk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(5 $\mu$M)</td>
<td>30 $\mu$g/ml</td>
<td>10 $\mu$M</td>
</tr>
<tr>
<td>% inhibition of apoptosis</td>
<td>100</td>
<td>63.38 ± 0.93</td>
<td>96.08 ± 1.14*</td>
<td>93.38 ± 1.14†</td>
</tr>
<tr>
<td>% inhibition of apoptosis</td>
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<td>96.08 ± 1.14*</td>
<td>93.38 ± 1.14†</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of five independent determinations (each with duplicate samples). Differences between VP-16-treated cells and VP-16 + annexin V-treated cells and VP-16 + z-VAD.fmk-treated cells were significant, with $p = 0.0317$ and $p = 0.0357$, respectively (Mann-Whitney test).

**In vivo assessment of the annexin V effect**

HN-2 or nitrogen mustard, a bifunctional alkylating agent still used in cancer chemotherapy, induced apoptosis in cultured lymphocytes at submicromolar concentrations (36) and a reduction of splenic weight and cellularity in mice (37). To examine the possible effect of annexin V on the apoptotic process in vivo, mice were treated by HN-2 in the presence or absence of annexin V. HN-2 induced a significant reduction of spleen weight, which was counteracted in annexin V-injected animals (Fig. 4). Comparable results were observed for cellularity (data not shown). It has to be noted that annexin V alone had no noticeable effect on spleen weight (Fig. 4a) or cellularity. These results suggest that annexin V exerts a protective effect in vivo.

**Discussion**

The induction of a death program is accompanied by a variety of characteristic changes of the cell morphology among which shrinkage, plasma membrane blebbing, nucleus condensation, and loss of microvilli are the most frequently evoked (1, 2, 38). Recent contributions point to the transverse redistribution of plasma membrane phosphatidylserine (3, 4) followed by the shedding of membrane particles being common features of apoptotic cell death (10). Links between the intracellular events leading to the cleavage of chromosomal DNA into nucleosomal units, the ultimate stage of this death process, and the external membrane remodeling have been established. There are converging evidences for a crucial role of the caspase cascade in the development of a death program, and the generation of caspase-3 appears to be a central step (5, 6, 39, 40). In this context, it was tempting to assess whether an external constraint exerted on the plasma membrane can interfere in this death cascade. For this purpose, annexin V was the selected tool owing to its ability to form two-dimensional arrays at the membrane interface (18–20), probably acting as a sort of “exoskeleton” preventing membrane vesiculation but not phosphatidylserine externalization as already observed in activated platelets (23).

When present during the execution of induced death programs, annexin V elicited a dose-response apparent inhibition of apoptosis. A maximum of about 45% reduction of events with hypodiploid DNA was achieved, at 18 h, at 30 $\mu$g/ml (850 nM) annexin V ($\sim$850 nM) or 10 $\mu$M z-VAD.fmk, and lysates corresponding to 4×10^{6} cells were subjected to caspase-3 cellular activity assay as described in Materials and Methods. Values are mean ± SEM of five independent determinations (each with duplicate samples). Differences between VP-16-treated cells and VP-16 + annexin V-treated cells and VP-16 + z-VAD.fmk-treated cells were significant, with $p = 0.0317$ and $p = 0.0357$, respectively (Mann-Whitney test).

In this study, annexin V was shown to be effective in vitro. This suggests that annexin V may also be effective in vivo. Further studies are needed to determine the exact mechanism of annexin V’s protective effect.
was additive to the intracellular antagonist effect of z-VAD.fmk, a potent irreversible inhibitor of caspase-1-like proteases, showing at the same time that annexin V did not significantly interfere in the cell permeability of relatively small molecules. The interference of annexin V in apoptosis was further demonstrated by a decreased proteolytic processing of CPP32 to its active form. None of the cells exposed to VP-16 or actinomycin D could be rescued by annexin V, z-VAD.fmk, or a combination of both agents after a 24-h culture period following the 18-h proapoptotic treatment. This suggests that annexin V effect takes place between the irreversible mitochondrial collapse (42) and the caspase checkpoint.

The obvious question arising from the above observation is: how can annexin V counteract apoptosis, resulting in reduced exposure of phosphatidylserine to which it precisely binds? The same holds true in respect of experiments performed in the presence of z-VAD.fmk. The answer takes into account the complex Ca$^{2+}$-mediated interaction between annexin V and phosphatidylserine; in this respect, the X-vivo 15 synthetic culture medium used in our experiments contains 1.8 mM Ca$^{2+}$. Annexin V has been shown to bind to unstimulated cells, but the number of binding sites is considerably lower when compared with stimulated cells (15). Several targets can be considered, but a proportion of phosphatidylethanolamine is accessible at the surface of a variety of unstimulated cells (7), which makes it a likely candidate allowing the binding of annexin V, with a half-maximal Ca$^{2+}$ concentration requisite of $\sim$0.9 mM (14). This suggests that bound annexin V molecules can be viewed as latent "nucleation" sites for the formation of two-dimensional arrays as soon as phosphatidylserine becomes exposed, allowing the binding of more annexin V molecules, even when the proportion of phosphatidylserine is low (20) in the early stages of the death program. The process must reach an asymptotic limit because the interaction of annexin V with phosphatidylserine has a delaying consequence on the progress of the cell death program, precisely resulting in a reduction of phosphatidylserine exposure. Such a mutual neutralization explains why the inhibitory effect of annexin V cannot be $>50\%$ (Fig. 1), and this is also true for the generation of caspase-3 activity (Table III).

In addition to an apoptosis-delaying effect, extracellular annexin V considerably reduced the degree of membrane particle shedding regardless of their Ag content. But cell permeable z-VAD.fmk had a limited action on the release of CD4$^+$ membrane fragments and no effect at all on PrP$^+$ ones, at least below 10–15 $\mu$M because at higher concentrations it might destabilize the membrane, leading to artifactual vesiculation. Interestingly, other investigators have reported the absence of morphological change and surface blebbing in apoptotic cells treated with peptic caspase inhibitors (5, 6, 33, 34, 43). The differential exportation of membrane proteins is suggestive of cluster formation before shedding, which may have consequences on the dissemination of particular Ags. Because CD4 and PrP do not have the same attachment to the membrane, it remains to be established whether this may account for discriminating shedding, especially for PrP, which could be preferentially associated with sphingolipid-cholesterol rafts as being GPI-anchored (44). CD4$^+$ particles, believed to reflect ongoing apoptosis of CD4$^+$ T cells, have been detected in peripheral blood samples from certain HIV-1-infected subjects (10). An intriguing issue is the part of apoptotic membrane particles in the possible conveying and addressing of PrP and its pathological misfolded form, scrapie prion protein, in the development of prion diseases. The converse points to the significance of the inhibition of the release of such particles by annexin V.

Hence, if an intracellular proapoptotic cascade of events leads to the external remodeling of the plasma membrane, symmetrically, an external constraint can delay the activation in the internal cascade. Although upstream coupling element(s), possibly of mitochondrial origin (45), remain(s) to be identified, these observations raise the question of a relevant role of annexin V in the process of apoptosis. In this respect, its discussed ability to generate Ca$^{2+}$ channels (46, 47) may be considered in the light of a possible impaired Ca$^{2+}$-mediated phospholipid scrambling (48). Despite the lack of a signal peptide, this canonical member of the annexin family (26, 47) may well be translocated to the exoplasmic leaflet attached to phosphatidylserine during the loss of asymmetry of the plasma membrane. Once externalized from cells that contain a substantial proportion, annexin V has been proposed to fulfill an anticoagulant function by neutralizing the procoagulant potential of phosphatidylserine (24, 25). More recently, an apoptosis-inducing activity was localized in the fraction of annexin V-binding Abs from 10 patients with lupus anticoagulant. Furthermore, annexin V neutralized the apoptotic potential of such Abs, but these authors

**FIGURE 4.** Effect of annexin V on spleen weight of HN-2 treated mice. At 24 h following the injection of HN-2 i.p. (6 mg/kg) and annexin V i.v. (50 $\mu$g), mice were sacrificed and their spleens weighed and processed as described in Materials and Methods. A, Spleen weight after 24 h treatment. Differences between spleen weight of HN-2-treated mice vs control and vs HN-2 + annexin V-treated mice were statistically significant: *, $p < 0.03$ and ‡, $p < 0.06$, respectively (Mann-Whitney test). B, Effect of annexin V on HN-2 treatment. The weight loss is the difference between the spleen weight of mice without HN-2 treatment and those submitted to HN-2 treatment in the presence or absence of annexin V. The protective effect of annexin V against spleen weight loss is significant with $p < 0.03$ (Mann-Whitney test). Values are mean ± SEM of four independent determinations.
did not consider that annexin V could interfere in the apoptotic process itself (49). It is also of interest to point to the modulation of cell growth or death by other members of the annexin family when exogenously added (50–53).

When injected in vivo, annexin V concentrates preferentially in kidney and spleen (54), which led us to examine its effect on spleen weight loss in mice treated by HN-2, a potent alkylating agent previously shown to induce lymphocyte apoptosis (36). Annexin V appeared indeed protective against spleen cell loss, in agreement with its ability to delay apoptosis in vitro when present in the extracellular medium as suggested from the above results.

These in vitro and in vivo observations are suggestive of a role of annexin V in the control of cell death, which deserves further attention.

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References