Cell injury releases endogenous adjuvants that stimulate cytotoxic T cell responses

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General immunostimulants (adjuvants) are essential for generating immunity to many antigens. In bacterial infections, adjuvants are provided by components of the microorganism, e.g., lipopolysaccharide. However, it is unclear what provides the adjuvant effect for immune responses that are generated to tumors and many viruses. Here we show that cell injury and death of tumor or even normal cells provide a potent adjuvant effect for the stimulation of cytotoxic T lymphocyte responses. This adjuvant activity is constitutively present in the cytoplasm of cells and is increased in the cytoplasm of cells dying by apoptosis. The release of these components stimulates immune responses both locally and at a distance, and provides a simple mechanism to alert the immune system to potential danger in almost all pathological situations.

Immunologists have long known that to elicit optimal antibody or T cell immune responses antigens must be admixed with adjuvants such as killed bacteria or components of their cell wall (1, 2). It is likely that adjuvants work by activating cells of the innate immune system (e.g., macrophages, natural killer cells, and dendritic cells) to become more stimulatory and to produce cytokines and inflammatory mediators, which, in turn, upregulate many of the components that are needed to stimulate lymphocyte responses. In the absence of adjuvants, lymphocytes may fail to respond to an immunization or even be tolerized.

Janeway (1–3) hypothesized that cells of the innate immune system would have receptors for unique components of microbial pathogens, which he termed pathogen-associated molecular patterns (PAMPs); these would allow a self-nonself discrimination and stimulate initial immune responses. As predicted, such receptors were identified in *Drosophila* (e.g., the toll receptor family; refs. 2 and 4) and more recently in mammals (5–8). Some of these receptors recognize PAMPs of bacteria whereas others react with unique molecular features of fungi. Engagement of PAMP receptors stimulates cells of the innate immune system, and this is thought to be the underlying mechanism of action of many microbial adjuvants.

The immune system continually monitors tissues for infections with microbes and cancerous cells and must respond rapidly to successfully eliminate these threats. The adjuvant properties of bacteria and some viruses (e.g., double-stranded RNA) promote this process, allowing the generation of robust immune responses in natural infections (1, 2). However, it has been unclear what, if anything, provides an adjuvant effect for immune responses to tumors and other viruses. These pathogens lack any obvious source of PAMPs to stimulate innate immune responses. Nevertheless, immune responses are generated to tumors and viruses, implying that an adjuvant effect is somehow provided.

On theoretical grounds, Matzinger (9, 10) has proposed that dying cells might provide a signal that alerts the immune system to danger. Recently, it has been shown *in vitro* that dying cells are recognized and avidly internalized by dendritic cells (11, 12). Through this process, antigen-presenting cells (APCs) would efficiently acquire and present antigens from dying cells to T lymphocytes. In addition, some (11, 13) but not other (14) studies find that dying cells can also stimulate dendritic cells to mature. Outside of these studies, the danger hypothesis remains largely untested. We now find strong experimental support for aspects of this hypothesis.

In this article, we demonstrate *in vivo* that cells contain in their cytoplasm endogenous adjuvants that can promote the generation of cytotoxic T lymphocyte (CTL) responses to particulate antigens. These adjuvants are released in situations where cells are injured and become necrotic. Moreover, when cells are injured and undergo apoptotic death, there is a marked increase in endogenous adjuvant activity present in the cytosol. This adjuvant activity can help to explain how CTLs are generated to pathogens that lack adjuvant activities.

Materials and Methods

Antigen Preparations. Iron oxide (BioMag; Advanced Magnetics, Cambridge, MA), latex (1 μM mean diameter; Polysciences, Warrington, PA), and CPG beads (unmodified highly porous glass beads, 0.5 μM mean diameter; CPG, Lincoln Park, NJ) were conjugated with ovalbumin (OVA, grade VI; Sigma or ICN) or HIV gp120 (HXBCII strain, GenBank accession numbers: K03454 and M38432, expressed in *Drosophila* cells; a gift from J. Sodrosky, Harvard Medical School, Boston, MA; protein purified by affinity chromatography), according to the manufacturers' instructions. Briefly, amine-terminated BioMag beads were first activated with 5% (vol/vol) glutalaldehyde and coupled with OVA in a purine coupling buffer. The reaction was quenched with a glycine solution. The end product was suspended in PBS with 0.02% azide and stored at 4°C; latex beads were coupled to either gp120 or OVA in 100 μM (pH 8.0) Borax buffer. The end product was suspended in PBS, aliquoted, and stored at −20°C without azide; CPG beads were coupled with either gp120 or OVA in a low-salt buffer (20 mM KH_2PO_4, pH 5.1) and subjected to a centrifugation (1,000 × *g*, 20 min) to remove the overly fine beads in the supernatant. The end product was suspended in PBS with 0.02% azide, and stored at 4°C antigen conjugations to BioMag and latex beads were performed overnight with shaking while CPG was incubated for 1 h. The coupling efficiency was determined by the differences in UV absorbance (at 280 nm) between pre- and postcoupling protein suspensions. Conjugated beads were sterilized by exposure to ionizing UV radiation. Antigen quantities given in this article were the equivalents of the weight of antigens contained on conjugated beads.

Animals, Cell Lines, and Reagents. Four-to-six-week-old BALB/c and some C57BL/6 mice were purchased from Taconic Farms. The remaining C57BL/6 mice were purchased from The Jackson

Abbreviations: CTL, cytotoxic T lymphocyte; APC, antigen presenting cell; HCM, hybridoma culture media; OVA, ovalbumin.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. K03454 and M38432).

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Laboratory. All mice were kept in the University of Massachusetts Medical Center animal facilities. EL4 (C57BL/6 T lymphoma), GL261 (C57BL/6 glioma), A20 (BALB/c B lymphoma), and NIH 3T3 (BALB/c) cell lines were routinely maintained in hybridoma culture media (HCM) consisting of RPMI medium 1640 plus 10% (vol/vol) FBS (Irvine Scientific, or Atlanta Biologicals, Norcross, GA, or GIBCO/BRL), 10 nM L-glutamine, Hepes, penicillin/streptomycin, and 50 μM 2-mercaptoethanol. EG7 (OVA gene transfected EL4 cells, a gift from M. Bevan, University of Washington, Seattle; ref. 15), 15–12 (gp120 transfected BALB/c 3T3 cells), and 18neo (vector control for 15–12, both gifts from J. Berzofsky, National Institutes of Health, Bethesda, MD; ref. 16) cells were grown in the same media plus 0.4 mg/ml G418 (GIBCO/BRL). Cells were found to be free of mycoplasma contamination by using a PCR-based assay kit (Roche Molecular Biochemicals).

Immunizations and CTL Analyses. For a typical cell adjuvant experiment, cells were washed (after trypsinization if adherent) and treated with 50 μg/ml mitomycin C (Sigma) or 8 μM of emetine (Sigma) in HCM at 37°C for 1 h. Cells were then washed and suspended in PBS at indicated densities and mixed with antigen preparations as needed. Mice were immunized with 50 μl of antigen beads $\pm$ cell preparations s.c. in both hind flanks (100 μl total, unless indicated otherwise). After 7–9 days (for OVA-immunized C57BL/6) or 14 days (for gp120 immunized BALB/c), splenocytes (4×10^7) were cultured in 10 ml of HCM with 10^6 mitomycin C-treated (at 100 μg/ml) syngeneic OVA-transfected (EG7) and gp120-transfected (15–12) cells, respectively, for 5 days. CTL assays were then performed with indicated target cells, as described (17).

Apoptosis/Necrosis Induction. For apoptosis induction, NIH 3T3 cells were grown in 6-, 10- or 15-cm Petri dishes to 70–80% confluence and were washed with PBS while still attached to the plastic surface. They were directly exposed to a UV source for a total of 5 min. EL4 cells at optimal growth phase or freshly obtained splenocytes were washed and suspended in less than 3 ml of PBS and plated out in Petri dishes for a similar UV exposure with frequent shaking and rotating. Control cells were treated exactly the same, except that they were shielded during the UV exposure. HCM media were added back to the dishes and cells were cultured further in a 37°C CO_2 incubator for 5 h, unless indicated otherwise. A20 cells were treated with 10–20 ng/ml Fas ligand (Alexis, San Diego) followed by 1 μg/ml anti-Fas ligand antibody for 4 to 5 h at cell densities less than 2×10^6/ml. NIH 3T3, EL4, and A20 cells were also treated with 8–20 μM emetine for 5–7 h at 37°C for apoptosis induction. Apoptosis was monitored by positive Annexin V and propidium iodine staining (PharMingen), and also by direct microscopic examination. For experiments testing apoptotic whole-cell lysates without fractionation, cells were quickly washed with cold PBS, removed gently from the dishes, and subjected to two rounds of freeze thawing before they were finally suspended in PBS. For necrosis induction, NIH 3T3, A20, and EL4 cells were treated with 25 μM oligomycin for at least 7 h.

Cell Fractionation. Untreated or UV-exposed NIH 3T3, GL261, or EL4 cells were washed with PBS and suspended in a low salt douncing buffer on ice at densities no higher than 6×10^7/ml. They were pressurized in a stainless steel nitrogen gas cavitation chamber (Parr Instruments, Moline, IL) at 500 psi for 5 min. The pressure was released over 1–2 min. Cavitated cells were collected and immediately subjected to 10 strokes in a tight-fitting douncing homogenizer. Tonicity was immediately restored with a high-salt buffer. Nuclei were isolated by centrifugation ($500 \times g$) for 5 min, washed once with 1% (vol/vol) Triton X-100 (Sigma; omitted for 3T3 nuclei because of nuclear lysis), three times with PBS containing 300 mM NaCl, and finally suspended in water. The post-nuclear fraction was centrifuged ($150{,}000 \times g$) for 90 min. The supernatant (cytosol) was rapidly frozen with liquid nitrogen, lyophilized, reconstituted in a small volume of water, and frozen immediately. All of the procedures were performed at 4°C.

Protein Synthesis Inhibition Assay. Three million cells at optimal growth phase were collected and cultured in fresh HCM for 2 h and washed twice with PBS. Cells were then cultured in 2 ml of methionine/cystine/L-glutamine-free RPMI Medium 1640 (Sigma) with 10% (vol/vol) dialyzed FBS for 30 min at 37°C, either in the presence of 8 μM emetine, 0.02% azide, or without inhibition. One milliliter of the same deficient media plus 0.6 mCi ^{35}S Easytag Express protein labeling mix (NEN, Boston, MA) was added. Ninety minutes later, 1 ml of normal HCM media was added for an additional 4 h culture. The incubation was stopped by washing with ice-cold PBS. One percent of the cells was suspended in 100 μl of 0.1% BSA/0.01% azide and precipitated twice with 1 ml of trichloroacetic acid. The pellet was further suspended in ethanol and one-fifth was taken for scintillation counting.

Results

Cells Constitutively Contain Endogenous Adjuvant Activity That Augments Priming of CTLs to Particulate Antigen. Dendritic cells and macrophages can internalize particulate antigens and degrade them into peptides that are presented on both MHC class I and class II molecules (17–19). Through this process they are able to acquire antigens from dying cells in peripheral tissues for subsequent transport to central lymphoid organs where they stimulate CD4 and CD8 T lymphocyte responses (20). Recent evidence indicates that this process is the major pathway for immune surveillance of viral infections and cancers of nonhematopoietic tissues (21, 22). To study how this process might be regulated by cytokines, we coinjected particulate antigen (OVA linked to iron oxide beads) together with irradiated or mitomycin C-treated syngeneic tumor cells that were transduced with various cytokine genes.

CTL responses to OVA beads were markedly enhanced by coinjection of the cytokine-producing B16 melanoma (data not shown). To our surprise, coinjection of control (nontransduced B16 cells) had very similar enhancing effects (data not shown). Moreover, the same effect was observed with other syngeneic tumor cell lines (GL261 glioma and EL4 lymphoma cells) that were not transduced with cytokine genes (Fig. 1 *A* and *B*) and with different OVA beads (latex and porous glass), although the magnitude of the augmentation varied slightly with different combinations (data not shown). Remarkably, coinjection of less than 10,000 cells (and in some cases as few as 500–1,000 cells) enhanced the response to OVA (Fig. 1*A* and data not shown). There was typically a 500- to 1,000-fold enhancement in the immune responses (based on the shift in the antigen-dose-response curve), and responses were observed with low doses (e.g., 40 ng) of conjugated antigen that otherwise failed to stimulate CTLs. We also observed a strong enhancement of CTL responses to another antigen (HIV-gp120 bound to latex beads) coinjected with syngeneic cells (NIH 3T3) in another mouse strain (BALB/c), although more cells were typically needed (Fig. 1*C*). In contrast, injection of the syngeneic cells without antigen did not prime CTLs to OVA or gp120 (data not shown). Therefore, the coinjection of cells is providing a potent adjuvant effect for the priming of CTLs to particulate antigens. This adjuvant effect is seen with multiple-cell types, mouse strains, and antigens.

Coinjection of cell-sized latex beads did not augment responses, indicating that the coinjected cells were providing something more than just an additional particulate stimulus (Fig. 2*A*). It was possible that bovine serum proteins or mutant gene

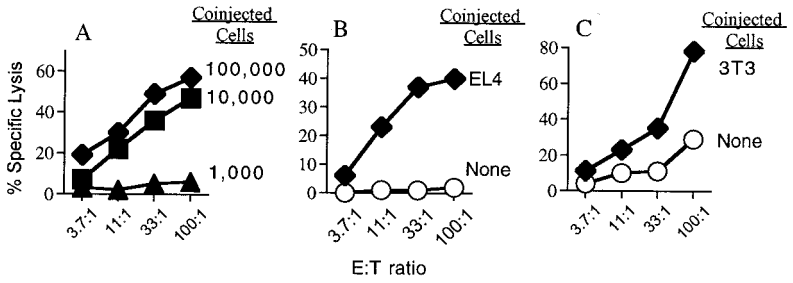

Fig. 1. Syngeneic cells provide a potent adjuvant effect for CTL induction. (*A*) OVA/BioMag beads (5 μg) were injected s.c. into the hind flanks of C57BL/6 mice with either 10^5, 10^4, or 10^3 mitomycin C-treated GL261 cells, or without cells in 100 μl of PBS. Seven days later, splenocytes were harvested and stimulated with OVA-transfected EL4 cells (EG7). CTL activity was measured against EG7 cells 5 days later in a ^{51}Cr release assay. Control mice immunized with beads alone had no detectable CTL. Killing of control EL4 cells in this assay as well as all of the following assays was minimal. (*B*) Similar to *A* except that 10^5 EL4 cells were used to replace GL261 cells. (*C*) Similar to *A* except that the antigen was gp120/latex beads (0.1 μg), the mice were BALB/c mice, and the coinjected cells were 5×10^5 syngeneic 3T3 cells. Mice were killed 2 weeks after the injection and splenocytes were stimulated with and assayed for killing against gp120-transfected 3T3 cells (15–12). Killing against a control cell line 18neo (control transfected 3T3) in this assay as well as all of the following assays was minimal. E:T ratio, effector-to-target cell ratio.

products in the cultured tumor cells might be stimulating an immune response that was augmenting the response to the coinjected bead-bound antigens. However, several findings argue against this possibility. Coinjected latex beads coated with FBS did not augment responses (Fig. 2*A*). Moreover, culturing cells in serum-free media did not alter their stimulatory capacity (data not shown). Furthermore, coinjection of noncultured syngeneic splenocytes augmented the responses to OVA (Fig. 2*B*) and gp120 (data not shown). Therefore, the adjuvant effect cannot be caused by an immune response to foreign antigens associated with the coinjected cells. We conclude that it must be arising from some component that is present in many and possibly all cultured and primary cells.

To examine whether the adjuvant effect might be mediated by cytokines that were being secreted by the coinjected cells, we treated these cells with an irreversible inhibitor of protein synthesis. As shown in Fig. 3*A*, a strong adjuvant effect was provided by cells that were treated with emetine, under conditions where protein synthesis was completely inhibited (Fig. 3*B*). Moreover, we also found that necrotic cells, which had been subjected to freezing and thawing, also augmented the response to particulate antigen. Therefore, these data rule out the possibility that the adjuvant effect is mediated by an actively secreted factor or factors. Furthermore, the finding of activity in cells killed immediately by freeze thawing indicates that there is an adjuvant moiety or moieties that is constitutively present in cells.

Cell Injury Increases Endogenous Adjuvant Activity. Interestingly, emetine-treated cells provided a more potent adjuvant effect than untreated control cells (Fig. 3*A*). This finding raised the possibility that cell injury or death was up-regulating or inducing new adjuvant activity or activities. To examine this hypothesis, 3T3 cells were injured by exposure to UV light and tested for adjuvant activity. The treated cells or untreated controls were then subjected to freeze thawing (so that the control and treated groups were in the equivalent form) and coinjected with gp120 beads into BALB/c mice. As shown in Fig. 4 *A* and *B*, the UV-treated cells contained about a 10-fold higher amount of adjuvant activity than the untreated controls. Similar effects were observed with A20 cells, another syngeneic cell line (data not shown). These results indicate that endogenous adjuvant activity in cells increases after certain kinds of cell injury. Moreover, because this effect is observed in emetine-treated cells, the increased adjuvant activity does not require *de novo* protein synthesis.

Injury can lead to cell death by either necrosis or apoptosis. We therefore investigated whether either of these death pathways up-regulated endogenous adjuvant activity. There was no

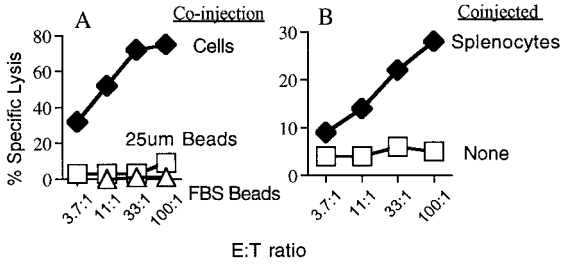

Fig. 2. Testing for adjuvant activity of cell-sized beads, foreign proteins, and primary cells. (*A*) Similar to Fig. 1*A* except that OVA beads were injected with GL261 cells (10^6), 25 μM latex beads (cell size, 2×10^6), or 10 μM of latex beads coated with FBS (2×10^6; 10^6 beads with FBS passively absorbed and the other 10^6 beads with FBS covalently conjugated). GL261 cells cultured in serum-free media gave nearly identical results as cells cultured with FBS (not shown). (*B*) Similar to Fig. 1*A* except 2 μg OVA/BioMag beads was injected with or without 10^5 C57BL/6 splenocytes. Syngeneic splenocytes also enhanced CTL responses to gp120 beads in BALB/c mice (not shown). E:T ratio, effector-to-target cell ratio.

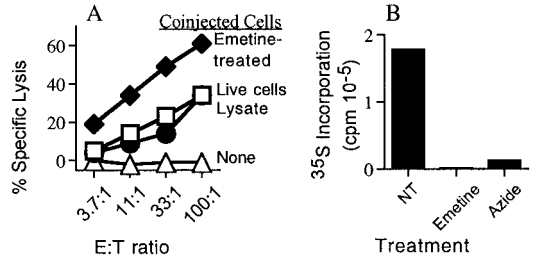

Fig. 3. The adjuvant effect is not mediated by secreted factors and does not require continued protein synthesis. (*A*) 10^6 GL261 cells were untreated (open squares), subjected to two rounds of freeze thawing (closed circles), or treated with 8 μM emetine (closed diamonds; leading to complete cell death overnight) and then injected into C57BL/6 with 5 μg OVA//BioMag; or OVA/BioMag beads without cells were injected (open triangles). CTL priming was evaluated as described in Fig. 1*A*. (*B*) Emetine completely inhibits *de novo* protein synthesis. A20 cells (see Fig. 5*B*) were either untreated or treated with 8 μM or 0.02% azide (control), and cultured with radio-labeled methionine and cystine. Trichloroacetic acid precipitation assay of ^{35}S-labeled cells was performed as described in *Materials and Methods*. E:T ratio, effector-to-target cell ratio.

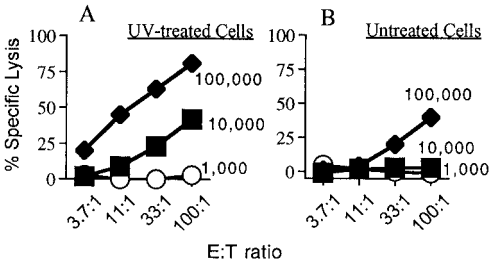

Fig. 4. UV treatment leads to higher adjuvant activity. (*A*) BALB/c 3T3 cells were UV-irradiated for 5 min, incubated at 37°C for 5 h, and then subjected to two rounds of freeze thawing. The indicated number of cell equivalents were mixed with 0.5 μg gp120/latex beads and injected into BALB/c mice. CTL priming was evaluated as described in Fig. 1*C*. (*B*) Identical to *A* except that the 3T3 culture was covered with plastic lids during the UV treatment. No response was seen in mice injected with antigen beads without cells. E:T ratio, effector-to-target cell ratio.

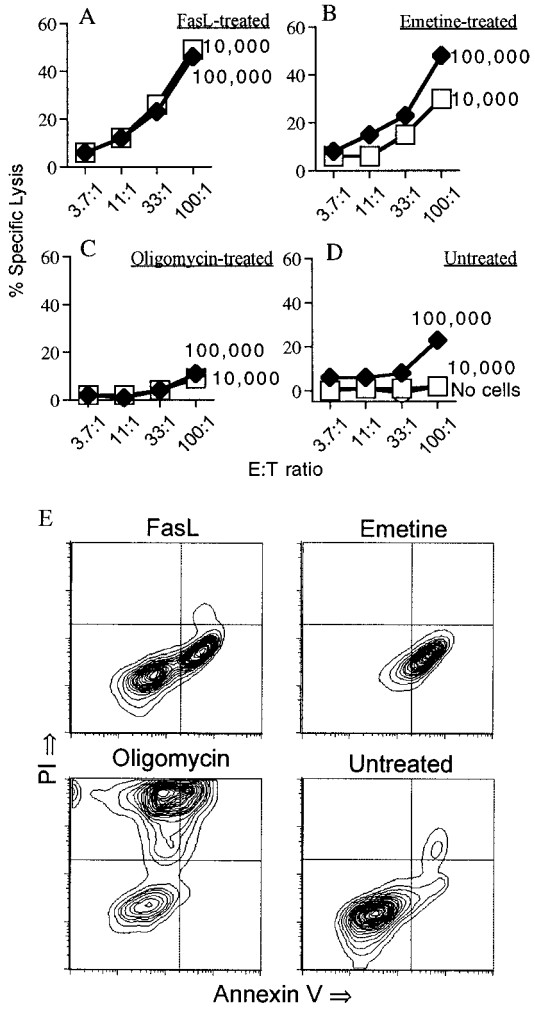

Fig. 5. Apoptotic cells provide a stronger adjuvant effect. (*A*) A20 cells were treated at 37°C with 10 ng/ml Fas ligand (Alexis, San Diego) followed by an anti-Fas ligand antibody and incubated for 4 h. A portion of the cells was used for flow cytometry (see *E*) and the remainder was frozen and thawed; the indicated number of cell equivalents were coinjected with gp120/latex beads into BALB/c mice. The priming of anti-gp120 CTLs was determined as described in Fig. 1*C*. (*B*) Same as *A* except cells were treated with emetine (20 μM). (*C*) Same as *A* except cells were treated with oligomycin (25 μM) for at least 7 h. (*D*) Same as *A* except cells were untreated. (*E*) Treated and untreated cells were stained with Annexin V and propidium iodine (PI) and analyzed by flow cytometry. All Fas ligand-treated cells were Annexin V positive with a 30 min longer incubation (at the time of harvesting for injection, data not shown). 3T3 cells treated with UV as in Fig. 4*A* also become Annexin V+, PI- (data not shown). E:T ratio, effector-to-target cell ratio.

increase in adjuvant activity when A20 (Fig. 5*C*) or 3T3 (not shown) cells were treated with oligomycin, an agent that induces necrosis (Fig. 5*E*). In contrast, there was an approximately 10-fold increase in adjuvant activity (Fig. 5*A*) after A20 cells were stimulated to undergo apoptosis by treatment with Fas ligand (Fig. 5*E*). Moreover, we found that emetine and UV treatment, which both increased adjuvant activity, were inducing A20 and 3T3 cells to undergo apoptosis (Fig. 5*E* and data not shown, respectively). In these experiments, all cells were subjected to freeze thawing so that all mice received comparable material. However, similar results were obtained if treated and control cells were injected without this step.

Constitutive and Induced Adjuvant Activities Are Present in the Cytosol. Because freeze thawing (or cell death) ruptures the plasma membrane, our findings raised the possibility that the intracellular contents that were released might be providing the adjuvant effect. To examine this possibility, cytoplasm was isolated from untreated 3T3 or EL4 cells and coinjected with antigen beads. As shown in Fig. 6*A*, there was a strong adjuvant activity present in the cytoplasm of these cells. Moreover, cytoplasm isolated from UV-treated 3T3 cells contained higher amounts of adjuvant activity (Fig. 6 *B* and *C*). In contrast, there was no activity in the nuclei of EL4 cells (Fig. 6*A*) or UV-treated or control 3T3 cells (data not shown, although adjuvant activity was detected in the nuclei of UV-treated EL4 cells). These results indicate that the cytoplasm of cells constitutively contains an endogenous adjuvant activity. Moreover, after cell injury, adjuvant activity is markedly up-regulated in the cytoplasm. It should be noted that live cells, when injected, also provide an adjuvant effect (Fig. 3*A*); however, it is likely that many of these cells die after injection into an ectopic site.

Endogenous Adjuvant Augments CTL Responses to Antigen at Distant Sites. To determine whether cells were mediating the adjuvant effect locally at the site of injection or systemically in host animals, we injected cells at the same site as the beads or on the opposite side of the animal. Surprisingly, we found that the cells were equally effective in augmenting immune responses, whether or not they were coinjected at the same site as the antigen beads (Fig. 7*C*). We also examined the timing of the adjuvant effect. We found that cells provided an adjuvant effect if they were injected up to at least 10 days before antigen injection or even after antigen injection (Fig. 7*A–C*). We conclude that the cell-based adjuvant effect works systematically, lasts for days, and can be effective even after antigen inoculation.

Discussion

Our data suggest the following model. All cells contain molecules with adjuvant properties that are normally sequestered by the plasma membrane. Loss of integrity of the plasma membrane, which occurs in the majority of pathological processes that lead to disease, would release these activities and would rapidly alert the immune system to a potential problem. This warning would be further heightened by cell injury increasing the amount of adjuvant that is released.

We do find that injection of untreated (live) cells also provides an adjuvant effect, although live cells are less potent than injured cells. Therefore, it is possible that even live cells release an adjuvant activity. However, in all cases (i.e., splenocytes and cultured tumor cells) these cell populations contain some dead

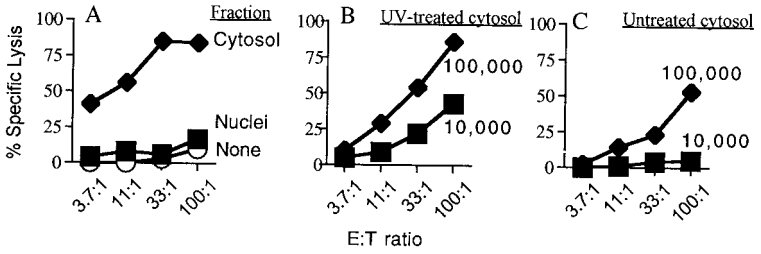

Fig. 6. The cytosol contains adjuvant activity. (*A*) EL4 cells were disrupted by nitrogen cavitation and fractionated by differential centrifugation. Cytosolic and nuclear fractions from 5×10^4 EL4 cells were mixed with 2 μg OVA/latex beads, injected into mice, and assayed as described in Fig. 1*A*. Fractionated GL261 cells and 3T3 cells (in BALB/c mice) gave similar results. (*B*) 3T3 cells were UV-irradiated as described in Fig. 4*A* and then were fractionated as in *A*. Cytosolic fraction in quantities equivalent to 10^5 and 10^4 initial 3T3 cells was mixed with 0.5 μg of gp120/latex beads and injected into mice. An assay similar to the one described in Fig. 1*C* was performed. (*C*) Identical to *B* except that 3T3 cells were covered with plastic lids during the UV treatment. E:T ratio, effector-to-target cell ratio.

cells (5–30%), and it is likely that many more die over time when injected into the ectopic (s.c.) location. Therefore, we favor the explanation that the adjuvant activity observed in populations of untreated cells is arising from injured and dying cells.

Tumor and virally infected cells generally cannot initiate specific immune responses by directly presenting their antigens to T cells (21, 22). Instead, antigens associated with these cells must be taken up and presented by professional APCs. Presumably, as the tumor or infected cells die they are rapidly engulfed by phagocytic APCs, and their antigens are presented on both MHC class I and class II molecules (11–13). The particulate antigens used in our study are believed to mimic this process (23). However, APCs that have simply acquired cellular antigens may not be able to stimulate strong immune responses and may even induce tolerance (13, 20). These cells must be stimulated to migrate, mature, process and present antigen, and provide costimulation (24). Adjuvants can stimulate these processes and promote the generation of productive immune responses. However, many viruses and tumors have no known source of adjuvant to promote immunity.

The release of endogenous adjuvant would allow the immune system to respond to any antigens that are present and prevent tolerance. This process could help to explain how immune responses are generated to pathogens that lack adjuvants. Moreover, the responses that would be generated would be more vigorous. Furthermore, because we find that endogenous adjuvants promote the generation of immune responses to concentrations of antigen that are more than 100-fold lower than usually required, they may be particularly important to the generation of immune responses when antigen is limiting, e.g., early in infections. Because endogenous adjuvants may be present in all cells, and cell damage and death occur in most pathological situations, the release of these activities would provide a very general mechanism to alert the immune system to danger and promote the generation of immune responses. It also needs to be considered that the release of adjuvant activities from injured cells might be involved in the pathogenesis of some autoimmune diseases.

This model may help to explain earlier reports that OVA expressed in pancreatic islet cells was either ignored or induced tolerance of OVA-specific CD8 T cells (25). However, if the islet cells were killed, then the bone marrow-derived APCs stimulated the OVA-specific CTLs (25). Although it was considered that this was caused by the more efficient uptake of OVA by APCs, our data suggest that the release of cytosol from the damaged cells would also provide an adjuvant effect to support the generation of CTLs. Moreover, because the islet cells were killed through apoptosis (by CTLs), this should have increased the amount of endogenous adjuvant activity released. Similarly, gene therapy that induces cell injury and death of a fraction of tumor cells has been reported to induce strong and protective immune response (26–29). The release of endogenous adjuvants along with particulate antigen from the dying tumor cells may underlie the generation of immunity.

There was already limited evidence that cell injury and death could affect some aspects of immune surveillance, but in ways that are distinct from, but potentially complementary to, what we now report. Specifically, the antigens in stressed or dying cells were reported to be preferentially taken up by dendritic cells through phagocytosis (11, 13). Also, macrophages have been described as acquiring antigen from heat shock protein-peptide complexes (30). It has also been observed by some (12), but not other labs (14), that when dendritic cells are exposed to necrotic cells they are stimulated to mature and become more immunostimulatory. These previously described phenomena are clearly distinct from the present report because they are acting locally at the site of cell damage, whereas we observe a systemic effect. Moreover, the earlier phenomena act by enhancing the uptake and presentation of antigens from the dying cells themselves, whereas the effects we report are not related to the presentation

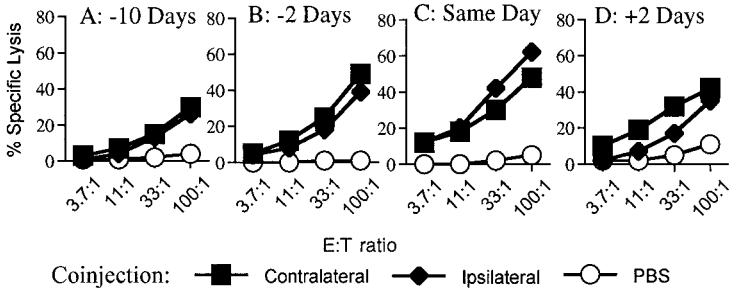

Fig. 7. The adjuvant effect is systemic and long lasting: 10^6 emetine-treated GL261 cells were injected either 10 days (-10 days) before, 2 days (-2 days) before, at the same time, or 2 days after the injection of 5 μg BioMag/OVA beads. The OVA beads and emetine-treated cells were either injected into the same flank (ipsilateral) or the opposite flank (contralateral). CTL priming was evaluated 9 days after injections as described in Fig. 1*A*. E:T ratio, effector-to-target cell ratio.

of specific antigens from the damaged cells. Finally, the previous studies found that only necrotic cells enhanced responses, whereas we find that both necrotic and apoptotic cells have adjuvant activity. However, the adjuvant effect we describe, although distinct, should augment immune stimulation through these other mechanisms. In fact, the activity we describe might have practical applications as an adjuvant for enhancing immune responses to vaccines.

Our data provide strong support for some aspects of the "danger hypothesis" that was proposed on theoretical grounds by Matzinger (9, 10). Specifically, our findings directly support the central concept that necrotic cell death is always abnormal and would alert the immune system to "danger". Our findings do not support the further prediction that apoptotic cells would not signal the immune system. We find that the amount of adjuvant activity in apoptotic cells is increased about 10-fold in some cells. It was proposed that apoptotic cells would not provide a danger signal because programmed cell death occurs in many nonpathological situations. However, many pathological processes, such as viral infections, induce apoptosis and, therefore, the release of a danger signal from apoptotic cells could be useful. It remains to be determined whether cells that undergo physiological programmed cell death up-regulate and/or release endogenous adjuvant. Because different stimuli can lead to apoptosis through different pathways (31–33), it will be interesting to determine whether these different forms of apoptotic death (i.e., physiological or pathological, transcription-dependent, or transcription-independent) lead to increased or decreased adjuvant activity.

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