Apoptosis-dependent Subversion of the T-Lymphocyte Epitope Hierarchy in Lymphoma Cells

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ABSTRACT

Tumor cells undergoing programmed death are an attractive source of tumor-associated antigens, and evidence is available for their therapeutic efficacy in vivo when used either alone or in association with dendritic cells. However, little is known about the specificity of the immune response induced by such antigen formulation. Indeed, activation of specific proteases during apoptosis may influence the cytoplasmic degradation of proteins and the generation of CTL epitopes. We show here that injection of C57BL/6 mice either with RMA lymphoma cells induced to apoptosis or bone marrow-derived dendritic cells pulsed with apoptotic RMA cells, a specific and protective CTL response is induced, which, however, is not directed against the immunodominant CTL epitope gag85–93. Lack of in vivo expansion of gag85–93-specific CTL in vaccinated mice is attributable to the apoptosis-dependent loss of gag85–93 in dying tumor cells. Indeed, we found loss of gag85–93 in RMA, MBL-2, and EL-4G+ lymphoma cells, which share gag85–93 as an immunodominant CTL epitope, induced to apoptosis by UV irradiation, mitomycin C, doxorubicin, or daunorubicin. This phenomenon appears to be caspase-dependent, because caspase inhibition by N-benzoyloxycarbonyl-Val-Ala-asp-fluoromethylketone prevents apoptosis of lymphoma cells and loss of gag85–93. Therefore, subversion of the epitope hierarchy in apoptotic tumor cells might be relevant in the induction of tumor-specific T-lymphocyte responses.

INTRODUCTION

Induction of apoptosis and clearance of apoptotic bodies by scavenger cells is a very efficient mechanism to physiologically dispose unwanted cells without causing inflammation and immunomodulated reactions (1). However, recent reports suggest that apoptotic cells may retain immunogenicity (1), which leads to the activation of immune effector cells and under certain circumstances is involved in the pathogenesis of immune-mediated diseases (2, 3). Furthermore, the demonstration that chemotherapy and radiation treatment often elicit apoptotic death of malignant cells (4), leading to the induction of antitumor effector cells, has prompted several investigators to use apoptotic tumor cells as a source of antigens for active vaccination (e.g., 5, 6).

Professional APCs, like DC, and macrophages, can uptake, process, and present antigens derived from apoptotic cells to CD8+ and CD4+ T lymphocytes (7, 8). Indeed, the in vivo injection of DC loaded previously with apoptotic tumor cells in vitro often elicits a CTL-mediated protective immune response (9, 10).

Apoptosis appears to be implicated in the modification of the antigenic content of dying cells (1–3). As an example, apoptosis causes the redistribution of intracellular autoantigens into cell surface blebs, which can detach as apoptotic bodies (11) and can be phagocytosed by APC. Of interest is the recent finding that during apoptosis triggered by cytotoxic lymphocyte granules, unique antigenic fragments are generated (12). In addition to causing generation of new epitopes, it is plausible that apoptosis might also cause the loss of dominant T-cell epitopes, subvert the epitope hierarchy of the cell, and perhaps lead to the generation of less effective antitumor responses.

In previous studies, we found that immunization of C57BL/6 mice with Apo-RMA (13) cells, or with DCs, which have phagocytosed Apo-RMA cells, elicited a protective CTL response, which was, however, less efficient than the one induced by live NR-RMA cells (9). Therefore, we sought to determine whether the mechanism(s) involved in the decreased efficiency in CTL generation by the presentation of antigens derived from apoptotic tumor cells, as compared with live cells, was related to an apoptosis-dependent subversion of the epitope hierarchy of the cell.

Here we show that the induction of apoptosis of RMA cells is associated with loss of the immunodominant virus-derived CTL epitope gag85–93 (14) leading to a less efficient CTL response. This phenomenon is common also to other two cell lines, MBL-2 (15) and EL-4G+ (16), known to be infected with a virus of the FMR family, which share gag85–93 as immunodominant CTL epitope (14). Furthermore, loss of gag85–93 is a common feature of apoptosis induced by UV irradiation, mit-c, or by some active agents against lymphomas like doxo and dauno (17). In addition, loss of gag85–93 appears to be caspase-dependent, because caspase inhibition blocks apoptosis induced by such treatments and prevents loss of gag85–93.

MATERIALS AND METHODS

Mice and Cell Lines. C57BL/6 (H-2b) female mice (Charles River, Calco, Italy), 8–10 weeks old, were housed in a specific pathogen-free animal facility and treated in accordance with the European Union guidelines. Experiments were approved by the Institutional Ethical Committee. RMA (H-2b) is a Rauscher virus-induced thymoma (13). EL-4 is a 9,10-dimethyl-1,2-benzanthracene-induced thymoma (H-2b; 18). EL-4G+ is a variant of EL-4 (16) known to be infected by and to express surface antigens of FMR-type viruses (14). MBL-2 is a Moloney Leukemia virus-induced lymphoma (H-2b; 15). RMA-ova cells were obtained from the transfection of the truncated OVA cDNA into RMA cells (10). Cell lines were cultured in RPMI 1640 supplemented with penicillin-streptomycin and 10% FCS. DCs were prepared from bone marrow as described previously (19). On days 5–7 of culture with recombinant granulocyte macrophage colony-stimulating factor and interleukin-4 (1000 units/ml; Pharmingen, San Diego, CA), nonadherent cells were used for in vitro cytotoxicity assays or mice immunization (4 intradermal injections of a total of 2 × 106 DCs unpulsed or pulsed with 1 μg of gag peptide; 19).

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The abbreviations used are: APC, antigen presenting cell; CTL, cytotoxic T lymphocyte; DC, dendritic cell; Apo-RMA, apoptotic RMA; NR-RMA, nonreplicating RMA; mitomycin C, mit-c; doxorubicin, doxo; daunorubicin, dauno; FMR, Friend-Moloney-Rauscher; z-vad-fmk, N-benzoyloxycarbonyl-Val-Ala-asp-fluoromethylketone; PI, propidium iodide; HPLC, high-performance liquid chromatography; mAb, monoclonal antibody; TCA, trichloroacetic acid; MS, mass spectrometry.
Apoptosis Induction and Cell Sorting. Apoptosis was induced by mit-c (100 μg/ml for 30’ at 37°C) or UV irradiation (UV G30W, for 30’ at a distance of 10 cm), followed by culture at 3 x 10⁶/ml for 8–72 h in RPMI 1640 containing 0.1% FCS. Alternatively, cells were cultured for 48 h in the presence of dixo (5 μg/ml) or dauno (2 μg/ml). To inhibit capasase activity, z-vad-fmk (Enzyme Systems Products, Livermore, CA) was suspended in DMSO at 10⁻³ M. RMA cells were incubated at 10⁶/ml in RPMI 1640 supplemented with 1–50 μM z-vad-fmk for 1 h at 37°C before treating them with the different drugs. The induction of apoptosis was verified according to morphological, biochemical, and cytometric features, as described (7). Early Apo-RMA cells were sorted by FACStarPlus after UV irradiation, 8–10 h after culture, and staining with annex V and PI (7). NR-RMA cells were obtained by treatment with mit-c (100 μg/ml for 30’ at 37°C), washed three times in PBS, and used immediately after. Mice were injected once i.p. with NR- or Apo-RMA cells, as described previously (9).

Generation of MHC Class I Tetramers and Cell Staining. Synthetic peptides gag₈₅₋₉₃ (CCLLTVFL) and LCMV gp33 (KAVYNFATM) were purchased from Research Genetics (Huntsville, AL). H-2-D¹-gag and H-2-D¹-gp33 complexes were refolded as described previously (20). The MHC complexes were quickly frozen in liquid nitrogen and stored at −80°C. A new set of tetramers were made fresh for every staining by mixing biotinylated MHC class I complexes with streptavidin-PE ( Molecular Probes, Eugene, OR) at a 4:1 molar ratio. For FACS analysis, mononuclear cells were isolated from splenocytes on a lympholyte-M gradient (Cedarlane, Hornby, Ontario, Canada) and additionally enriched in CD₈⁺ cells by magnetic beads sorting, following the manufacturer instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells (1 x 10⁸) were resuspended in PBS with 2% FCS. Fc receptors were blocked by incubating the cells with the anti-CD16/32 mAb (PharMinGen). Without additional washing, cells were incubated with the Cy-conjugated anti-CD8 antibody (PharMinGen) plus the indicated tetramer complex for 45 min at +4°C. Tetramers were used at a nonspecific mAb concentration of 250 ng/million cells.

In Vitro CTL Induction. Spleen cells from mice at day 14 after vaccination were resuspended in RPMI 1640 containing 10% heat-inactivated FCS, 50 μM of 2-mercaptoethanol, 2 mM of l-glutamine, 10 mM of HEPES, 1 mM of sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin, and coinoculated with 3 x 10⁶ NR-RMA cells at an effector to stimulator ratio of 10:1 or 1 μM of gag₈₅₋₉₃ peptide. After 4 days of culture, blasts were isolated on a lympholyte-M gradient (Cedarlane), cultured for an additional day in medium supplemented with 20 IU/ml human recombinant interleukin 2, and tested for cytolytic activity in a 4-h ⁵¹Cr release assay. For induction of the 9-4-99 CTL line, blasts from spleen cells of mice vaccinated with NR-RMA cells (9) were weekly restimulated with the synthetic peptide gag₈₅₋₉₃ (1 μM) pulsed on irradiated spleen cells in culture medium. After 4–5 in vitro restimulations, >90% of the cells were CD₈⁺ and Vα₃/Vβ₅.1, as depicted by FACS analysis with Vα₃/Vβ₅.1-T-cell receptor-specific mAbs (PharMinGen). To obtain OVA₂₅₇₋₂₆₄-specific CTL, spleen cells of OT-1 mice (21) were stimulated in vitro with 1 μM of OVA₂₅₇₋₂₆₄ peptide, as described above. The characteristics of the gag₈₅₋₉₃-specific CTL clones 48 and 76 are described (22).

Cytotoxicity Assays. Clones and the 9-4-99 CTL line were tested for cytolytic activity in a standard 4-h ⁵¹Cr release assay (7). DCs at the 5-6 day of culture were incubated overnight with a 1:5 ratio of apoptotic tumor cells, as described previously (9), before ⁵¹Cr labeling. Alternatively, DC or EL-4 cells were ⁵¹Cr labeled and pulsed with 1 μM of gag₈₅₋₉₃ or OVA₂₅₇₋₂₆₄ or HPLC fractions dissolved in DMSO for 30’ at 37°C. In cold target inhibition assays, unlabeled inhibitor tumor cells were seeded together with labeled RMA (10⁶/well) cells at ratios of 3:1 to 100:1. Apo-RMA induced CTL were then added at Effector/Hot target ratios of 25:1. The percentage of inhibition by cold targets was calculated as follows: % inhibition = 100 x (1 – % lysis of CTL with cold targets/% of lysis of CTL in the absence of cold targets). In inhibition experiments using mAb, target and effector cells were preincubated at 37°C for 15’ with anti-K⁺, anti-D⁺, or anti-CD4 mAb (PharMinGen), respectively, before addition to the cytotoxicity assay at an E/T ratio of 50:1.

HPLC and MS Analyses. HPLC separations were performed on a reverse phase Nucleosil 300-S column C18 (250 x 4.6 mm; Machery-Nagel, Duren, Germany) using a Jasco HPLC (Jasco Spectroscopic Co., LTD, Tokyo, Japan) equipped with a single pump (mod. 880-PU) and a ternary gradient mixer (mod. 880-2). The UV detector (Jasco, mod. 875-UV/VIS) was set at 210 nm.

The column was equilibrated with 5% aqueous acetonitrile containing 0.1% trifluoroacetyl. Bound proteins were eluted with a gradient from 5% to 95% acetonitrile over 25 min, starting 5’ after sample injection (flow rate, 1 ml/min). The synthetic peptide gag₈₅₋₉₃ (50 μg) was dissolved in 10% DMSO and 90% TCA solution (10%) in methanol. Live, NR-, or Apo-RMA cells (10 x 10⁶) were pelleted and resuspended in 700 μl of TCA. Each preparation (50 μl) was injected into a C18 column. Fractions were collected manually from 19’ to 23’, dried using a SpeedVac System (Savant), and stored at −20°C. Purified fractions were used for cytotoxicity assays or analyzed by micro-LEC-EEL-MS. Dried fractions were dissolved with 20 μl of methanol and injected into a WakoSil C18 column (150 x 1 mm; 5 μm; 120A; GE, Rome, Italy), directly connected to the ion source of the MAT95, or with a Jupiter C18 column (150 x 0.5 mm; 5 μm; 300A; Phenomenex, Torrance, CA), directly connected to the ion source of a LCQ-Deca. The column was eluted for 2’ with 10% of acetonitrile, containing 0.25% of trifluoroacetyl, followed by a linear gradient from 10 to 80% over 12 min (flow rate, 10 μl/min). Products ions of ion at m/z 1012 were recorded.

RESULTS

Immunization with NR-RMA Cells Induces a Vigorous gag₈₅₋₉₃-specific CTL Response. Expansion of gag₈₅₋₉₃-specific CTL in the lymphoid organs of NR-RMA-vaccinated mice is detectable by staining with Vα₃/Vβ₅.1-T-cell receptor-specific mAbs after in vitro restimulation but not ex vivo (23, 24). Soluble tetramers of MHC class I molecules complexed to defined CTL epitopes are a very accurate and sensitive means to visualize antigen-specific T lymphocytes ex vivo (25). Therefore, we used D¹⁰/gag₈₅₋₉₃ and the irrelevant D⁰/gp33 tetramers to follow the fate of gag₈₅₋₉₃-specific CD₈⁺ CTL in the spleen of mice vaccinated i.p. 2 weeks before with either NR-RMA (5 x 10⁶) cells or Apo-RMA (10⁵) cells. As seen in Fig. 1, a and b, a significant expansion of gag₈₅₋₉₃-specific CD₈⁺ T lymphocytes was only observed in NR-RMA-vaccinated mice. In sharp contrast, the percentage of D¹⁰/gag₈₅₋₉₃ and CD₈⁺ cells in the spleens of mice vaccinated with either Apo-RMA cells (Fig. 1c) or

Fig. 1. Only the vaccination with NR-RMA cells induces the in vivo expansion of CD₈⁺ T lymphocytes specific for the tetramer D¹⁰/gag₈₅₋₉₃. The panels depict one representative experiment, where enriched CD₈⁺ splenocytes from mice injected with 2 weeks before with either NR-RMA (a) and Apo-RMA cells (c and d) were analyzed by FACS with a Cy-conjugated anti-CD₈ mAb and PE-conjugated D¹⁰/gag₈₃ (a and c) or D⁰/gp33 (b and d) tetramers, and analyzed by FACS. The average ± SE of the percentage of double-positive cells in three independent experiments were as follows: a, 8.8 ± 2.1; b, 1.7 ± 0.5; c, 1.5 ± 1.0; d, 1.2 ± 0.3.

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PBS (not shown) was comparable with D9/gp33+ CD8+ cells (Fig. 1d), suggesting that only vaccination with live RMA cells elicits a gag85–93-specific CTL response. This lack of expansion of gag85–93-specific CTL correlates with the immunogenic potential of the two vaccines. Indeed, all of the mice vaccinated with 5 × 105 NR-RMA cells were protected by a challenge with a lethal number of live RMA cells (9). Conversely, 20-fold more Apo-RMA cells (i.e., 106) were needed to obtain a tumor-specific CTL response and some protection (i.e., 29%) of the vaccinated mice (9). Of relevance, NR- and Apo-EL-4 cells (i.e., an apparently nonrelated H-2b lymphoma) were not able to induce in vivo expansion of RMA-specific CTL and protection against a lethal challenge of live RMA cells (9, 23).

Vaccination with Apo-RMA Cells Elicits a Less Efficient CTL Response, Which Is Not Directed against gag85–93. To additionally characterize the specificity of the CTL induced by vaccination with Apo-RMA cells, splenocytes from vaccinated mice were tested in a standard cytotoxicity assay 5 days after in vitro restimulation with RMA cells. Although these CTL recognized and killed RMA cells (Fig. 2a), they were less efficient than CTL from NR-RMA-vaccinated mice (Fig. 2b). Furthermore, Apo-RMA-induced CTL did not kill EL-4 cells pulsed with the synthetic peptide gag85–93 as well as unpulsed EL-4 cells (Fig. 2a) and the natural killer targets YAC-1 (not shown). This was the case also when restimulation was performed with gag85–93 (Fig. 2, c and d). To additionally substantiate the specificity of the immune response induced in mice vaccinated with Apo-RMA cells, a cold-target inhibition assay was performed. Killing of RMA by Apo-RMA-induced CTL was inhibited by cold RMA cells and not by cold EL-4 cells unpulsed or pulsed with gag85–93 (Fig. 2e). Moreover, in antibody blocking assays, RMA killing by Apo-RMA-induced CTL was inhibited by anti-Kb and anti-D b antibodies, and not by anti-CD4 antibodies (Fig. 2f); therefore, suggesting that the cytolytic activity induced in mice by vaccination with Apo-RMA cells is MHC class-I restricted and CD4-independent.

Lack of Expansion of gag85–93-specific CTL on Vaccination with Apo-RMA Cells Does Not Depend on the Phase of Cell Death. A limitation of the experiments shown above is that late Apo-RMA cells (i.e., cells triggered to apoptosis 36–48 h before) were used. This was done because both UV irradiation and mitogen treatment cause an asynchronous induction of programmed death in RMA cells (7), which results in survival of small amounts of live cells during the first 24 h on treatment. Therefore, any detectable immune response could be elicited by contaminating viable cells (9) and not be the result of vaccination with apoptotic cells. Conversely, 36–48 h after apoptosis induction, all of the cells are in advanced apoptosis (7). However, the long culture time may result in apoptosis-independent cleavage of gag85–93. Moreover, in vivo cells committed to apoptosis rapidly expose cell surface signals and are swiftly recognized and phagocytosed by scavenger and neighboring cells (1). To overcome this experimental limitation, we sorted annexin V+PI − Apo-RMA (early Apo-RMA) cells 8–10 h after UV light exposure (Fig. 3a). After cell sorting, all of the cells were annexin V+PI −/PI − Apo-RMA (Fig. 3b). Tumor-specific CTLs were induced when mice were injected with 105 early Apo-RMA cells and not with 5 × 105 cells, as shown previously for late Apo-RMA cells (9; Fig. 3, c and d, respectively). However, RMA-specific CTL did not kill EL-4 pulsed with gag85–93 (Fig. 3c). Therefore, these results indicate that late and early Apo-RMA cells have similar antigenic and immunogenic potential.

Inability to Generate gag85–93-specific CTL Responses on Vaccination with Apoptotic Cells Is Not Attributable to Impairment in APC Function, Is Not Restricted to RMA Cells, and Is Associated with Several Proapoptotic Stimuli. Despite the lack of expansion of gag85–93-specific CTL vaccination with Apo-RMA cells still elicited CTLs specific for RMA (Figs. 1 and 2), suggesting, therefore, that professional APCs in vivo are fully capable to efficiently present antigens other than gag85–93 from Apo-RMA cells.

The ability of DCs to efficiently phagocytose apoptotic cells and process/present their antigens was confirmed by the finding that DCs pulsed with Apo-RMA cells expressing the antigen ovalbumin (Apo-RMA–OVA; 10) were efficiently killed in vitro by CTLs specific for the K b-restricted epitope OVA257–264 (Fig. 4d).

To confirm in vivo that DCs may efficiently uptake and process/present antigens other than gag85–93 from apoptotic cells, mice were injected once with DCs pulsed either with gag85–93 or Apo-RMA cells, and their splenocytes restimulated in vitro with RMA cells. Both treatments allowed induction of RMA-specific CTL, but only the
vaccination with gag85-93-pulsed DCs caused the in vivo expansion of epitope-specific CTL (Fig. 4, b and c).

To investigate whether the lack of expansion of gag85-93-specific CTL was a feature restricted to Apo-RMA cells, apoptosis was induced in MBL-2 and EL-4G+ cells. The gag85-93-specific CTL 9-4-99, which lysed RMA and MBL-2 targets to the same extent, and EL-4G+ cells even more efficiently (Fig. 5a), did not kill DCs pulsed with apo-RMA, MBL-2, and EL-4G+ cells (Fig. 5b). Comparable results were obtained with the gag85-93-specific CTL clone 76 (Fig. 5c; 22), thereby demonstrating that the phenomenon is a common characteristic of apoptotic FMR-induced tumors.

We also verified whether proapoptotic stimuli other than mit-c were associated with lack of presentation of gag85-93 by DCs. Indeed, DCs that have phagocytosed RMA cells induced to apoptosis by UV irradiation, doxo, or dauno were not recognized by 9-4-99 CTL (Fig. 5d).

Apoptosis-induced Loss of gag85-93 and Subversion of the T-Lymphocyte Epitope Hierarchy. The inability of DCs, after phagocytosis of apoptotic FMR lymphomas, to present gag85-93, may well explain the lack of expansion of gag85-93-specific CTL in mice vaccinated with Apo-RMA cells (Figs. 1 and 2). This lack might be because of an altered antigen processing/presentation of apoptotic-cell-derived antigens by DCs, or to the loss of gag during programmed death. Therefore, we sought to characterize the antigenic content of Apo-RMA cells.

HPLC analysis allows the identification and separation of relatively small amounts of peptides in complex mixtures like tumor cell lysates (26). When the synthetic peptide gag85-93 was injected into a C18 column and analyzed by HPLC, dominant peaks within the fraction at 20' appeared (Fig. 6a), which were confirmed by MS analysis to be the peptide of interest (Fig. 6b). Furthermore, fraction 20' was recognized by 9-4-99 CTL when pulsed on EL-4 cells (Fig. 6f). RMA cells were left untreated (Fig. 6c) or treated with mit-c, and immediately after (NR-RMA; Fig. 6d) or 48 h later (Apo-RMA; Fig. 6e), lysed in TCA and analyzed by HPLC. A peak at 20' was evident at the HPLC analysis of untreated and NR-RMA cells, and not of Apo-RMA cells. The corresponding fractions were collected from each cell preparation and pulsed on EL-4 cells, or analyzed by MS. As expected, gag85-93-specific CTL killed EL-4 cells pulsed with fractions from untreated and NR-RMA cells, and not from Apo-RMA cells (Fig. 6f). Moreover, MS analyses showed the presence of gag85-93 in the fraction derived from NR-RMA cells and not from Apo-RMA cells (not shown), thereby demonstrating a selective loss of gag85-93 in Apo-RMA cells. Similar results were obtained with cell preparations from MBL-2 and EL-4G+ cells, as well as RMA cells induced to apoptosis by dauno and doxo (not shown).

Loss of the Immunodominant CTL Epitope Appears to be Caspase-dependent. Most of the proapoptotic stimuli lead to the activation of caspases, cysteine proteases involved in essential apoptotic signaling pathways (27), which might also be involved in antigen processing (2). To evaluate the direct involvement of caspases in the apoptosis-dependent loss of gag85-93, we treated RMA cells with the different proapoptotic drugs in the presence of the caspase inhibitor z-vad-fmk. As shown in Fig. 7, z-vad-fmk totally blocked apoptosis induced by mit-c (Fig. 7, a and b), doxo (Fig. 7, c and d),

Fig. 3. Lack of expansion of gag85-93-specific CTL on vaccination with Apo-RMA cells does not depend on the phase of cell death. RMA cells were induced to apoptosis by UV irradiation (a) and sorted by FACStar Plus (b) 10 h later. Early apoptotic cells, which expose phosphatidylserine for annexin V binding but do not allow incorporation of PI, are double positive and are depicted in the upper right quadrant of the two panels. Spleen cells from each mouse injected i.p. with 10×10^6 (c) or 5×10^6 (d) early Apo-RMA cells were cultured for 5 days in the presence of NR-RMA cells and tested in standard cytotoxicity assays. Curves represent the cytotoxicity against RMA ( ), EL-4 pulsed with gag85-93 ( ), and unpulsed EL-4 cells ( ). Data are means of triplicates of the percentages of specific 51Cr release at the indicated E:T ratios; bars, ± SE. The data shown is representative of at least two independent experiments.

Fig. 4. Inability to generate gag85-93-specific CTL responses on vaccination with Apo-RMA cells is not because of impairment in APC function. a, CTL obtained from OT-1 mice and restimulated in vitro with the synthetic peptide OVA 257-264 were tested in a 51Cr release assay against DCs after overnight culture with RMA-OVA cells induced to apoptosis ( ), RMA-OVA cells ( ), or DCs unpulsed ( ) or pulsed with the peptide OVA257-264 ( ). Vaccination with DCs pulsed with Apo-RMA cells elicits a CTL response, which is not directed against gag85-93. Spleen cells from mice vaccinated 2 weeks before with DCs pulsed with gag85-93 (b) or Apo-RMA cells (c) were collected and cultured for 5 days in the presence of NR-RMA cells and tested in cytotoxicity assays. Curves represent the cytotoxicity against RMA ( ), EL-4 pulsed with gag85-93 ( ); and unpulsed EL-4 cells ( ) of splenocytes from single animals. Data are means of triplicates of the percentages of specific 51Cr release at the indicated E:T ratios; bars, ± SE. Vaccination with unpulsed DCs did not generate a specific CTL response (data not shown). The data depicted in the panels are representative of three independent experiments.
and dauno (Fig. 7, e and f), thereby suggesting that loss of gag<sub>85-93</sub> depends on caspases.

To verify whether treatment with z-vad-fmk also prevented loss of gag<sub>85-93</sub>, RMA cells were treated with mit-c in the presence of z-vad-fmk. Later (8–10 h), dying cells were separated from live cells, lysed in TCA, and analyzed by HPLC. A peak at 20 mV was evident at the HPLC analysis of RMA cells treated with mit-c in the presence of z-vad-fmk (not shown). Even more strikingly, DC pulsed with HPLC fractions from those cells were recognized and killed by the CTL clone 48 (Fig. 8a). The results were confirmed by the gag<sub>85-93</sub>-specific CTL clone 48 (Fig. 8b; 22).

DISCUSSION

Our findings demonstrate that during apoptosis a dramatic subversion of the T-cell epitope hierarchy in lymphoma cells may occur, resulting in reduced immunogenicity and inability to expand in vivo gag<sub>85-93</sub>-specific CTL. Worth noting, the loss of gag<sub>85-93</sub> is not found in other forms of cell death like necrosis induced by exposure to TCA or rapid freeze-thaw cycles.

Loss of gag<sub>85-93</sub> during programmed cell death is supported by the finding that gag<sub>85-93</sub>-specific CTL cannot be visualized ex vivo in the spleens of mice vaccinated with Apo-RMA cells, and on in vitro specific restimulation, CTLs can be induced, which are specific for RMA and not for gag<sub>85-93</sub>. A similar phenomenon occurs on vaccination with DCs pulsed with Apo-RMA cells. Moreover, Apo-RMA-pulsed DCs do not become targets for gag<sub>85-93</sub>-specific CTL, and gag<sub>85-93</sub> is not found in the HPLC and MS analyses of RMA cells induced to apoptosis by several drugs. Finally, loss of the gag epitope seems to be a relatively early event during programmed death, because early and late Apo-RMA cells have similar antigenic and immunogenic potentials.

Apoptosis is an active cellular process, which requires de novo protein synthesis and gene transcription. Several catabolic hydrolases are activated during programmed cell death, which allow full development of the cell suicide. Caspases are responsible for a highly selected pattern of apoptosis-specific protein degradation (28). A direct involvement of caspases in the cleavage of gag<sub>85-93</sub> is unlikely because of the absence of aspartic acid residues, sites of selective cleavage by caspases (27, 28), within the sequence of the peptide and in the flanking regions. Conversely, the indirect role of caspases is...
strongly suggested by the fact that apoptosis and loss of gag_{85-93} are prevented by the caspase inhibitor z-vad-fmk.

Other enzymatic complexes are activated during apoptosis, like serine proteases, calpains, and proteasomes (28, 29). Many of those catalytic complexes may have, or institutionally have, as for the ubiquitin-proteasome machinery (30), a relevant role in the processing of intracellular protein and the generation of CTL epitopes. Increase, as well as decrease of ubiquitin expression has been reported in apoptotic cells, and proteasome inhibitors may stimulate or inhibit apoptosis as well (29). It is possible that the loss of gag_{85-93}, of which the processing/presentation is proteasome-dependent (31), is also because of an altered processing mediated by apoptosis-dependent modifications of the proteasome machinery.

The epitope gag_{85-93} resides within the leader sequence of the gag protein (14). The M_{r} 72,000 component of the signal recognition particle, of which the function is to facilitate the translocation of nascent transcripts from the ribosome to the endoplasmic reticulum, is cleaved during apoptosis (32). Worthy of note, cleavage of M_{r} 72,000 is prevented by caspase inhibitors (32). Therefore, it is tempting to hypothesize that loss of gag_{85-93} is linked to an impaired translation of the gag protein into the endoplasmic reticulum.

Other post-translational modifications of gag_{85-93} might drastically modify its immunogenicity, as has been described for other cysteine-rich epitopes (33).

During ontogenesis as well as during the normal tissue turnover, many cells undergo programmed death and are silently disposed by scavengers. In physiological conditions these events are supposed to generate ignorance/tolerance of the immune system against self-antigens contained in the dying cells (1, 3, 34). Also apoptosis of tumor cells during the first “silent” phases of neoplastic growth might cause tolerance against tumor-associated antigens.

It might also be that the context (absence or presence of danger signals) in which these antigens are presented by APC determines whether priming versus tolerance would ensue. Indeed, massive apoptosis, mediated by chemotherapy and/or radiotherapy, occurring in an inflammatory milieu, might favor the activation of a tumor-specific immune response (1, 3, 34).

Finally, apoptosis may differ depending on the proapoptotic stimulus (1, 2) that may drastically influence the intracellular protein synthesis and degradation (2, 12, 28). Therefore, it could be hypothesized that during the physiological cell death of normal and neoplastic cells, tolerance occurs against antigens different from the ones released during apoptosis induced by chemotherapeutic agents and radiation, as well as during other forms of cell death.

Although our findings demonstrate a subversion of the T-lymphocyte epitope hierarchy in FMR virus-induced tumors, apoptosis-dependent loss of CTL epitopes does not appear to be a universal phenomenon. Indeed, we and others have found that known CTL epitopes are conserved in apoptotic cells (8, 10, 35). However, Berard et al. (35) recently analyzed DCs by confocal microscopy after phagocytosis of human melanoma cells expressing the antigens gp100 and Melan A/MART-1. They reported that, whereas the gp100 antigen was present in the cytoplasm of DCs that had phagocytosed apoptotic melanoma cells, the Melan A/MART-1 epitope recognized by the A103 antibody was lost. Similarly, Labarriere et al. (36) found loss of Melan A/MART-1 in two human melanomas undergoing programmed death.

In conclusion, during apoptosis a dramatic subversion of the epitope hierarchy might occasionally and selectively occur for different antigens and in different neoplastic tissues. A relevant question that stems from our results is whether subdominant epitopes are “promoted” during this apoptosis-dependent editing to become more immunogenic. Additional studies are warranted to fully understand the molecular bases of such a mechanism and to clarify the role of apoptosis in the shaping of the antigen-specific immune response.

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REFERENCES

Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22–24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 891; substitute for the last paragraph:

The data in Table 8 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O2 consumption, it is seen that the amount of glucose "cleavage products" exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if M = +0.27 and L = -0.16 and the normal differential is 65 per cent M and 35 per cent L, then

\[
0.65(0.27) + 0.35(-0.16) = +0.12
\]

a figure identical to the observed +0.12 for normal leukocytes.
Apoptosis-dependent Subversion of the T-Lymphocyte Epitope Hierarchy in Lymphoma Cells

Paola Castiglioni, Alfonso Martin-Fontecha, Gabriella Milan, et al.


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