EXPERIMENTAL CELL RESEARCH 215, 23–27 (1994)

Interferon-γ (IFN-γ) Induces Programmed Cell Death in Differentiated Human Leukemic B Cell Lines

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Interferons (IFNs) are cytokines that exert an antiviral effect on target cells and possess immunomodulatory and antitumor properties. In this study we have investigated the effects of human recombinant IFN-γ on human leukemic B cell lines at different stages of maturation. Our data show that in Burkitt's lymphoma RAMOS-1 B cells IFN-γ induces a reduction of cell growth and a clonal selection via programmed cell death; in contrast, IFN-γ treatment of KM-3 pre-B cells does not induce biochemical and morphological changes as shown by electron microscope analysis and DNA gel electrophoresis. © 1994 Academic Press, Inc.

INTRODUCTION

Several cytokines, including interferons (IFNs), act on a variety of immune and nonimmune cells, inducing changes in their proliferation and differentiation. Cytokines can alter gene expression by modulating transcription factors that regulate specific genes [1].

Human interferon-γ, or immune interferon, is a polypeptide of 20–25 kDa, produced by T lymphocytes following stimulation with mitogens xenoantigens or alloantigens [2, 3]. It has been shown that activated B cells are able to stimulate *in vitro* the production of IFN-γ by NK cells [4]. In addition to its anti-viral activity, IFN-γ has been shown to enhance TNF cytotoxicity [5] and to induce MHC class I and II expression in target cells [6–8]. Pleiotropic effects of IFN-γ also consist in the upregulation of T cell activating protein in resting murine T cells [7–9], induction of B cell differentiation, enhancement of Ig secretion by resting B lymphocytes, and inhibition of some IL-4-induced B cell responses [7–14]. These findings suggest that IFN-γ plays an important role in the regulation of the immune response [14, 15].

Cell death may occur via two different pathways: necrotic death, caused by interference with cellular metabolism, and apoptotis, in which an active biochemical pathway leads to cell death [16, 17]. Apoptotic death has been widely studied and recognized as an important pathway for clonal deletion in the immune system [18]. Since IFN-γ is thought to be a mediator of apoptotic signals in a model of tolerance acquisition [19, 20], in this study we have evaluated the effects of human recombinant IFN-γ on two human leukemic B cell lines at different stages of differentiation, in order to detect changes in chromatin structure and DNA cleavage related with the process of apoptosis that eventually produces clonal selection.

MATERIALS AND METHODS

Cell cultures. The terminal deoxynucleotidyl transferase (TdT)-positive human pre-B cell line KM-3 described by Schneider *et al.* [21] is a common acute lymphoblastic leukemia that has undergone rearrangements both of heavy and light chain immunoglobulin genes and that consequently is arrested at the stage of pre-B cell. The TdT-negative cell line RA-1 (Ramos, ATCC CRL 1596) is an EBV-negative Burkitt lymphoma in which B lymphocytes express surface-associated mu and kappa chains. Cell lines were maintained in continuous suspension culture in RPMI 1640 medium supplemented with 10% FBS, 4 m*M* L-glutamine, 100 m*M* Na pyruvate, and 25 m*M* Hepes. Cells were grown at 2.5×10^5/ml, and were more than 98% viable as determined by trypan blue dye exclusion. During the log growth phase, cells were treated with 500 units/ml of human recombinant IFN-γ for a time ranging between 0 and 48 h. Cell growth was evaluated both as total cell number and by [^{3}H]thymidine uptake, 48 h after culture with IFN-γ.

DNA extraction. DNA was extracted with chloroform/isoamyl alcohol (24/1) and precipitated with 2.5 vol of absolute ethanol. Ten micrograms of DNA/sample were loaded into a 1.8% agarose gel, run at 20 V, and observed under uv light.

Morphological analyses. Treated and untreated KM-3 and RA-1 cells were fixed with 1.25% glutaraldehyde in cacodylate buffer, pH 7.6, for 1 h at 4°C, postfixed with OsO_4, stained overnight with uranyl acetate, and processed for electron microscope analysis.

[^{3}H]Thymidine uptake. DNA synthesis in KM-3 and RA-1 cells was measured by adding 2 μCi of [^{3}H]thymidine (6.7 Ci/mmol) for the final 6 h of the culture period. Cells were harvested, and [^{3}H]-thymidine uptake was measured in a liquid scintillation beta-counter.

Immunocytochemistry. The immunocytochemical localization of TdT in KM-3 TdT-positive cells was carried out as described previously [22]. Briefly, cytocentrifuge preparations were fixed for 45 min at room temperature with 1% paraformaldehyde. Samples were incubated with a rabbit polyclonal anti-TdT antibody for 30 min and in-

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0014-4827/94 $6.00

FIG. 1. Electrophoretic analysis of DNA from IFN-γ-treated (lane A) and untreated (lane B) KM-3 cells that do not show the presence of DNA fragmentation. Analysis of IFN-γ-treated (lane C) and untreated (lane D) RA-1 cells. The ladder-like pattern of DNA banding indicating apoptosis is evident only in IFN-γ-treated RA-1 cells.

cubated with a goat anti-rabbit IgG peroxidase-conjugated antibody. To detect sites of antibody reaction, a solution of 0.05% diaminobenzidine tetrahydrochloride containing 0.015% H_2O_2 in 0.05 *M* Tris–HCl buffer, pH 7.6, was used.

Enzyme assay. TdT activity was determined as described previously [23]. Briefly, cells (2×10^7), washed with RPMI 1640 medium, were lysed in 250 μl of 0.3 *M* KP_i, pH 7.4, by freeze–thawing. The lysate was cleared by centrifugation at 15,000 rpm for 30 min at 4°C and aliquots were assayed using 0.5 m*M* d(pA)50, as initiator, 5 m*M* [^{3}H]dGTP (50–150 cpm/pmol) as substrate, 8.0 m*M* $MgCl_2$, 1.0 m*M* β-mercaptoethanol, 0.1 mg/ml BSA, and 0.2 *M* potassium cacodylate, buffer, pH 7.5. The reaction products were scored as acid-insoluble radioactivity on glass fiber filters. One unit of enzyme has been defined as 1 n*M* dGMP incorporated in 60 min at 36°C.

Fluorescence-activated cell sorter analysis. Treated and untreated KM-3 and RA-1 cells were stained for 30 min with Dr, CD10, CD20, and CD25 monoclonal antibodies at 1:400 dilution and revealed using goat anti-mouse FITC-conjugated antibody. Quantitative fluorescence analysis of stained cells was performed on a FACStar^{plus} flow cytometer (Becton–Dickinson, Mountain View, CA).

Source of materials. Human recombinant IFN-γ with a specific activity of 2.5×10^7 unit/mg was from Boehringer Mannheim (Mannheim, Germany). Rabbit polyclonal antibody against 32-kDa calf thymus TdT protein was produced as described [24]. Monoclonal antibodies recognizing Dr, CD10, CD20, and CD25 antigens where from Becton–Dickinson (Mountain View, CA). All cell lines culture and reagent grade materials were from Sigma (St. Louis, MO). Reagents for electron microscopy were from Polyscience (Warrington, PA). Radiolabeled materials were from Amity PG (Geneva, Switzerland).

RESULTS

Human recombinant IFN-γ induces apoptosis of RA-1 cell as shown by DNA electrophoresis and by the typical features of apoptosis recognizable by morphological analysis. Agarose gel electrophoresis of DNA from IFN-γ-treated and untreated cell lines displays the ladder-like pattern of DNA banding only in treated RA-1 cells (Fig. 1, lane C). There is no evidence of DNA banding in treated (lane A) and untreated (lane B) pre-B KM-3 cells and in untreated RA-1 cells (lane D). [^{3}H]-Thymidine uptake (Fig. 2) shows that IFN-γ treatment exerts an anti-proliferative effect in RA-1 cells but not in KM-3 cells. The results (data not shown) of the immunocytochemical analysis and the specific TdT activity do not differ between IFN-γ-treated and untreated TdT-positive KM-3 cells; more than 98% of treated and untreated cells are TdT positive with 98 U/ml of specific enzymatic activity. Morphological analysis (Fig. 3) does not show significant differences between untreated (Fig. 3C) and treated KM-3 cells (Fig. 3D), but, in contrast, reveals a significative presence, more than 35%, of apoptotic cells evident in treated RA-1 (Fig. 3B). RA-1-untreated cells do not show evidence of programmed cell death. (Fig. 3A). Electron microscopy analysis of IFN-γ-treated RA-1 cells shows all of the features of programmed cell death (Fig. 4). The ultrastructure of untreated RA-1 cells is shown in Fig. 4A. Treated samples allow identification of the earliest apoptotic features showing the nuclear chromatin condensed in masses abutting on the nuclear envelope and displaying characteristic crescent appearance (Fig. 4B). Different cells display a progression of apoptotic process and we have noted typical nuclear changes and subplasmalemma densities in the cleavage furrows (Fig. 4C). Some cells show nuclear fragmentation with the formation of apoptotic bodies. In Fig. 4D we demonstrate the late stage of programmed cell death, showing the nucleus broken into a number of discrete apoptotic bodies; micronuclei, surrounded by plasmalemma with roughly spherical or ovoid shape, were often released. IFN-γ is able to induce in RA-1 cells changes of phenotype along B-cell lineage, as demonstrated by flow cytometric analysis. For this purpose, we show a noticeable increase of CD20 antigen after 48 h of treatment (Fig. 5). KM-3 cells do not show significant changes of phenotype during IFN-γ treatment.

DISCUSSION

The differentiation of B lymphocytes proceeds through a number of well-characterized steps from

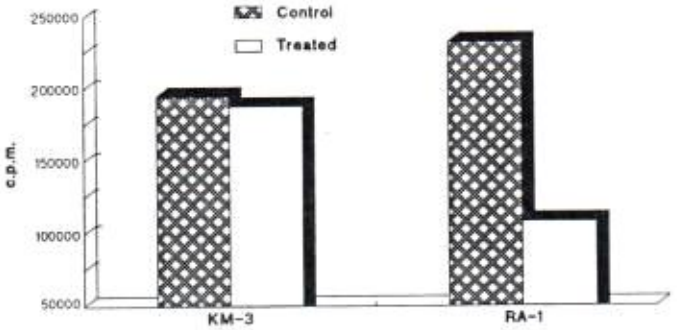

FIG. 2. [^{3}H]Thymidine uptake in IFN-γ-treated and untreated KM-3 and RA-1 cells. After 48 h of IFN-γ treatment RA-1 cells display a significant inhibition of cell growth. The cpm is defined as the mean of triplicate wells from four different experiments ± SD.

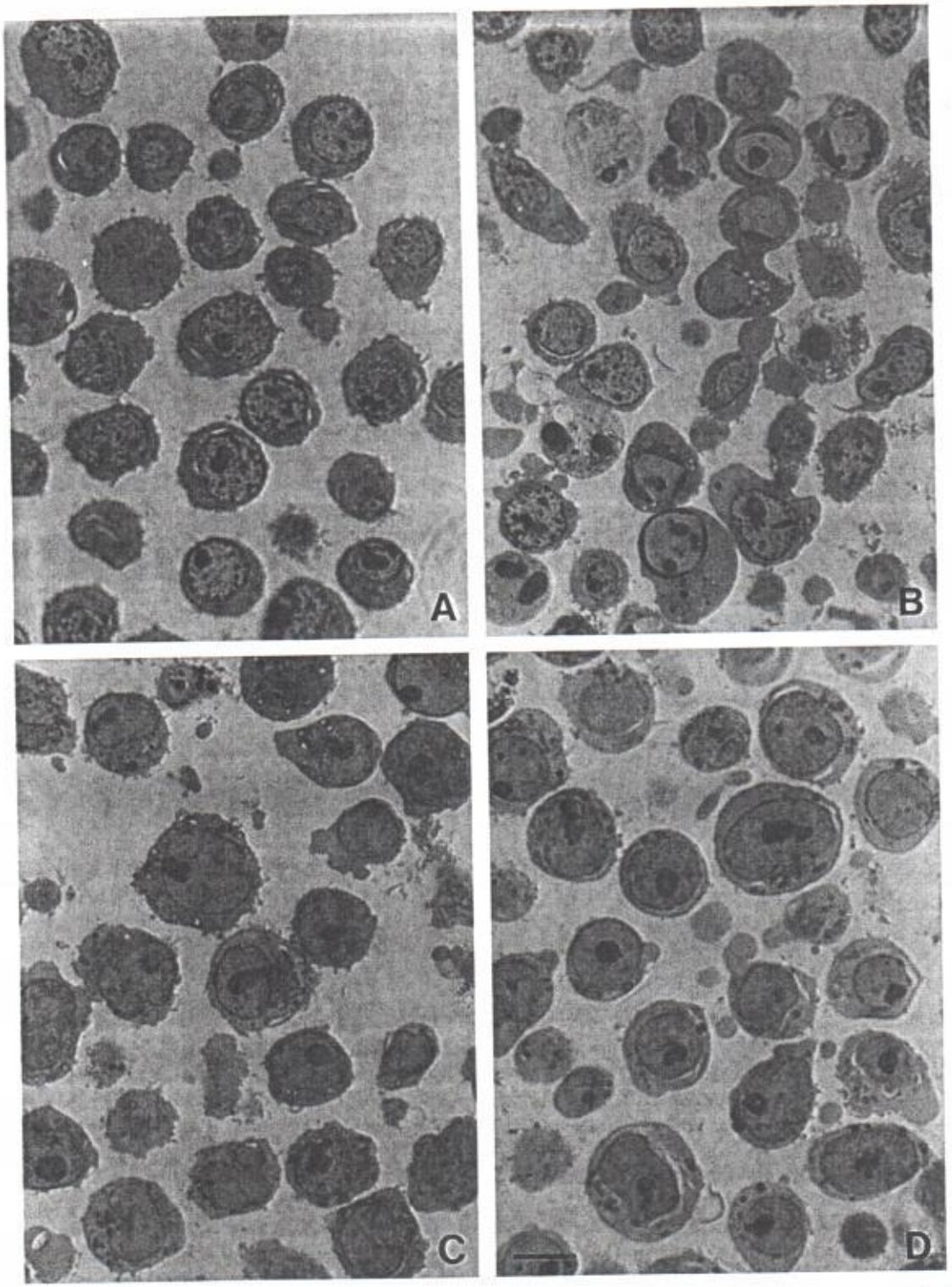

FIG. 3. Morphological analysis of untreated (C) and treated (D) KM-3 cells does not show structural differences. Typical features of programmed cell death are shown in IFN-γ-treated RA-1 cells (B) but not in untreated cells (A). Bar, 10 μm.

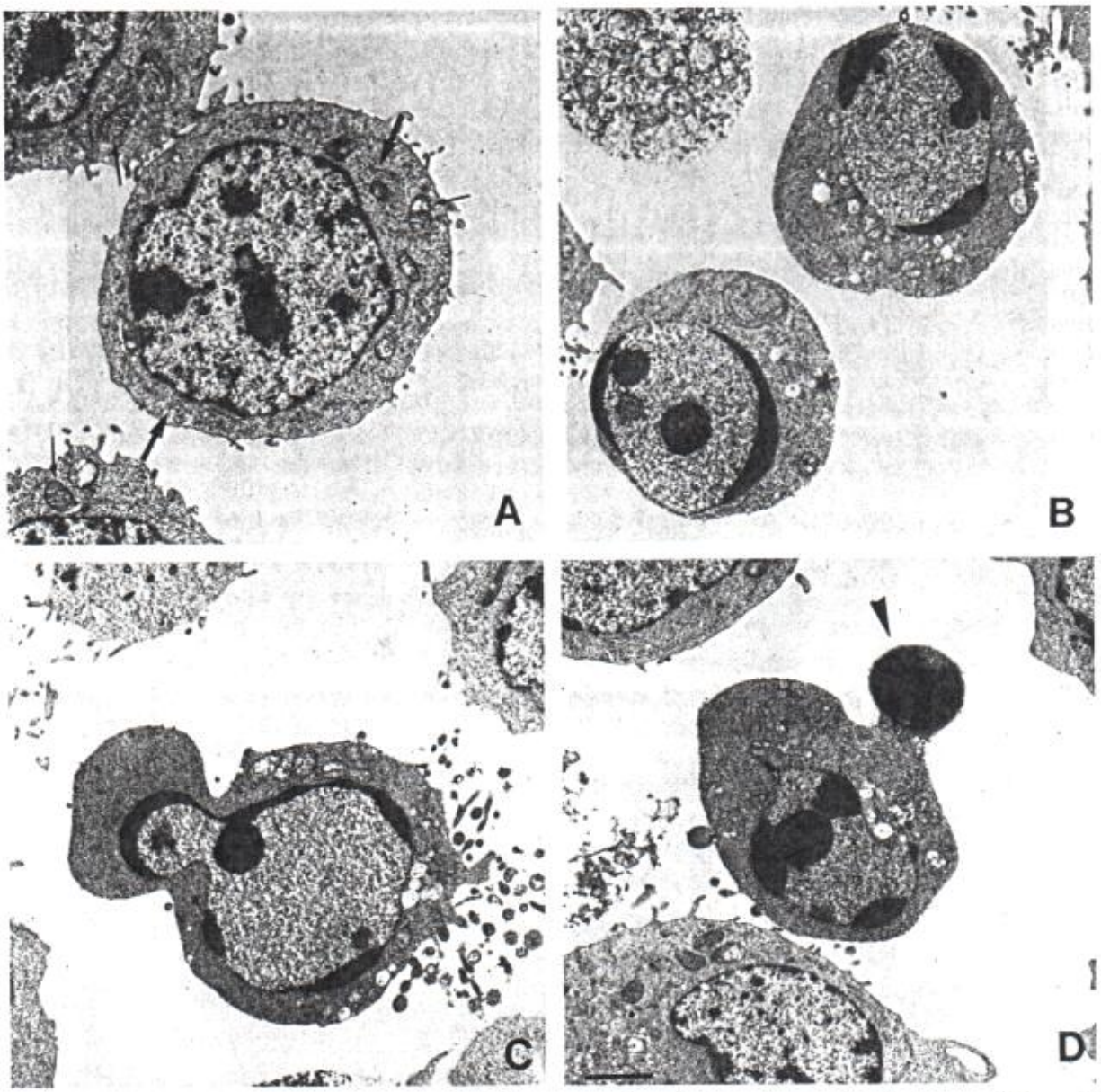

FIG. 4. Electron microscopic analysis of RA-1 cells. (A) Ultrastructural morphology of untreated cells. Arrows, RER; small arrows, mithocondria. (B, C, D) Various morphological features of programmed cell death as described under Results. Note the condensed chromatin around the nuclear periphery. Arrowhead, micronucleus. Bar, 3 μm.

pluripotent stem cells to Ig-secreting plasma cells. Several factors have been shown to mediate, at the various levels, the process of B cell differentiation. IFN-γ exerts immunoregulatory effects and *in vivo* interaction of IFN-γ and B cells may occur without the involvement of other cell types or signals that induce terminal B cell differentiation. Affinity maturation of antibodies in response to T-cell-dependent antigens is mainly attributable to modification of the immunoglobulin variable region sequence by somatic mutation operated by TdT activity [25]. TdT is a DNA polymerase present only at the pre-T and pre-B stages during immunopoiesis [26]. In the bone marrow, the phenotypic analysis of B lymphocyte precursors and the study of immunoglobulin gene expression in the corresponding leukemic cells indicate that B cells derive from TdT-positive precursors and that the development of immature B cells is associated with acquisition of cytoplasmic and surface IgM. IFN-γ, at late stages of B cells differentiation, is able to drive non-Ig-secreting to actively-secreting cells [9]. Germinal centers in lymph nodes represent the site of B cell exposure to antigens and specific immunoglobulin

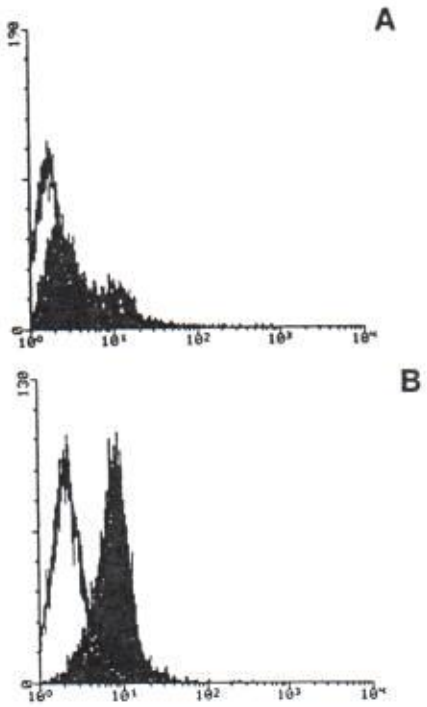

FIG. 5. Expression of CD20 antigen as analyzed by flow cytometry. (A) Untreated cells. (B) IFN-γ-treated cells evaluated after 48 h of incubation. We report histograms of KM-3 cells (white area) and RA-1 cells (black area). Cell number is indicated on the ordinate and fluorescence intensity, in arbitrary units, is indicated on the abscissa.

production and, at this level, a mechanism of clonal selection occurs in order to ensure that high-affinity antibodies are produced. B cells producing low-affinity immunoglobulins or self-reactive clones, are eliminated by programmed cell death [4, 13, 14, 27].

The phenotype changes, the antiproliferative and apoptotic effect of IFN-γ shown in this study, may depend on the maturational stage of target cells. Our results, in accordance with those of Granwunder *et al.* [27] showing that IFN-γ inhibits cell proliferation and induces apoptosis of normal pre-B cells suggest that clonal selection occurs by a mechanism of programmed cell death that occur in B cells during the late stages of the maturation process. In TdT-negative RA-1 B cells, IFN-γ selects cells via apoptosis. TdT-positive KM-3 pre-B cells do not respond to IFN-γ, probably because at this early stage of differentiation cells do not rearrange immunoglobulin genes and therefore a clonal selection mechanism does not seem essential. These findings may contribute to the understanding of the opposing influence of IFN-γ in B cell development at the stage of transition from common/pre-B (TdT-positive) to Ig secreting (TdT-negative) B cells.

Received March 1, 1994
Revised version received July 1, 1994

The authors express their gratitude to Professor Carlo E. Grossi for the critical reading of the manuscript. This study was supported by Italian MURST and CNR grants.

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