Lysis by Activated Lymphocytes of Melanoma and Small Cell Lung Cancer Cells Surviving in Vitro Treatment with Mafosfamide

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ABSTRACT

Six short term-cultured melanoma cell lines and one small cell lung cancer cell line were treated in vitro with the alkylating agent mafosfamide. The sensitivity of the surviving cells to in vitro lysis by recombinant interleukin 2-activated autologous and allogeneic lymphocytes was then investigated. In no case did chemo-surviving tumor cells appear less sensitive to lymphocyte-mediated lysis than untreated counterparts. In three of seven cases (two of which were derived from the same patient), chemo-selected cells were even more sensitive to cytotoxic lymphocytes, a difference not explained by a different distribution of neoplastic cells in the various cell cycle phases. We also studied the inhibitory activity of activated lymphocytes on the clonogenic potential of chemo-surviving tumor cells by the human tumor clonogenic assay. Inhibitions of tumor cell growth in the two patients tested were 100 and 94%, respectively; the activity of lymphocytes was dependent on the coculture time and the effector/target cell ratio. These data indicate that in vitro treatment with mafosfamide does not select cells resistant to the action of activated lymphocytes and that, given the right experimental conditions, these immune effectors can completely lyse tumor cells.

INTRODUCTION

It is well known that lymphocytes from many cancer patients activated in vitro with interleukin 2 or by allostimulation can efficiently lyse autologous tumor cells (1-4).

Many experimental models have also been developed to assess the efficacy of the infusion of activated lymphocytes (adoptive immunotherapy) in tumor-bearing animals. The results of these experiments show that a high percentage of animals can be cured provided the tumor burden is small and a large number of effector cells is infused (5-7); often the addition of a cytotoxic drug to the treatment schedule is needed to achieve optimal results (8). Similar data have been obtained in nude mice xenografted with human tumors and treated with autologous and allogeneic alloactivated lymphocytes (9). Based on these premises, some clinical trials of adoptive immunotherapy have begun and the preliminary results appear promising (10-12). However, since adoptive immunotherapy can be effective in the presence of a relatively small number of cancer cells (6, 7, 13), a rational clinical application of adoptive immunotherapy could be capable, in conjunction with other treatments such as chemotherapy, of eradicating tumor burden.

At present chemotherapy represents the most commonly used treatment modality in medical oncology. In fact, some tumors are permanently cured by chemotherapy; for many others, however, the existence of drug-resistant tumor cells determines whether the cytotoxic drugs will fail to eradicate the tumor (14, 15). Therefore, agents with a more specific antitumor activity and able to destroy surviving tumor cells are needed (16).

In an effort to provide an experimental basis for the combination of chemotherapy and adoptive immunotherapy, we have tested in vitro whether and to what extent human tumor cells surviving a treatment with the cytotoxic metabolite of a commonly used cytotoxic drug can be lysed by activated lymphocytes. Here we report that in six melanoma patients and one SCLC patient, tumor cells surviving in vitro treatment with the antineoplastic drug mafosfamide were lysed by activated lymphocytes to the same extent as untreated counterparts. Moreover, we show that activated lymphocytes can abrogate the in vitro clonogenic potential of tumor cells pretreated with mafosfamide.

MATERIALS AND METHODS

Mafosfamide. This drug (formerly AZ 7557) was kindly given by Dr. Peter Hilgard (Hasta Werke, Bielefeld, Federal Republic of Germany); it was dissolved in sterile water at 1 mg/ml and filtered immediately before using.

Patients. Six melanoma (stages II-IV) patients and one SCLC (stage IV) patient entered this study. None of the melanoma patients had received prior chemotherapy while the SCLC patient had been treated with 6 cycles of cyclophosphamide-Adriamycin-vincristine chemotherapy. The code numbers for melanoma patients are 1906, 4039, 8427, 0151, 2125, and 7301; the SCLC patient code is 0004. For patient 4039 two samples were simultaneously obtained from different anatomical sites and were designated 4039-2 (right supraclavicular node) and 4039-3 (right axillary node). In the case of cells treated in vitro with mafosfamide the code of the patient is followed by the dose of the drug used (for example 4039-3/2 µg or 4039-3/20 µg).

Tumor Cells. Surgery specimens were treated mechanically as described previously (17); cell suspensions contained more than 80% of viable tumor cells (as evaluated by trypan blue exclusion and routine staining techniques). In all experiments with melanoma cells, primary or less than fourth passage cultures were used; SCLC cells were xenotransplanted and grown in Swiss athymic (nude) mice before being cultured in vitro. All cultures were checked for Mycoplasma contamination by electron microscopy and were found to be negative.

In Vitro Treatment with Mafosfamide. Tumor cells resuspended in RPMI 1640 (MA Bioproducts, Walkersville, MD) plus 1% FCS (Flow Laboratories, Irvine Ayrshire, United Kingdom) were incubated at 37°C with 2, 20, or 200 µg/ml of mafosfamide; after 60 min cells were washed twice, resuspended in RPMI 1640 plus 10% FCS and antibiotics, and seeded in 35-mm plates at 1.5 x 10⁶ cells/plate. After 7-10 days cells were used for ³¹Cr release assay, cytofluorography, or coculture with activated lymphocytes.

PBL Separation and ³¹Cr Release Assay. Mononuclear cells were obtained from peripheral blood by Ficoll centrifugation. Monocytes were removed by plastic adherence for 90 min. Nonadherent cells were seeded with 50 units/ml of rIL-2 (Biogen SA, Geneva, Switzerland) for 6 days or with irradiated (4000 rads) allogeneic lymphocytes pooled from 6 healthy donors for 2-3 days followed by culture in rIL-2 (50 units/ml) for 3-4 days. Cell concentration at the beginning of cultures was 1 x 10⁶ responding cells/ml; the stimulation was performed in 15-mm wells (Costar, Cambridge, MA) or in small flasks (Corning, Cambridge, MA) maintained in an upright position. The medium used was RPMI 1640 (MA Bioproducts) plus 10% FCS, 2 mM glutamine (MA Bioproducts), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

1 The abbreviations used are: SCLC, small cell lung cancer; PBL, peripheral blood lymphocytes; rIL-2, recombinant interleukin 2; E/T, effector/target cell ratio; HTCA, human tumor clonogenic assay; SR, spontaneous release; TR, total release.

Received 10/13/86; revised 1/5/87; accepted 2/16/87.

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1 This work was partially supported by "Associazione italiana per la ricerca contro il Cancro" of Milan, Italy.

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acid buffer (MA Bioproducts), penicillin (100 units/ml; Farmitalia, Milan, Italy) and streptomycin (100 \( \mu \)g/ml; Farmitalia). Allogeneic PBL were obtained from normal single donors and were processed as described for patients' PBL. When used after the 6th day of culture, lymphocytes were maintained in culture with rIL-2 (50 units/ml). The \( ^{31} \)Cr release assay was performed as described previously (17); briefly, \( ^{31} \)Cr-labeled tumor cells were admixed at various ratios (1/100 to 1/10; 3 replicates for each ratio) with lymphocytes in 96-well plates and incubated at 37°C. After 16 h aliquots of supernatants were collected and radioactivity was counted. Results were expressed as

\[
\text{% of specific release} = \frac{\text{cpm sample} - \text{cpm SR}}{\text{cpm TR} - \text{cpm SR}} \times 100
\]

TR was obtained by adding 100 \( \mu \)l of Nonidet P-40 detergent (BDH, Poole, United Kingdom) to the incubation wells. SR was always less than 35%.

Cytofluorography. It was performed using a 30L cytofluorograph (Ortho Instruments, Raritan, NJ) as described previously (18). The percentage of cells in the cell cycle phases was calculated by the method of Baiss et al. (19). For each sample 50–100 \( \times 10^3 \) cells were counted.

Tumor-Lymphocyte Coculture. Treated and untreated tumor cells were seeded in 15-mm Costar wells (15 \( \times 10^5 \) cells/well) with RPMI 1640 plus 10% FCS and left for 2 h at 37°C in a humidified 5% CO\(_2\) atmosphere to permit adherence to the plastic. Activated autologous lymphocytes (7.5 \( \times 10^5 \)/well) plus rIL-2 (50 units/ml) and nonactivated autologous lymphocytes (7.5 \( \times 10^5 \)/well), or rIL-2 alone (50 units/ml) were then added and the cultures were continued for 24–72 h. Control wells contained tumor cells only. At the end of coculture, the media of two wells containing activated PBL plus rIL-2 were replaced without adding rIL-2. Lymphocytes thus died in 3–4 days and culture was continued for up to 100 days to allow regrowth of surviving tumor cells.

HTCA. After tumor-lymphocyte coculture, medium was removed and cells were detached by treatment with 0.05% trypsin/0.02% EDTA (Flow Laboratories). The cells in control wells (tumor alone) were counted and the cell concentration of all samples was adjusted on the basis of this value. (Tumor cell count in wells containing rIL-2 or nonactivated lymphocytes did not differ significantly from controls, while in wells containing activated lymphocytes the counts were very low and often no tumor cells were noted.) HTCA was performed according to the micromethod of Thompson et al. (20). Briefly, an underlayer (bottom medium) of 100 \( \mu \)l well of 0.5% agar in Ham's F-10 medium (MA Bioproducts) containing 10% FCS, penicillin (100 units/ml), and streptomycin (100 \( \mu \)g/ml) was seeded in the 60 interior wells of 96-well microtiter tissue culture trays (Costar), while the 36 outside wells were filled with 0.3 ml of Ham's F-10 to preserve humid atmosphere to permit adherence to the plastic. Activated autologous lymphocytes (7.5 \( \times 10^5 \)/well) and rIL-2 (50 units/ml) in 10 medium (MA Bioproducts) containing 10% FCS, penicillin (100 units/ml), and streptomycin (100 \( \mu \)g/ml) was seeded in the 60 interior wells of 96-well microtiter tissue culture trays (Costar), while the 36 outside wells were filled with 0.3 ml of Ham's F-10 to preserve humid culture. The plating layer (top medium) consisted of 0.3% agar in the same medium as the underlayer, with freshly added insulin (Sigma Chemical Co., St. Louis, MO; 1.54 units/ml), glutamine (Flow Laboratories; 0.45 \( \mu \)g/ml), pyruvate (Sigma; 0.34 \( \mu \)g/ml), and mercaptoethanol (0.77 mm). For each sample 5 wells were seeded with 2000 tumor cells/well in 80 \( \mu \)l of top medium. After 14–16 days the number of multicellular growth units (>2 cells) was scored as suggested by Meyskens et al. (21).

For each patient tested and for each drug concentration, decreasing numbers of control tumor cells were seeded in order to determine the sensitivity of the method.

Statistical Analysis. All statistical comparisons were made by one way variance analysis. Multiple comparisons were performed with the Dunnet test. \( P < 0.01 \) was considered to be statistically significant.

RESULTS

In Vitro Activity of Mafosfamide. This drug has been selected since it represents a cytotoxic metabolite of one of the most used antineoplastic drugs, cyclophosphamide, and is highly active in vitro (22). To evaluate the growth-inhibitory activity of mafosfamide on melanoma and SCLC cells, cultures of such cells were exposed to increasing amounts of the drug. It is evident from Fig. 1 that the percentage of reduction of tumor cells after treatment with 2–200 \( \mu \)g/ml of mafosfamide ranged from 28% (4039; from 110 \( \times 10^4 \) to 80 \( \times 10^4 \) at 2 \( \mu \)g/ml) up to 100% (0004 and 151 at 200 \( \mu \)g/ml), in a dose-dependent fashion. With 20 \( \mu \)g/ml it was impossible to continue the culture since the few cells still viable 7–10 days after treatment died during further culture. With 20 \( \mu \)g/ml at least 1 log reduction in the number of cells was obtained in 4 of 6 samples, but the remaining cells were able to regrow. With 2 \( \mu \)g/ml a limited reduction (±50% in 4 of 6 patients) was obtained. In conclusion, by using mafosfamide it was possible to reduce the number of tumor cells in a dose-dependent way and to select surviving cells for subsequent tests.

Lysis of Chemoselected Cancer Cells. For 7 tumors (6 melanomas and 1 SCLC) it was possible to compare the lysis of either untreated or mafosfamide-pretreated tumor cells exposed to rIL-2-activated autologous or allogeneic PBL. As reported in Table 1, in no case did treated cells appear less sensitive to killing by activated PBL than control cells; for melanoma 1906 a slightly reduced lysis of pretreated cells was apparent at lower E/T ratios, but the difference was not statistically significant. In 3 tumors (4039-2, 4039-3, 8427) treated cells were even more susceptible to lysis than their untreated counterparts in a dose-dependent fashion. This cannot be explained by a difference in cell viability after treatment with mafosfamide since the spontaneous releases of \( ^{51} \)Cr of both treated and control cells were similar. These results thus indicate that cells surviving drug treatment are not selected for resistance to the lytic effect by activated lymphocytes.

In order to evaluate different types of activation protocols, rIL-2-activated autologous PBL were compared with autologous PBL stimulated with irradiated allogeneic PBL plus rIL-2 (see "Materials and Methods"). As shown in Table 2 no major differences in the killing of both untreated and mafosfamide-pretreated target cells were noted between rIL-2-activated and allo/rIL-2-activated autologous PBL.

These results indicate that both types of protocols (rIL-2 or allo/rIL-2) are equally efficient in activating autologous effector PBL to kill tumor cells. Moreover, rIL-2-activated autologeneic PBL were as efficient as rIL-2-activated autologous PBL, as shown by their similar cytotoxic activity on pretreated tumor cells (Table 2).

Cytofluorographic Analysis. Since many cytotoxic drugs exert their activity in a particular phase of cell cycle, we investigated whether the higher tumor killing observed in some drug-treated cells by activated PBL could reflect a difference in the cell cycle phase distribution of tumor cells, possibly caused by mafosfamide. Three tumors were analyzed: one (1906) in which no...
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Table 1 Lysis of melanoma and SCLC cells surviving in vitro treatment with mafosfamide by autologous or allogeneic rIL-2-activated lymphocytes, respectively.

| Tumor cells were treated with various doses of mafosfamide and cultured in RPMI 1640 plus 10% FCS for 7–10 days. Then they were labeled with 51Cr and tested with IL-2-activated PBL. Values are expressed as percentage of specific lysis at E/T ratio of 100/1. |
|---|---|---|---|---|
| Patient | Dose (µg/ml) | % of specific lysis at E/T ratio of 100/1 | 50/1 | 25/1 | 10/1 |
| 1906 | 0 | 75 | 81 | 80 | 67 |
| | 2 | 69* | 67 | 73 | 71 |
| | 20 | 78* | 62 | 63 | 57 |
| | 200 | 75* | 68 | 59 | 58 |
| 4039-2 | 0 | NT | 17 | 8 | 7 |
| | 2 | 18 | 14* | 2 | 0 |
| | 20 | 49 | 52* | 40 | 21 |
| 4039-3 | 0 | 23 | 10 | 9 | 0 |
| | 2 | 35* | 35 | 18 | 4 |
| | 20 | 49* | 42 | 30 | 18 |
| 8427 | 0 | 13 | NTa | NT | 2 |
| | 2 | 23* | 20 | 16 | 12 |
| | 20 | 26* | 24 | 18 | 16 |
| 151 | 0 | 17 | 14 | 15 | 7 |
| | 2 | 29* | 28 | 23 | 17 |
| | 20 | 25* | 27 | 20 | 16 |
| 2125 | 0 | 49 | 34 | 31 | 14 |
| | 2 | 35* | 33 | 20 | 12 |
| | 20 | 38* | 28 | 16 | 13 |
| 0004 | 0 | 17 | 15 | 10 | 6 |
| | 2 | 16 | 23 | 9 | 11 |
| | 20a | 37* | 29 | 30 | 22 |
| | 20b | 33* | NT | NT | 18 |

* P > 0.01 in comparison with the respective control.
* P < 0.01 in comparison with the respective control.
a NT, not tested.
b Allogeneic PBL.

Table 2 Comparison of cytotoxic activity of rIL-2- or allo-IL-2-activated autologous PBL and rIL-2-activated allogeneic PBL.

| Tumor cells were treated with various doses of mafosfamide and cultured in RPMI 1640 plus 10% FCS for 7–10 days. Then they were labeled with 51Cr and tested against IL-2-activated PBL. Values are expressed as percentage of specific release. PBL were activated with rIL-2 or with allogeneic irradiated PBL plus rIL-2 (see Materials and Methods); all values reported are at an E/T ratio of 50/1. |
|---|---|---|---|---|
| Patient code | Dose of mafosfamide (µg/ml) | rIL-2 | Allogeneic PBL | rIL-2-activated allogeneic PBL |
| 4039-3 | 0 | 10 | 16.9a | 37.2a |
| | 2 | 34.8 | 23.6a | 30.1a |
| | 20 | 41.7 | 42.6a | 52.4a |
| 1906 | 0 | 81 | 69a | 61a |
| | 2 | 67 | 70a | 74a |
| | 20 | 62 | 57a | 64a |
| | 200 | 68 | 59a | NT|

a NT, not tested.

and Methods." At the end of tumor-lymphocyte coculture, 2 wells containing activated PBL and cancer cells were left in liquid medium without rIL-2 to permit the growth of tumor cells which survived the effect of cytotoxic lymphocytes. Activated PBL died in 3–4 days and were removed by subsequent medium changes; each well was cultured for up to 100 days. Tumor regrowth was never observed except for one well of 4039-3/2 µg in which 3 foci of melanoma cells appeared after 30 days. These cells (named 4039-3/2 µg S) were cultured for 2 additional months to allow further expansion and were then used as targets in a 51Cr release assay performed with autologous rIL-2-activated PBL. 4039-2/2 µg S cells were well lysed by activated autologous lymphocytes (43 and 13% specific lysis at E/T ratios of 100/1 and 10/1, respectively). This experiment therefore shows that those tumor cells which escaped lytic activity of autologous rIL-2-activated PBL were not intrinsically resistant to a subsequent exposure to the same cytotoxic effectors.

Effect of Tumor-Lymphocyte Coculture on the Clonogenic Potential of Cultured Melanoma Cells. A clonogenic assay was performed in two cases (2125 and 7301) to evaluate the number of clonogenic cells surviving the in vitro tumor cell-lymphocyte coculture. Preliminary experiments to assess the sensitivity of the assay, in which serially decreasing numbers of neoplastic cells were seeded, indicated that an approximately 2-log diminution in the number of cells plated (from 2000 to 31) produced few but discernible growth units (Fig. 2). In the first patient [2125] activated lymphocytes exerted a high cytotoxicity against the autologous tumor in the 51Cr release assay [49% at E/T 100/1 (Table 1)]. Table 4 shows that coculture of tumor cells with rIL-2 alone did not influence the number of growth units while nonactivated PBL slightly impaired the clonogenic capacity of tumor cells, although these effectors were not cytotoxic in the 51Cr assay (≤10% specific release at an E/T ratio of 100/1). Coculture of these melanoma cells for 3 days with rIL-2-activated autologous PBL at an E/T ratio of 50/1 abrogated the clonogenic potential of tumor cells. On the basis of the sensitivity of the HTCA, we can conclude that in this patient activated PBL eliminated at least 2 logs of untreated clonogenic melanoma cells. In this case we tested by HTCA the growth-inhibitory activity of activated PBL on melanoma cells pretreated with mafosfamide (Table 5); in both 2-µg- and 20-µg-pretreated cells the coculture completely inhibited the clonogenic potential of melanoma cells. The clonogenic capacity of 20-µg-pretreated cells was, however, lower than that of 2-µg-treated and control cells. In the second patient (7301) the cytotoxicity of autologous rIL-2-activated PBL on tumor
cells was lower in the $^{51}$Cr release assay (26% at E/T 100/1); this was paralleled by a lower inhibition of clonogenic potential of melanoma cells in HTCA, since a maximal inhibition of 94% was obtained (Table 4). In this second case it was also possible to compare the effects of 3 versus 1 day coculture time, the

**DISCUSSION**

Adoptive immunotherapy with in vitro-activated lymphocytes is now emerging as a possible new cancer treatment modality (23). A clear limitation of this approach, which may be overcome by the addition of a chemotherapeutic treatment, is represented by tumor burden. Thus, it is important to study the combination of adoptive immunotherapy, which does not require the full immunological competence of the host (23), with other anticancer therapies, mainly chemotherapy. In this study we have investigated whether low passage melanoma and SCLC tumor cells surviving in vitro treatment with the alkylating agent mafosfamide can be lysed by in vitro-activated autologous or allogeneic lymphocytes. Although in vitro treatment with a cytotoxic drug represents an artificial and simplified setting in comparison with the in vivo situation, this type of experiment can provide useful data on the possible interactions between drug-treated tumor cells and activated lymphocytes.

The results reported here clearly indicate that chemo-surviving tumor cells were not less sensitive to lysis by activated autologous and/or allogeneic PBL than their untreated counterparts. In some cases the drug treatment rendered the neoplastic cells even more sensitive to lymphocyte killing. At present we have no clear explanation for this increased sensitivity of chemo-surviving cells. However, treatment with cytotoxic drugs is known to induce the expression of new antigenic specificities on plasma membranes (24), some of which are possibly related to the drug-resistant phenotype (25). This fact could offer new target structures to activated PBL, thereby enhancing their cytotoxic activity. Alternatively, the drug may affect some other membrane characteristics (26), which may lead to a higher sensitivity to the lysis by activated lymphocytes. The only conclusion we can draw presently is that the increased lysis of mafosfamide-pretreated cells is not dependent on differences in the cell cycle kinetics.

Since some data suggest that allostimulation could trigger more types of lymphocyte subpopulations than IL-2 (27), we
evaluated the cytotoxic potential of lymphocytes activated by rIL-2 alone or by allogeneic irradiated PBL followed by rIL-2. We have found that lymphocytes activated by these protocols exerted similar cytotoxic and tumor growth-inhibitory activity against tumor cells in both the $^{51}$Cr release assay and the HTCA. We have also shown that different types of effector PBL (autologous versus allogeneic) can produce similar tumor cytotoxicity; this can have practical implications for trials with non-autologous PBL in which activated PBL could be prepared from normal donors.

Another important issue regarding adoptive immunotherapy and its combination with chemotherapy is how complete the lysis of tumor cells by activated PBL can be. In fact, by using the $^{51}$Cr release assay it is usually difficult to obtain cytotoxicity values of 90% or more, probably due to the relatively low sensitivity of the method and to the short incubation time required by this assay. Thus, the question, “Can activated PBL lyse 100% of tumor cells?” cannot be answered by using the $^{51}$Cr release assay only. For this reason we used the HTCA after coculture of tumor cells with activated PBL in liquid medium. This method has been shown previously to correlate with the $^{51}$Cr release assay, but with a higher sensitivity (28, 29). Our results indicate that HTCA appears to be a more sensitive test and that, given the right experimental conditions (high cytotoxicity of lymphocytes, sufficient coculture time, and E/T ratio), it is possible to obtain a 100% inhibition of tumor cell growth.

These data, together with the fact that tumor cells surviving the in vitro coculture with activated PBL remained sensitive to the cytotoxic activity of these immune effectors, suggest that it may be possible to destroy all tumor cells by cytotoxic PBL. Other data from melanoma clones (30) and from animal systems (23) tend to support this hypothesis. It is important to note that all tumors were used for HTCA within the first three culture passages, with the aim of retaining the original tumor heterogeneity as much as possible, since it is frequently lost in long term lines. Our data also permit an approximate evaluation of the number of tumor cells which could be killed by adoptive immunotherapy. In patient 2125, 100% inhibition of tumor growth was obtained; in this patient, 7.5 x 10$^5$ activated PBL could augment or diminish this value; for example, an in vivo expansion of the infused lymphocytes during in vivo rIL-2 administration could be beneficial, whereas a nonhomogeneous distribution of effector cells could determine greater tumor cytotoxicity in some organs, such as lungs where an initial preferential homing of lymphocytes has been shown to occur (31), but a lower or absent effect in other body sites.

In conclusion, we have shown that tumor cells from six melanoma and one SCLC, surviving an in vitro treatment with the antineoplastic drug mafosfamide, are not resistant to activated PBL; moreover, the clonogenic potential of chemosurviving melanoma cells can be abrogated by the same effectors.

These data could be useful in planning future trials of adoptive immunotherapy in conjunction with chemotherapy in the treatment of human neoplasms, in particular of those tumors which show a good initial response to chemotherapy but retain a high risk of recurrence.

ACKNOWLEDGMENTS

The authors wish to thank Professor Luciano Lombardi for electron microscopy examination, Drs. Marco Bregni and Salvatore Siena for helpful discussions, and Edoardo Marchesi for skillful technical assistance.

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