

The selective anticancer agent PB-100 inhibits interleukin-6 induced enhancement of glioblastoma cell proliferation *in vitro*

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Abstract. The multifunctional cytokine interleukin-6 behaves as a growth factor for various malignancies. It is produced in significant amounts by glioblastoma cells. When exogenous IL-6 is added (pg/ml) to culture medium of human glioblastoma cells and normal (non malignant) astrocytes used as controls, it exerts a dose dependent and differential effect on these two cell lines. Enhancement of cell proliferation is twice as high for glioblastoma cells as for astrocytes. *In vitro*, the novel anticancer agent PB-100 ($\mu\text{g/ml}$) dose dependently inhibits this stimulatory activity. In addition, increasing PB-100 concentrations finally induce death of the malignant cells, yet do not impede multiplication of normal astrocytes. PB-100 does not abolish IL-6 production by cells, but keeps its level down to physiological values. PB-100 should therefore find its place in therapies requiring control of IL-6 production.

Introduction

Interleukin-6 (IL-6) is a key multifunctional cytokine. It plays a part in a wide range of physiological processes, some of them normal, like regulation of hemopoiesis or T and B lymphocyte activation, but others pathological, such as induction and/or maintenance of inflammatory and autoimmune diseases as well as proliferation of a number of malignancies (1,2). IL-6 can be produced by many kinds of mammalian cells, generally in response to various activation factors, some of the most potent being IL-1, TNF and bacterial LPS (3). Monocytes/macrophages, endothelial cells and fibroblasts are major IL-6 producers (2).

In tumors such as myeloma (4), lymphoma or plasmocytoma (3), IL-6 behaves as an endogenous growth factor. Derived from the lethal and most frequent brain tumor,

glioblastoma, the SK-MG4 cell line also constitutively produces IL-6. Much of our recent research has focused on *in vitro* inhibition of the proliferation of a human BCNU-resistant glioblastoma cell line, U 251, using as a control a normal astrocyte cell line, CRL 1656. We demonstrated recently that our selective anticancer agent PB-100 efficiently inhibits glioblastoma cell multiplication (5).

In this study, using the above-mentioned malignant and normal cell lines, we first investigated IL-6 induced increase of cell multiplication, which is much higher for glioblastoma cells than for astrocytes. Then we demonstrated that this activity may be efficiently checked by PB-100.

Research described in this report was carried out in the light of our long-standing experimental results and theoretical views concerning the cancer process, and using the basic techniques which led to these findings (6). We briefly summarize the part relevant to the present report.

Fifteen years ago, after comparing numerous DNAs from malignant tissues and their normal counterparts, we came to the conclusion that the essential feature of cancer DNA, at the root of the characteristic behaviour of tumor cells, is its highly relaxed, destabilized secondary structure. This was demonstrated by measuring UV absorbance of DNAs at 260 nm, which is known to increase as more hydrogen bonds are broken inside the molecule. Cancer DNA UV absorbance was always distinctly higher than that of normal DNA. This hyperchromicity stands out as its fundamental character (6,7).

While strands of normal, i.e. non-cancer, DNA only separate locally and temporarily for replication or transcription, multiple and extensive areas of cancer DNA strands remain permanently separated as a result of H-bond breakage. This makes them easily accessible to numerous kinds of molecules, many of which can break still more H-bonds: for this reason these agents may be considered as carcinogenic. They form a long list comprising, besides exogenous chemical agents, a number of physiological molecules, such as steroid hormones.

On separated strand areas of cancer DNA, new initiation sites for replication and transcription become exposed in an uncontrolled fashion. This accounts for the increased proliferation and dysregulated protein synthesis of malignant tissues, gene expression being additionally disturbed as some genes are silenced by chain tightening on either side of relaxed areas (6). Experimentally, the high *in vitro* cancer DNA synthesis, and the resultant increase of *in vitro* and *in vivo* cancer cell multiplication, closely correlate with cancer DNA hyperchromicity.

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Abbreviations: IL-6, interleukin-6; PB-100, flavopereirine; DNA, deoxyribonucleic acid

Key words: astrocyte, glioblastoma cell, DNA, interleukin-6, PB-100 alkaloid

We were able to find a number of agents of natural origin, such as the plant-derived PB-100, which are specifically able to restabilize cancer DNA by bringing its strands back together into their normal position, thus closing its open chains. This is experimentally evidenced by decrease of cancer DNA UV absorbance, which is brought down to normal values, and by a matching decrease of *in vitro* DNA replication and of *in vitro* and *in vivo* cell multiplication.

Access to the non relaxed, closed chains of normal DNA is much more difficult; this makes the latter far less receptive both to destabilizing and restabilizing agents, although persistence and/or high doses of carcinogens may, on the long run, overcome the normal cell's defenses and finally induce malignancy. Our anticancer agents being unable to bind to normal DNA, they interfere neither with its replication nor with expression of its genes, and thus are not toxic for non-cancer tissues. Binding selectively to cancer DNA, they act specifically on malignant cells.

PB-100 thus had the makings of a good agent for inhibiting the stimulation of cancer cell multiplication by a physiological molecule such as IL-6 without upsetting balanced production of this cytokine by normal cells.

Materials and methods

Reagents. IL-6: Boehringer Mannheim (Mannheim, Germany). IL-6 EASIA: Medgenix Diagnostics (Fleurus, Belgium). Trypsin, RPMI 1640 culture medium, fetal calf serum, glutamine: Gibco (Grand Island, NY, USA). Other chemicals: Prolabo (Paris, France). PB-100 was purified in our laboratory.

Cell lines and culture techniques. The established human BCNU-resistant glioblastoma cell line U 251 was obtained from the Swedish Cell Collection (Uppsala, Sweden), and the normal astrocyte line CRL 1656 Mpf (cell repository line) from the American Type Culture Collection (Rockville, MD, USA). Cells were grown at 37°C in an incubator. Stock cultures were maintained by continuous passage in RPMI 1650 culture medium containing 10% fetal calf serum. Culture medium was renewed three days after seeding and thereafter twice a week. Absence of mycoplasma and bacteria was checked every three months. For experiments, each cell type was subcultured in 6-well tissue culture plates (9.8 cm² wells), starting from a 4x10⁴ inoculum. Cell viability was determined using trypan blue; it was 99-100% at start of experiments.

Cell growth inhibition and stimulation. Increasing concentrations of filtration-sterilized tested substances (IL-6 and PB-100) were added to the cell suspensions in the wells. After a 48 hour incubation, 0.05 ml/well of trypsin+0.05% EDTA were added; then, after a 5 minute incubation, cells were detached from the culture plates. Trypsinization was stopped using 4 ml of fresh RPMI 1640 per well. Cells were then counted with a Coulter counter. All experiments were performed three times, each time in triplicate.

Isolation of DNAs. DNAs from the U 251 glioblastoma cells and CRL 1656 astrocytes were isolated and purified by the

method described elsewhere (6). The amount of DNA was determined by measurement of weight and of DNA UV absorbance at 260 nm, then purity was checked by conventional methods (260/280=2.05) (8). Following incubation with 0.1 N KOH, DNAs exhibited a 45-52% hyperchromicity.

DNA UV absorbance measurement during experiments. DNA was dissolved in 0.01 M Tris-HCl buffer (pH 7.3). Increasing concentrations of substances under test were dissolved in 10 µl of the same buffer and added to both the blank and DNA solutions. Mixture was gently agitated at room temperature and its 260 nm UV absorbance was determined.

IL-6 assay. The enzyme amplified sensitivity immunoassay (EASIA) is based on the oligoclonal system which uses several monoclonal antibodies directed against distinct IL-6 epitopes. It is available as an immunoenzymetric assay kit for use in serum, plasma or culture medium.

Results

Effect of IL-6 on cell proliferation. In a first series of assays, we showed that IL-6 production by U 251 glioblastoma cells was over 8-fold that of CRL 1656 astrocytes; in parallel, growth rate of the glioblastoma cells was 30-40% higher than that of normal astrocytes. We found, moreover, that most of the cytokine was contained in the cell lysate, much less remaining in the culture medium. IL-6 content of glioblastoma cell lysate was 5-fold that of the medium in which the cells had grown; astrocyte IL-6 content was 4-fold that of medium (Table I).

This led to closer investigation of IL-6 effects on proliferation of glioblastoma cells and astrocytes *in vitro*. Addition of exogenous IL-6 to culture medium increased multiplication of both cell lines in a dose dependent, but differential way. Maximum increase, induced by 200 pg/ml IL-6, was 80% for glioblastoma cells, compared to 30-40% for astrocytes (Fig. 1). Maximum increase of DNA *in vitro* synthesis, obtained with 2400 pg/ml IL-6, was 250% for glioblastoma DNA and no more than 150% for astrocyte DNA (Fig. 2). These results are matched by UV absorbance values: maximum hyperchromicity, induced by the above IL-6 concentrations, was 39% for glioblastoma DNA and 29% for astrocyte DNA (Fig. 3).

When testing various mitogens, we consistently observed a similar discrepancy between the activities of these compounds on cell cultures and on isolated DNAs (7). In the living cell, mitogen activity is limited by numerous physiological regulation processes (some of which are mentioned below in Discussion), involving a coordinated response of a number of cell components. No such regulations protect isolated DNA, and straightforward mitogen activity can thus extend to much higher concentrations.

Effect of PB-100 on cell proliferation

In the absence of exogenous IL-6. Previous experiments, carried out in the absence of exogenously added IL-6, had

Table I. Endogenous IL-6 production by human glioblastoma (U 251) and normal astrocyte (CRL 1656) cell lines.

	IL-6 pg/ml	
	U 251	CRL 1656
Culture medium control	58.33	8
Cell lysate control	296.66	35
+PB 100 12.5 µg/ml		
Culture medium	12	7.5
Cell lysate	24.5	35.5
+PB 100 25 µg/ml		
Culture medium	14	17
Cell lysate	23	16.5

Human BCNU-resistant glioblastoma (U 251) and normal astrocyte (CRL 1656) cell lines were grown for 48 h at 37°C. Culture medium (2 ml) was separated from cells and 0.1 ml was used for IL-6 determination. Each type of cell (10^7 /well) was trypsinized, washed with HBSS buffer solution, centrifuged and resuspended in 2 ml of Tris-HCl, pH 7.5. Lauryl sulfate solution (0.15 ml of 20% solution) was used to lysate cells and 0.1 ml of lysate was used for IL-6 assay using Medgenix kit (see Methods). The results are mean values of three independent experiments (U 251: SD±7.49; CRL 1656: SD±4.58).

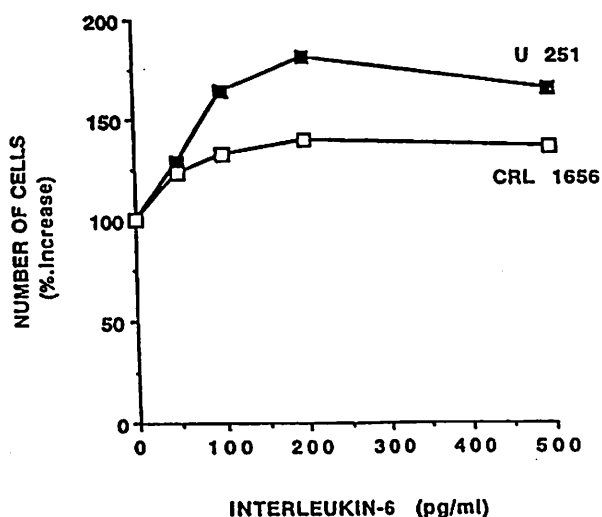


Figure 1. Both normal astrocyte (CRL 1656) and human BCNU-resistant glioblastoma (U 251) cell lines were grown in the absence and presence of indicated concentrations of interleukin-6. Numbers of cells (% increase) are mean values of three separate experiments. (U 251: SD±5.09; CRL 1656: SD±4.36).

shown us that increasing concentrations of PB-100 efficiently inhibit glioblastoma cell *in vitro* proliferation: using 100 µg/ml PB-100, all glioblastoma cells are killed (5).

Astrocyte multiplication is not decreased at all; it increases very slightly, probably due to the presence of some factor such as ATP in the culture medium, because synthesis

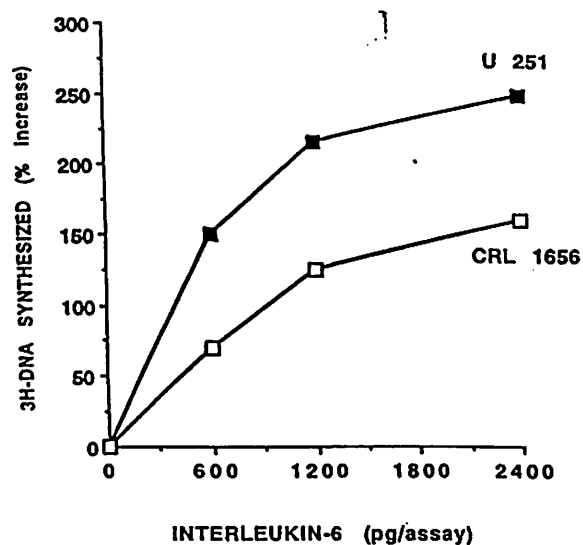


Figure 2. Effect of IL-6 on *in vitro* synthesis of DNA isolated from normal astrocyte (CRL 1656) and human BCNU-resistant glioblastoma (U 251) cell lines. DNA synthesis was measured by incorporation of ^3H -TTP into acid precipitable material as described elsewhere (5). Data are mean values of three separate experiments. (U 251: SD±9.35; CRL 1656: SD±13.77).

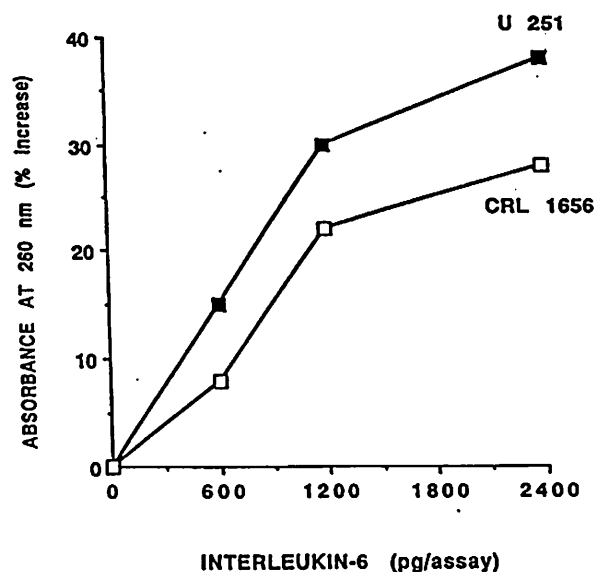


Figure 3. Effect of Interleukin-6 on UV absorbance of DNA isolated from normal astrocyte (CRL 1656) and human BCNU-resistant glioblastoma (U 251) cell lines. DNA (10 µg) was dissolved in Tris-HCl buffer solution, pH 7.3. Substances to be tested were dissolved in 1 ml of the buffer and added to the blank and to the DNA solution. Each mixture was gently agitated at room temperature and its UV absorbance at 260 nm was measured, for each different concentration of IL-6. Data are mean values of three separate experiments. (U 251: SD±3.27; CRL 1656: SD±1.36).

of astrocyte DNA is not increased by PB-100. In contrast, glioblastoma DNA synthesis is drastically inhibited (5). Correspondingly, PB-100 cancels glioblastoma DNA hyperchromicity, while astrocyte DNA UV absorbance remains unmodified (Fig. 4).

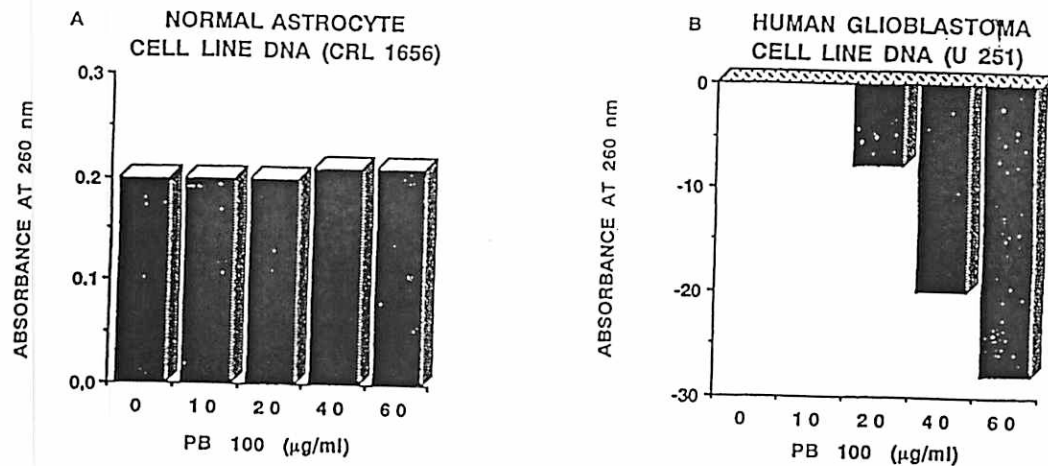


Figure 4. Effect of PB-100 on UV absorbance at 260 nm of DNA isolated from normal astrocyte (CRL 1656) and human BCNU-resistant glioblastoma (U 251) cell lines (5). See legend to Fig. 3.

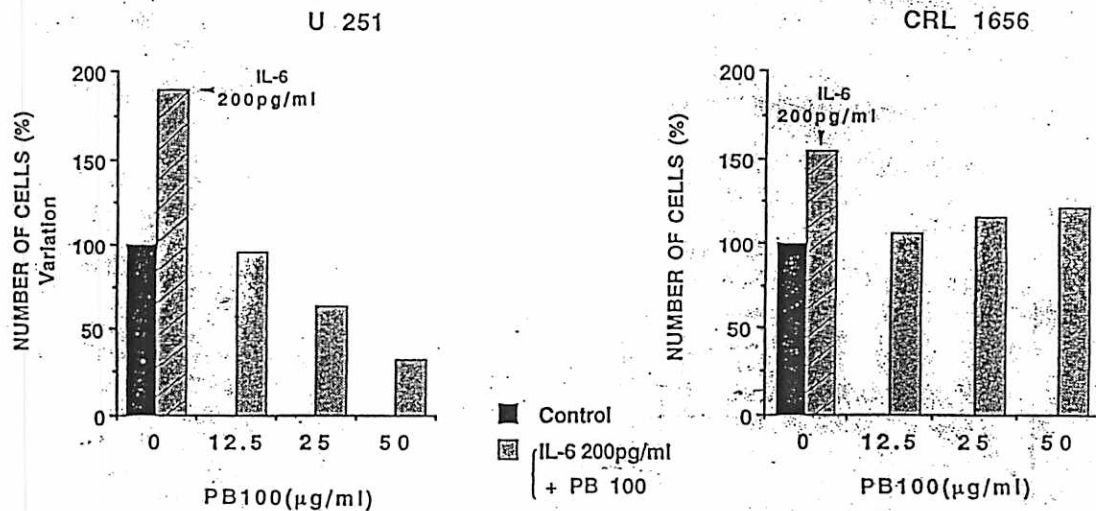


Figure 5. Human BCNU-resistant glioblastoma (U 251) and normal astrocyte (CRL 1656) cell lines were grown for 48 h at 37°C in the absence and presence of IL-6 either alone (controls) or added prior to increasing concentrations of PB-100. After 48 h, cells were trypsinized and counted. Data are mean values of three independent experiments expressed as percent of variation. (U 251: $\text{SD} \pm 2.89$; CRL 1656: $\text{SD} \pm 3.48$)

Addition of 12.5 $\mu\text{g/ml}$ PB-100 causes a spectacular 10-fold drop of IL-6 production by glioblastoma cells, down to the normal level produced by astrocytes. Under these conditions, the number of malignant cells concomitantly decreases by only 10 to 20%, as previously reported (5). Astrocyte IL-6 production remains unaffected. Using 25 $\mu\text{g/ml}$ PB-100, there is practically no further change of cytokine production by glioblastoma cells, while production by astrocytes slightly decreases and the total amount of IL-6 is now equally shared between cells and medium (Table I).

In the presence of exogenous IL-6. Comparable inhibitory effects are obtained in the presence of exogenous IL-6. PB-100 dose dependently suppresses the stimulatory effect of IL-6 on cell multiplication. As can be seen from Fig. 5, 12.5 $\mu\text{g/ml}$ PB-100 abolish IL-6 induced stimulation of both glioblastoma cell and astrocyte proliferation; 50 $\mu\text{g/ml}$ PB-100

then decrease the number of malignant cells by a further 75%, while the amount of astrocytes very slightly increases. Correspondingly, using, per assay, 20 μg PB-100 and 2400 pg IL-6, (i.e. the cytokine dose inducing maximum hyperchromicity increase of isolated DNA), glioblastoma DNA hyperchromicity is largely suppressed, while astrocyte DNA UV absorbance remains slightly above normal, indicating that the chosen dose of PB-100, while actively preventing malignant DNA replication, does not totally cancel IL-6 stimulation of normal cells; this is indeed the prerequisite for conservation of the physiological activity of the cytokine (Figs. 6 and 7).

Discussion

The aim of this research was, first, to investigate the activity of interleukin-6 as a growth factor for glioblastoma cells and,

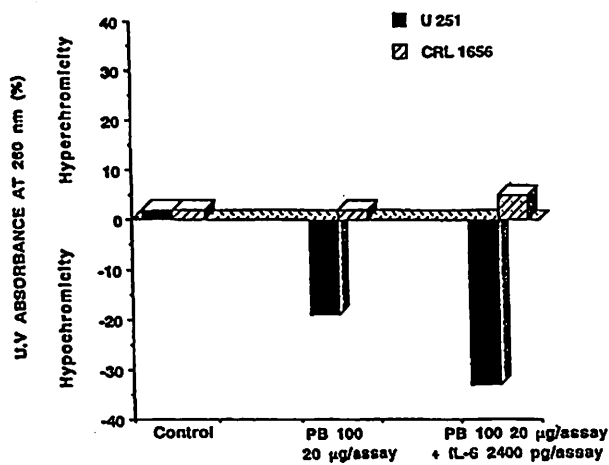


Figure 6. UV absorbance at 260 nm of human BCNU-resistant glioblastoma (U 251) and normal astrocyte (CRL 1656) cell line DNA was measured in the absence (controls) and presence of PB-100 and then using PB-100 together with IL-6. Data are mean values of three independent experiments.

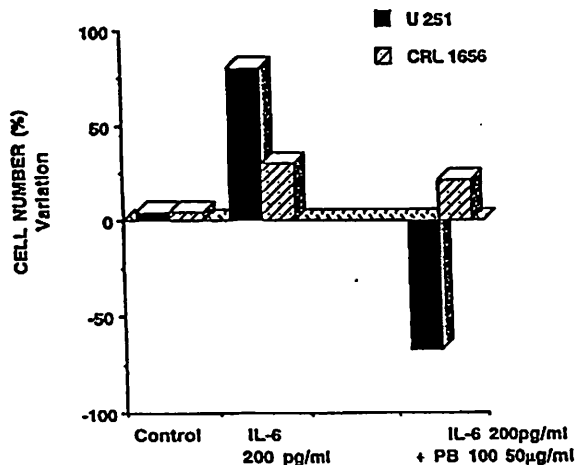


Figure 7. Effect of PB-100 on the multiplication of human BCNU-resistant glioblastoma (U 251) cells and normal astrocytes (CRL 1656) grown in the absence (controls) and presence of IL-6 for 48 h at 37°C. Data are expressed as variation of cell number (%). (U 251: SD±9.53; CRL 1656: SD±5.95).

second, to explore the possibility of suppressing IL-6 induced enhancement of malignant cell multiplication using our selective, nontoxic anticancer agent PB-100. This research was carried out *in vitro* on the BCNU resistant human glioblastoma cell line U 251 and the normal astrocyte line CRL 1656, chosen as the noncancer counterpart of the brain tumor cell line and used as a control.

The experimental data presented herein, while confirming the fact that glioblastoma cells are much more active IL-6 producers than normal astrocytes, demonstrates that i) addition of exogenous IL-6 to cell culture medium induces a differential, dose-dependent increase of both these malignant and noncancer cells, matched by increase of their respective DNA replication *in vitro* and DNA 260 nm UV absorbance,

and ii) the selective anticancer agent PB-100 efficaciously suppresses these IL-6 induced stimulatory effects, without compromising the physiological role of the cytokine in normal cells.

Cytokines are renowned for their ability to behave 'like angels or devils' and IL-6 is no exception. Besides its beneficial activities, such as promoting-immune defense at various levels (1,3,9) or fostering differentiation of blood platelet stem cells and nerve cells, it has also won recognition as a tumor growth factor for blood cell tumors (3) like T or B lymphoma, myeloma and plasmocytoma (4).

In the human U 251 cells derived from the highly malignant brain tumor, glioblastoma, we detected a significant IL-6 production, which renders their proliferation self-perpetuating. Our work indicates that the cytokine is almost entirely confined to the cells themselves (where it presumably behaves as an autocrine growth factor), the amounts remaining in the culture medium being about 3- to 4-fold smaller. As for control astrocytes, they contain the more modest, physiological amounts of IL-6, i.e. 40-50 pg/ml. Measurement of UV absorbance at 260 nm also confirms that glioblastoma DNA, when compared to normal astrocyte DNA, exhibits hyperchromicity, as is the case for every cancer DNA we compared with its normal counterpart in over 15 years of research on this subject (see Introduction).

Further probing into IL-6 activity called for investigation of the effects of exogenously added cytokine. After addition of increasing IL-6 concentrations to the cell culture medium, proliferation of both malignant and normal cell lines was enhanced. Maximum enhancement of glioblastoma cell multiplication, obtained with 200 pg cytokine/ml, was 2-fold that of astrocytes (respectively 80% and 30-40%). Obtained using 2400 pg/ml IL-6, maximum increase of DNA *in vitro* synthesis, respectively 250% and 150%, and maximum DNA hyperchromicity, 39% and 29%, follow the same trend.

These results call for several comments. The first concerns mediation of IL-6 activity. IL-6 was shown to bind to a cell surface receptor; the latter, when cloned and sequenced, was seen to be made up of two glycoproteins, one of which carries a G protein binding site (3). Therefore IL-6 is assumed to trigger a second messenger pathway inside the cell.

In the course of our work, we showed that, during *in vitro* tests using isolated DNA, IL-6 itself can bind *in vitro* to the destabilized malignant DNA molecule, where it breaks further H-bonds, as evidenced by a rise of DNA hyperchromicity. According to the conclusions of our previous research (see Introduction), this may be defined as carcinogen-like activity, not to say carcinogenic. It parallels, and is confirmed by, the observed enhancement of glioblastoma cell proliferation.

The second remark regards the extent of observed IL-6 activity. The 80% increase of glioblastoma cell multiplication, using the cytokine in amounts much higher than physiological ones (which remain in the range of 40-50 pg/ml) must be considered as a moderate response. This might be accounted for in several ways. For one, glioblastoma cell response to exogenous IL-6 might be partly saturated due to the high endogenous IL-6 autocrine activity. On the other hand, in some cells, such as hepatocytes, the

presence of IL-6 decreases the number of receptor molecules on the cell surface (3). A similar process may occur in the glioblastoma cells, receptor expression being perhaps already limited by endogenous cytokine production.

Now, exogenously added IL-6 also exerts a similar, though half as large effect on astrocytes. Being noncancer cells, these should respond but very slightly to the carcinogen-like activity of the cytokine. However, the relationship between IL-6 and astrocytes is complex, being that of a physiological mediator and its target cells. Astrocytes not only carry IL-6 receptors on their surface, a property they share with numerous other kinds of mammalian cells, but they are also recognized producers of this cytokine (10). No more considered as mere neuron feeder cells, they are now seen to play a part in various fundamental processes, including development of the central nervous system, neuromediator metabolism and even immune defense of the brain (11); in this regard, some of their activities are akin to those of monocytes/macrophages, which are main IL-6 producers (12). Astrocyte synthesized IL-6 appears to exhibit autocrine activity, at least as concerns enhancement of nerve growth factor release by these cells (10). Astrocytes may thus be expected to behave as target cells for the normal physiological activity of IL-6. Being, so to speak, designed to respond to small variations in the amount of IL-6, astrocytes should be particularly sensitive to the cytokine, down to DNA level. We must remember that, in a similar way, other physiological compounds, a case in point being the steroid hormones, become carcinogenic for their target cells when present in excessive or simply unbalanced amounts. Indeed, we showed that this well-known effect of steroid hormones is matched by an increase in DNA hyperchromicity (6). Therefore, we should keep in mind that IL-6 in excessive amounts not only enhances proliferation of glioblastoma cells, but also, even if in a more limited way, that of normal astrocytes, which means that IL-6 is potentially carcinogenic for these latter cells. Increased IL-6 production by glioblastoma cells may affect neighboring astrocytes, and increased IL-6 production by astrocytes may also become menacing in various ways (see below). Neither should we forget that cytokines work together with, and are influenced by, numerous other cytokines.

The nontoxic anticancer agent PB-100, a plant-derived beta-carboline alkaloid, dose dependently suppresses the IL-6 induced enhancement of *in vitro* cell proliferation. Using 12.5 µg PB-100/ml, a) the high glioblastoma cell endogenous IL-6 production is restored to normal, i.e. it is decreased by about 90% (Table I); b) enhancement of glioblastoma cell proliferation induced by addition of 200 pg of exogenous IL-6/ml is completely suppressed; c) in control astrocytes, the physiological endogenous IL-6 production is not affected; d) astrocyte proliferation induced by 200 pg of exogenous IL-6/ml is abolished (Fig. 5, Table I). Using 50 µg PB-100/ml in the presence of 200 pg of exogenous IL-6/ml, 75% of the glioblastoma cells are now killed, whilst astrocyte multiplication slightly increases (Fig. 5). In parallel, and using the latter concentrations, IL-6 induced glioblastoma DNA hyperchromicity is abolished by PB-100, but that of astrocyte DNA remains slightly above normal (Fig. 6).

Ongoing research in our laboratory indicates that entry of the small PB-100 molecule into a cell is not receptor mediated; therefore, competition with IL-6 is not exhibited at receptor level. One of the areas where dysregulated DNA strand separation is normalized by PB-100 presumably contains the IL-6 gene.

At DNA level, PB-100 forms a complex with glioblastoma DNA (7), but only binds to astrocyte DNA insofar as its chains have been abnormally opened by exogenous IL-6. If PB-100 is added to cell culture medium before exogenous IL-6, it prevents the stimulatory activity of the cytokine on malignant cell proliferation, because glioblastoma DNA chains are preventively closed by the anticancer agent. Thus PB-100 not only suppresses excessive endogenous IL-6 production by glioblastoma cells, but also makes them unresponsive to the stimulatory activity of the cytokine, whether exogenous or endogenous; furthermore, as concentrations (µg/ml) of the anticancer agent are increased, these cancer cells are unable to survive the PB-100 induced unavailability of their abnormal replication and transcription initiation sites which had been exposed by strand separation (see Introduction). In the case of astrocytes, our results show that PB-100 decreases DNA receptivity to excessive amounts of IL-6, but impairs neither the physiological production of the cytokine, nor normal cell multiplication (Table I, Fig. 5).

These data indicate that PB-100 is not only a good candidate for the treatment of glioblastoma, but also that it might serve to regulate IL-6 effects and production in other cases. IL-6 is implicated in many different illnesses beside cancer (13). It is found in various diseases involving non malignant cell proliferation (keratinocytes in psoriasis, mesangial cells in some forms of glomerulonephritis) and in certain auto-immune diseases, such as lupus erythematosus. IL-6 is widely present in inflammatory diseases: a striking example is the report that synovial fluid of rheumatoid arthritis patients may contain from 10- to 1000-fold more IL-6 than normal (14-16); damaged synoviocytes and chondrocytes further contribute to IL-6 production.

Elevated amounts of IL-6 are also found in biological fluids in the course of infectious diseases, a case in point being HIV infection. IL-6 and TNF-alpha were shown to enhance HIV virus multiplication (17). It was suggested that IL-6 released by activated B lymphocytes could reactivate HIV virus inside monocytes (3). In addition, inflammation of the hematoencephalic membrane may facilitate brain infection by HIV virus as well as by other pathogens. Recent observations showed that monocytes/macrophages from HIV infected patients produce large amounts of IL-6. *In vitro*, about 80% of this production may be inhibited using PB-100 (Professor J. Cahn, personal communication); this ensures that there remains the required cytokine synthesis for physiological purposes.

It may be hoped that PB-100 can find its place in therapy whenever excessive IL-6 production is involved.

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