CERN/SPSC 2002-040
SPSC-P-324-Add. 1
January 6, 2003

Addendum to Proposal P-324
Biological Effectiveness of Antiproton Annihilation

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Abstract

The proposal P-324 to study the potential biological effectiveness of antiprotons for cancer treatment was submitted to the SPSC on October 12, 2002 and discussed at the open session on November 5, 2002. As a result of careful analysis of the presented facts the SPSC formulated a series of comments, questions, concerns and requests, which we address in the current document.

In the original proposal we inadvertently gave the impression that we will measure an absolute Relative Biological Effectiveness (RBE) for antiprotons and protons. Being such a complex concept, which depends on many variables that are beyond our control in this experiment, RBE was clearly the wrong measurement for us to be proposing. We thank the committee for pointing this out to us, and hope we can make our intention more clearly understood through this addendum. We do not propose to perform an absolute measurement of RBE of antiprotons, or to establish a final conclusion as to the therapeutic potential of antiprotons. This proposal is for an enabling experiment asking the narrowly focused question:

Does the high effectiveness for cell killing of the densely ionizing particles emanating from the annihilation of antiprotons produce an increase in biological dose in the vicinity of the narrow Bragg peak for antiprotons compared to protons for identical physical dose delivered to the entrance channel?

In other words, as a first step, we aim for a proof of principle that the increase in "biological" dose for antiprotons compared to protons in a narrow Bragg peak is significantly greater than the already known increase in "physical" dose, thus differentiating the radiobiology of antiprotons from that of protons. A positive result to this answer would then warrant more detailed investigations of this issue, including the development of clinically relevant beams (with spread-out Bragg peaks), absolute dosimetry, RBE measurements for different biological systems, and many more.

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The questions and comments of the SPSC can be grouped in the following subsections:

I. Issues concerning the biology:

1) It should be clearly explained which are the expected advantages of using antiprotons in radiotherapy with respect to protons and heavy ions beams. The vast body of literature describing the measurements with these beams in many laboratories should be consulted and referred to.

2) RBE depends on the biological system considered: the choice of cell lines should be discussed and explained. In general the description of the biological measurements and the cell handling deserves more details.

3) The effect of the particular structure of the AD beam on biological systems should be discussed and compared with the different beam structures used in other radiobiological approaches.

4) Before "determining the therapeutic potential of antiprotons for radiosurgery", many biological variables influencing RBE should be discussed and experimentally studied.

II. Issues concerning the physics:

5) The non-linearity/saturation of the proposed detectors to high LET radiation should be determined experimentally and its relevance to the dose measurements and to the biological effects should be discussed.

6) Quantitative estimates on the precision of the measurements (dosimetry and radiobiology) should be clearly stated.

7) Recent experimental data on antiproton stopping power and on the differences in the slowing down processes of protons and antiprotons should be taken into consideration.

III. Questions concerning the organization of the collaboration:

8) The structure, the policy, the goals of the Pbar Medical Inc. company and its financial plan in the experiment should also be stated.

9) It could be beneficial to strengthen the collaboration involving more persons with high experience in particle radiobiology.

In addition, the SPSC suggested we first demonstrate the proposed method with a measurement at a proton accelerator under conditions similar to those expected at the AD.

The following document is organized along these subsections and will describe the biology, specific physics issues, the organization of the collaboration, and finally the test experiment performed with protons at the TRIUMF facility in Vancouver, British Columbia, Canada.

I. The expected potential advantages of antiprotons in radiotherapy with respect to proton and heavy ion beams and the biological methods proposed to establish quantitative estimates of those advantages.

A. Definition of the problem

The advantages of protons and heavy ions over X-rays for cancer treatment have been clearly demonstrated in many experiments worldwide and predominantly consist of the feature of a well-defined Bragg peak. This feature allows precise deposition of the energy of a particle beam in a tumor buried inside the human body with minimal damage to overlying healthy tissue. First proposed by R. R. Wilson in 1946 [Wi46] the use of heavy charged particles in radiotherapy of deep seated tumors, which began in the 1970's, entered a phase of more rapid development in the 1990s, and has been well documented in a series of proceedings of the International Symposium of Hadrontherapy [Am94, Am97] and in related review articles [Am99, Kr00]. The scientific background and methods of advanced hadron therapy is given in a number of publications too numerous to be listed here and the reader is referred to a short list of selected publications (and references therein) most relevant to our proposal [Am96, Be00, Br01, Ka02, Kr95, Kr00a, Sk98, Su00].

In short, the effect of irradiating a tumor does not depend solely on the total absorbed dose (absorbed energy per unit mass), which describes the deposition of energy at a macroscopic level, but also on the mechanism of energy transfer from the radiation to the tissue, described by the concept of linear energy transfer (LET). LET is defined as the ratio of the Energy, ΔE, deposited by a charged particle in a short track element, Δx. LET values for hadrons range from 4 keV/μm for 10 MeV protons to 15 – 200 keV/μm for 400 – 10 MeV/u carbon ions [Am99]. Due to the increased capability to induce DNA double strand breaks and other substantive DNA lesions, high LET charged particle beams have the capacity to produce much more tumor cell killing than conventional X-rays (or protons), for the same amount of damage to overlying normal tissues, which under most circumstances limits the maximum treatment dose.

These discussions introduce the concept of RBE and make it clear that absorbed dose is not a good indicator of the biological effects. The RBE of a given radiation is defined as the ratio between the absorbed dose of a reference radiation (typically ^{60}Co) and that of the test radiation required to produce the same biological effect (tumor inactivation being the most important parameter for our application). Belli et al. [Be95] gives RBE values for a range of LETs from 1 to 10^4 keV/μm, which shows a pronounced maximum of RBE values around 100 keV/μm (C, Ne).

Antiprotons are expected to provide the following advantages over current treatment techniques:

a) Higher physical dose than protons in the Bragg peak due to the annihilation events, and

b) The ability to deliver this higher dose in forms of fragments with a high ionization density, which increases the biological effect.

In addition, antiproton radiotherapy will provide real-time imaging of the annihilation events. Utilizing the high-energy gammas and pions which exit the tissue with minimal interaction, and employing particle detectors developed to an extremely high technological standard at CERN for high-energy experiments, will allow the reconstruction of the annihilation vertex in the tissue. This would provide true real-time imaging of the annihilation events and therefore assist a medical professional to monitor the proper aim of a potential treatment beam to the previously established tumor position and shape. While this is not a direct aim of our current proposal, this future possibility adds substantially to the perceived value of antiprotons for cancer therapy, provided the outcome of our preliminary studies demonstrates the validity of the fundamental concept.

Our proposed experiment is aiming at a proof of principal demonstration of the second of the above points. It was originally proposed by L. Gray and T. E. Kalogeropoulos in 1984 [Gr84] and then experimentally verified at CERN by A. H. Sullivan in 1985 [Su85] that the ratio between the energy deposited in the Bragg peak and the energy deposited in the entrance channel (the "plateau" region) for antiprotons is roughly two times that for protons, i.e. for the same dose in the plateau the physical dose deposited in the Bragg peak for antiprotons is twice that for protons. While the additional total energy deposited in the Bragg peak was found to be "only" 30 MeV, out of the total 2 GeV annihilation energy, this enhancement in physical dose alone constitutes a 100% improvement in localized energy deposition, a dramatic increase on the biological scale.

Most of the 2 GeV energy of the annihilation is carried away by the charged pions or high-energy gammas (resulting from the immediate decay of neutral pions) with minimal interaction with the surrounding tissue. The higher energy neutrons emitted in the annihilation process have intermediate ranges and result in a diffuse neutron radiation background centered on the tumor, but extending beyond the targeted region. Similarly, the higher energy protons and pions can produce some background radiation beyond the immediate region of annihilation.

However, there is potential for significantly increased biological efficacy of an antiproton beam in the vicinity of the Bragg peak. This increase stems from the heavy recoils and fragments that result from a fraction of the many annihilation events where one of the pions may interact with a proton or neutron in the nucleus to cause nuclear excitation with subsequent break-up. These heavy fragments and recoils have a very short range and deposit all their energy in a localized region around the annihilation vertex. This localization of densely ionizing radiation close to the Bragg peak has the potential to significantly increase the differential in biological dose

between tumors that are in a Bragg peak and more remote normal tissues that are outside the peak. To quantify this effect we will measure the Biologically Effective Dose Ratio (BEDR) by comparing the ratio of doses, peak to plateau, producing the same level of biological effect. The BEDR will depend on dose, and the comparison can be performed for any dose level in the plateau region. Of special interest is the value of the $BEDR_{2Gy}$, the Biologically Effective Dose Ratio for a 2 Gy dose to the entrance channel, since this value is of interest to radiation oncologists because of its clinical relevance and can be compared with existing data from other particle beams. As for all radiation delivery systems, the clinical application of antiprotons will be limited by the response from normal tissue as encountered by the antiprotons in the entrance channel. Standard clinical radiation therapy employs (plateau) dose fractions of 2 Gy, which is generally assumed to be tolerated by normal tissue when delivered daily in a multi-fraction delivery schedule to a total dose as high as 50 – 80 Gy. This scheme allows normal tissue to repair the damage caused by low LET radiation between the individual fractions.

When total doses delivered to a tumor are already achieving a measurable percentage of cures, a relatively small escalation of biological dose can produce a meaningful improvement in tumor control rates. For example, the rate of control of prostate cancer can increase steeply with dose [Ha02], on average by about 3% per Gray. In other sites the increase per Gy has been less (e.g. 1-1.5% per Gy for head and neck cancer) presumably because of heterogeneity in factors other than dose that determine outcome. Thus, if a standard X-ray equivalent dose of 70 Gy (in 2 Gy fractions) were enhanced by a factor of only, say, 1.5 within a Bragg peak, an increase from 10% to greater than 90% control rate for prostate cancer could be achieved. Even if the peak to plateau Biologically Effective Dose Ratio were only 1.25, the increase in tumor control rate could exceed 50%. Since control rates are already commonly above 50%, the clinical significance of such an enhanced therapeutic ratio would be major. Since spreading the Bragg peak by modulating the beam energy will reduce the biologic effectiveness of peak relative to plateau, a useful therapeutic gain could be achieved for BEDR values of 3 – 4 for a narrow peak like we propose to use for these tests. Many indications point to BEDR values much larger than this and the primary goal of the proposed experiment is the quantitative assessment of the concept of an enhanced biological dose within the Bragg peak of an antiproton beam. Only after this has been done, the technically difficult investigations of using clinically useful beams capable of depositing doses to larger tumor volumes within a spread-out Bragg peak can be reasonably undertaken.

The fundamental question we would like to address with our experiment is the following: Is there an additional increase in biological dose in the vicinity of the narrow Bragg peak for antiprotons compared to protons for identical physical dose delivered to the entrance channel because of the high relative effectiveness for cell-killing of the densely ionizing particles emanating from the annihilation of

antiprotons? Beams of heavy ions (e.g.carbon) have already demonstrated an increase in relative biological dose over protons and have been used successfully in experimental clinical treatments since 1995 at GSI in Darmstadt, Germany [Kr95, Kr00a] and at HIMAC in Chiba, Japan [Su00]. The success of this work has led to a proposal to construct a dedicated Heavy Ion Therapy Center at the University of Heidelberg [Gr98] and at the University of Stockholm [Br01]. The magnitude of the ratio of peak to plateau "biological" dose achievable with an antiproton beam is expected to be even higher than with heavy ions, since the heavy ion fragments are produced locally within the targeted tumor.

We propose to conduct a first investigation of the potential improvement in therapeutic ratio to be gained from using an antiproton beam. The limited resources available for this study in terms of beam time and variety of beam specifications precludes us from conducting a full study of the therapeutic potential of antiprotons, especially including questions of RBE values for treatment planning. The first necessary step is to obtain a proof of principle that there is an increase in "biological" dose ratio for antiprotons in a narrow Bragg peak of antiprotons compared to protons, which is significantly greater than the already known increase in physical dose ratio for the same number of particles due to the annihilation effect [Su85], thus differentiating the radiobiology of antiprotons from that of protons.

An additional important piece of information we propose to obtain concurrently with these measurements is the degree of localization achievable by measuring the biological effects on cell samples at a certain distance away from the Bragg peak.

This first step will quantitatively assess the potential of an antiproton beam to improve treatment of cancer. Without establishing such proof of principle, the formidable future task of detailed studies of absolute RBE values for different indications, treatment schemes, biological systems and different biological end points, as well as the task of developing antiproton beams for radiation therapy would not be warranted.

Relative Biological Effectiveness (RBE) requires absolute knowledge of the physical dose delivered to the tissue. Due to the intensity and time structure of the antiproton beam available from the AD, and the mixed radiation field consisting of high LET particles produced in the annihilation, the physical dose in the Bragg peak cannot be reliably determined, given the limited time scale available to us. The dose in the plateau region can, however, be measured with reasonable certainty (see a discussion of dosimetry effects later in this addendum). We therefore propose to base our investigation on "biologically effective dose" (BED) in (and immediately beyond) the Bragg peak to be measured relative to the "biologically effective dose" along the incident plateau of an antiproton beam, thus establishing a Biologically Effective Dose Ratio (BEDR).

That is, we propose to measure the ratio of peak to plateau "biological doses" by determining how the efficiency of cell killing changes with defined distance along an antiproton beam as it penetrates a tissue-equivalent cell-containing medium (Figure 1).

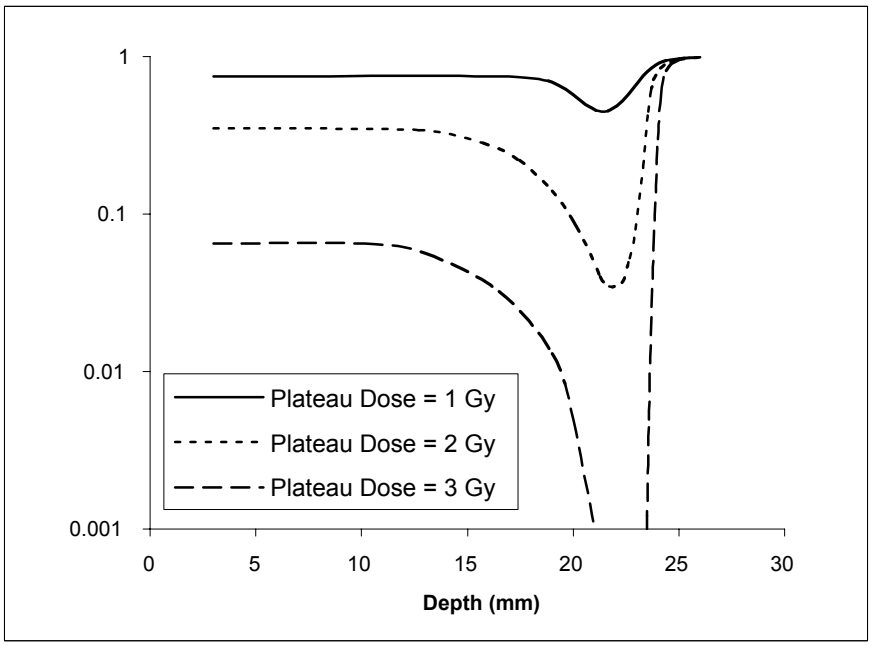

Figure 1: Conceptual plot of survival fraction of cells at different penetration depth of the beam into the sample tube (for illustration purpose only).

Two complimentary approaches can be used to extract biologically significant information from a given data set from antiproton irradiation of cells at CERN. Using reasonable estimates for the physical plateau doses we can cross-plot the survival vs. depth data to obtain the survival vs. (plateau) dose for both the entrance channel and the Bragg peak. We can plot averaged survival values for plateau and peak vs. entrance dose by using a sufficient number of slices in the entrance channel to achieve statistical significance and two slices in the (narrow) Bragg peak (see Figure 2). From this plot we can establish the BEDR for the entire range of entrance dose by comparing the "effective" dose values in peak and plateau producing the same biological effect.

In the antiproton peak, a proportion of the dose to the tumor will be from high LET particles, and the cell survival curve is expected to have a narrow or nonexistent shoulder (see Figure 2), unlike protons. Therefore, the biological differential between tumor and normal tissue will be greater at 2 Gy individual dose than at higher dose, and can be amplified by fractionation. While the RBE of antiprotons compared with protons would vary with dose, the BEDR, the ratio of doses for a given biological effect, will remain constant for multi fraction survival curves, with a value determined

by the size of dose per fraction being used. This is because multi fraction survival curves are linear exponential if equal doses fractions produce equal effects.

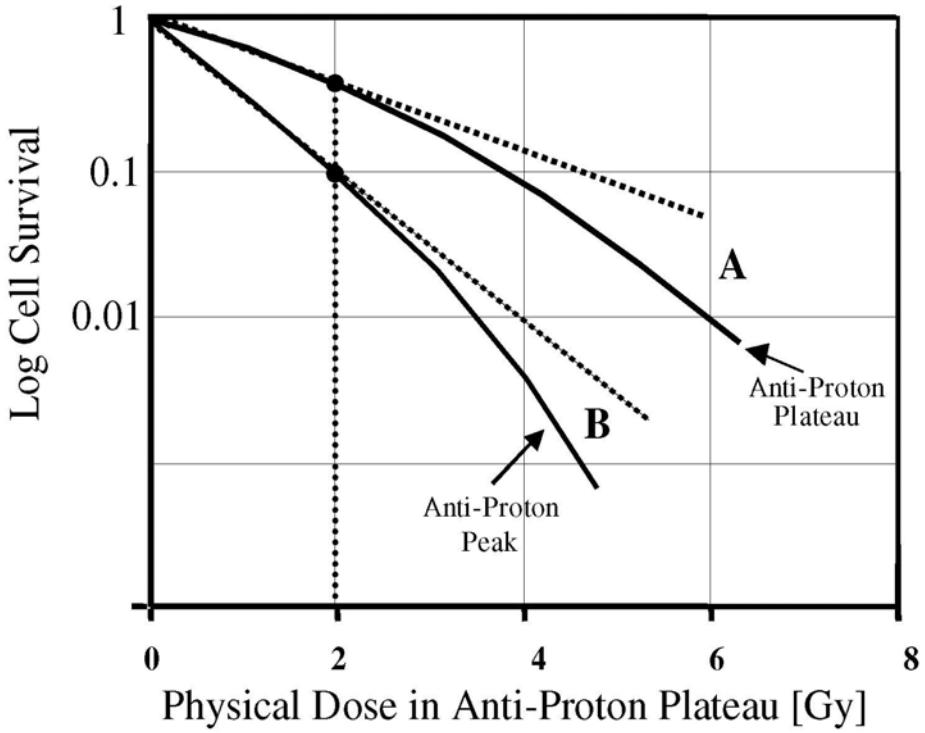

Figure 2: Conceptual survival curves for antiproton irradiation in the plateau and the Bragg peak (Solid lines represent single dose survival curves and dotted lines are multi fraction survival curves for 2 Gy individual doses per fraction in the entrance plateau region.)

To determine the $BEDR_{2Gy}$, cells will be exposed to a range of plateau (and peak) doses and cell survival levels at 2 Gy plateau doses will be determined from our experimental survival curves fitted with a linear quadratic model. (Survival in the peak will be lower than in the plateau. Therefore a wide range of doses will be required to develop useful dose survival curves from both the plateau and peak simultaneously.) The survival curves can be plotted as a function of plateau doses with the actual linear exponential slope of the multi fraction curves being defined in terms of that plateau dose. The $BEDR_{2Gy}$ is then represented by the ratios of slope for survival from doses between 0 Gy (100% survival) and 2 Gy for both peak and plateau survival. (Although we will use results from 2 Gy fractions because of clinical relevance, any dose could be chosen.) For example, if cell survival fractions after 2 Gy in the plateau of antiprotons were 0.5, compared with 0.1 in the peak, the BEDR would be derived from a straight exponential line from 0 Gy through survival

fractions of 0.5 and 0.1 Gy (curves A and B in Figure 2), regardless of the physical dose in the peak. (Although accurate measurement of physical peak doses is not necessary to achieve our initial limited goals, it is appreciated that these doses must be measured in order to know "true" RBE and to further develop the dosimetry of spread-out peaks.)

Additionally, we will perform proton irradiation experiments (using both a standard proton beam for which good absolute dosimetry is available and a proton beam with profile and time structure closely modeling the AD beam), as well as irradiation with a standard ^{60}Co beam to further enhance our analysis.

For the standard proton beam, absolute dosimetry is available and survival in peak and plateau vs. plateau dose can be plotted directly to produce a graph similar to Figure 2. For the antiproton beam at the AD as well as for the proton beam designed to model the radiation delivery at the AD we can use either the standard proton beam or the ^{60}Co reference radiation to connect the biological dosimetry (same survival fractions) to the physical dosimetry (energy deposited per unit mass).as follows:

a) For a given proton beam profile, determine the average survival for the plateau region and for the Bragg peak.
b) Locate the average survival values on either the survival curve from the standard proton beam or the ^{60}Co curve and read off the corresponding doses on the x-axis. The ratio of these two doses is then the BEDR $=(BED)_{pk}/(BED)_{pl}$ for protons.
c) Repeat the same procedure for the antiproton measurements and plot the BEDR values for protons and antiprotons vs. plateau dose, for which we expect a result similar to that in Figure 3.

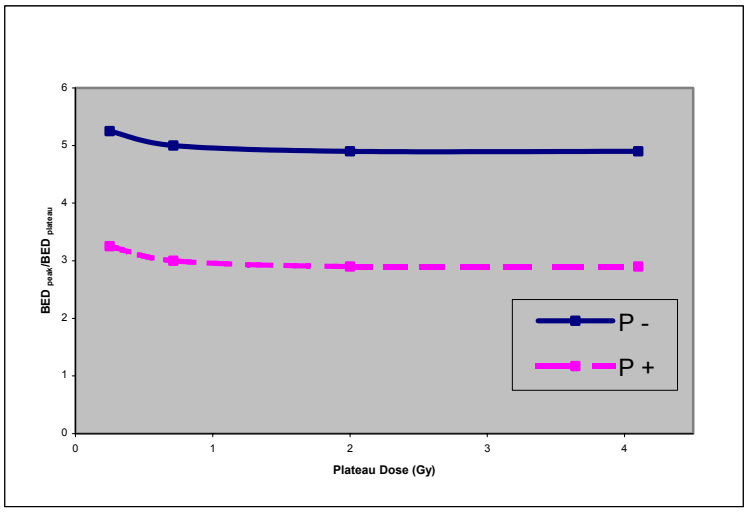

Figure 3: Conceptual graph of Biologically Effective Dose Ratios (BEDRs) for protons and antiprotons (for illustration purposes only).

It is important to note that both analysis methods can be applied to the same set of data. The difference is simply in the representation. The concept of BEDR for all plateau doses may be more interesting to the radiobiologist while the method using the $BEDR_{2Gy}$ concept is more attractive to the radiation oncologist, and while it is not a true RBE, this information is clinically relevant. Using the antiproton irradiation alone and assuming the dosimetry problem in the plateau region is identical for protons and antiprotons and can be done reliably, no further information is necessary to extract meaningful biological results. In contrast, using the proton and/or ^{60}Co reference irradiation will allow us to use the biological response of the cells as a "biological dosimeter", making the measurement of the BEDR independent of physical dosimetry in the AD beam.

To increase the validity of this comparison we plan to study the proton survival curves both for the "standard" beam structure which allows accurate absolute dosimetry and can be linked to the cobalt irradiations, as well as for a proton beam with spatial and temporal characteristics as close to the antiproton beam at the AD as possible to quantify (or eliminate) the possibility of dose rate effects due to the time structure of dose delivery. Due to the fact that we plan to keep our cell samples at low temperature, proliferation and repair by the cells are strongly suppressed. Furthermore, we have shown that the plating efficiency varies only negligibly over a 48 hour time period. For these reasons we expect no effect due to the time period of 2 hours required to deliver an 8-Gy dose.

Experimental Priorities

Given the limited resources we can expect from the AD, the experimental design aims to capture enough data for an initial evaluation of the maximum potential of antiprotons for radiotherapy. Knowing that we have a narrow, sharply peaked beam and that the number of antiprotons available is limited, we have designed a very conservative experiment. It is clearly not all we need for a definitive assessment of possible therapeutic applications of antiprotons, but it will determine if such studies are warranted and be a guide as to the potential of antiproton therapy. To ensure we get at least the bare minimum of data we need, we propose the following:

a) Measure cell survival in the peak and plateau of the antiproton beam. The primary concern is to accurately quantify cell survival along the beam path using estimated doses ranging from 0.5 to around 4 Gy plateau dose. The cell surviving fractions will be measured by fitting a linear quadratic model over the experimental dose range.
b) Perform the same experiment as for a) but using protons of comparable energy, and a beam with time structure and modulation characteristics (spread out peak width) as similar to the antiproton beam as possible. This experiment would allow us to experimentally compare the BEDR for antiprotons to

protons. Additional measurements comparing a well-defined proton dose to ^{60}Co irradiations will help to further solidify our results.

c) Determine the lateral dose effects for the antiproton beam by placing cell samples at locations away from the beam axis and study cell survival in these samples for identical beam currents as used for the previous studies.

Measurement c) will be a good guide in establishing the conformity that could be achieved for a given tumor geometry, which is a critical requirement for eventual clinical applications of antiprotons.

This set of measurements can be achieved within the requested beam time allocated and will be sufficient to reach the primary goal of our proposal, to determine if the effect on cell survival is significantly higher for antiprotons than for other particle beams.

B. Choice of biological system, end-point, and methods

For X-rays (and most other forms of radiation) the biological response is non-linear in dose to varying degrees and can be described by the linear-quadratic expression for the survival of irradiated cells: $S = S_0 \exp\{\alpha D + \beta D^2\}$. A typical way to visualize this relationship is to plot the survival of cells, S, vs. dose on a semi-logarithmic plot. This curve typically exhibits a "shoulder" at low dose, where the α term dominates, and then falls more steeply with increasing dose, as the quadratic term β becomes more important. For particle radiation of increasing linear energy transfer (LET) the β term becomes smaller and finally the response is given by a pure exponential dose relationship for the entire dose range. The Relative Biological Effectiveness (RBE) is defined as the ratio of X-ray dose to particle dose producing the same effect: $RBE = D_{X\text{-}ray}/D_{particle}$ and is typically found most pronounced in the region of the "shoulder" defined above.

Inherent to its definition is the fact that RBE depends on a variety of factors. Since RBE is referenced to a linear-quadratic dose response curve it is dose dependent. It also is dependent on the chosen end-point (e.g. survival level), the atomic number of the incoming particle (due to differences in LET), and especially on the biological system under study [Kr99]. If RBE is plotted vs. LET for a beam of a specific atomic number one obtains a family of curves for different biological endpoints, which exhibit a maximum for the RBE at a given LET value. While the location of the RBE peak only depends on LET as long as the identical atomic number is chosen, its height depends strongly on the biological system chosen. It has been found experimentally that systems with a large repair capacity have a large RBE maximum while for systems of low or absent repair capacity only a shallow or no RBE peak is found [We99].

In a real treatment situation the beam may encounter different types of tissue and this variation of RBE with tissue type is of great importance in treatment planning. Here, where we are concerned only with a proof of principle experiment, it is natural to pick a biological system that has been studied in a large variety of experimental settings and would therefore allow a direct link to a large data set available from proton and heavy ion irradiation tests. Our choice of the V79 Chinese Hamster cell line was determined according to the following:

a) Chinese hamster V79 cells have been used in more RBE studies, including proton and ion beams, than any other cell line, and therefore have the advantage that measurements of biological responses for a new beam type can be directly compared to published RBE data for many other particle beams (see, for example, ref. Sk98, Table II and references Be01, Be02, Ch02, Ri00, Ri02, Su00 for some of the more recent work).
b) The very low dose rate anticipated with antiprotons from the AD makes it necessary to carry out irradiations at reduced temperature to avoid cell cycling and certain other biological processes which would lead to a dose-rate effect. When our cells are suspended in gel-containing medium, they tolerate the cold exposure very well, better than in medium alone. But, of course, the gel is mainly present for another reason, to hold the cells in a semi-rigid matrix so that they do not move about during irradiation. For this reason, our cells are irradiated, transported and held at 2˚C until just before plating. The total time at 2˚C in a recent experiment collaborators Lloyd Skarsgard and Bradly Wouters performed at the Massachusetts General Hospital (MGH) in Boston (with plating in Vancouver) was up to 33 hours. The cell line used, V79-WNRE, tolerates this cold exposure extremely well, as can be seen in Figure 4 below. Many other cell lines do not.
c) Wouters et al. have previously shown that the V79-WNRE sub-line gives reliable results under experimental conditions very similar to those that will be encountered at CERN and we have personally characterized its radiobiological response to protons and gamma rays. We measured an RBE of 1.12 +/- 0.02 for a 160 MeV clinical proton beam and 1.21 +/- 0.05 for TRIUMF's 70 MeV beam [Wo96].

Below, we discuss a number of issues of interest that led to our decision on the sliced gel technique with cell sorting as developed by Lloyd Skarsgard and his colleagues and described in detail in [Wo96].

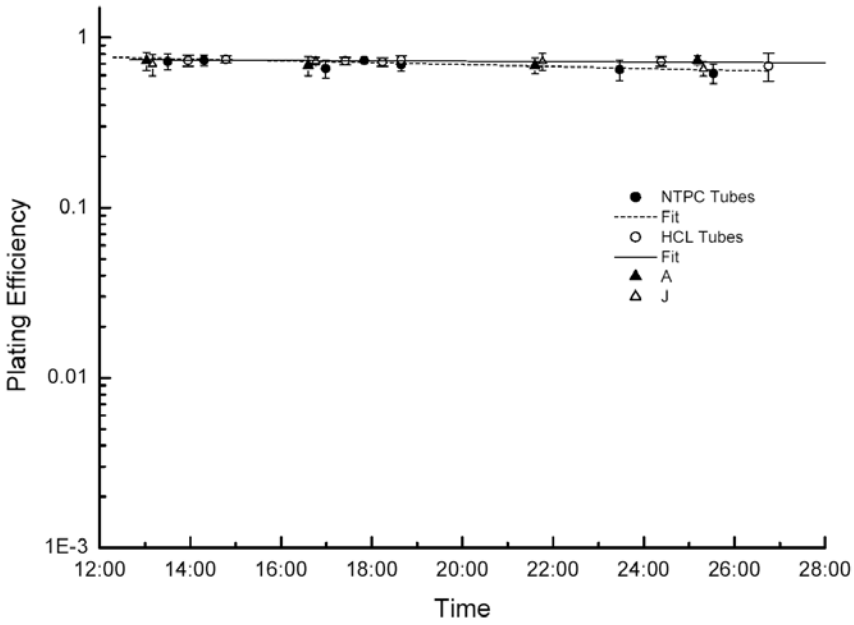

Figure 4: Zero-dose plating efficiency during the 14hr plating period back in Vancouver. Note the cells had been on ice for ~18 hours before plating began in Vancouver, for a total of ~33 hours for the last samples.

Tissue equivalence – when the gel matrix is made up to contain 25% gelatin, its elemental composition (H, C, O, N, etc.) is almost identical to soft tissue (not surprising since it is derived from animal soft tissue). This is important because of the pion reactions occurring after annihilation. The spectrum of reaction products that would be expected in human tissue is much better simulated in our gel system than it would be in the mix of medium and plastic cover-slips that is present in a conventional non-gel system (such as that used for the cell-stack technique in the recent paper by Kagawa et al. [Ka02]).

Feasibility, cell numbers – The small useful diameter of the antiproton beam (~ 6 mm transverse diameter to +/- 5% dose uniformity) would make a cover-slip technique extremely difficult: 6 mm diameter cover-slips are not available as far as we are aware, and the small number of cells which they would accommodate would severely limit the dose to which we could extend survival measurements. By comparison, slicing the gel as it is extruded from the ABS tube is very rapid and gives good numbers of cells. Kagawa et al. used the standard 25 mm cover-slips and only a single dose, because trypsinizing cells off the cover-slips is a laborious procedure. They used 25 square-cm flasks for his full dose responses, irradiating 1 flask (i.e. 1 depth) at a time. We have neither the dose rate nor the field size (>>25 sq-cm) for that type of very conventional measurement.

Resolution – Some concern has been raised as to whether we could reliably slice our gel at 1 mm intervals, as would be necessary to achieve adequate resolution of the antiproton Bragg peak. In the week of December 16th some of our collaborators visited Prof. Lloyd Skarsgard in his laboratory at TRIUMF and conducted an irradiation experiment using the gel technique and a 70 MeV proton beam, which was modulated by one step to produce a calculated Bragg peak of 2 mm Full Width at 90% of maximum. At the time of this writing we have positive information that slicing the gel in 1 mm steps did yield an adequate cell population to achieve good counting statistics and a good depth resolution of the Survival vs. Depth profile. The incubation, staining and counting took place through the Christmas holidays and we will present the final analysis at the meeting of the SPSC in January 2003.

Precision – The group of Prof. Lloyd Skarsgard is the only group in the world to use cell sorting to improve the precision of RBE measurements. In this technique, a cell sorter is used to precisely count out and collect in a test tube the cells to be plated for survival measurement in each sample. This eliminates much of the statistical and experimental error that is associated with the serial dilution and micro-liter plating volumes of conventional survival assays. This technique has been used since 1994, and RBE values have been obtained with uncertainties of less than +/- 2 to 4%. This is significantly better than is normally available with conventional dilution assays (see the review mentioned above).

In short, the gel technique is clearly the method of choice, and is in our opinion the ONLY system that could give useful information on the biological effects of the antiproton beam at CERN.

Detailed description of the biological experiment

For preparing the biological samples, cells will be maintained as monolayers in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 2.2 g/L sodium bicarbonate and 0.122 mg/ml sodium pyruvate. For each experiment, appropriate numbers of cells will be seeded approximately 48 hours prior to irradiation at cell densities designed to give 50 – 75% coverage of the culture surface at the time of harvest. Cell monolayers are harvested with trypsin, neutralized with growth medium, centrifuged and re-suspended in medium containing 12% gelatin and 20% serum at 37˚C at a concentration of approximately $2x10^6$ cells/ml. At this temperature the gelatin is a viscous fluid, which can be poured into ABS (acrylonitrile butadiene styrene) plastic tubes 0.6 cm in diameter and 6 cm long with a piston at one end. After solidification of the gel at 4˚C the tubes are sealed and stored on ice.

For irradiation, one of the tubes of live cells will be placed within a circulating water bath phantom, approximately 30 cm x 30 cm x 30 cm. A refrigerated water bath will be used to circulate the water at 2˚C. The phantom, a volume of tissue-equivalent

material, simulates the effect of backscattering and energy absorption in a human body. The ABS, the 12% gelatin, and the phantom have the same density and stopping power. This ensures a constant stopping power throughout the entire phantom and thus avoids any artifacts that could result from scattering of protons or antiprotons from points outside the gel. The experimental system will be very similar to what we have used previously to successfully measure the RBE for heavy charged particles [Sk82, Wo96]. The phantom and the tube will be held at 2˚C from the time of insertion into the tube until the cells are plated after irradiation. This means the cells are immobilized within the gel and that cell cycling and DNA repair, which would otherwise lead to a dose rate effect when the irradiation is delivered over an exposure period of several hours, is inhibited.

As stated previously, our experience shows that V79-WNRE cells can be held at 2˚C for up to 48 hours without a significant decrease in plating efficiency. Since logistics dictate that the harvesting and culturing of the cells for the clonogenic assay will be performed at a facility remote from CERN, this is a major advantage of V79-WNRE over most other cell lines.

We are currently in contact with Prof. Gerd Beyer from the Hospital Cantonal in Geneva to define capabilities in the local area and assume that cell preparation can be performed in the Geneva area. This will allow us to react much more flexibly to sudden changes in the AD schedule. To a certain extent we can accommodate unforeseen delays in the AD beam schedule by starting several 48-hour harvest cultures at different times and then choosing the one appropriate for harvest when we know the beam will be ready. It requires only 4 – 6 hours to harvest the cells and load our gel-tube samples.

If it is not feasible to culture our cells and prepare our biological samples in the Geneva area, it can be done at the University of Maastricht, the Netherlands, where collaborator Dr. Bradley Wouters (Head of Experimental Radiation Oncology) maintains his laboratories. Those laboratories, which are only 2 – 3 hours from Geneva by air, would be very suitable for our needs.

The beam pulses and repetition rate of the AD can provide radiation dose rates in the cell-containing volume of interest of approximately 2 Gy/hr for a 1-cm2 spot size. The total doses of biological interest will be in the range of 0.1 – 8 Gy. Again, the small useful diameter of the antiproton beam (about 6 mm in diameter for +/- 5% dose uniformity), the variation of dose with depth, the length of time required for irradiation, and the number of cells that need to be accommodated at given depths makes cover-slip or flask irradiation techniques unfeasible.

A number of endpoints have been used to measure the biological effects of irradiation. These include clonogenic cell survival, apoptosis, DNA strand breaks, micronucleus

formation, chromosome aberrations and many others. Historically, the clonogenic assay is the method of choice, especially for the measurement of survival curves to study the RBE of charged particles and heavy ions, and is considered the "gold standard" in the field.

To maximize the chances of experimental success, we will use the sliced gel technique we have used on many previous occasions to measure radiobiological parameters of protons and pions reproducibly and reliably [Sk82, Wo96, Sk98].

After irradiation, and under sterile tissue culture conditions, the gel will be extruded from the tube using a plunger connected to a delivery mechanism that advances the gel by 1 mm each time. The gel will be sliced every 1 mm using a taut wire and collected. The cells will then be dispersed in MEM culture medium containing 10% fetal calf serum and antibiotics. In the test experiment at TRIUMF, using a 70 MeV proton beam we demonstrated that this will yield adequate depth resolution for our goals. Each slice will represent a different position along the path of the beam. The number of cells in each sample will be counted using a cell sorter (flow cytometer) which identifies individual cells on the basis of forward and perpendicular light scatter without the use of a cell stain [Du91]. We have shown previously that this is an extremely accurate way of determining the number of cells plated per dish [Wo96]. The number of cells plated in Petri dishes will be chosen to likely give 400 colonies per 10 cm dish (which requires using many different starting cell densities). Using this technique has improved precision in our measurement of RBE values to +/- 2 to 4%. After incubation for 5 – 6 days in a controlled environment, the colonies that develop will be stained and counted. Only those colonies having more than 50 cells will be counted as having been derived from a single surviving cell (smaller colonies are due to cells that successfully negotiated one to five doublings before their reproductive death). Survival curves will be fitted using the standard non-linear curve-fitting routines and Biologically Effective Dose Ratios of antiprotons as a function of plateau and peak dose will be calculated as described above.

We are also discussing the cell analysis capabilities of the local laboratories but would like to point out that even though flow cytometry has undergone a rapid development in the last decade, the original instrument which is available to us in Vancouver is superior in performance for our task than more "modern" versions. Additionally, as demonstrated by our test experiment, we have assembled a well-orchestrated team to assure the highest quality of cell analysis for our experiments.

The stability of our cell samples at low temperatures will definitely allow the analysis to take place in Vancouver, but if we can identify a laboratory team close by we will certainly take advantage of this opportunity.

For test and calibration experiments we will send samples from our preparation to a

proton accelerator. Possible choices at this time include the University of Aarhus, Loma Linda in California, and TRIUMF in Vancouver. We also will explore other possibilities closer to CERN (e.g. the Paul Scherrer Institut in Villigen, Switzerland).

II. Physics issues

A. Dosimetry of intense antiproton beams

The measurement of a true Relative Biological Effect (RBE) depends on many factors including absolute dose, cell line, biological end point, dose rate, density of ionization, track structure, temperature, environment, cell plating efficiency, etc. We have choices and control of some of these factors, but the inherent limitations of the antiproton beam available to us from the AD impose restrictions on how well we can approach the ideal measurement of the absolute dose. For reasons discussed in the original submission of proposal P-324, specific characteristics of the CERN AD beam preclude absolute dosimetry via commonly used means. The absolute number of antiprotons per pulse, the time structure of the beam, and the total beam time are not variables we can control. We have therefore optimized the experimental design to ensure we obtain a biologically meaningful result relying only minimally on absolute dosimetry. Unfortunately the compromises are not consistent with the ultimate measurement of RBE based on absolute dose.

The preferred measurement of absolute dose based on calorimetry is not possible because the total number of antiprotons available to us is insufficient for accurate calorimetric measurements within the separate plateau and Bragg peak regions of an antiproton beam stopping within a phantom. Briefly, the average dose rate is several orders of magnitude lower than that required for accurate calorimetry.

The next best alternative, a small, calibrated ionization chamber such as those routinely used in proton therapy centers will not work because of the time structure of the beam (200 nsec pulses of 3×10^7 antiprotons/pulse every two minutes). The high instantaneous dose rate of the beam saturates ionization chambers.

In addition to specific beam characteristics, antiproton dosimetry is complicated by the complex nature of the post-annihilation radiation constituents. Localized dosimetry within the Bragg peak is further complicated by a mixture of significant high LET components. Antiproton dosimetry is most straightforward within the plateau region, where the problem is approximately equivalent to proton dosimetry because the contribution from in-flight annihilations is relatively small. Outside of the primary beam, the challenge to dosimetry is similar to that for pions.

The AD and DEM beam line can provide the following:

1) Total circulating antiproton current/pulse (+/- 1%).
2) Total extracted beam current/pulse from a current pick-off detector (10%).
3) The extracted spot size and position of the beam deposited in the phantom with spatial resolution of 1 mm. The divergence of the beam will be determined by optics calculations and the AD operations group.
4) Pulse-to-pulse monitor detectors with wide range linear response (hybrid photo diode detectors and fine mesh Cherenkov photomultipliers (+/- 1%)).
5) An experimental measurement of the DEM beam line extraction efficiency based on Aluminum activation (10%) [Fu00].

The multiplicity of reference detectors means we can measure the integrated number of antiprotons on target with relative precision from dose to dose of a few percent. In the incident plateau region, an absolute dose can be calculated that depends on the absolute integrated number of antiprotons, spot size, and Monte Carlo calculations. This inferred dose would have relatively large error bars (20%). Any similar attempt to infer an absolute dose within the Bragg peak region would have even larger uncertainty and any RBE based on these calculated doses would not be biologically meaningful.

Because the absolute dose within the entire primary antiproton beam cannot be determined experimentally within the available beam time, the proposed radiobiological studies are not intended to characterize the radiobiological effect (in the traditional sense), but rather to evaluate the cell killing within the antiproton Bragg peak relative to a chosen reference dose in the plateau region. This concept of the Biologically Effective Dose Ratio is discussed in detail in section I.A. of this addendum. The physical dosimetry part of this experiment is aimed primarily at determining antiproton beam characteristics within the plateau region relative to detector responses within a similar plateau region of a reference proton beam and through comparison to a reference beam of ^{60}Co X-rays. Absolute dosimetry (1%) is available for both proton and ^{60}Co reference radiations.

The primary method for characterizing the dose in the plateau region is thermoluminescent dosimetry. Thermoluminescent Dosimeters (TLDs) have been used extensively in the characterization of proton, pion and heavy ion beams. Thermoluminescent dosimetry of proton beams, and to a lesser extent pion beams, is relatively straightforward. Of particular relevance to this project is the work of Vatnitsky et al. [Va99], who demonstrated the usefulness of TLDs in determining dose characteristics in proton beams with similar profiles to those proposed for experimental work at the AD.

The fundamental approach to dosimetry for this experiment is based on the observation that in the high-energy plateau region, most of the locally deposited dose is due to the slowing down of the incident antiprotons. The response of TLDs will be

the same for this component of the antiproton radiation as for protons. Figure 5 is a survival plot of 50 MeV antiprotons as a function of depth in a water phantom. It shows that less than 5 % of the incident antiprotons annihilate in flight in the first 1 cm of the phantom. The magnitude of the average additional locally deposited energy due to annihilation in flight is approximately 1 MeV or 7% of the normal dE/dx for an antiproton. This is in the plateau region where all measurements, both biological and physical, are referenced.

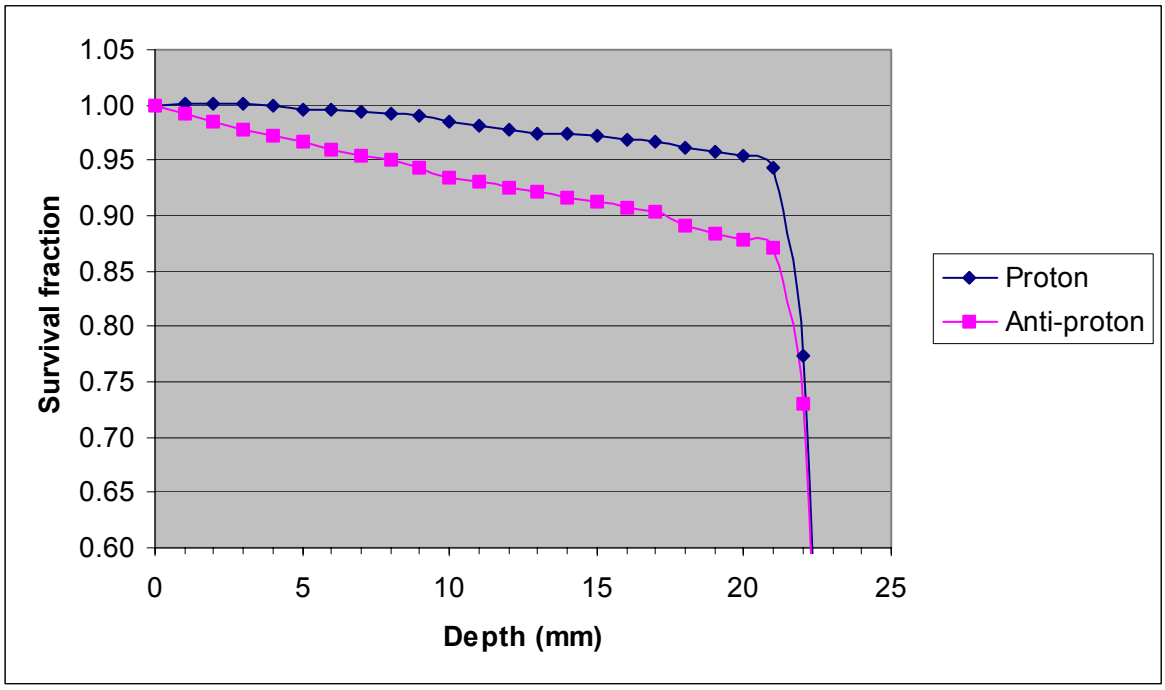

Figure 5: Calculated survival fraction for 50 MeV protons and antiprotons as a function of depth for our specific experimental geometry.

It has been well established in the literature that, for a given dose, TLD efficiency decreases as a function of increasing LET and the Z of the charged particle [Ge98]. Therefore, TLDs cannot be used for *a priori* absolute dose measurement in locations where there is significant high LET radiation or high Z charged particle contributions to the total dose, i.e. the dose in the Bragg peak region of a stopped antiproton beam cannot be measured with a TLD. However, a TLD can be useful in a location of low LET radiation or where the response has been experimentally determined.

Previous results suggest TLDs are an appropriate dosimeter to characterize the plateau region of proton beams because below 5 keV/µm the efficiency of LiF is essentially unity [Bn00], an observation reiterated by Vatnitsky et al. [Va99]. If there is no significant high LET or high Z component of charged particle radiation in the plateau region for antiprotons, TLDs may be used for dosimetry in the plateau region in a manner similar to their use with therapeutic proton beams. Figure 6 below shows the results of Monte Carlo calculations for the average LET of the proton and antiproton

components of a 50 MeV antiproton beam in water. The model includes an accurate representation of the AD beam profile based upon a Gaussian in the x- and y-dimension with a sigma=0.749cm. This produces our intended full-width 80% maximum of 10 mm. The energy distribution across the profile is assumed monoenergetic (50 MeV). The data were obtained by scoring the average energy deposited normalized by the average track length within the scoring volume as a function of depth. With only contributions from incident antiprotons and recoil protons included, the data are consistent with previous proton investigations [Co97, Pa97]. Except for the high-energy annihilation in flight, the stopping of antiprotons and protons in the plateau region is the same [Mo02] and we are proposing to use TLDs in a similar fashion. Finally, the reference proton dosimetry described in the proposal will utilize the facilities and expertise at Loma Linda where the work of Vatnitsky was conducted.

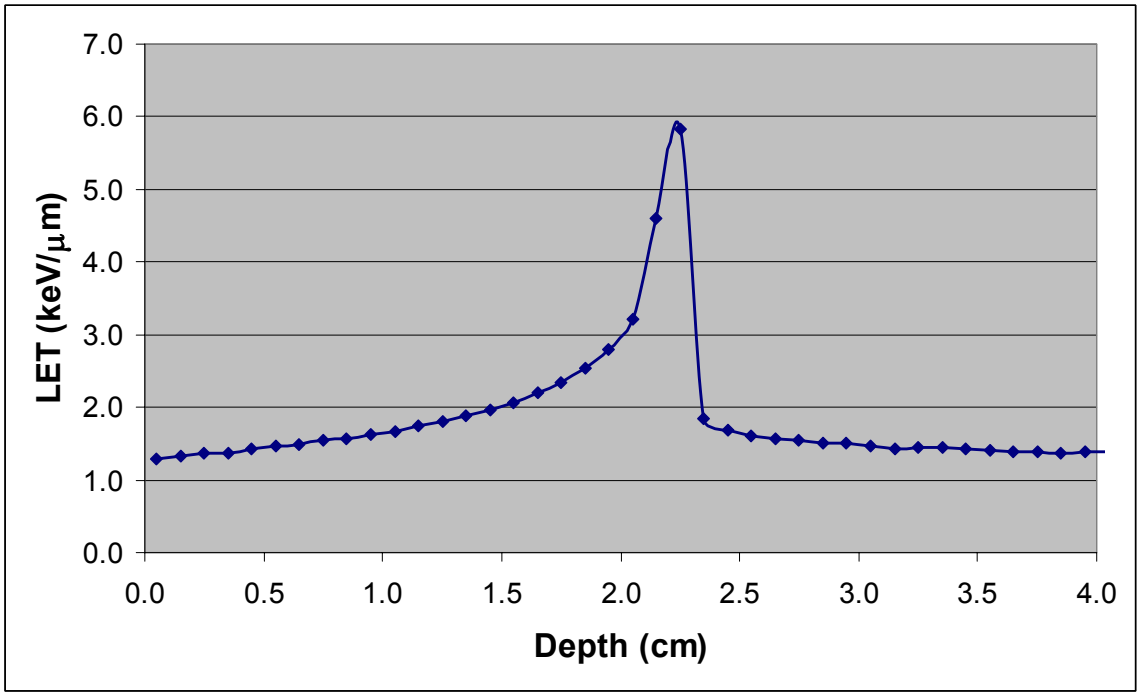

Figure 6: Linear energy transfer (LET) as a function of depth for a 50 MeV antiproton beam in water. Only the contributions from protons and antiprotons are shown.

TLD dosimetry is complicated by the high LET nature of charged particle beams. Furthermore, within the Bragg peak region for stopped antiproton beams, there are contributions from both the primary beam and nuclear fragments. Detailed model calculations have shown that TLD response can be predicted with great accuracy without the use of any free parameters. "However, because of the integration of radial dose distribution as well as over the contribution from primary ions and fragments it is not possible to determine the absolute dose from a TLD measurement in a complex particle field." [Ge98] TLDs cannot be used for absolute dosimetry because of their charge and energy dependent efficiency. Mitaroff et al. [Mi98, Ge98a] have concluded that thermoluminescent dosimetry is useful for verification of the dose

distribution in high LET situations provided changes in detector efficiency as a function of LET are taken into account. It is possible to use TLDs for absolute dosimetry in the case of monoenergetic heavy ion beams when using calculated TLD efficiencies in agreement with comprehensive data sets. However, in mixed radiation fields, only dose verification is possible.

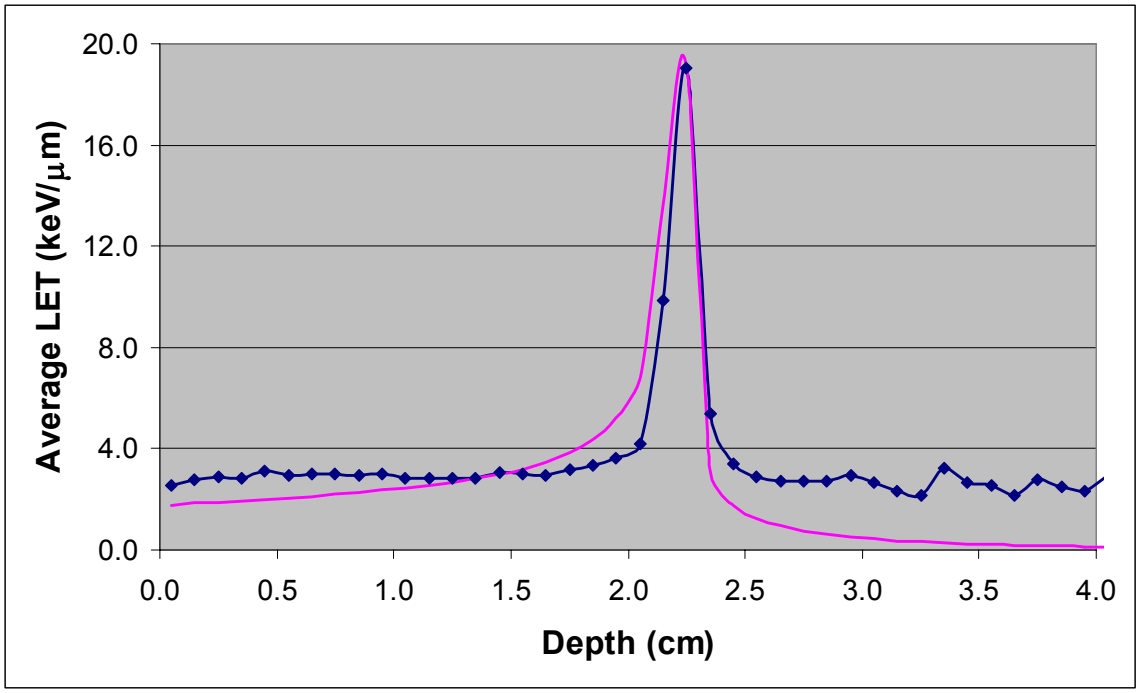

Figure 7: Average linear energy transfer (LET) as a function of depth for a 50 MeV antiproton beam in water. LET is weighted by the dose fraction contributed by each individual particle. A scaled depth-dose distribution is shown for comparison.

Though accurate dosimetry within the Bragg peak is not the main goal of our dosimetric efforts, we can apply Monte Carlo calculations to estimate the peak dose. Figure 7 shows average linear energy transfer, weighted by the dose fraction contributed by each individual particle, for a 50 MeV antiproton beam in water. In contrast to Figure 6, all secondary particles are included. Proximal to the Bragg peak, the contribution comes almost exclusively from protons and antiprotons, while within the first few cm distal to the peak, protons and pions contribute approximately equally. Within the Bragg peak there is a significant population of ^{4}He, ^{3}He, tritons, and deuterons.

It is not obvious that this is the most appropriate way to calculate an average LET relevant to predicting a biological effect much less estimating TLD efficiency. The various micro-dosimetric approaches such as dose weighting, track length weighting, and the local effects model can be compared after we obtain the data from the experiment. To estimate efficiency for TLD response in the Bragg peak region for antiproton annihilation would be highly speculative at this time.

Dosimetry using film, both radiographic and radiochromic, is also subject to saturation and non-linear effects [Sp01]. We will follow a similar procedure, namely, systematic absolute dosimetry in a proton beam with responses in the plateau region correlated with responses in the plateau region of the AD antiproton beam. Radiochromic film has one advantage in that it does not require chemical processing. Radiographic film will be developed off site and analysis will be facilitated using a dedicated film scanner (100 μm resolution) located at UCLA. Given that the efficiency of film response drops more sharply with LET than TLDs (<50% at 10 keV/μm), analysis in the peak region of the AD beam will require knowledge of the antiproton LET spectrum within the film. Spielberger et al. [Sp01] have previously evaluated film efficiency in KODAK X-Omat V film as a function of proton and carbon ion energies. Again, film dosimetry of proton beams has been well established at the Loma Linda facility [Va97].

BANG gels have unique characteristics for radiation dosimetry. They exhibit a 3-dimensional nature and high spatial resolution, but like TLDs and film, also suffer from saturation effects with increasing LET. Nevertheless, Ramm et al. [Ra00] have shown that gel dosimetry can predict the distal edge of the Bragg peak with reasonable accuracy, provided the data properly account for the entrance window of the gel container. Additionally, the authors have suggested that gel dosimetry is useful for evaluating beam profiles. Antiproton dosimetry using BANG gels is intended to be qualitative only – beam characteristics will be evaluated in much the same manner as Ramm et al. MR scanning and subsequent analysis of the gels will be facilitated by Dr. Lawrence Reinstein and colleagues at the State University of New York, Stony Brook. Dr. Reinstein's group also provides dosimetry support to Brookhaven National Laboratory.

In summary, in lieu of a direct determination of absolute dose using standard methodologies, we have proposed two alternate methods. First, absolute dose can be calculated using Monte Carlo codes provided an appropriate means of measuring integrated beam current is available. Second, we propose a systematic evaluation of beam characteristics using a variety of detectors with the antiproton response in the plateau region correlated to that in a proton beam of a similar quality. Additionally, the peak-to-plateau response ratio will be evaluated experimentally using TLDs, supplemented by information on LET obtained from Monte Carlo calculations and TLD efficiency data from the literature. This may facilitate an approximate determination of the RBE in addition to the $BEDR_{2Gy}$ for which the biological experiments are designed. Finally, this initial characterization of the response of various integrating detectors is an essential first step for the eventual goal of utilizing antiprotons in radiotherapy.

B. Differences in stopping power of antiprotons and protons

The energy loss of antiprotons passing through matter has been measured at CERN by a number of different collaborations (PS194, PS196, PS200, PS201, AD1, AD2 and AD3). For high-energy antiprotons and protons, the difference in stopping power was found to be small and mostly affected the very end of range. The most precise and systematic measurements were performed by the collaborations PS194 [Mo97] and AD3 [An02, Mo02]. The energy range investigated by those collaborations extended from 1 keV to 3 MeV, and the target materials were C, Al, Si, and a number of heavier elements. The stopping power of antiprotons was compared to that for protons with equal velocities, which is well known [ICRU93].

Broadly speaking, the stopping power of antiprotons is identical to that of protons for energies down to a few MeV, within a few percent. At energies below approximately 300 keV, however, the stopping power of antiprotons is close to half the stopping power of protons. This means we can estimate the range "R" of 50 MeV antiprotons in a biologically equivalent material such as water according to the following:

R(50 MeV pbar) = R(50 MeV protons) + R(300 keV protons)
= 21.9 mm + 5 micrometer

where the proton range stems from the Monte Carlo program SRIM [Zi85]. As can be seen, the difference between the ranges of 50 MeV protons and antiprotons is negligible in the context of the proposed experiment.

With respect to the energy deposited by antiprotons, in the above approximation the last 300 keV is spread over 10 micrometers instead of the 5 micrometers, which is the range of 300 keV protons.

The range straggling and angular scattering for a beam of 50 MeV antiprotons are expected to be determined by collisions taking place during the first parts of the slowing down, and are therefore expected to be almost the same as for 50 MeV protons.

III. Organization of the collaboration

A. The role and vision of PBar Medical, Inc.

PBar Medical, Inc., located in Orange County, California and Los Alamos, New Mexico, is a scientifically based organization dedicated to the experimentation, exploration, and development of antiproton applications in medical, environmental, and other human-serving areas.

PBar Medical, Inc. is highly interested in collaborating with the general scientific community to determine the feasibility of using antiprotons for various applications, primarily in the medical field. PBar Medical, Inc. has initiated a collaboration with world leading research institutions and has attracted senior scientific, medical, and executive talent to further this effort.

Given the early stage of the research and the lack of detailed information on production economics at this time, it is largely impossible to define a long-term commercial strategy for antiprotons, beyond simply funding experiments to make some initial investigations as to the general validity of the basic concepts. PBar Medical, Inc. is funded by individual, wealthy investors who are interested in exploring powerful solutions to a devastating disease, namely cancer. These individuals recognize they are funding basic science research. They further recognize that any financial return or commercial feasibility is highly speculative and will certainly not be realized in the near future.

PBar Medical, Inc. is committed to assuring that the money received from its sponsors is used in the most productive way to further the scientific basis of understanding the true potential of antiprotons for cancer therapy and other applications in related areas. For this purpose we have built a collaboration amongst several renowned institutions from Europe and the United States and enable members of these institutions to involve themselves in a highly interdisciplinary scientific exchange and collaboration. This collaboration has approached CERN with the request to provide the necessary antiproton beam and technical infrastructure support to conduct an enabling experiment, a measurement that shall answer the narrowly focused but important question:

Do antiprotons have the potential to treat cancer effectively, and are the advantages over other methods significant enough to warrant further studies and development in this area?

To date, PBar Medical's executive team has secured the requisite funds to fully conduct the entire experimental program laid out in our proposal. There is no funding uncertainty with respect to PBar Medical or its ability to support its collaborators in pursuit of its mission and in support of its ongoing experiments. In addition, additional funds for further developments following a successful execution of the proposed experiment at CERN are actively sought, and will enable PBar Medical, Inc. to provide the necessary infrastructure and support to a growing international collaboration in pursuit of this important goal.

B. New collaboration members

Since the date of submitting our proposal we have added a number of key persons to our diverse team of physicists, radiobiologists, and radiation oncologists to guarantee that we have all capabilities needed for a successful execution of this interdisciplinary experiment.

Prof. Dr. Lloyd Skarsgard from the British Columbia Cancer Research Centre and the University of British Columbia recently joined our team. Dr. Skarsgard headed the Department of Medical Biophysics at the British Columbia Cancer Research Centre for 24 years until his recent retirement and still holds an Honorary Professor position in both the Department of Physics and the Department of Pathology of the University of British Columbia. He has published numerous papers on the topic of biological effects of particle radiation on biological media and has been an invited speaker at many international conferences on this topic.

Some selected publications of Prof. Skarsgard are given below:

Evidence for two forms of substructure in the cell survival curve: Mechanisms and clinical consequences. Skarsgard L. D., Hill A. A., Acheson D. K. Acta Oncologia **38**(7), 895-902 (1999).

Radiobiology with heavy charged particles: A historical review. Skarsgard L. D. Physica Medica **14**(4), 179-180 (1998).

Measurements of Relative Biological Effectiveness of the 70 MeV proton beam at TRIUMF using Chinese hamster V79 cells and the high-precision cell sorter assay. Wouters B. G., Lam G. K. Y., Oelfke U., Gardey K., Durand R. E. and Skarsgard L. D. Radiation Research **146**(2), 159-170 (1996).

Through collaboration with Dr. Skarsgard we also have attracted Dr. Brad Wouters, the head of Experimental Radiation Oncology at the University of Maastricht, The Netherlands, to our collaboration. Dr. Wouters has collaborated with Prof. Skarsgard for a number of years and was responsible for the data analysis and interpretation of many particle irradiation experiments performed in collaboration with Dr. Skarsgard.

A selection of relevant publications of Dr. Wouters is given below:

Low-dose radiation sensitivity and induced radioresistance to cell killing in HT-29 cells is distinct from the "adaptive response" and cannot be explained by a subpopulation of sensitive cells. Wouters B. G. and Skarsgard L. D. Radiation Research **148**(5), 435-442 (1997).

Substructure in the cell survival response at low radiation dose: Effect of different subpopulations. Skarsgard L. D. and Wouters B. G. International Journal of Radiation Biology **71**(6), 737-749 (1997).

Low-dose hypersensitivity and increased radioresistance in a panel of human tumor cell lines with different radiosensitivity. Wouters B. G., Sy A. M. and Skarsgard L. D. Radiation Research **146**(4), 399-413 (1996).

Measurements of Relative Biological Effectiveness of the 70 MeV proton beam at TRIUMF using Chinese hamster V79 cells and the high-precision cell sorter assay. Wouters B. G., Lam G. K. Y., Oelfke U., Gardey K., Durand R. E. and Skarsgard L. D. Radiation Research **146**(2), 159-170 (1996).

Substructure in the radiation survival response at low dose in cells of human tumor cell lines. Skarsgard L. D., Skwarchuk M. W., Wouters B. G. and Durand R. E. Radiation Research **146**(4), 388-398 (1996).

In addition, we are in contact with Dr. Gerd Beyer from the Hospital Cantonale in Geneva and have been discussing possible collaborations with him in the area of cell preparation and analysis.

Dr. Beyer is a radiochemist who has worked for more than 25 years on medical application of radio-isotopes and radiation. In Rossendorf, Germany he was responsible for medical isotope production using the cyclotron. His team contained a strong biophysical component that studied radiation effects in cells using different particles for bombardment (deuterons, alpha particles and heavy ions). They found for the first time some indications, and proposed a corresponding mechanism, of a repair mechanism of the DNA after double strand breaks. He also has a strong link to the department of Radiation Bio-Physics in Dubna. He initiated collaborations between the Physicists from Rossendorf and the Heavy Ion Therapy Project in Darmstadt, to develop a dedicated PET system for measuring the dose deposition of heavy ions during therapeutic treatments.

We are well aware of the major developments in the direction of proton and heavy ion therapy in Europe, specifically with the GSI Heavy Ion Project and the collaborations around the TERA Foundation in Italy. We visited Professors T. Haberer, G. Kraft, and W. Henning in June 2002 and spent a day in very interesting and useful discussions. Our impression was that a strong interest exists to apply the theoretical models developed at GSI over the last decade to this new problem, once our first experiments have moved the question on antiprotons for cancer therapy from the level of a curiosity item to a realistic future possibility.

We will continue this contact once we have gained approval at CERN and at that time will also initiate discussions with the TERA group to explore common interests. If our expectations for the enhancement of biological effectiveness of antiprotons for tumor therapy are proven to be right, we envision it will lead to significant research activity, both theoretically and experimentally, which will involve many groups and institutions worldwide.

IV. Proton Test Experiment performed at TRIUMF, Vancouver, BC, Canada

The collaboration has performed an experiment with an external beam of protons that demonstrates that the proposed antiproton experiment at the AD will produce biologically meaningful data. Because of the very short time between the request of the committee for a demonstration proton experiment and available accelerator beam time, the proton demonstration experiment was only able to address the four most important biological questions relevant to the antiproton experiment:

1) What will the data look like and is the concept of the Biologically Effective Dose Ratio (BEDR) meaningful for the proton and antiproton experiments?
2) Does the proposed sliced gel technique combined with cell sorting have adequate depth resolution to resolve the difference in biological response between the plateau and peak regions of a two step spread out Bragg peak?
3) Does a 1 mm slice of dispersed cells in gel contain enough cells to measure the cell survival in the peak region with sufficient sensitivity and precision to determine a biologically meaningful result?
4) What is the estimated precision and accuracy of the proposed experiment?

We did not have sufficient time to fabricate the phantoms for holding TLD arrays, photo chromic and photographic films, and BANG gels, nor was sufficient beam time available at the accelerator before the scheduled shutdown to perform a complete demonstration of the physical dosimetry part of the experiment. We consider this to be less important from a biological perspective at this time because there is extensive literature covering these aspects of the experiment. Furthermore, the physical dosimetry was calibrated with a certified ionization chamber that is used routinely for therapy on the specific beam line that we used.

During the week of December 15, 2002 we performed a proton experiment with the external 70 MeV proton beam at TRIUMF. The beam line is the same that is used to treat eye melanomas. The biological work involving the growth of cells, dispersal in growth medium containing gelatin (gel/medium) at the appropriate concentrations, insertion into sample tubes, certified reference irradiations with ^{60}Co, post irradiation gel slicing, cell sorting and counting using flow cytometry, cell plating, incubation, fixation, staining, and counting of the surviving colonies was performed in the laboratories of Dr. Lloyd Skarsgard at the British Columbia Cancer Research Centre

in Vancouver, British Columbia, Canada.

Figure 8 shows the relative dose as a function of depth for the two-step spread out Bragg peak produced for the 70 MeV proton experiment performed at TRIUMF. A total of 15 separate 1 mm slices of the solidified gel/medium containing living cells were analyzed for cell survival after irradiation in the proton beam. The sample depths were chosen to measure biological response in the plateau and peak regions of the dose vs. depth curve. Separate exposures of sample tubes containing cells were made at proton doses of 1, 2, 3, 4, 5, 6, 10 and 14 Gy in the peak region. The dose profile was measured with a Markus parallel plate ion chamber scanned in the water tank. The absolute dose was measured using an Exradin T1 ion chamber (0.05 cc) which has been calibrated by a standards lab using Co-60. This is the same ion chamber that we use for proton therapy calibrations.

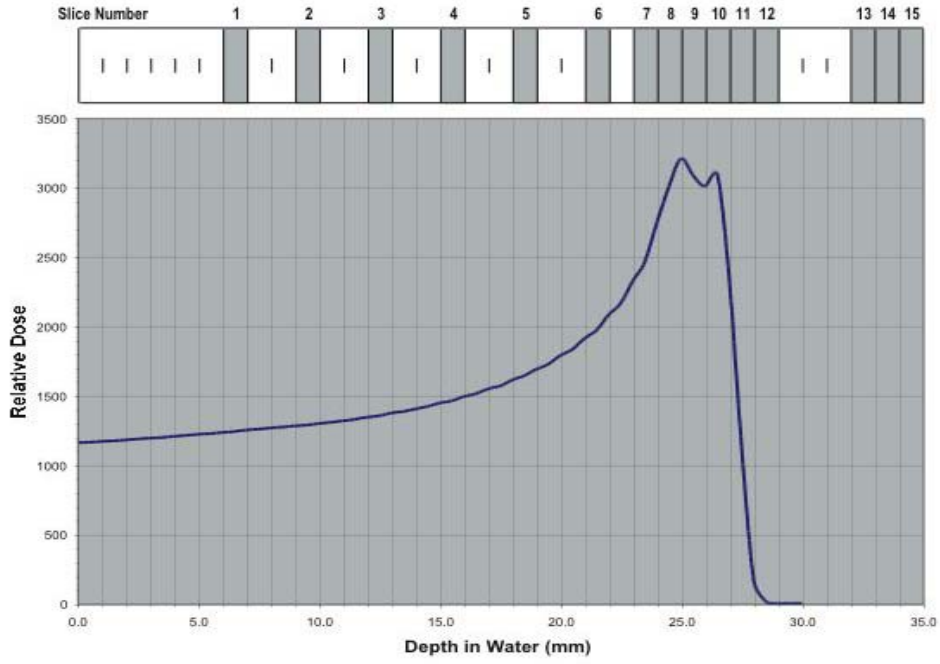

Figure 8: Dose vs. depth for the TRIUMF 70 MeV proton experiment. The sample depths for subsequent cell survival measurement are indicated.

For preparing the biological samples, cells were maintained as monolayers in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 2.2 g/L sodium bicarbonate and 0.122 mg/ml sodium pyruvate. Appropriate numbers of cells were seeded approximately 48 hours prior to irradiation at cell densities designed to give 50 – 75% coverage of the culture surface at the time of harvest. Cell monolayers were harvested with trypsin, neutralized with growth medium, centrifuged and re-suspended in medium containing 12% gelatin and 20% serum at 37˚C at a

concentration of approximately $2x10^6$ cells/ml. At this temperature the gelatin was a viscous fluid, which was poured into ABS (acrylonitrile butadiene styrene) plastic tubes 1.2 cm in diameter and 18 cm in length with a piston located so as to accommodate a 6 cm deep cylinder of gel/medium. After solidification of the gel at 4˚C the tubes were sealed and stored on ice.

For irradiation, each tube of live cells was placed within a circulating water bath phantom, approximately 18 cm x 18 cm x 24 cm. A refrigerated glycerin and water solution of the same density as the gelatin and ABS tubes was used to maintain the cells at 2˚C. The phantom simulates the effect of backscattering and energy absorption in a human body. The ABS, the 12% gelatin, and the phantom have the same density and stopping power. This ensured a constant stopping power throughout the entire phantom and thus avoids any artifacts that could result from scattering of protons from points outside the gel. The experimental system was very similar to what we have used previously to successfully measure the RBE for heavy charged particles [Sk82, Wo96]. The sample tubes were held at 2˚C until the cells were plated after irradiation.

After irradiation, and under sterile tissue culture conditions, the gel was extruded from the tube using a plunger connected to a delivery mechanism that advanced the cylinder of gel in 1 mm steps. The gel was sliced every 1 mm using a taut wire. The slices from selected depths were dispersed in MEM culture medium containing 10% fetal bovine serum and antibiotics. Each slice represents a different depth along the path of the beam. The dispersed cell samples were analyzed using a cell sorter (flow cytometer) which identified individual cells on the basis of forward and perpendicular light scattering without the use of a cell stain [Du91], and dispensed the prescribed number of cells into a test tube for plating. We have shown previously that this is an extremely accurate way of determining the number of cells plated per dish [Wo96]. The numbers of cells plated in Petri dishes were chosen to likely give 400 colonies per 10 cm dish (which required using many different starting cell densities). Using this technique is known to improve precision in our measurement of RBE values to +/- 2 to 4%. After incubation for 5 – 6 days in a controlled environment, the colonies that developed were fixed, stained and counted. Only those colonies having more than 50 cells were counted as having been derived from a single surviving cell (smaller colonies are due to cells that successfully negotiated one to five doublings before their reproductive death). As of December 30, 2002 the cell colonies have been counted, but the tabulation and analysis of the data has not been completed. The initial results indicate that this measurement technique has sufficient depth resolution and sensitivity to measure the difference in biological response between the plateau and Bragg peak regions of the samples.

Survival curves will be fitted using the standard non-linear curve fitting routines and Biologically Effective Dose Ratios of antiprotons, as a function of plateau and peak

dose, will be calculated as described previously in this technical addendum. The detailed results with estimates of the precision and biological significance will be presented at the SPSC meeting on January 14, 2003.

V. References

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