PRODUCTION AND CHARACTERIZATION OF ANTIBODIES AGAINST IRRADIATED HUMAN ERYTHROCYTE MEMBRANE PROTEINS.

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ABSTRACT

Gamma irradiation affects people in several situations, with few if any sensitive biological assay of its action. Nucleic acids and proteins are affected by radiation, but only the former was used in most dosimetric techniques. The irradiation of proteins promotes structural modifications attributed to free radicals from water radiolysis. Theoretically, antibodies induced by irradiated proteins could recognize these radical-related new epitopes, allowing their use as a probe. Human erythrocyte membrane proteins(HEMP), few and well defined molecules, are certainly exposed to radiation, being the ideal target. With this rationale, we study the production of antibodies in mice immunized with ^{60}Co irradiated HEMPs. Membranes from hypotonic lysis with differential centrifugation of A+ erythrocytes, were irradiated in a Gammacell 220 with 400, 800 and 1600Gy, and used as immunogen for Balb/c mice, after SDS-PAGE. Irradiated HEMP induced antibodies recognize only irradiated human erythrocytes in an intact cell indirect immunofluorescence assay(ICIIFA). When used in Western-blot against non-irradiated HEMPs, those sera recognize most proteins, suggesting a pool of abs directed both to native, as detected by Western Blot, or irradiated, as detected by ICIIFA, HEMPs. Those data confirmed our assumptions, allowing the use of those abs in the search for a method of biological dosimetry.

I. INTRODUCTION

The effects of gamma radiation on people exposed to its action, in accidents or therapeutic uses, are difficult to estimate in an biological approach[1]. The estimates of physical dosimetry underscores interpersonal biological diversity[2]. Nucleic acids and proteins are affected by radiation, both directly or by free radical action due water radiolysis[3]. Most of available biological dosimetry techniques deals with the damage of the DNA of the cell, using time-consumpting cytogenetical methods, discarding dying or anucleated cells[4]. Proteins, despite its quantity, long turn-over time and stability, had been rarely considered as a probe or an biological dosimetric model[5].

During its radiation, proteins are also affected by free radicals from water radiolysis, and some oxidative changes could occur, generating aggregates or molecular modifications, creating new epitopes or chemical entities[6]. Theoretically, if those new epitopes could be recognized by an specific antibody, a powerful probe will be created, especially if the irradiated protein had an uniform distribution in the body and were easily accessed for testing.

Human erythrocyte membrane proteins are the perfect target using this rationale. They comprises relatively few proteins, some exposed in the surface of the erythrocyte and are easily analyzed by SDS-PAGE and immunofluorescence microscopy[7]. Otherwise, erythrocytes are homogenous circulating cells, with a relatively long turn-over of 45 days, certainly exposed in therapeutic or accidental radiation[8].

Specially, two erythrocyte membrane proteins, band 3 and spectrin, are clearly identified on SDS-PAGE, and presented diverse cell localization, one exposed in the erythrocyte surface and the other, immediately above the membrane, inside the cell[9]. Despite several studies dealing with irradiation of these proteins, we cannot found a one report dealing with the existence of

radiation induced new epitopes[10,11].In this logical basis, we study the production of specific antibodies against human erythrocyte membrane proteins irradiated by ^{60}Co in isogenic mice.

II.MATERIALS AND METHODS

Materials:

All reagents used in the experiments were from pro-analysis quality, and the solutions used were made with MilliQ high purity water. Human red blood cell(RBC) were kindly furnished from Fundação Hemocentro de São Paulo, as blood bank donor bags, maintained in ACD solution. Anti-mouse fluorescent conjugates were bought from Sigma Co.

Methods

Human erythrocyte membrane isolation: The membrane fraction of human erythrocytes was obtained according as described[12]. Briefly, phosphate buffered saline(PBS) washed RBCs were diluted in 20 vol. of 1/20 PBS saline and centrifuged to 1000g/5 min. The supernatant was recentrifuged to 17000g/20 min. The loose pellet was resuspended in 1/20 PBS and recentrifuged. The process was repeated until the absorbance at 540 nm was less than 0.1. The creamy suspension had its protein content determined according Bradford[13], and stored at -70°C until use.

SDS-PAGE and Western Blotting: For analyzing molecular weight and protein structure we used the vertical slab system described by Laemli[14], for discontinuous SDS polyacrylamide gel electrophoresis. The running gel acrylamide concentration was 7.5% and 4M Urea was added to sample buffer. Commercial molecular weight standards were run together in separate lanes. Protein bands was revealed both by Coomassie Blue G-250 or silver staining. Adequate transfer to nitrocellulose membranes was accomplished with selected samples, according reported methods[15].

Irradiation and immunization schedule: The creamy suspension was adjusted to a protein concentration of 1 mg/ml in PBS pH 7.2, and distributed in adequate vials. The irradiation was accomplished homogeneously in a Gammacell 220(Atomic Agency of Canada Ltd), achieving 400Gy, 800Gy and 1600Gy, at 400Gy/h, in the presence of oxygen and at room temperature. After the irradiation samples were submitted to SDS-PAGE or used in immunization schedule. The basic immunization schedule involves three biweekly subcutaneous injections of 50 µg/total protein/ Balb/C mice dissolved in complete(the first one) and incomplete(the others) Freund's adjuvant. At least five animals were injected and tail blood samples were collected in standardized filter paper and stored dry at -20°C[15].

Intact Cell Indirect Immunofluorescence Assay: PBS washed A+ human RBCs were submitted to 800Gy irradiation similarly to above described. After a new washing, the irradiated red cells or non-irradiated controls were layered over immunoflurescence slides and dried gently at room temperature. The intact cells were rehydrated immediately before use. Dried filter paper containing immunized mice sera were suspended in PBS in order to give an 1/20 dilution and serial dilution were applied over to irradiated or controls erythrocytes. After washings, the bound antibody was revealed by an anti-mouse fluorescein conjugate, with adequate photomicrography in a Zeus Axiophot immunofluorescence microscope[15].

Immunoassays: Nitrocellulose strips, containing transferred bands of SDS-PAGE of human erythrocyte membrane proteins adequately blocked with skim milk, was assayed with immunized mice sera diluted as described. The bound antibody was revealed with an anti-mouse IgG peroxidase conjugate, using Diaminobenzidine/cobalt enhanced detection system[16].

III. RESULTS

Purification and irradiation of ghosts: We purified erythrocyte membranes of a blood bank bag, containing washed human A+ RBCs(200ml), as described in Methods. The purified ghosts were then submitted to ^{60}Co irradiation(400, 800 and 1600Gy) in a GammaCell 220 and the profile of SDS-PAGE electrophoresis of processed and control antigens could be seen in fig. 1

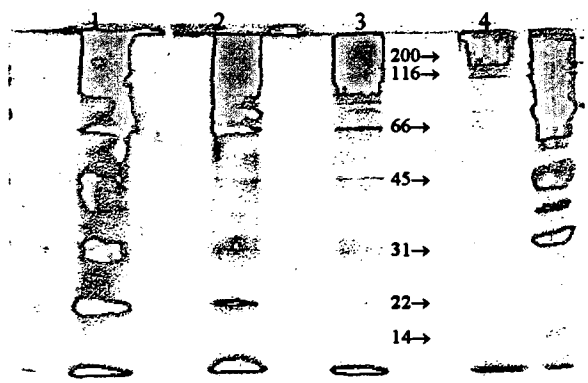

Figure 1. SDS-PAGE of proteins of erythrocyte membranes submitted to ^{60}Co irradiation. Lanes 1: Controls; 2: 400Gy; 3: 800Gy; 4: 1600Gy. MW of markers are added between lanes 3 and 4.

A clearly defined bands are only seen in samples irradiated with less than 400 Gy. In others samples, a poorly defined high molecular weights aggregates appears., with a poor definition of regular bands that are also underexpressed. The protein amount in each lane was the same, as detected by Bradford assay, with an increase staining in the high molecular weight area in the gel. Mice were immunized with irradiated erythrocytes membranes, the same as submitted to electrophoresis as above cited. The immunization was performed according methods and tail blood was collected in dried filter paper. We use this samples in antibody detection by ICIIFA, with some dilution in order to titer quantification. We shown the results of mice immunized with 800 Gy irradiated HEMPs, that was similar to the other irradiated samples tested.

ICIIFA: Eluted antibody from immunized mice was used in ICIIFA assay as described, against intact or irradiated erythrocytes. The results could be seen in figure 2, showing that mice immunized with irradiated HEMP produced few amount, (1/4 titer), of specific antibodies against intact RBCs, but easily detectable(1/64 titer) antibodies against irradiated RBCs.

Figure 2. Intact cell indirect immunofluorescence assay.
A. Intact erythrocytes non irradiated vs Abs from mice immunized with 1600 Gy irradiated HEMP
B. Irradiated erythrocytes vs Abs from mice immunized with 1600 Gy irradiated HEMP.

The mice immunized with native HEMP show few reaction with both type of antigen in this test.(data not shown).

We also tested those sera in an ELISA with microplates adsorbed with irradiated and non-irradiated HEMPs, with detection of antibodies in all tested samples. As we cannot ascribed this result to inespecific adsorption , we performed Western Blot analysis using nitrocellulose membranes adsorbed with previously SDS-PAGE separated HEMP. All immunized mice produced antibodies against erythrocyte membrane proteins, as shown in Figure 3, without significant difference of bands recognition or other qualitative analysis, unrespect to the antigen used in the immunization

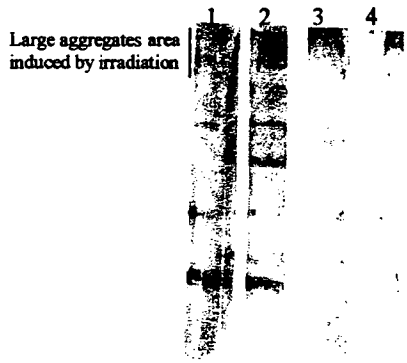

Figure 3 - Western blot analysis. Lane 1: abs of irradiated HEMP immunized mice, 2: abs of native HEMP. immunized mice .3 and 4 control non-immunized mice. All lanes were from SDS PAGE of 800 Gy HEMPs.

IV.DISCUSSION

Our results of the irradiation of purified HEMPs were similar to those already described, except by the fact of high molecular weight ill defined bands were found in our results[10,11]. We could explain this phenomenon to the fact that we never try to centrifuge our samples after irradiation, in order to maintain the protein amount. In others systems, when proteins were irradiated, some sort of precipitation could occur. Probably, as shown in venom's irradiation, aggregation of protein molecules could occurs, explaining the decreasing of low molecular weight bands[17].
The irradiated HEMP are antigenic both to native epitopes and the new generated by irradiation as demonstrated by our data in the ICIIFA, when clearly it was seen that some antigenic determinants in irradiated erythrocytes could be detected. Unfortunately, those specific antibodies are, in fact, only a minor fraction of the antibody induced by the

irradiated HEMPs, as shown by our Western Blot analysis, when multiple bands are stained.
Those data could be explained by the exposition of protein molecules in the external surface of the RBC to the water radiolysis free radicals, generate new epitopes, without the high scavenger activity from the RBC cytoplasm[6]. Those new epitopes could be shown only by the ICIIFA, that maintains the original structure of the RBC, avoiding the proteins in the inside of the cell. When techniques that expose those internal epitopes are used, as the ELISA and Western Blot , sera both from native or irradiated immunized mice reacts similarly. We cannot avoid the possibility that the irradiation induces some modifications in the structure of RBC[18], allowing the penetration and reaction of the antibodies with internal structures, but this fact probably are incompatible with the maintenance of the morphological structure of the RBC, as shown in our ICIIFA assay, and we carefully washed the cells before assay in order to eliminate heavily damaged RBC during the irradiation process.
The antibodies induced by irradiated HEMP presenting promising characteristics , with the possibility of affinity purification, allowing its use in the detection of RBCs that were irradiated *in vivo*, during accidents or radiotherapy, as a possible biological dosimetry tool.

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