c/a Glaser

IN-VITRO RESPONSE OF LYMPHOCYTE CULTURES EXPOSED TO RF RADIATION: PROGRESS REPORT ON FEASIBILITY AND DETERMINATION OF CRITICAL VARIABLES

Richard H. Lovely, Thomas J. Sparks and A.W. Guy

University of Washington
School of Medicine
Department of Rehabilitation Medicine
Bioelectromagnetics Research Laboratory
Seattle, Washington 98195

Contract No. F41609-75-C-0021

April 15, 1976

Prepared for

USAF School of Aerospace Medicine
Aerospace Medical Division (AFSC)
Brooks Air Force Base, Texas 78235

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) IN-VITRO RESPONSE OF LYMPHOCYTE CULTURES EXPOSED TO RF RADIATION: Progress report on feasibility and determination of critical values		5. TYPE OF REPORT & PERIOD COVERED Technical 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Richard H. Lovely, Thomas J. Sparks, and A.W. Guy		8. CONTRACT OR GRANT NUMBER(s) F41609-75-C-0021
9. PERFORMING ORGANIZATION NAME AND ADDRESS Bioelectromagnetics Research Laboratory Department of Rehabilitation Medicine RJ-30 University of Washington School of Medicine Seattle, Washington 98195		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Airforce School of Aerospace Medicine Brooks Air Force Base, Texas 78235		12. REPORT DATE April 15, 1976 13. NUMBER OF PAGES Eleven (11)
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

lymphocytes, radio frequency fields

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

In preparation for in-vitro microwave exposure of cultured primate lymphocytes, preliminary determination of the most effective values of the requisite biological parameters were determined and are outlined herein. In addition, preliminary results from the initial lymphocyte exposures are presented.

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE
S/N 0102-014-6601

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

IN-VITRO RESPONSE OF LYMPHOCYTE CULTURES EXPOSED TO RF RADIATION: PROGRESS REPORT ON FEASIBILITY AND DETERMINATION OF CRITICAL VARIABLES

Richard H. Lovely, Thomas J. Sparks and A.W. Guy

A number of recent studies have shown that electromagnetic radiation in the RF band frequencies, as well as in the microwave range, can significantly affect the number of mitotic figures obtained in PHA-stimulated lymphocyte cell cultures whether the radiation exposure per se is carried out in-vivo [1][2], or in-vitro [2][3]. The in-vitro effects, however, have only been obtained with microwave and not RF band stimulation. Since only in-vivo effects had been obtained with RF band radiation at the time, it seemed highly desirable to examine the effects of RF band electromagnetic radiation on in-vitro lymphocyte cell cultures and to do so in such a manner that the power deposition in the culture vessel could be quantitatively related to the absorbed power that might occur in man and laboratory animals. The specification of power deposition during in-vitro RF radiation seemed most likely to be achieved by the development of a standardized culture vessel with known absorption properties. Such a vessel would allow not only for precise quantitation of in-vitro power deposition in our laboratory, but could also be employed for similar preparations in other laboratories studying electromagnetic radiation effects on lymphocyte (or other cell) cultures as well as suspended cells in general. The advantages of a standardized culture vessel for comparisons of research findings from different laboratories should be obvious.

The research and development of the standardized culture vessel under the subject contract was the second task of the three major work efforts to be conducted and has been reported on to the sponsor elsewhere [4]. Briefly, it can be indicated here that the research and development of the culture ring was a more formidable task than originally anticipated. Thus, the originally proposed time necessary to develop such an exposure vessel significantly underestimated the effort that finally eventuated. Since the completion of the culture ring was not finalized until the end of the time period allowed for the subject research contract, there was very little opportunity to employ the culture ring in the exposure of freshly obtained primate lymphocytes.

Even though the unexpected delays in finishing the standardized culture ring precluded carrying out the extensive work effort on cultured primate lymphocytes exposed to RF radiation, a number of milestones were nevertheless achieved which now places our laboratory in an excellent position to answer the original questions posed, as well as related questions dealing with microwave and RF band radiation effects on hematopoiesis. These achievements are described in the following paragraphs.

A biological laboratory technician has been trained in the laboratory of Dr. Stanley Gartler (Genetics and Biochemistry) in the techniques of lymphocyte cell cultures. In addition, the requisite equipment and supplies necessary for lymphocyte culture work have been purchased and a base of operations has been established in Dr. Lovely's laboratories. Five monkeys (M. mulatta) housed in the Health Sciences vivarium complex are now being used as blood donors.

The initial work centered largely on growing non-radiated cultures so as to establish the best technique for the radiation work to eventually be done. For example, various media, mitogens, mitotic blocks, and hypotonic solutions have been used in combination with various size samples (ranging from micro-cultures to macro-cultures) to determine the best set of conditions that reliably produce the lymphocyte cultures with a minimal amount of variance. In addition, whole blood as well as sedimented "plasma" has been compared to determine which will give the highest yield.

These parametric comparisons have yielded a set of values which seems most reliable in producing lymphocyte cell cultures with maximum replicability. The subset of techniques which seem to work best includes the use of 10 cc of whole blood drawn from one of the donor monkeys with a heparinized syringe. The blood is then centrifuged at 600 RPM for 2-5 min. periods and the "plasma" is removed. Media is added to the cell suspension in a ratio of 4:1 and PHA-p is then added to the culture in a ratio of 50:1 (taken from a 50:1 stock dilution). The semi-open culture system is then placed in a CO_2 environment at 37°C for 72 hr. and 3-4 hr. prior to the culture harvesting, colcemid (1 µg/ml concentration) is added (0.2 ml per culture). Cultures are then harvested using a 0.075 molar KCl hypotonic, methanol-acidic acid fixative, and stained with giemsa. The baseline mitotic rate obtained from the procedure described above ranges from 1.5 to 2%.

In May, 1975, while Dr. Frazer (USAFSAM) visited our laboratories, blood was drawn from two monkeys exposed to radio frequency radiation and was cultured by the techniques described above. The two monkeys

were exposed twice to a magnetic field strength of 40 A/m for 30 sec. at a frequency of 143 MHz. Rectal temperatures taken before and after each of the two radiations indicated a mean increase of 1.2°C in one monkey and 0.5°C in the second monkey. In addition to the sample drawn following radio frequency radiation, a second 6-8 cc sample of blood was drawn and cultured 48 hr. after radiation, as well as a control sample of 6-8 cc of blood drawn immediately prior to radiation. Blood samples obtained from the two monkeys prior to radiation showed approximately 1.5% rate of mitotic activity (which is in agreement with our baselines obtained to date) while, on the other hand, samples drawn immediately or 48 hr. after radiation showed no mitotic activity at all.

These findings, obtained from the two monkeys described above, are interesting findings that have at least two possible explanations for their occurrence. The first possibility could be that the effect of RF radiation (at the levels reported) was to totally suppress PHA-stimulated mitotic activity, which would be diametrically opposed to the findings of Prince, et al. [2]. An alternative explanation, and one which would accommodate the findings of Prince, et al., is that the effect of these particular exposures was to induce a phase-shift [5] in peak mitotic activity, i.e., metaphase has undergone a significant temporal shift and to capture the mitotic figure change reported by Prince, et al., one must harvest and "colcemid-block" at a different point in time, e.g., 96 hr., instead of 72 hr., as was done in these samples.

The second interpretation proposed above was somewhat anticipated at the time of the original proposal which is why we proposed to harvest

all lymphocyte cell cultures on a time-dependent basis. More specifically, in the original proposal [6] the culture vessel to be developed was to consist of an rexolite ring subdivided into several compartments from which cultured lymphocytes could be harvested every 8 hr. following RF radiation without disturbing the balance of the samples. However, the proposed design for the culture ring did not work out as originally proposed [6], and the culture vessel actually developed [4] consists of a single compartment stainless steel and teflon ring, which is interfaced with the RF source. This single compartment ring has necessitated the adoption of a completely new experimental design wherein plasma stimulated with PHA is placed in the ring only for the duration of RF exposure. It is then removed and returned to a standard culture test tube for the remainder of the incubating period.

When the latter (stainless steel) culture ring was finally developed, the first requisite for its continuous utilization was that it be compatible with lymphocyte cell cultures. Surprisingly, we encountered substantial difficulty growing such cultures. After a number of attempts, which entailed PHA-stimulated plasma being placed in the culture ring for up to one hour before being placed in a standard culture test tube and incubated in a CO_2 environment, we were finally able to determine that the culture ring was, in fact, compatible with lymphocytes in culture. The problems we had encountered, it turned out, were due to the extra time (between 40 min. to 1 hour) that the stainless steel ring's cultured cells were exposed to oxygen as were the non-irradiated culture ring control (both rings continuously failed to yield viable mitotic figures),

when compared to the standard lymphocyte cultures which had been placed immediately in a CO_2 environment following PHA stimulation. Subsequently, it was determined that if the total oxygenation time was reduced to 30 min., or less, viable lymphocyte cultures were obtained in the stainless steel and teflon ring. However, even these cultures appeared to have undergone a phase-shift such that peak mitotic activity occurred at 96 hr., while the control cultures (standards) do better at 72 hr. It is entirely possible that the ring-compatibility test cultures originally exposed for up to an hour (i.e., over-oxygenated) would have yielded mitotic figures if harvested at another point in time, e.g., 120-150 hr., since these cultures clearly went to metabolism and numerous blast cells were seen in these preparations.

These interpretations were suggested by the following experiment. Blood plasma, drawn from three monkeys, was divided equally into three sub-samples. One sample from each monkey was cultured by the standard culture-tube procedure. A second sample was cultured in the stainless steel culture ring but was placed immediately in a CO_2 environment rather than going to the RF radiation source, while the third subset of samples were placed in a standard culture test tube but left outside the CO_2 environment for the period of time it typically took to irradiate a culture in the ring. Thus, the experiment treated one set of standard culture test tubes like the culture ring in terms of time out of the CO_2, while treating the culture rings like standard culture tubes by going directly to the CO_2 incubator. All samples were then harvested at 97 hr.

The results of this experiment were that the standard culture technique yielded 1.2% mitotic activity and those cells cultured in a

CO_2 environment (but in a culture ring) yielded 0.9% mitotic activity, while the third set of cultures grown in standard culture tubes but allowed to remain outside the CO_2 environment for approximately 40 min. showed 2.13% mitotic activity. While these mean differences appear to be small, an analysis variance indicated that they were marginally significant ($F = 5.22$, $df = 2.5$, $.05 > p < 0.1$). Exposure for as little as 40 min. to an oxygenated environment induces a significant phase shift in peak mitotic activity for cultured lymphocytes harvested at 97 hr. The probable mechanism for the significant shift is a change in Ph, i.e., increased acidity due to over-oxygenation.

Subsequent studies conducted tangential to verifying the compatibility of the stainless steel culture ring have by-and-large suggested three major parameters which will probably affect the metaphase time, i.e., phase-shift for peak mitotic activity, and these factors are 1) Ph (acid-base relationship), 2) environmental temperature during the first hour of PHA-stimulation, and 3) PHA itself (not only the amount, but that point in time relative to radiation at which the PHA is added).

Since the standardized stainless steel culture ring has taken so much more time than originally anticipated, and since even the ring-lymphocyte interface compatability tests took well over a month, we have not been able to generate as much in-vitro data consequent to RF exposure as we would have like to. However, with the ring now finished, and with a better understanding of the relationship between metaphase time and the three critical variables outlined above (Ph, temperature and PHA), we have been able to initiate the first RF exposure trials using the new

ring to expose cultured lymphocytes.

The first such exposures were conducted while this report was being prepared. The cultures were exposed to 30 MHz RF radiation at 5 V/cm for 20 min duration. A constant temperature circulator maintained the culture temperature between 36.1°C and 36.6°C in one sample, while in a second sample the temperature reached 41°C within 2 min. and 44°C within 5 to 10 min. in response to RF exposure. In both cases, each lymphocyte sample had two pair-matched controls - one incubated as the "standard" and one that had its access to the incubator (CO_2 and temperature-controlled environment) delayed the same length of time as it took to process the sample undergoing RF irradiation. During this delay period, the CO_2-delay controls were maintained in a water bath at 37°C.

The plasma sample exposed with the temperature maintained at 36.5°C, unfortunately, yielded too few cells to obtain any viable index of mitotic activity. The two pair-matched controls, harvested at 72 hr., yielded 1.3% and 0.7% mitotic indices. The lower value was for the water bath (CO_2-delay) control. On the other hand, the sample that underwent a 7°C (to 43.8°C) temperature rise in about as many minutes did yield viable cells and a mitotic index of 0.4% consequent to RF radiation. The two pair-matched controls yielded mitotic indices of 0.7% and 0.2% for the standard and water-bath (CO_2-delay) control, respectively. All of the latter values were obtained following harvesting at 96 hr. While all the latter values are low (harvested at 96 hr.) relative to the first set of samples and our typical baseline of 1.5% - 2.0%, they do not appear to differ significantly from each other.

More importantly, these latter data suggest two obvious conclusions. First, that viable lymphocytes can be captured at metaphase when exposed to RF radiation in the newly developed stainless steel culture ring reported on by Guy [4]. Secondly, it would appear from these data that exposure to 5 V/cm at 30 Mhz does not induce any significant change in mitotic activity - at least when harvested at 96 hr. It is, of course, conceivable that harvesting at some other point in time might yield significant differences between samples.

During the current contract with the U.S. Air Force School of Aerospace Medicine, we will be continuing with our evaluation of RF exposure parameters on lymphocytes in culture, as well as expanding our effort to include RF exposure effects on immune sensitization in the rat following chronic (over 800 hours) irradiation. Finally, and as time permits, we will be initiating a series of studies employing immunofluorescent techniques to evaluate lymphocyte responses to RF radiation.

REFERENCES

1. Baranski, S., "Effect of chronic microwave irradiation on the blood forming system of guinea pigs and rabbits," Aero. Med., 1971, 42, 1196.
2. Prince, J. E., L. H. Mori, J. W. Frazaer, and J. C. Mitchell, Cytologic aspect of RF radiation in the monkey," Aero. Med., 1972, 43, 759.
3. Stodolnik-Baranska, W., "Lymphoblastoid transformation of lymphocytes in-vitro after microwave irradiation," Nature, 1976, 214, 102.
4. Guy, A. W., "A method for exposing cell cultures to EM fields under conditions of controlled temperature and field strength," Scientific Report Nc. 9, Bioelectromagnetics Research Laboratory, Dept. Rehabilitation Medicine, Univ. Wash. School of Medicine, Seattle, Wa. Prepared for: USAFSAM Contract No. F41609-75-C-0021, April 15, 1976.
5. Dr. James W. Frazer - Private Communication.
6. U.S. Air Force School of Aerospace Medicine, Brooks Air Force Base, Texas, Contract No. F41609-75-C-0021, "Critical Comparison of RF Field Delivery Techniques and Measurable Results in Cell Culture of Whole Animals."