IMMUNOLOGICAL EFFECTS OF MICROWAVES, ULTRASOUND, AND HYPERTHERMIA: B-LYMPHOCYTE CAPPING

BY

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THESIS

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A la mémoire de mon père

EXPRESSION OF APPRECIATION

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I. INTRODUCTION

Microwave and ultrasound devices have found an increasing number of industrial, medical, consumer, and military applications over the past decades. Microwave and ultrasound hyperthermia are presently being used to treat cancer and may become a widely used clinical procedure. Consequently, there has been a growing concern in both the public and scientific communities regarding the deleterious effects of microwave or ultrasound radiation on biological systems, particularly the immune system. Various microwave or ultrasound immuno-effects have been reported. These will be reviewed in the next chapter. Originally, these phenomena were considered to be a direct result of the heating properties of microwave or ultrasound radiation, but many scientists are now convinced of the existence of non-thermal field specific effects.

The present study was undertaken to evaluate the effects of microwaves and ultrasound on the capping of antigen-antibody (Ag-Ab) complexes on the surface of freshly isolated splenic mouse B-lymphocytes. Capping is an active process which occurs after specific antibodies bind to cell membrane receptors. Originally, these membrane-bound receptors are uniformly distributed on the cell surface. Following binding of specific antibodies, the receptor-antibody complexes are redistributed and regrouped under normal conditions (37°C, no drugs, no radiation) into a polar cap on a small portion of the cell membrane. The remaining larger portion of the membrane is thus devoid of any of that particular receptor.

Before assessing the possible effects of microwave or

ultrasound radiation, it was necessary to determine whether heat alone had any effect on capping. A series of experiments was done for that purpose in the temperature range between 37 and 43°C. Possible thermal and non-thermal field specific effects of microwaves or ultrasound were later decoupled by designing experiments where both irradiated and control cell suspensions were maintained at the same regulated temperature.

While the exact immunologic role of capping has not yet been fully determined, it has been suggested that, for the case of cell surface immunoglobulin (Ig) molecules, capping may be the triggering signal for B-lymphocyte proliferation. Also it has been speculated that capping and shedding of membrane receptors may help the antigen bearing cells escape the immune surveillance system; such a phenomenon may occur with cancer cells. Any thermal or non-thermal effect on capping would then seem to have important consequences on immunity.

II. GENERAL REVIEW

II A. HYPERTHERMIA

A1. General biological effects of hyperthermia

The last two decades have witnessed an increased interest in hyperthermia and its possible application as a therapeutical agent against cancer (1-3). In 1967, Cavaliere et al. (4) reported the effects of perfusion hyperthermia on 22 patients who had malignancies of the extremities. The temperature in the tumors was raised to 41.5 - 43.5°C for several hours in 25 regional perfusions with prewarmed blood free of any chemotherapeutical agents. A complete disappearance of the tumor was observed in 10 out of 22 patients and a significant decrease in tumor volume was observed in 5.

Pettigrew (1974) treated 82 cancer patients with several repeated exposures to hyperthermia at 42°C (5). The treatment was well tolerated as long as the temperature was kept below 41.8°C. Clinical and radiologic evidence of tumor regression was often obtained, but no long-term tumor-free survival was reported.

At present, the mechanisms of hyperthermic cell killing are poorly understood. Many experiments have been conducted and many theories are suggested, but none of them can be regarded as conclusive.

Some of the best documented results show that there is a selective heat sensitivity of cancer cells (4, 6-8). Cancer cells are apparently more heat sensitive than normal cells. Cavaliere (4) reported that the oxygen uptake of Novikoff

hepatoma and Ehrlich ascites carcinoma cells was considerably less at 42°C than at 38°C, whereas for normal cells there was little difference in respiration at these two temperatures.

Different phases in the cell cycle also show different sensitivities to hyperthermia. By determining the survival reduction caused by a constant dose of heat on synchronized cells, Bhuyan et al. (7) showed that the mid and late S-phase CHO cells were more sensitive than cells in the M-phase (mitosis) or early S-phase (DNA Synthesis). G_1 and G_2 cells were the least sensitive. Other studies on HeLa cells (9) showed similar results, with the S and M phases being the most heat sensitive relative to cells in other phases of the cell cycle. In contrast, the S phase is the most radio resistant in X-ray treatment.

Harris (10) was the first to quantitate hyperthermic killing. His heat-survival curves show an initial shoulder followed by an exponential decline of the surviving fraction. These curves show that there is an accumulation of heat damage at low doses that results in cell killing. At these sublethal doses, the damage inflicted by heat can be repaired. At the higher doses that correspond to the exponential decline, the cells have accumulated all the damage they can tolerate, and a single damaging event would then be irreversibly lethal. Similar results were obtained by Parlzer and Heidelberger on HeLa cells (9).

In 1963, Crile (11) found that the destructive effects of heat on tumors implanted on the feet of mice began at 42.° C. He also found that the increase in temperature above 42°C which

halves the time required to elicit the same lethal damage is 1.0°C.

Other related studies show that hypoxia (poor oxygenation) increases the thermal sensitivity of cells (12, 13). Schulman (12) found that under hypoxic conditions, killing of cultured mammalian cells by hyperthermia started at 41°C whereas 43°C was necessary to produce substantial cell killing.

The environmental acidity is another determining factor in the hyperthermic effect (13,14). Gerweck (14) observed an enhanced cell killing when the cells were exposed to reduced pH and elevated temperature simultaneously.

It is not yet known how hyperthermia causes cell death. Several targets have been suggested: proteins, RNA, DNA cellular membranes, lysosomes or any combination of these might be directly damaged by heat. Overgaard (15) observed a pronounced lysosomal activity after a few hours treatment of a murine mammary carcinoma, and hypothesized that a primary, lysosomally conditioned selective destruction of the malignant cells occurs and that this reaction is intensified by a high acidity in the tumor milieu. Mondovi (8) suggested that the first damage in heat treated cancer cells resides in some organized system such as lysosomal or surface membranes. In support of his conclusion is the fact that tumor membranes have a different composition than those of normal cells. They have in general higher levels of cholesterol. Other investigators have suggested that hyperthermia may cause tumor cell death indirectly, either by affecting the vascular system (16,17), or by sensitizing the immune system (18-30).

A2. Immunological effects of hyperthermia

Hyperthermia may have an indirect effect--beneficial or adverse--on tumors via the immune system. Regression of human malignancies has been reported to occur spontaneously, after episodes of fever and infection. In 1975, Stehlin et al. (18) observed disappearance of distant metastases in some patients treated with hyperthermic perfusion, and suggested that such effect might be related to the stimulation of antitumor immunity of the patient. Many other investigators now correlate an elevation of body temperature with a better function of host defense mechanisms.

Experiments on bacterially infected rabbits show that there may be an optimum febrile range for an animal to resist infection, with moderate fevers beneficial and abscence of fever or presence of high fever detrimental (19). Other studies on lizards and fish show that the absolute temperature itself is not as important as is the elevation of temperature above that normal for the species.

The beneficial effects of hyperthermia on response to viral infection have been shown in different animal species. New born piglets infected with gastroenteritis virus (20), newborn puppies challenged with canine herpes virus (21), and mice inoculated with herpes virus (22) show increased resistance to infection when treated with hyperthermia.

Direct effects of hyperthermia on isolated immune functions have been reported in in vitro studies. Mitogenic responses of human lymphocytes appear to be enhanced by hyperthermia. Roberts and Steigbigel (23) found that lymphocyte transformation

responses to the mitogen phytohemagglutinin (PHA) and the antigen streptokinase-streptodornase were enhanced at 38.5°C relative to 37°C. The elevated mitogenic response was not accompanied by its acceleration, and the effect was not seen at 40°C. Similar results were obtained by Ashman and Nahmias (24) who found a significant increase in (^{3}H) Thymidine uptake in human lymphocytes incubated with PHA or CON-A at 39°C. Their results show that hyperthermia not only enhanced, but also caused an earlier onset of the mitogen response. Bruce et al. (25) also reported an enhancement of DNA systhesis in mixed lymphocyte cultures incubated with PHA, CON-A, or pokeweed mitogen (PWM) at a temperature of 40°C. Cytotoxic T-cell responses to allogeneic cells were also enhanced.

Roberts and Sandberg (26) reported that hyperthermia at 38.5°C was consistently associated with greater spontaneous and stimulated LIF (Leukocyte Migration Inhibition Factor) production by human mononuclear leukocytes. Migration at 38.5°C after exposure to LIF produced at 38.5°C was greatly reduced relative to migration at 37°C in response to LIF produced at 37°C.

Recently, it has been reported that heat-treatment of tumor cells modifies cellular plasma membranes resulting in the enhancement of antibody-complement cytotoxicity against human colon tumor cells (27), and against virus-transformed hamsters PARA-7 cells (28).

In vivo experiments have demonstrated that local hyperthermia at 43°C (tumor temperature) results in the stimulation of the macrophage and T-lymphocyte systems in the

treatment of Guerin epithelioma (29) and Mc7 sarcoma (30) in rats.

Although most of the reports cited so far show the beneficial effects of hyperthermia, other studies indicate that hyperthermia may have adverse effects on the immune response. Harris (31,32), and MacDonald (33) found that hyperthermia reduced the cytolytic activity of cytotoxic T-lymphocytes (CTL's), and that the P 815 mastocytoma cells did not show any increased susceptibility to immune lysis, when treated with heat. The CTL's were shown to recover dramatically after being inactivated by a brief hyperthermic exposure. Neither suppression of protein synthesis nor damage to membrane lipids seemed to be the causative mechanism.

The view that heat lowers hot immune response is supported by Schechter et al. (34) who reported that heating isolated spleen cells in vitro reduces their capacity for in vitro tumor cells cytotoxicity. However, local heat treatment of metastasizing carcinoma in Wistar/Furth rats caused a decreased growth rate of the primary tumor as well as distant metastases. Also these authors observed no detrimental effect on cell-mediated tumor immune response of heated rats as tested by an in vitro lymphocytotoxicity assay 1 day later.

Other reports show that local heating of tumors may be beneficial, whereas whole-body hyperthermia may have adverse effects on the immune response. Shah and Dickson (35,36) observed that local heating of VX2 tumor-bearing rabbits, was followed by tumor regression and a marked increase in cell-mediated immunity, as measured by skin reactivity to tumor

extract and dinitrochlorobenzene. Total body hyperthermia, however, led to temporary restraint of tumor growth, followed by a return to an exponential increase in tumor volume. This was accompanied by abrogation of the enhanced immune responsiveness that followed local heating.

Discrepancies in the response of various species with different neoplasms to heat-induced hyperthermia indicate that more work is needed in this area. Whether local hyperthermia or whole-body hyperthermia induce a better immune response is not yet known. What is known is that different components seem to be highly heat sensitive. This suggests that in future experiments the temperature rise induced by hyperthermia in tumors or tissues must be uniform, well controlled, and well characterized. Microwaves and ultrasound are now being used as non-invasive modalities to induce thermal rise by heat generation in specified tumor or tissue volumes.

II B. MICROWAVES AND ULTRASOUND

B1. Use of microwaves and ultrasound as hyperthermia-inducing agents

Microwaves and ultrasound are relatively new clinical tools that are being used in medicine. Because of their wave nature, they can be applied non-invasively to different parts of the human body. Absorption of the waves in tissues results in heat generation which can be therapeutically beneficial. If the intensity of the wave is properly selected, the temperature could reach the levels needed for hyperthermic applications. For a complete understanding of the mode of interaction of waves

with biological media, one must start by defining the electrical and acoustic properties of various tissues. Absorption coefficients, reflection at interfaces between different tissues, and scattering properties must be known, so that an approximate temperature-rise profile in the irradiated tissues can be estimated.

Because standing waves are known to exist at and near interfaces, undesirable "hot spots" with temperatures exceeding the required levels, might be found. These hot spots might interrupt the uniformity of the temperature profile, and might induce unwanted micro-thermal damage.

Various microwaves and ultrasound bio-effects have been reported. These effects might interfere with their primary function as clinical devices. The possible radiation hazards can be thermal or non-thermal in origin. The thermal effects occur whenever the intensity of the wave is high enough. These effects seem to be of the same nature as those obtained when heat is directly applied to the tissue. Non-thermal effects are reported to occur at different intensity levels, even at sub-thermal levels. However, these reports are still controversial in nature, and the mechanisms by which they operate are not well understood, even though many mechanisms have been suggested so far.

B2: Possible mechanisms for microwaves bio-effects.

The interaction of microwaves with tissues are classified according to the Radiation Protection Guide (RPG) into "strong" and "weak" interactions. "Strong" interactions are defined as those which take place at intensity levels beyond the 10

mWatts/cm^2 implied by the RPG. They are mainly of thermal origin, but they also include non-thermal effects, such as field-induced force effects, dielectric dispersion, and denaturation of biomolecules.

Field-induced forces arise from the fact that the electrical potential energy of a particle or a system of particles depends on the geometric arrangement with respect to the applied field. The field-induced forces lead to the reorientation of individual particles in the field, and to the rearrangement of a set of particles, such as in the "pearl chain formation" phenomenon (37). This effect is frequency dependant and has thresholds much larger than 10 mWatts/cm^2.

Dielectric dispersion and denaturation of biomolecules occur at field strength levels effective in completely overcoming Brownian motion, resulting in complete orientation of polar molecular subgroups of biomolecules (38,39).

"Weak" interactions are defined as those taking place when the microwave intensity is less than 10 mW/cm^2. They are of non-thermal origin, and the suggested mechanisms include the excitation of cellular membranes, and the local amplification of electric fields.

Several theoretical models have been proposed to explain the excitation of cellular membranes by microwaves (40-42), and by low frequency amplitude modulated radio frequency fields (43-45). Experimentally, Seaman and Wachtel (46) reported that low energy microwaves were able to alter the firing rates of individual pacemaker neurons, while Bawin et al. (47) found an enhanced release of calcium ions from brain tissue exposed to

amplitude modulated radio frequency radiation.

Even though the microwave intensities in many reported effects are in the non-thermal range, microthermal heating due to hot spots in microwave field distribution might be responsible for these effects. Nilsson and Petterson (48) suggest that microscopic-wedge-shaped boundaries between regions with different dielectric constants, which are likely to be present in the mammalian body, may give rise to local fields about 100 times larger than the macroscopic electric fields. The field-induced local damage could then occur at incident radiation intensities 10^4 times smaller in magnitude than intensities needed for regional damage.

B3. Possible mechanisms for ultrasound bioeffects.

The bioeffects of ultrasound are classified into thermal and non-thermal interactions. Thermal effects usually occur at ultrasound intensity levels at and above the threshold needed to induce destruction of tissues. Non-thermal interactions require generally high intensities, but many effects were reported at intensities lower than 100 mW/cm^2. Some of the mechanisms which have been suggested as responsible for non-thermal ultrasound effects include cavitation, acoustic streaming and microstreaming, and radiation force effects.

Cavitation occurs when microbubbles in liquid suspensions respond to an acoustic field by executing expansion-contraction pulsations. This causes gas to diffuse radially inward and outward during each cycle in an asymmetrical manner, so that net inward flow results. The bubble will then grow by rectified diffusion, and collapses after sufficient expansion, leading to

very high temperatures and pressures. Bioeffects can then be produced by mechanical shocks or by chemical changes. Some of the bioeffects which have been attributed to cavitation include cell membrane damage in cultured mammalian cells (49), reduction of growth rate of plant roots (50), pyknosis of cultured human lymphocytes (51,52), and the stimulation of collagen synthesis in human embryonic fibroblasts (53).

Acoustic microstreaming might occur when shear stresses are set up near an oscillating bubble lacking spherical symmetry or resting on a solid boundary. Large velocity gradients arise, which might act on a cell by elongating it and then lysing it. There is experimental evidence that these microflows play an important role in the degradation of macromolecules, destruction of cells, and disturbance in the physiochemical states of the cell membrane (54).

Radiation force effects arise when there is a net change in the momentum of the wave, either by reflection or by absorption. Cells in suspension are subjected to such a radiation force. This may explain the stasis phenomenon reported by Dyson et al. (55), who have shown that red cells in blood vessels of chick embryo collect into parallel bands spaced one-half wavelength apart in an ultrasonic standing wave field.

In many reports, the mechanisms responsible for the bioeffects are not yet understood. These reported bioeffects occur sometimes at intensity levels much lower than 100 mW/cm^2. In cultured human cells, cellular attachment was reported to be significantly reduced after 0.50 minute of exposure for a total power output of 1.76 mW, which is equivalent to an average

intensity of 0.62 mW/cm^2 (56). In another experiment, the ultrasonic intensity reaching the uterus of a mouse was less tha 40 mW/cm^2, and the exposure resulted in induced contractions of the uterine smooth muscle (57).

It is important to note that the experimentally measured rise of temperature in irradiated tissues is an extracellular one, and that the intracellular rise in temperature may be higher than that outside of the cell. Love and Kremkau studied this problem theoretically (58), and found that for an intensity of 100 mW/cm and frequency of 1-MHz, and for the thermal properties of cellular components they assumed, the maximal differential rise was of the order of 10^{-5}°K only, and occurred at the center of the spherical cell model they used. Other microthermal effects, similar to those suggested for microwaves, are not to be excluded.

B4. Immunological effects of microwaves

There have been many studies on the effects of microwaves on lymphocytes and the immune system. As in hyperthermia, one of the most consistent findings is increased lymphocyte formation and activity (59,60), suggesting that the effects are mainly thermal. Prince et al. (61) reported an enhanced mitotic response of monkey's peripheral blood lymphocytes stimulated in vitro with PHA, three days after the monkeys were exposed to 1.32 W/cm^2 pulsed radiation.

Increased "spontaneous lymphoblastoid transformation" is reported by Czerski (60) to occur in pheripheral blood lymphocytes from irradiated rabbits, following in vitro

cultivation. He also reported that irradiated mice had significantly greater numbers of antibody-producing cells and higher serum antibody titers following immunization with sheep red blood cells.

Wiktor-Jedrzejeczak et al. (62-64) exposed mice in a rectangular waveguide to 2.45-GHz microwaves and found a significant increase in the proportion of complement-receptor positive (CR+) lymphocytes, immunoglobulin positive (Ig+), Fc receptor postive (FcR+), and Thy-1 negative spleen cells. However, no change in the total number of Ig+ cells in spleens of these mice was observed. They concluded that microwave irradiation did not stimulate lymphoid cell proliferation per se, but appeared to act as polyclonal B-cell activator, which led to early maturation of noncommitted B-cells. Their results were later substantiated by Sulek et al. (65). Liburdy reported that the acute transient lymphopenia in mice was induced by whole-body exposure to thermogenic radiofrequency radiation. These mice also showed a relative increase in splenic T- and B-lymphocytes (66). In another study he reported alterations in lymphocyte distribution and function which are characteristic of a state of immunosuppression (67).

It is now believed that the functional integrity of lymphocytes can be changed following in vitro exposure to microwave radiation. It is difficult to interpret the immunological significance of these changes, because of the inconsistencies that exist among various reports. While many studies indicate that microwaves increase responsiveness to lymphocytes and potentiates the immune response to antigen,

others indicate depressed responsiveness. For a better understanding of the effects of microwave radiation on the immune and thermoregulatory systems, the characterization and the control over the temperature profile must be accurately determined, whether it is a case of local or whole-body irradiation.

Many in vitro experiments were done to investigate the possibility of a direct action of microwaves on the responsiveness of lymphocytes to different mitogens. When the temperature of the cultures was left to reach hyperthermic levels, lymphoproliferative response of the cells was reported (23-25). However, when the temperature of the irradiated cells was controlled so that it did not differ greatly from controls, no effect on the proliferative response of lymphocytes was observed (68,69). This strengthens the belief that the microwave effects are mainly thermal in origin.

B5. Immunological effects of ultrasound

A variety of ultrasound effects on the immune system has been reported in the last few years. DNA damage followed by DNA repair was observed after in vitro sonication of human lymphocytes (70,71) and HeLa cell in the G1 phase (72). However, other reports failed to confirm any chromosomal damage in human lymphocytes and HeLa cells, while heating of the sonicated medium was avoided (73,74). Hedges and Leeman (51), and Graham et al. (52) observed a degeneration of the cell nucleus followed by the eventual disruption of the entire cell in human lymphocytes irradiated with ultrasound. They suggested

that sonication may damage lysosomal membranes via a cavitation process.

Ultrasound effects taking place at the cell membrane level have also been reported. Martins et al. (49) observed that sonicated mammalian cells were rounded and had characteristic vesicles around them which did not appear on the smoother surface of control cells. Repacholi and Kaplan (75) found that the number of concanavalin A (CON-A) receptors on the surface of human peripheral lymphocytes treated with ultrasound was significantly less than that found on the surface of the untreated controls. Anderson and Barrett observed a state of immunosuppression (76), and depression of phagocytosis (77) in mice insonated with a low energy wave applied over the area of the spleen. These investigators suggested that the effects they observed were caused by cell membrane damage during exposure to ultrasound.

The mechanisms by which hyperthermia, microwaves, or ultrasound may affect cellular functions are not yet fully determined. However, it seems very likely that many of the reported effects are initiated at the cell membrane level. Immune recognition and response are modulated by a variety of membrane-antigen and membran-membrane interactions. Thus any effect on cellular membranes may have adverse consequences on immunocompetence. It is therefore necessary to have a good understanding of the cell membrane biology. this will be the subject of the next section.

C1. Cell membrane structure and properties

All living cells are delimited from their environment by a surface membrane, the plasma membrane, which has vital biological functions due to its strategic location. The cell membrane receives information from and transmits signals to the environment, controls the permeation of nutrients, regulates the action of certain drugs and hormones, modulates cell to cell recognition, interaction and communication, and influences cell locomotion, growth and differentiation. The most satisfactory current model for natural membranes that accounts for the latest experimental observations is the fluid lipid-globular protein mosaic model presented by Singer and Nicholson in 1972 (78). They consider that the basic structure of biological membranes is a two-dimensional arrangement of globular proteins dispersed in a matrix of fluid lipid bilayer (Figure 1). Lipids are amphipathic molecules, i.e., they contain both hydrophobic (non-polar) and hydrophilic (polar) regions. The hydrophilic-polar head groups prefer an aqueous environment, whereas the hydrophobic hydrocarbon tails are only stable in a non-aqueous environment. In the Singer-Nicholson model, the lipids are arranged in a bilayer with their ionic head groups in direct contact with the aqueous salt solutions on each side of the membrane, and with their hydrocarbon tails aligned back to back to minimize their contact with water. This leads to a configuration which is the most thermodynamically stable. Globular proteins are also amphipathic biomolecules. Their hydrophobic regions are embedded in the hydrocarbon core of the lipid bilayer, and the more polar regions are exposed to the

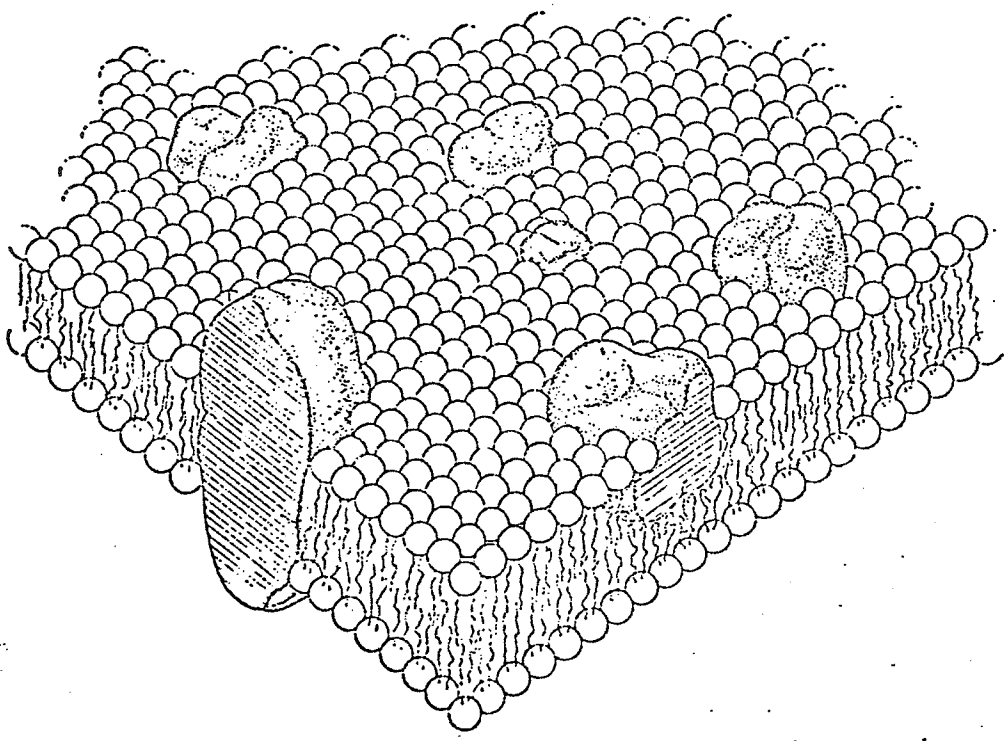

Fig 1— Representation of three-dimensional organization of plasma membranes. A bimolecular film of phospholipids forms the matrix of the membrane, and globular proteins are embedded in the lipid core. Some proteins span the membrane; others are embedded in one of the lipid monolayers. (Courtesy of Singer, S. J., and Nicolson, G. L.: The fluid mosaic model of the structure of cell membranes, Science 175:720, 1972. Copyright 1972 by the American Association for the Advancement of Science.)

aqueous solutions at one (peripheral proteins) or both sides (integral proteins) of the membrane.

To fulfill its vital functions, the membrane must operate differentially on the two compartments it separates, and thus it must by asymmetric. The asymmetrical placement of the proteins within the lipid matrix has been studied extensively. The topological asymmetry of phospholipids in membranes has been studied more recently (79, 80).

The biological functions of the cell membrane may be affected by environmental factors such as temperature, pH, and concentration of different chemical or biochemical constituents. Non-thermal microwaves or ultrasound effects are not yet clearly established. Membrane proteins have fairly precise environmental requirements for optimum activity, some requiring a fluid environment, whereas others require much more rigid surroundings. The transition of the membrane lipids from a fluid, liquid-crystalline phase to a solid, gel phase is not sharp, but occurs over an appreciable range of temperatures (81, 82). Associated with any continuous phase transition process, there are a variety of pre- and post-melting phenomena (83). Since the lipids in a biological membrane exist within a few degrees of their phase transition temperatures, they may be at the temperature at which pre- and post-transition phenomena are expected to be biologically important. It is possible that mixed lipid phases could occur at physiological temperatures. If patches of solid lipids are present in mammalian cells at 37 C, they could play an important role in controlling the mobility and the arrangement of a wide range of membrane

receptors such as antigen proteins. In an experiment on mouse and human surface antigen mixing, Petit and Edidin (84) found that the mobility of the antigens was decreased as the temperature was lowered from 42°C to 21°C, but with further cooling, the mobility was increased with a new maximum occurring at 15°C. They suggested that the decreased mobility in the 42°C to 21°C interval is due to increases in viscosity of membrane lipids with decreasing temperatures, and that between 21 and 18°C the effects were due to phase separations.

Lipid phase transition temperatures are pH -dependent. Trauble and Eibl (85) found that when the pH was increased from 7 to 9, the transition temperature was lowered by 20°C. Fluid-ordered transition at constant temperature can be induced by use of divalent cations (Mg^{++} and Ca^{++}) which increase the transition temperature. Monovalent cations (Li^{+}, Na^{+}, K^{+}) lower the transition temperature and therefore have antagonistic effects to the effects of divalent cations.

C2: Lipid-protein interactions.

Lipid-protein interactions are important in establishing the function of the proteins included in a membrane. The lipids which surround a protein experience hydrophobic forces which are different than those found in portions of the membrane far from a protein. Jost et al. (86) demonstrated a highly restricted or immobilized component of the lipid corresponding to about 0.2 mg of lipid per mg of protein. They suggested that this restricted "boundary lipid" corresponds to a single solvation layer of lipid about the circumference of protein where it is in contact with lipid bilayer. The existence of a boundary lipid

has been confirmed by many other investigators (87-91). Marsh and Barantes (88) estimated that the proportion of lipid in the immobilized component is greater than calculated for a single boundary layer. Other investigators (87) found that the amount of lipid in the restricted environment is strongly temperature dependant at temperatures well above the phase transition temperature of the pure lipid. This implies that the higher temperatures which can be biologically important are differrent than those at which phase transitions of the pure lipid occur. These temperatures would correspond to the range in which the boundary lipids start their phase transition, because a change in the fluidity of the annular lipids might bring about changes in protein conformation and so changes in protein activity.

Although lipids in immediate contact with protein are somewhat immobilized, Grant and McConnell (89) and Overath et al. (90) reported that the entire lipid-protein complex has a tendency to occupy fluid regions of the bilayer. Very few reports found no evidence for the existence of "boundary lipids" (92). Many theoretical models for protein-lipid and protein-protein interactions in bilayer membranes have been presented, based on thermodynamical arguments (93-95).

Lipid-protein interactions are found to depend on many environmental factors such as temperature, pH, and the presence of different chemical or biochemical constituents. Protein mobility in membranes depends also on similar environmental factors (96). Aggregation of a class of proteins (antigens) on cell surfaces following antibody binding is related to the mobility of these proteins. The antigen-antibody complexes may

aggregate together to form a single aggregate. This phenomenon is called capping.

C3: Redistribution of surface receptors: capping.

Antigens found on the surface of cells are proteins which are embedded on the lipid bilayer and which carry one or more "antigenic determinants". An antigenic determinant is that portion of the antigen to which an antibody binds by molecular complementarity. The antigens are initially diffusely scattered all over the surface of the cell. The binding of antibody initiates a redistribution of the antigen-antibody complexes on the cell surface. The redistribution depends on the type of receptor and on the type of the cell that carries this particular receptor. On many types of cells, the redistribution starts with a regrouping of the antigen-antibody complexes into patches. Patch formation is a passive phenomenon arising from the diffusion of small complexes which upon collision with one another become progressively larger. Patching is followed by capping of the complexes: all patches coalesce into a polar cap, and the remaining part of the membrane is devoid of any of that particular antigen. In contrast with patching, capping is an active process which requires metabolic energy.

Capping has been very well characterized under normal conditions (37 C, no drugs, no radiation) on the surface of B-lymphocytes (97,98). B-cells carry the immunoglobulin receptor, surface Ig. The number of Ig molecules presumably serving as antigen receptors has been estimated to be 10^5 molecules on the average per B human lymphocyte cell. Surface Ig is found diffusely throughout the plasma membrane in a

nonrandom distribution. The molecules are distributed in a lacy discontinuous network with very small clusters which are often interconnected by strands of a few molecules. This short-range organization does not necessarily negate the fluid mosaic model of membrane structure. It may be due to protein-protein or protein-phospholipid interactions on the membranes, or to the attachment of the Ig molecules in variable numbers to a given anchoring structure. When anti-Ig antibodies bind to the Ig receptors, patching followed by capping takes place in a matter of minutes, if the environmental factors are favorable. Capping is inhibited at 4 C, in the presence of metabolic inhibitors, or in the presence of high doses of cytochalasin B which is a microfilament inhibitor. Colchicine, a microtubular inhibitor, has some enhancing effects on capping (97). Cis-dichlorodiammineplatinum II (cis-DDP), an anticancer agent, has been found to inhibit capping of Ig and of concanavalin A (CON-A) receptors on mouse B-lymphocytes (99).

CON-A is a lectin to which many cell types carry surface receptors. CON-A receptors are known to cap on B-lymphocytes (97,98). They also show remarkable redistribution on the surface of normal and SV40-transformed human fibroblasts. The changes in the transformed cells occur more quickly than in the normal cells, suggesting that the membranes of the transformed cells are more fluid (100).

Cancer cells also show capping: Rosenthal et al. (101) have found that antibodies specific for carcinoembryonic antigen (CEA) induce polar redistribution of CEA which is expressed on the surface of human intestinal carcinoma cells. While

colchicine had no effect on capping, inhibition of redistribution was observed following cytochalasin-B treatment.

The exact mechanisms of capping are not yet fully understood. However, better capping has been observed many times to occur under conditions where the cell membrane exhibits higher fluidity, whereas poor capping has been attributed to increased membrane viscosity, such as in lymphoma cells. Upon binding of the antibody, increased microfluidity may be obtained by phospholipid methylation, which would then facilitate capping (102). It also appears that the cytoskeletal system may be directly or indirectly involved in the redistribution process. This suggestion is supported by observation of the effects of cytochalasin-B and colchicine which are known to inhibit microfilaments and microtubules--the building blocks of the cytoskeletal system.

C4. The cytoskeletal system: involvement in capping.

Microfilaments (MF)

MF are fine structures of various lengths and diameters. The 5 - 8 nm thick MF are made of actin, while the 13 - 25 m thick MF are made of myosin. Actin has very conserved composition and characteristics throughout all Eukaryotic cells. At least two classes of MF systems can be found: some MF constitute a loose network of short interconnected MF forming a lattice localized at the anterior part of the cell, just beneath the plasma membrane (PM), and may be associated with it. They are disrupted by cytochalasin but they do not seem to bind heavy meromysin (HMM). The other set of MF consists of parallel MF, organized as bundles of fibres in the posterior part of the

cell, they are not disrupted by cytochalasin, but they bind HMM (HMM is the globular ATPase portion of skeletal muscle myosin obtained by trypsin digestion). Several investigators seem to support the idea that MF may be anchored to membrane. In fibroblasts, both sets of MF are linked to the PM; the whole cell length is spanned by long fibres which converge to focal points (98, 103).

Microtubules (MT)

MT appear as separate fibres of uniform thickness (outer diameter = 24 nm) which run through the cytoplasm. They have a 15 nm hollow core, and a dense cortex which is made up of 13 adjacent protofilaments, 4 - 5 nm wide. These protofilaments are built of subunits of tubulin dimers, which can bind upon denaturation one molecule of colchicine and one molecule of vinblastine at separate sites. Colchicine and vinblastine reversibly disrupt MT. Low temperature, hydrostatic pressure and high calcium concentration ($>10^{-5}$M) can induce a reversible MT dissolution into subunits. The equilibrium of assembly-disassembly between MT and tubulin can be shifted to MT formation in vitro by D_2O, which supports the concept of a hydrophobicity-driven association of the subunits. There are many lateral cross-bridges (2 - 5 nm thick by 10 - 40 nm long) between adjacent MT or between a MT and all kinds of membranous components, including membrane vesicles and PM. Cell shape is reversibly destroyed by disruption of MT.

Involvement of the cytoskeleton in capping

Early B cells show higher rates of capping and even

spontaneous capping of the Ig receptors, as compared to capping on mature B cells. This might be due to a balance of MT and MF activities quite different from that of mature cells (104). Many models related to the involvement of the cytoskeleton in capping have been proposed (98, 105-107).

Loor (98) proposes that a contractile MF system, anchored directly or via intermediate pieces to some membrane components and responsible for their polar migration, would be antagonized by an MT network also associated with some membrane components and conferring to the cortical cytoplasm of the cell "thixotropic" gel-like properties (A system, like the MT-MF system, is said to have thixotropic gel-like properties when it becomes liquid when and as long as a force is applied, and solidifies immediately after).

Berlin et al. (105) suggest that a primary role of microtubules is to influence the distribution of membrane associated microfilaments; the membrane subtended by microfilaments has different properties from bulk membrane: it has an increased "microviscosity" with an increased affinity for antigen-antibody complexes. By this model direct microtubule-receptor connections are not an absolute requirement for microtubule regulation of surface topography. Albertini and Clark (106) reported that CON-A capping induced redistribution of cytoplasmic MT and colchicine binding proteins. Tubulin was found preferentially concentrated in the capped area of the cell. They suggest that the capped membrane domain may be a preferred site of MT polymerization.

In contrast with what Albertini and Clark reported, Singer

et al. (107) found no evidence of a redistribution of tubulin after CON-A capping. Instead, they found that capping was always accompanied by a redistribution and concentration of actin and myosin under the cap. They proposed a model for capping in which clusters of receptors become linked across the membrane to actin- and myosin-containing structures. After the linkage, these clusters of receptors are still mobile in the plane of the membrane, and are actively collected into a cap by an analogue of the muscle sliding filament mechanism.

Finally, Emerson and Cone (108) found that different categories of membrane proteins on the same cell surface may be associated either with MF or MT.

Scientists have not yet agreed on a unified model for capping, which would explain all of the reported facts. However one fact may be agreed upon: whether via MF, MT, or both, the cytoskeleton seems to be either directly or indirectly involved in the capping process.

III. MATERIALS AND METHODS

ANIMALS: Young adult (12 to 16 weeks old) outbred female ICR Swiss mice were obtained from Harlan Laboratories, Indianapolis, and were used as the source of B-lymphocytes.

MEDIA: Minimal Essential Medium (MEM) was used throughout the study. MEM was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 gm/ml streptomycin, 2 mM L-glutamine, and buffered with 20 mM HEPES, and 0.075% $NaHCO_3$.

CELL SUSPENSIONS: In each experiment, 3 to 5 mice were sacrificed by cervical dislocation. A suspension of spleen cells was obtained by mincing the organs in complete Minimal Essential Medium (MEM) containing 10% heat inactivated fetal-bovine serum (MEM + FBS), and dispersing the cells through a fine wire stainless steel mesh. The cell suspension was then washed in MEM + FBS and centrifuged at 400 g for 30 minutes on Ficoll-Hypaque (123). The mononuclear leukocyte band was removed from the gradient and washed three times in MEM + FBS. For antibody-complement cytotoxicity experiments, T-cells were lysed by treatment with monoclonal anti-Thy 1.2 antibodies (New England Nuclear, Boston, MA) and guinea pig complement (Grand Island Biological Company, Grand Island, NY) for 1 hour at 37°C. The remaining B-lymphocyte-enriched cell population was then washed 3 times and resuspended in MEM + FBS. Viability of cells was better than 90% as determined by trypan blue exclusion. These cells served as the standard cell population.

HEAT TREATMENT: Approximately 1 x 10^6 spleen cells per 12 x 75 mm test tubes were suspended in 300 µl complete MEM or in 100 µl diluted antibody depending on the experiment. The test tubes were sealed with rubber stoppers and immersed in a water bath. The heater coils were fed from a proportional temperature controller YSI model 72 (Yellow Springs Instrument Co., Yellow Springs, Ohio). The temperature was sensed by means of a thermistor probe (model YSI 403) which was connected to the input of the controller. The temperature was monitored at all times on a digital thermometer, model BAT-8 (Bailey Instruments Inc., Saddle Brook, NJ). The temperature variation inside the test tubes was less than 0.1°C.

CYTOCHALASIN-B TREATMENT: Lyophilized cytochalasin-B obtained from SIGMA Laboratories, St. Louis Missouri--was dissolved in dimethylsulphoxide (DMSO), 10 mg/ml, and stored in 0.1 ml aliquots at -20°C. Before each experiment, the drug was diluted in complete MEM to the desired final concentrations (10, 50, and 100 µg/ml). Approximately 1 x 10^6 spleen cells per 12 x 75 mm test tubes were suspended in 300 µl complete MEM + cytochalasin-B. The test tubes were capped with rubber stoppers and immersed in a water bath at 37°C for 40 minutes. The temperature was controlled as above. After 40 minutes treatment, the cells were either washed in cold PBS or left with the drug, then tested for capping using a direct immunofluorescence technique.

MICROWAVE IRRADIATION: Three and a half ml of spleen cells were placed in cellulose nitrate tubes and sealed with parafilm as

described above. The test tubes were irradiated in an exposure system described previously (124). Briefly, the test tubes were placed vertically in a microwave-absorber-lined exposure chamber 45 cm below the aperture of a 15 x 20 cm horn antenna. The source of microwaves was a Litton Industries L-3501 Microtron, operating at 2.45-GHz. Both water-bath and microwave-exposed cells were constantly agitated by the same shaker mechanism at 75 cycles/min. A thin plastic test tube holder in the microwave chamber was mechanically linked to the shaker bath platform by a wood dowel passing through the chamber wall. Temperature-controlled water from the water bath was circulated through a shallow acrylic tray which supports the moving flask holder. With agitation, the internal test temperature was maintained to within 0.1°C of the water bath temperature. Amplitude of sinusoidal platform displacement was 3.8 cm peak to peak.

MICROWAVE DOSIMETRY: The specific absorption rate (SAR) in the cell suspension was estimated from a series of temperature-rise measurements made after irradiation intervals of 15, 30, and 60 sec. The test tubes were insulated with a 2 mm thick waterproof polyurethane foam sleeve. The test tubes were irradiated with the shaker moving but with the circulating pump off. Final temperature measurements were made within 3 sec following the irradiation periods with a digital thermometer. SAR was estimated from the initial slope of the heating curves.

RF EXPOSURE SYSTEM: Samples were exposed in a specially designed Crawford cell (Figure 2). The cell has an upper cutoff

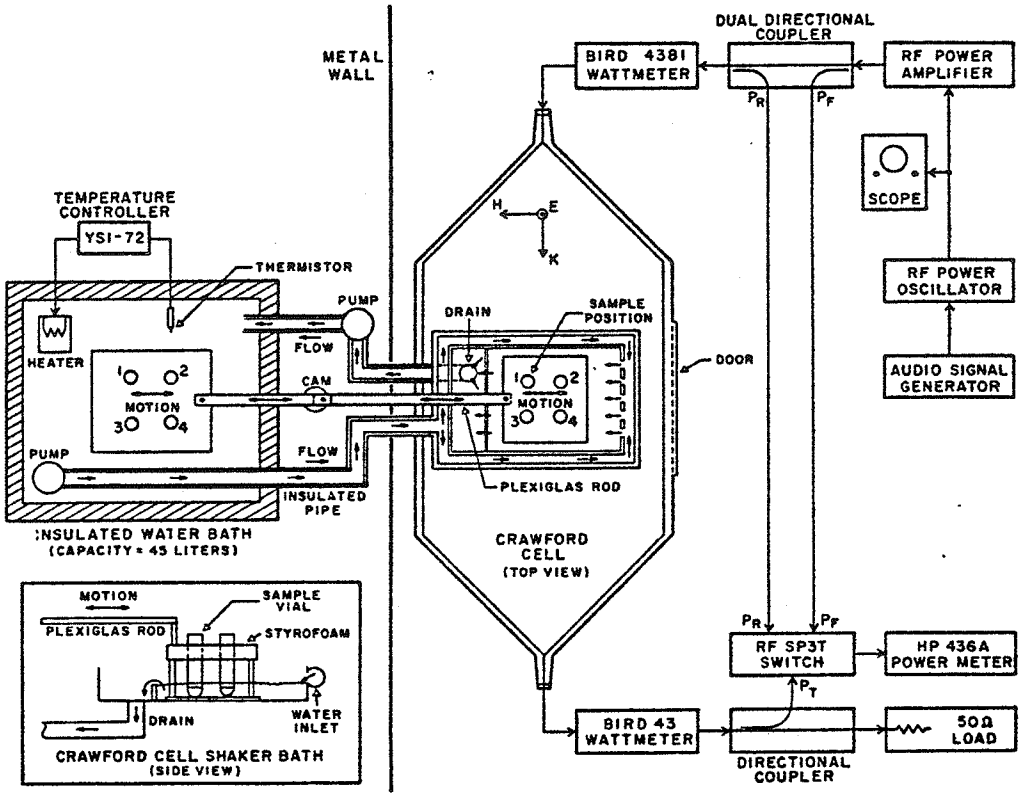

Figure 2: Diagram of the temperature control and amplitude modulated RF exposure system. (Not to scale).

frequency of 300 MHz. Below the cutoff frequency, an incident power signal will propagate only in the TEM mode, thus establishing a quasi far-field plane wave throughout the test area of the cell (125). An HP 3311A function generator provided a low frequency sine wave which was used to amplitude modualate (>90%) a 147-MHz carrier from an RF sweep generator (Textronix TM 504). The modulated signal was monitored on an oscilloscope (Textronix type 585), then amplified by a 200-watt broadbanded amplifier (Amplifier Research, model 200L). Forward, reflected, and transmitted powers were simultaneously sampled through different directional couplers and displayed on a digital power meter (HP 436A) through a Transco 14704 SP3T coax RF switch and an HP 8481A power sensor.

The cell was terminated with a 50Ω load impedance. The SWR on the input of the cell was less than 1.10, as measured with a digital BIRD RF Power Analyst model 4381.

Inside the Crawford cell, two round bottom thin wall cellulose nitrate tubes sealed with Parafilm and containing 3 ml of cell suspension were placed 6.4 cm apart in sample positions 1 and 2 (Figure 2). Non-irradiated controls were placed in a temperature-controlled water bath with 45-liters capacity. Both irradiated and non-irradiated cells were constantly agitated by the shaker bath platform at 1.25 cycles/sec. The Styrofoam test tube holder in the Crawford cell was mechanically linked to the water bath platform by a thin Plexiglas rod passing through the cell wall. The platform moved sinusoidally with a 3.8 cm peak to peak amplitude.

Water was circulated through the Plexiglas tray which

supported the moving test tube holder. The depth of water surrounding the test tubes was 1.3 cm. With a platform 1.25 cycles/sec movement and an 8 l/min flow rate of temperature controlled water, the internal test tube temperature was maintained within 0.1°C of the desired temperature.

A Styrofoam shelf placed in the center of the Crawford cell supported the Plexiglas tray such that the cell suspensions were 12.5 cm above the septum. In that region, the variations in electric (E) field strength are minimal (125), thus reducing the 1.25 Hz amplitude modulation component due to movement of the shaker bath platform.

RF DOSIMETRY: Although the quantity of energy being absorbed by the samples was too small to be detected by our instruments at any of the power densities used, an upper limit of the average specific absorption rate (SAR) for the samples was estimated. The total absorbed power was calculated after measuring the incident, reflected, and transmitted powers. the net power absorbed by the samples and surrounding fluid was estimated after performing two series of power measurements, with the samples and fluid either inside or outside the Crawford cell. The difference between the two measurements was less than the standard deviation of any of the two measurements. Taking the largest standard deviation as an upper limit for the net power absorbed in the samples, the average SAR for the samples was estimated to be less than 42 μW/gm per mW/cm^2 of incident intensity.

This upper limit for the SAR was confirmed by another method for estimating SAR's based on temperature-rise

measurements in the cell suspension. The test tubes were insulated with a 2 mm thick water proof polyurethane foam sleeve. A thermocouple microprobe (Bailey Model IT-18) was inserted in one of the test tubes. Thermocouple wires were perpendicular to the E-field. Final temperature measurements were made within 3 sec following irradiation with a Bailey ditital thermometer (model BAT-8). Less than $0.1^{o}C$ temperature-rise was observed following a 60 sec 1-KW irradiation. The SAR was thus estimated to be less than 35 $\mu W/gm$ per mW/cm^2 incident radiation, confirming the upper limit for the SAR estimated earlier.

ULTRASOUND IRRADIATION AND TEMPERATURE CONTROL: A 1-inch diameter unfocused transducer was used to insonate the cell suspensions. The transducer was driven at its resonant frequency (0.99 MHz) by a frequency synthesizer and an amplifier system. The transducer was mounted in a rubber-lined Plexiglas tank containing 36 liters of temperature-controlled degassed Ringer's solution. The sound intensity at the level of the cell suspensions was calibrated by means of a thermocouple junction. Primary calibration of the thermocouple was carried out by a radiation force method using displacement of a stainless steel ball in the sound field (126).

Cell suspensions were placed in 6 mm diameter Spectra/Por 2 membrane tubes (Spectrum Medical Industries Inc., LA-90054) which are usually used for dialysis. These tubes were used because the unique structure and 0.002-inch thickness of the membranes offered good thermal conduction between the cell suspensions and the temperature-controlled Ringer's solution.

Furthermore, the same characteristics allowed the membranes to act as acoustic windows thus minimizing reflections and standing waves. Dialysis effects were minimal during the 20 minute immersion in the Ringer's solution.

Inside the tubes, the cell suspensions were layered on top of a 6 cm column of Ficoll-Hypaque solution, and below a 6 cm column of heavy mineral oil. The reason behind such an arrangement was to avoid any solid-fluid interfaces (such as between container and cell suspensions) and fluid-air interfaces (such as between cell suspensions and air), thus minimizing reflections of the ultrasound beam at interfaces between cell suspensions and surrounding media.

The tubes were immersed in the irradiation tank 5 minutes before starting the sonication to allow for equilibration of the temperature in the cell suspensions to that of the surrounding Ringer's solution. The tubes were positioned vertically such that the volume displaced by the cell suspensions (0.6 cm diameter, 0.7 cm tall) was centered in the focal volume of the beam. In thant region, the beam width (0.9 cm) was greater than the largest dimension of the cell suspension (0.7 cm), thus insuring a uniform field distribution.

Control samples were prepared in the same way and immersed in the Ringer's solution simultaneously with the irradiation samples. These controls were positioned behing the transducer in an ultrasound-free region.

The temperature in the Ringer's solution was adjusted by feeding the heater coils from a proportional temperature controller YSI model 72. The actual temperature was sensed by

a thermistor probe YSI model 403, which was connected to the input of the controller. Temperature variations in the Ringer's solution were less than 0.1 C during experiments.

DIRECT IMMUNOFLUORESCENCE ASSAY AND CRITERIA FOR CAPPING: Treated or untreated spleen cells were washed in cold PBS and centrifuged at 500 x g. The test tubes were then transferred to 4°C. Then 100 µl of FITC--labeled goat anti-mouse Ig diluted in PBS were added to the cell pellets. After allowing 10 minutes for the antibody to bind to surface Ig, the test tubes were transferred from 4°C to the desired temperatures. Samples were taken at different time intervals and fixed with 2.5% cold paraformaldehyde. Following three washes in PBS, the cells were resuspended in two drops of glycerol-PBS (10:1), mounted under a glass coverslip, and examined for capping with a Carl Zeiss fluorescence microscope. Most experiments were done in triplicate. For each sample of the triplicates, two hundred cells showing fluorescence (Ig+ cells) were randomly selected and scored for capping.

ANTIBODY-COMPLEMENT (Ab-C) CYTOTOXICITY ASSAY: Heat-treated or untreated B-lymphocytes (2×10^6 cells per 12 x75 mm test tubes) were washed and resuspended in 80 µl of goat anti-mouse-Immunoglobulin (anti-Ig) antibodies (Grand Island Biological Company, Grand Island, NY). After a 10 minute incubation at 4°C, the cell preparations were transferred to water baths preset at the desired temperature. After 12 minutes of incubation, 20 µl of guinea pig complement (Grand Island Biological Company, Grand Island, NY) were added to the cell

suspensions. The Ab-C reactions were stopped at different intervals by transferring to 4°C. The assays were done in duplicate. For each replicate, two hundred cells were randomly selected and viability was measured by trypan blue exclusion.

IV. RESULTS

A. EFFECTS OF HYPERTHERMIA ON CAPPING OF THE ANTIGEN-ANTIBODY (Ag-Ab) COMPLEXES ON THE SURFACE OF B-LYMPHOCYTES

A1. KINETICS OF CAPPING DURING HYPERTHERMIC EXPOSURE

B-lymphocytes collected from normal mice spleens were first incubated with 100 μl FITC-labeled anti-Ig antibodies for 10 minutes at 4°C. This allowed the antibody to bind to B-cell surface Ig, while preventing capping of the Ig- anti Ig complexes. Different cell suspensions were then transferred immediately from 4°C to 37°C, 39°C, 40.5°C, or 42°C. Because the volume of the cell suspension was small (100 μl), the temperature in each cell preparation reached its designated value in less than a minute. After incubation for different intervals of time, various cell preparations were fixed with 2.5% cold paraformaldehyde. The percentage of Ig-positive cells with capped Ig-anti Ig complexes was estimated under a fluorescence microscope. The results indicated that less than 4 minutes were sufficient for the completion of capping on the surface of those cells that had the ability to cap surface Ig (Figure 3). Endocytosis of the redistributed Ig-anti Ig complexes was observed by 12 minutes of incubation. Figure 3 also shows the kinetics of Ig-anti Ig capping at different temperatures above 37°C. The percentage of cells capping Ig-anti Ig complexes was gradually reduced from 85% at 37°C to about 10% at 42°C.

The data in Figure 3 indicates that heat treatment of B cells with prebound anti Ig inhibited capping. To examine the

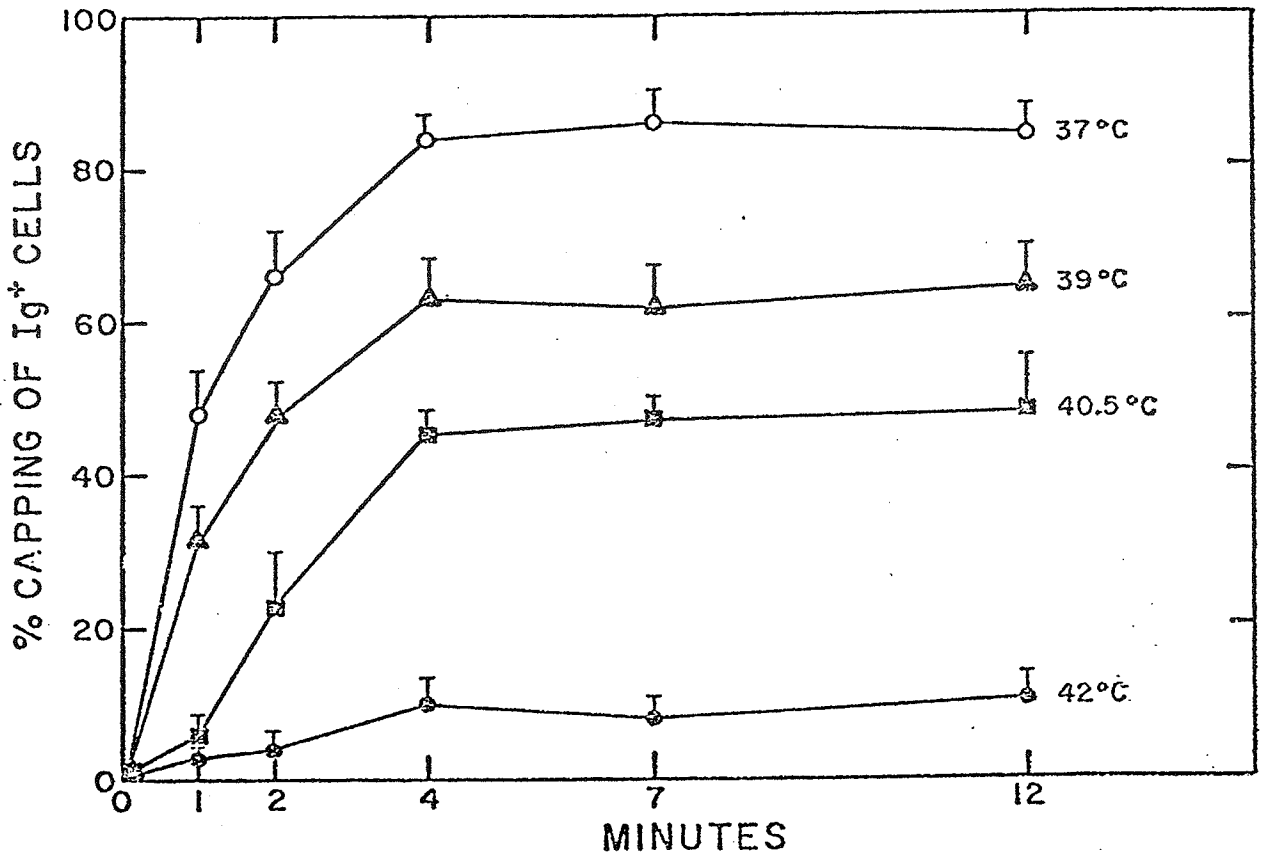

Figure 3: Kinetics of capping of Ig-anti-Ig complexes. B cells were first incubated at 4°C with anti-Ig antibodies, and transferred to 37°C (0), 39°C (▲), 40.5°C (■), or 42°C (●) for the times indicated in the horizontal axis. The reaction was stopped by fixation with 2.5% cold paraformaldehyde. Points represent the mean ± SD of triplicate assays.

effects of heat on Ig-anti Ig binding and capping in more detail, three sets of conditions were set up: 1) cells were incubated with anti Ig for 10 minutes at 4°C and then transferred to the desired temperatures and observed for capping (Figure 4c); 2) cells were incubated with anti Ig for 10 minutes at 4°C, washed to remove unbound antibody and then transferred to the desired temperatures to observe capping (Figure 4a); 3) cells were heated at the desired temperatures for 60 minutes, washed and incubated at 4°C for 10 minutes with anti Ig and then transferred to 37°C for capping to occur (Figure 4b). The results shown in Figure 4 demonstrate that in all three cases (4a,b,c) capping of the Ig-anti Ig complexes was gradually reduced from about 90% at 37°C, to less than 10% at 42°C. The percentage of capping on cells that were transferred to the indicated temperatures after washing out the unbound antibody (4a) suggests that the inhibition of capping was not due to an inefficiency in binding of the antibody to surface Ig at the hyperthermic temperatures. This obsevation was further supported by the fact that pretreatment of the cells at hyperthermic temperatures for 1 hour in the absence of antibody, followed by 10 minutes incubation with anti-Ig at 37°C, resulted in a similar inhibition of capping (4b).

A2. RECOVERY OF B-LYMPHOCYTES FROM HEAT TREATMENT AS MEASURED BY THE ABILITY TO CAP Ig-ANTI Ig COMPLEXES.

To determine whether hyperthermia caused reversible or irreversible inhibition of Ig-capping, B-lymphocytes were preheated for 30 minutes at temperatures up to 43°C. They were

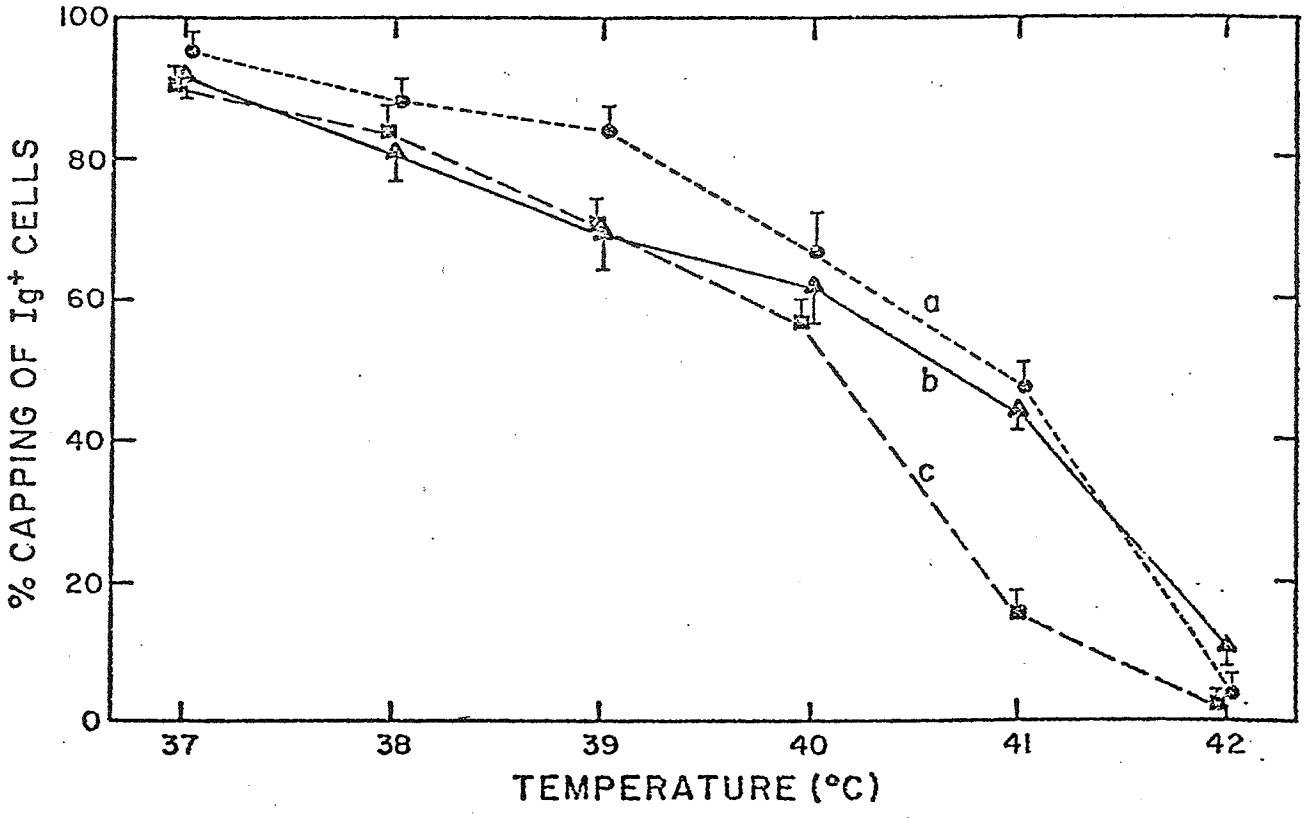

Figure 4: Inhibition of capping of Ig-anti-Ig complexes at hyperthermic levels. B cells were incubated at 4°C with anti-Ig antibodies, and transferred directly (c) or after washing (a), to the temperatures shown on the horizontal axis. Other sets of B cells (b) were pretreated for 60 minutes at the indicated temperatures, washed, incubated in the cold with anti-Ig, and transferred to 37°C. All reactions were stopped after 10 minutes incubation by fixation with cold paraformaldehyde. Points represent the mean ± SD of triplicate assays.

then transferred to 37°C and allowed to recover for different intervals of time, after which they were incubated at 37°C with FITC-labeled anti-Ig for 10 minutes, then fixed and scored for capping. The results showed that less than two hours were sufficient for the cells to recover the ability to cap Ig after 30 minutes heat pretreatment at 41°C or 42°C (Figure 5). However, cells that were preheated at 43°C did not recover the ability to cap even after 3 1/2 hours incubation at 37°C (Figure 5). After heat treatment, the viability of the cells was determined by trypan blue exclusion and was found to be better than 90%, even for cells heated for 30 minutes at 43°C. In another experiment in which cells were preheated at 42°C, a direct correlation was found between the length of the time of pretreatment and the decrease in percentage of capped cells, and the time required for recovery of capping (Figure 6).

A3. EFFECT AND RECOVERY OF B-LYMPHOCYTES FROM CYTOCHALASIN-B TREATMENT AT 37°C

Cytochalasin-B is a drug which disrupts microfilaments (111) and as a result has a variety of effects on cell in vitro. One particular effect is the consistent, although in most cases only partial, inhibition of capping of surface immunoglobulin (112). To examine the possibility that heat inhibits capping by impairing microfilament function in the same manner as it is inhibited by cytochalasin-B, the rates of recovery from cytochalasin-B treatment at 37°C were compared with the rates of recovery from heat treatment. B cells were incubated for 40 minutes at 37°C with different concentrations of cytochalasin-B.

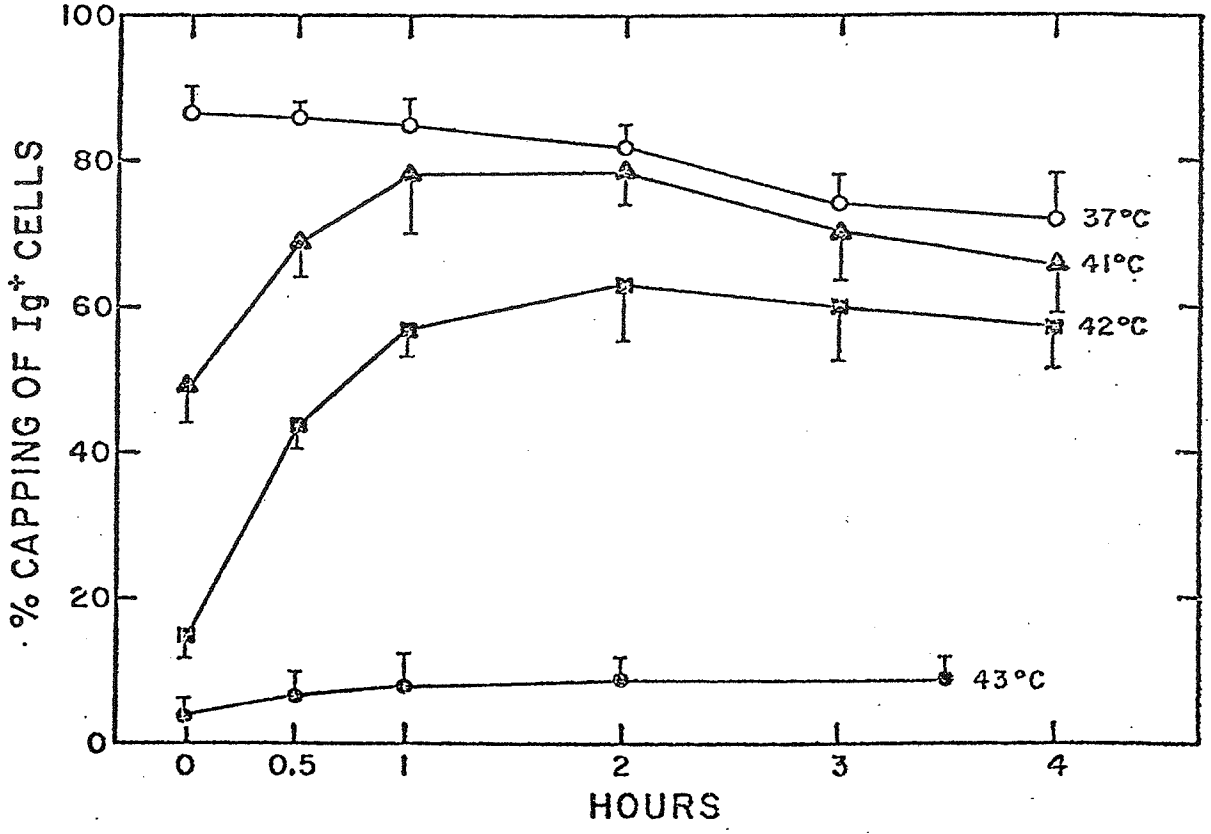

Figure 5: Recovery of B cells from heat treatment as measured by their ability to cap Ig-anti-Ig complexes. B cells were pretreated for 30 minutes at 37°C (○), 41°C (▲), 42°C (■) and 43°C (●). Cells were then transferred to 37°C, and samples removed at intervals to determine the percentage of capping at 37°C. Points represent the mean ± SD of triplicate assays.

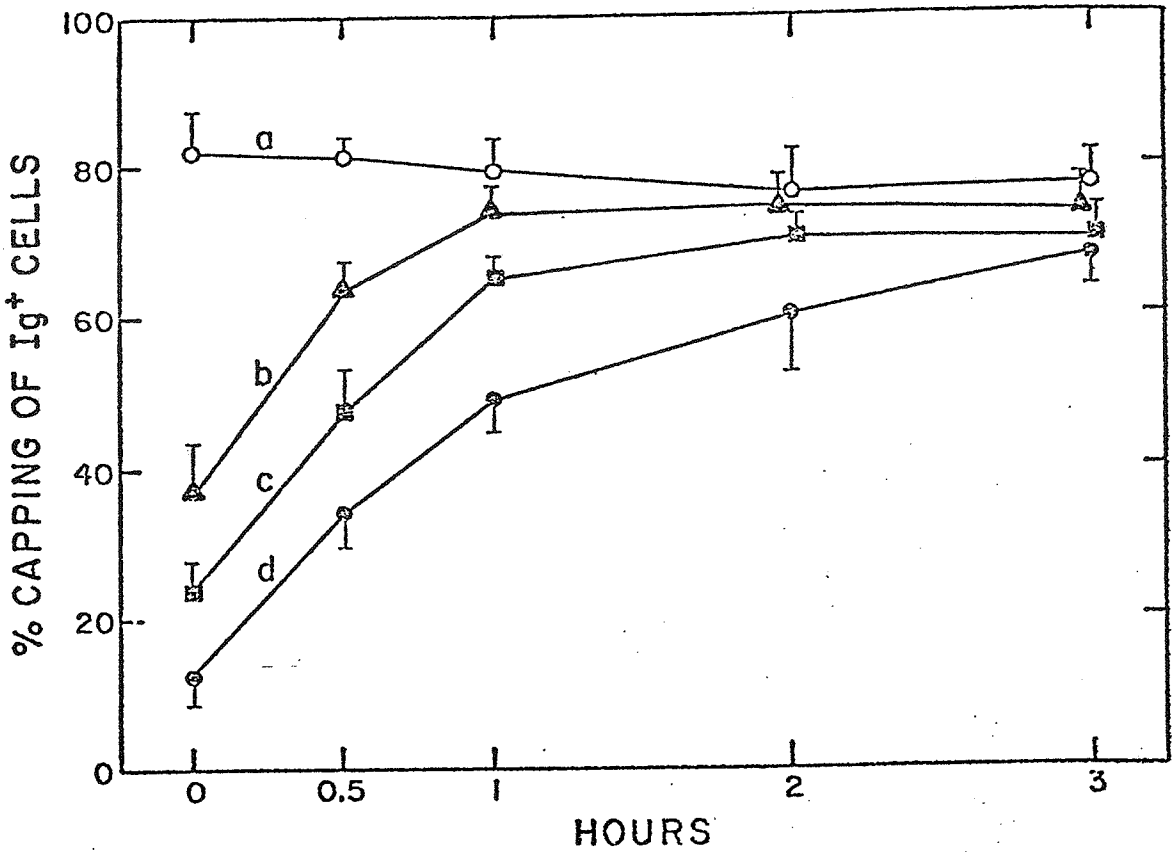

Figure 6: Recovery of B cells from heat treatment as measured by their ability to cap Ig-anti-Ig complexes. B cells were incubated at 37°C for 40 minutes (a), or at 42°C for 10 minutes (b), 20 minutes (c), and 40 minutes (d). Cells were then transferred to 37°C, and samples removed at intervals to determine the percentage of capping at 37°C. Points represent the mean ± SD of triplicate assays.

Part of the cells was then tested for capping at 37°C in the presence of the drug, another part was washed once at 4°C with PBS to remove the drug and was immediately tested for capping, and a third part was washed with cold PBS, resuspended in 300 µl MEM + FBS, and tested for capping after allowing 30 minutes for recovery. Cells that were prevented from capping (<10% capping) recovered the ability to cap surface Ig within 30 minutes (Figure 7). In fact, the large variability in the percentage of capping of pretreated cells which were immediately tested for capping (10 minutes at 37°C) after washing out cytochalasin-B (Figure 7), suggests that the cells were recovering while they were capping. In any case, it takes less than 30 minutes for the cells to recover from cytochalasin-B treatment, while heat treated cells need about 2 hours for complete recovery (Figure 5). It can therefore be suggested that microfilament function is not affected by heat in the same manner or magnitude it is affected by cytochalasin-B. Also it is possible that heat affects other cellular functions required for recovery of an intact microfilament system.

B. EFFECTS OF HYPERTHERMIA ON ANTIBODY-COMPLEMENT (Ab-C) CYTOTOXICITY AGAINST B-LYMPHOCYTES AND ITS RELATION TO CAPPING

B1. SENSITIVITY OF B-LYMPHOCYTES TO Ab-C CYTOTOXICITY DURING HEAT TREATMENT: ANTIBODY AND COMPLEMENT TITRATIONS

A cytotoxic antibody titration was performed to obtain a quantitative measure of the relative amounts of anti-mouse-Ig required to lyse unheated or heated mouse B-lymphocytes, in the absence or presence of a fixed complement dilution (1:40). The

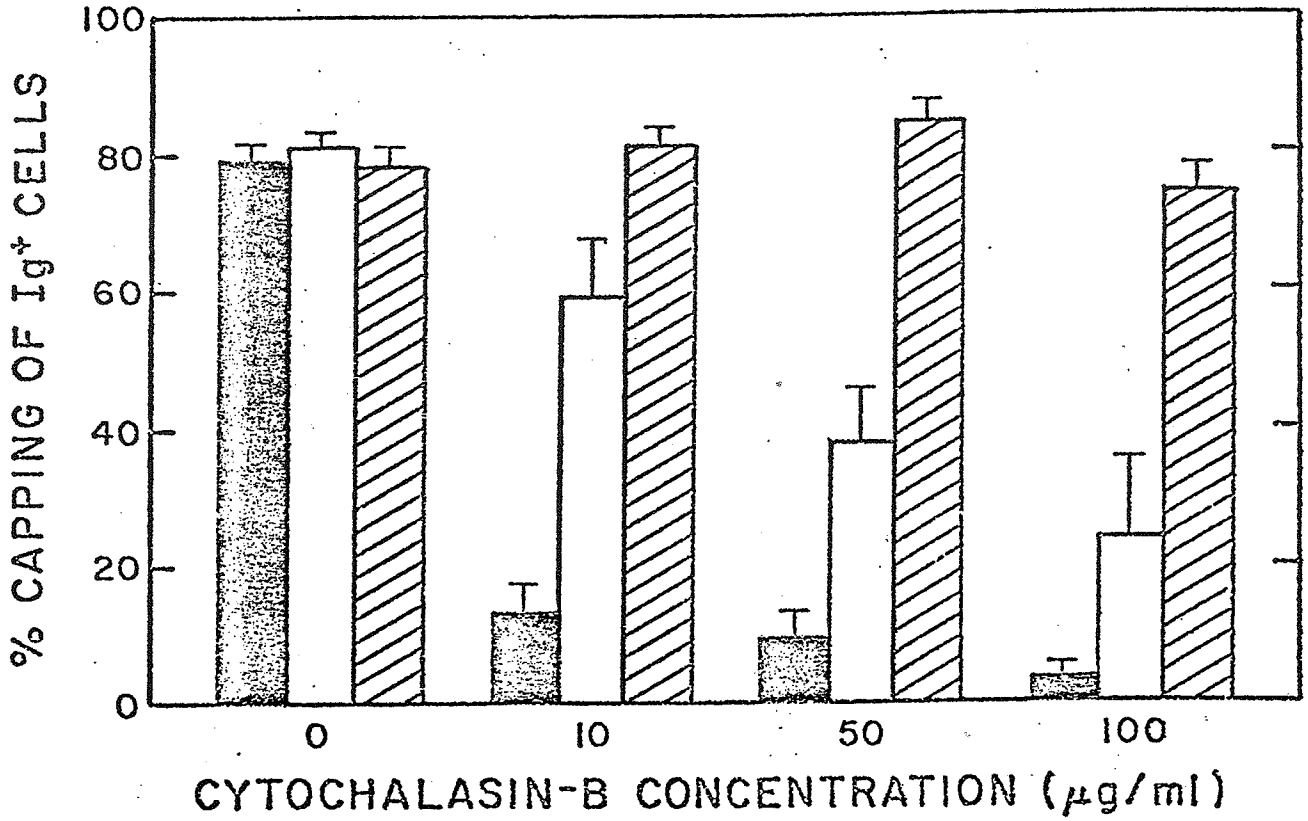

Figure 7: Effect of cytochalasin-B on capping of Ig-anti Ig complexes at 37°C, in the presence of cytochalasin B at the indicated concentrations (■). Other cells were incubated with cytochalasin-B for 30 minutes, washed, and incubated for 10 minutes with anti-Ig directly after washing (□), or 30 minutes after washing (▨). All reactions were stopped with 2.5% cold paraformaldehyde. Points represent the mean ± SD of triplicate assays.

assay was performed according to the standard procedure described in MATERIALS AND METHODS, and is further detailed in the caption of Figure 8. At the highest concentration used (1:10), antibody was cytotoxic in the presence and even in the absence of complement activity, for both heated or unheated cells (Figure 8). Complement alone had no observalbe effect in the absence of antibody (= CONTROLS). In the absence of antibody and complement, viability of the cells dropped from 91% at 37°C to 72% at 42°C, demonstrating that 42°C hyperthermia was causing direct cytotoxic effects on cells. For antibody diluted between 20 and 80 times, Figure 8 clearly demonstrates that Ab-C cytotoxicity was enhanced at 42°C relative to 37°C. An intermediate antibody dilution of 1:30 was selected and used as the standard antibody dilution in subsequent Ab-C cytotoxicity assays.

With the antibody dilution fixed, another Ab-C cytotoxicity assay was performed at 37°C or 42°C with different complement dilutions. Once again, the results shown in Figure 9 clearly demonstrate that 42°C hyperthermia enhances the cytotoxicity of Ab-C against normal mouse B-lymphocytes. Cytotoxicity at 42°C was most significant at the lowest complement dilution (1:10), but at that dilution some Ab-C lysis occured at 37°C. An intermediate complement dilution of 1:30 was used in all subsequent studies. Complement alone was not cytotoxic at all dilutions which were used ($p > .1$; Student's t-test).

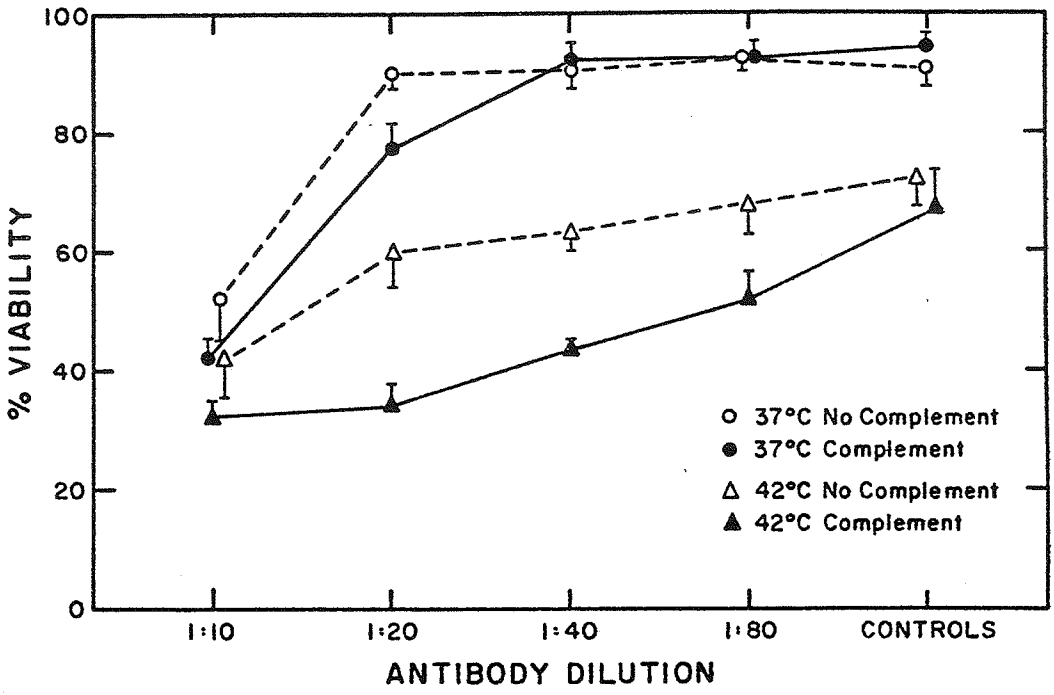

Figure 8: Sensitivity of B-lymphocytes to Ab-C cytotoxicity during heat treatment: Antibody titration. Cells were incubated for 10 minutes at 4°C with different antibody dilutions (CONTROLS = no Ab), then transferred to 37°C or 42°C. Complement (final dilution = 1:40) or MEM + FBS were added after 12 minutes. The Ab-C reaction was stopped after 45 minutes by transferring to 4°C. Viability was measured by trypan blue exclusion. Error bars represent one SD.

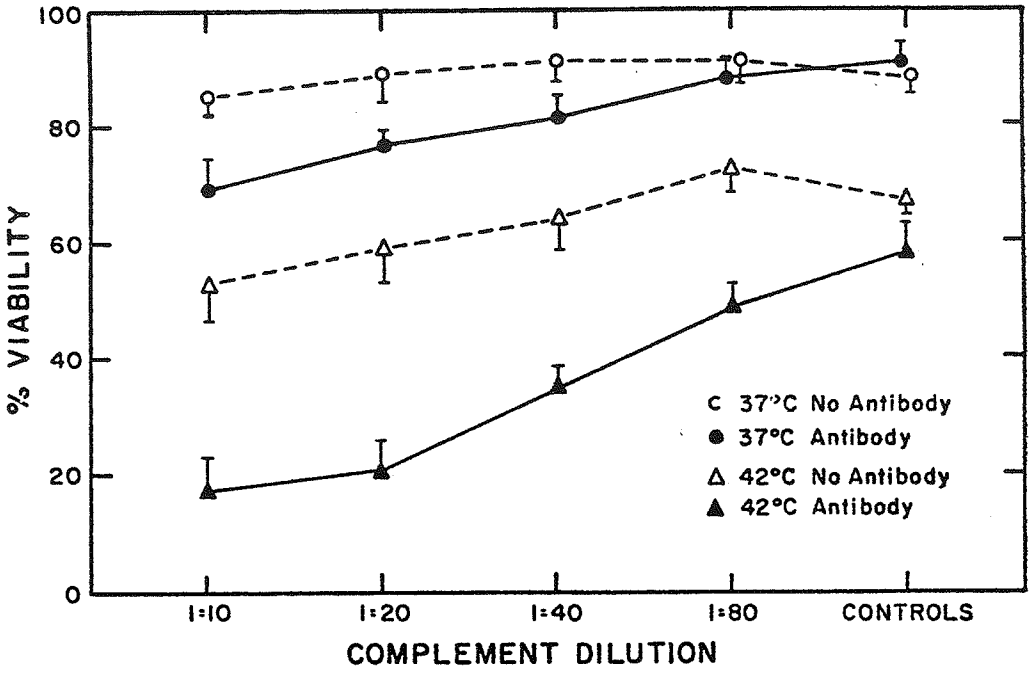

Figure 9: Sensitivity of B-lymphocytes to Ab-C cytotoxicity during heat treatment: Complement titration. Cells were incubated for 10 minutes at 4°C with or without antibody (1:30), then transferred to 37°C or 42°C. Different dilutions of complement (CONTROLS = no C) were added after 12 minutes. The Ab-C reaction was stopped by transferring to 4°C. viability was measured by trypan blue exclusion. Error bars represent one SD.

B2. SENSITIVITY OF B-LYMPHOCYTES TO Ab-C CYTOTOXICITY WITH DURATION OF OF INCUBATION WITH COMPLEMENT

Cells were incubated with antibody for 10 minutes at 4°C, then transferred to 37°C or 42°C. Complement was added after 12 minutes incubation with antibody. The Ab-C reaction was stopped after different intervals of time by transferring the cells to 4°C. Viability measurements demonstrated that hyperthermic enhancement of Ab-C cytotoxicity was more significant the longer the cells were incubated with complement (Figure 10). A statistical analysis (Student's t-test) showed that a 45 minute incubation with complement at 42°C was sufficient for a significant enhancement of Ab-C cytotoxicity ($p < 0.05$). Further experiments were performed with cells incubated for 45 minutes with complement.

B3. SENSITIVITY OF B-LYMPHOCYTES TO Ab-C CYTOTOXICITY AFTER HYPERTHERMIA

The Ab-C cytotoxicity assays described above were performed during hyperthermia treatment and, therefore, did not distinguish between direct effects of heat on particular cellular components, or on antibody-complement chemistry. To determine whether hyperthermia enhanced Ab-C cytotoxicity against B-lymphocytes by affecting some cellular function directly rather than affecting the activity of antibody or complement molecules, B-lymphocytes were preheated for 40 minutes at 37°C or 42°C, then transferred to 37°C. After different periods of time, a standard Ab-C cytotoxicity assay was performed at 37°C. The results demonstrate that Ab-C cytotoxicity was enhanced immediately after 42°C hyperthermia (Figure 11), implying that heat sensitized the cells directly

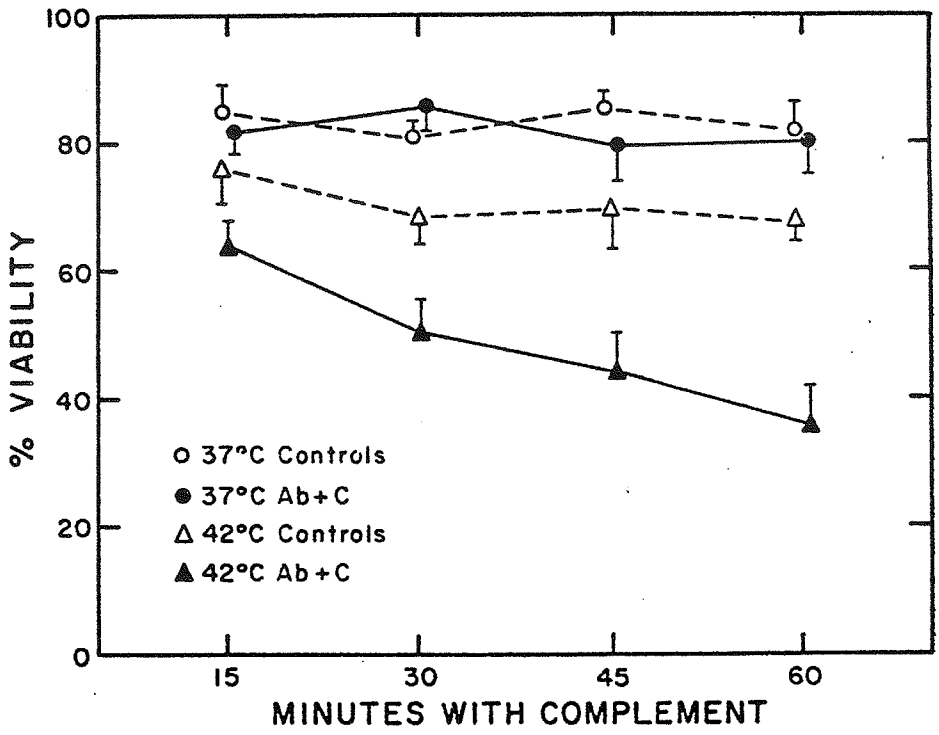

Figure 10: Sensitivity of B-lymphocytes to Ab-C cytotoxicity with duration of incubation with complement. Cells were incubated for 10 minutes at 4°C with or without antibody (1:30). Complement (final dilution = 1:30) or MEM + FBS were added after 12 minutes. The Ab-C reaction was stopped after different time intervals by transferring to 4°C. Viability was measured by trypan blue exclusion. Error bars represent one SD.

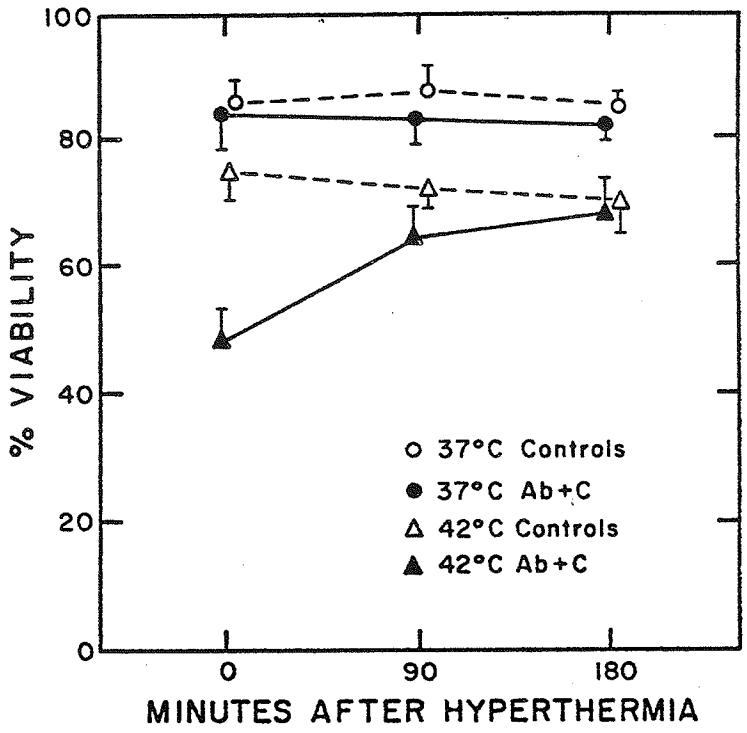

Figure 11: Sensitivity of B-lymphocytes to Ab-C cytotoxicity after hyperthermia. Cells were heated for 40 minutes at 37°C or 42°C, then transferred to 37°C. At different time intervals after hyperthermia, a standard Ab-C assay was performed at 37°C (12 min with 1:30 Ab, then 45 min. with 1:30 C). At the end of each reaction, viability was measured by trypan blue exclusion. Error bars represent one SD.

since it could not have affected Ab or C molecules. Furthermore, sensitivity to Ab-C cytotoxicity returned to normal levels by 2-3 hours post exposure to 42°C (Figure 11), suggesting that the target cells had the ability to repair whatever cellular mechanism was affected by heat.

B4. RELATION BETWEEN HYPERTHERMIC ENHANCEMENT OF Ab-C CYTOTOXICITY AND HYPERTHERMIC INHIBITION OF CAPPING

Kinetics of recovery from 42°C hyperthermia (Figures 5, 6, and 11) strongly suggest that the increased sensitivity of B-lymphocytes to Ab-C lysis was due to inhibition of capping by heat treatment. To investigate further this possibility, B-lymphocytes were heated for 40 minutes at 37°C, 42°C, or 43°C. A fraction of the cell suspension was then tested for sensitivity to Ab-C lysis at 37°C immediately or 2.5 hours after hyperthermia. Another fraction was tested for capping at 37°C during the same periods. Immediately after heat treatment, a direct correlation was found between the hyperthermic enhancement of Ab-C cytotoxicity and the hyperthermic inhibition of capping (Figure 12). By 2.5 hours post exposure to 42°C, both the sensitivity to Ab-C cytotoxicity and the ability to cap Ab-Ag complexes returned to normal levels. Cells heated at 43°C were still sensitive to Ab-C cytotoxicity and did not recover the capping ability even 2.5 hours after heat treatment (Figure 12). The reversibility of these effects after 42°C hyperthermia and their irreversibility after exposure to 43°C provide a strong argument in support of the hypothesis that hyperthermic enhancement of Ab-C cytotoxicity is significantly correlated to

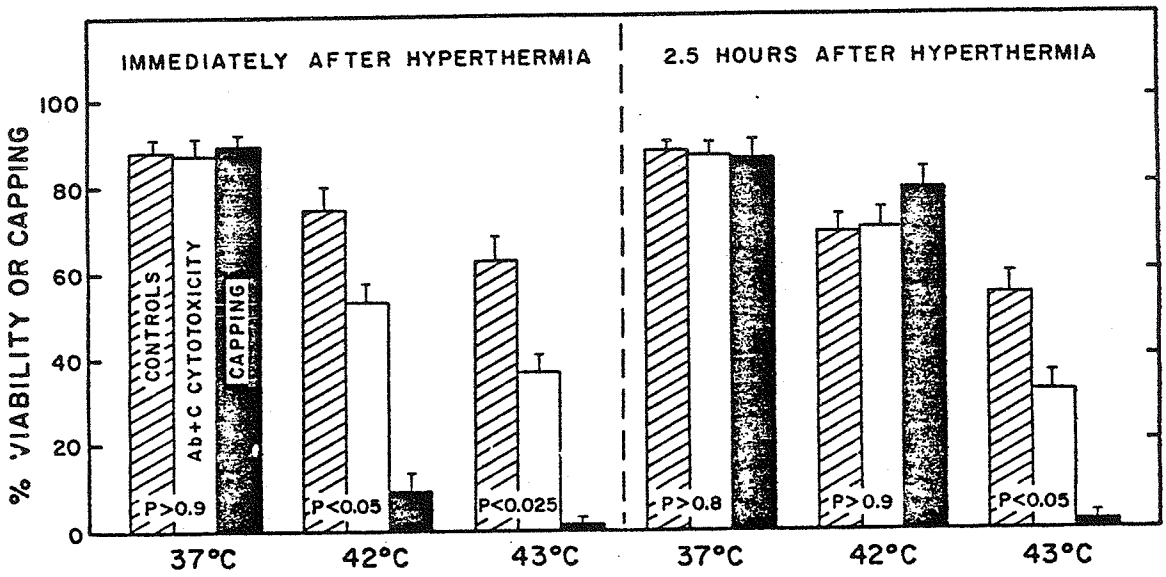

Figure 12: Relation between hyperthermic enhancement of Ab-C cytotoxicity and hyperthermic inhibition of capping. B cells were heated for 40 minutes at the indicated temperatures then transferred to 37°C. Immediately or 2.5 hours after hyperthermia, a standard Ab-C assay was performed at 37°C on part of the cells, while other cells were simultaneously tested for capping at 37°C. Error bars represent one SD. Statistical significance of Ab-C cytotoxicity was examined using the Student's t-test (differences considered significant if $p < 0.05$).

hyperthermic inhibition of capping.

C. EFFECTS OF MICROWAVES ON CAPPING OF Ag-Ab COMPLEXES ON THE SURFACE OF B-LYMPHOCYTES

C1. KINETICS OF CAPPING FOLLOWING 30 MINUTES HEAT TREATMENT

Before assessing the effects of microwave radiation on the ability of B lymphocytes to cap plasma membrane antigen-antibody complexes, it was necessary to determine how fast capping occurs at 37°C following heat treatment at different temperatures. Cells were heated for 30 minutes at 37°C, 41°C, and 42.5°C. following incubation at these temperatures, the viability of the cells was determined by trypan blue exclusion and was better than 90%. Immediately after the treatment, the cells were washed and incubated at 4°C for 10 minutes with 100 µl anti-Ig, and then transferred to 37°C to allow for capping. The temperature in each cell preparation reached 37°C in less than a minute because of the small volume of the cell suspension (100 µl). After incubation for different intervals of time, various cell preparations were fixed, washed, and scored for capping. The results indicated that less than 4 minutes were sufficient for the completion of capping on the surface of the cells that had the ability to cap surface Ig (Figure 13). Endocytosis (internalization) of antigen-antibody complexes started after 12 minutes of incubation at 37°C. While more than 90% of the Ig-positive cells treated at 37°C exhibited capping, it was found that cells pretreated at 41°C lost partially their ability to cap, and that capping on the surface of cells pretreated at 42.5°C was almost totally inhibited (Figure 13).

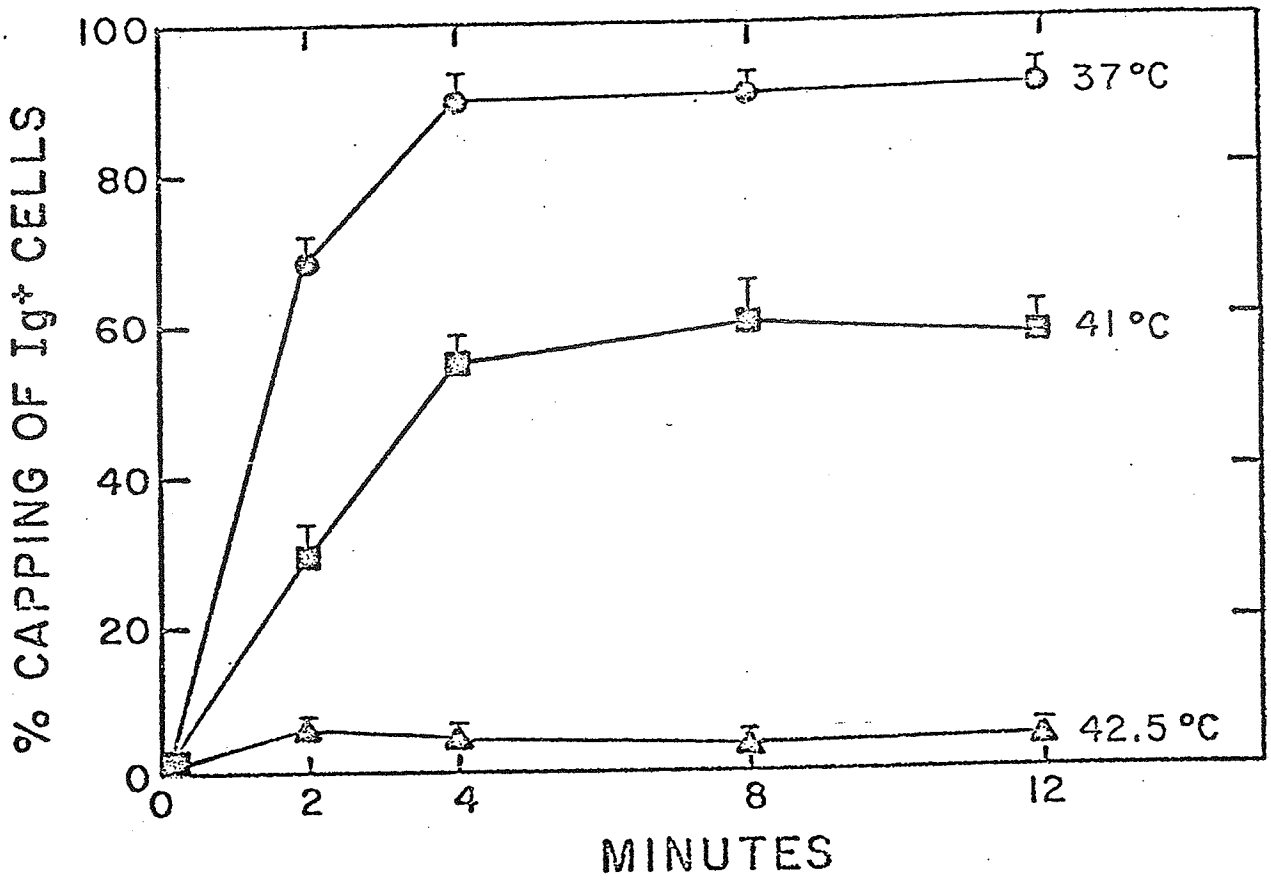

Figure 13: Kinetics of capping of Ig-anti Ig complexes at 37°C following heat treatment. Cells were preheated for 30 minutes at 37°C (●), 41°C (■), or 42.5°C (▲), washed, incubated with anti-Ig at 4°C, and transferred to 37°C for the times indicated in the horizontal axis. The reaction was stopped by fixation with 2.5% cold paraformaldehyde. Points represent the mean ± SD of triplicate assays.

C2. EFFECTS OF MICROWAVES AND/OR HYPERTHERMIA ON CAPPING

Cell suspensions were irradiated for 30 minutes with 2.45 GHz CW microwaves at intensities up to 100 mW/cm^2 at 37°C, 41°C and 42.5°C. The specific absorption rate (SAR) was obtained by measuring the initial rate of temperature rise in the irradiated suspensions when no heat exchange was allowed between the irradiated cell preparations and the surrounding. The SAR was 0.45 ± 0.04 Watt/kg per mW/cm^2 of incident radiation. The temperature in the microwave-exposed suspensions and the non-irradiated controls was taken just before the onset and immediately after the shut-off of the microwave field. The temperature variation between the irradiated suspensions and the non-irradiated controls was on the order of ± 0.1°C. Immediately after the microwave exposure, various preparations were tested for capping after 9 minutes incubation at 37°C with 100 µl anti-Ig. Figure 14 shows that for the nonirradiated controls, the results are in agreement with those shown in Figure 13, that is an inhibition at the hyperthermic levels. The percentage of capping of Ig-positive cells was gradually reduced from 90% at 37°C, to 52% at 41°C, to less than 5% for cells pretreated at 42.5°C. Figure 14 also shows that there is no significant difference in the percentage of capping between the microwave-treated cells and the non-irradiated controls as long as both were maintained at the same temperatures. ($P > 0.1$; Student's t-test).

In another experiment, the possibility of a direct action of the microwave field on capping was investigated, while capping of the Ig-anti Ig complexes was taking place. Cells

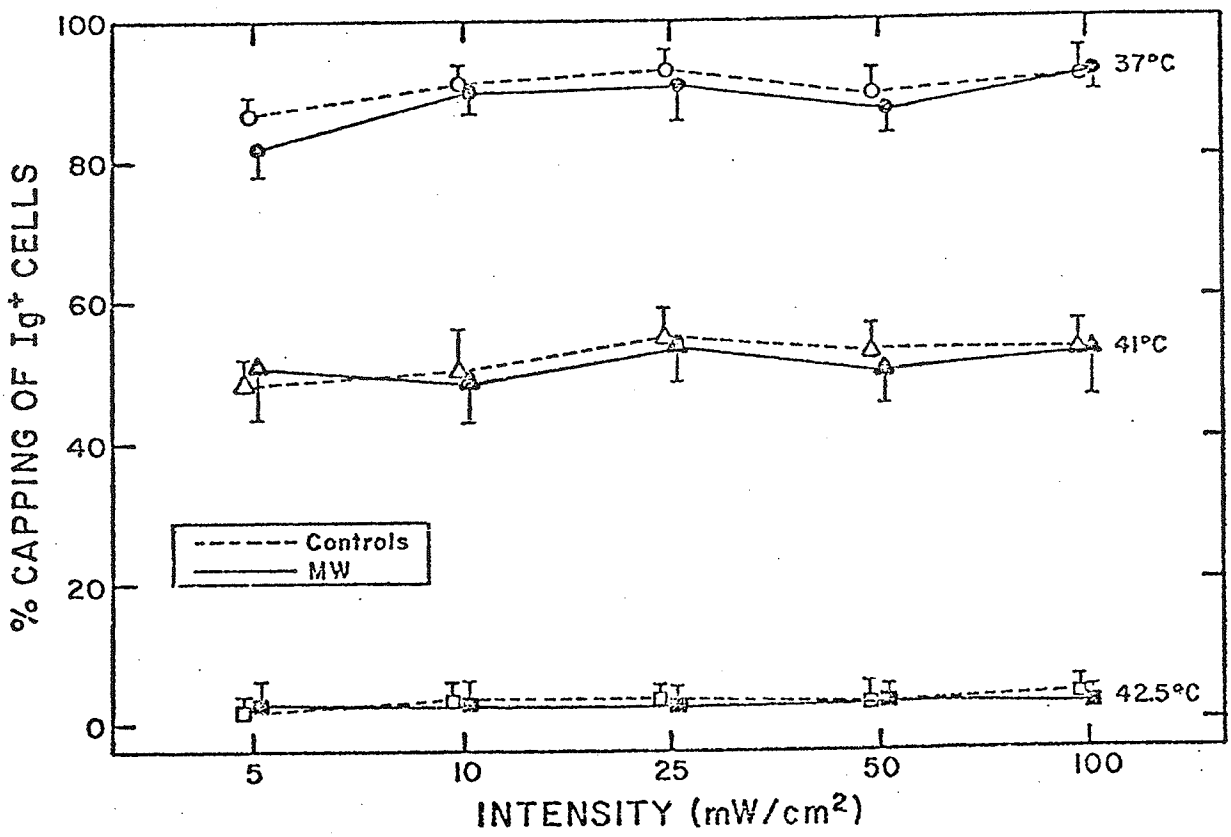

Figure 14: Percentage of capping of Ig-positive B cells pre-exposed to water bath (---) and microwaves (——). Suspensions of B-lymphocytes were exposed to 2.45 GHz microwaves for 30 minutes at different intensities and temperatures. The non-irradiated controls were maintained at the same temperatures at all times. Samples were then washed and tested for capping at 37°C. Points represent the mean ± SD of triplicate assays.

were incubated with 100 µl FITC-labeled anti-Ig for 10 minutes at 4°C. Incubation at 4°C allowed the antibody to bind to surface Ig, while preventing the antibody-antigen complexes from capping. After addition of 3.5 ml of cold PBS, the cell suspensions were immediately transferred to the microwave chamber or to the control water bath, both adjusted to operate at 38.5°C. After 10 minutes exposure to microwaves at different intensities, the cells were fixed with 2.5% cold paraformaldehyde, washed, and scored for capping. As shown in Figure 15, the results demonstrate that there is no significant difference in the percentage of capping between the microwave-exposed cells and the controls, even at intensities as high as 100 mW/cm^2.

D. EFFECTS OF AMPLITUDE MODULATED RADIO FREQUENCY (RF) ON CAPPING OF Ag-Ab COMPLEXES ON THE SURFACE OF B-LYMPHOCYTES

D1. EFFECTS OF AMPLITUDE MODULATED RF FIELDS AND/OR HYPERTHERMIA ON CAPPING

Cell suspensions were irradiated for 30 minutes with low frequency amplitude modulated 147 - MHz RF fields. The intensities ranged between 0.1 and 48 mW/cm^2. Sham and exposed groups were treated simultaneously and maintained at the same temperature. Temperature measurements were taken just before the onset and immediately after the shut-off of the RF field. Temperature variation between the irradiated suspensions and controls was less than 0.1°C. Immediately after the exposure, both control and irradiated groups were tested for capping at 37°C following the procedure outlined in MATERIALS AND METHODS.

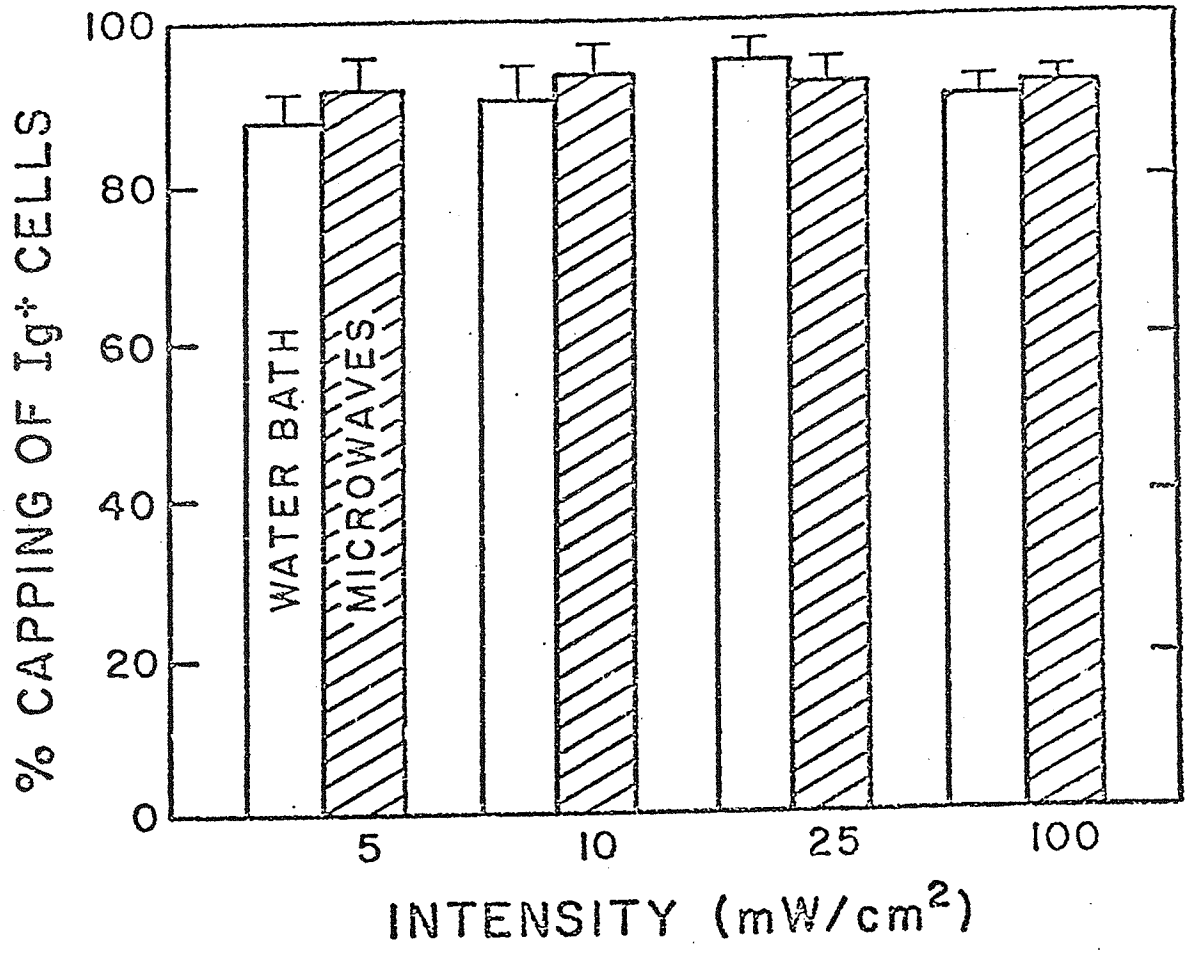

Figure 15: Percentage of capping of Ig-positive B cells during exposure to water bath and microwave at 38.5°C. B cells were incubated at 4°C with 100 µl anti-Ig for 10 minutes. After antibody binding, 3.5 ml of cold PBS were added, and the cell suspensions were transferred to the water bath (□) or to the microwave exposure chamber (▨), both at 38.5°C. All reactions were stopped after 10 minutes incubation by fixation with cold paraformaldehyde. Points represent the mean ± SD of triplicate assays.

The results are shown in Figures 16, 17, and 18 for 9, 16, or 60 Hz modulation frequency, respectively. The numbers in parentheses represent the actual intensity that was measured during the RF exposure of that particular sample. The results demonstrate that at any of the modulation frequencies and power densities used, there was no significant difference in the percentage of capping between the RF-treated cells and the non-irradiated controls as long as both were maintained at the same temperatures ($p>0.4$, student's t-test). While more than 90% of Ig-positive cells preheated at 37°C capped membrane-bound Ag-Ab complexes (upper set of curves in Figures 16, 17, and 18), less than 10% capping occurred when cells were preheated at 42°C (lower set of curves in Figures 16, 17, and 18).

E. EFFECTS OF ULTRASOUND ON CAPPING OF Ag-Ab COMPLEXES ON THE SURFACE OF B-LYMPHOCYTES

E1. EFFECTS OF ULTRASOUND AND/OR HYPERTHERMIA ON CAPPING

Samples were prepared as described in MATERIALS AND METHODS and immersed in the temperature-controlled water bath. The temperature in the cell suspensions reached its designated value in less than 2 minutes (Figure 19). Exposure to ultrasound was initiated 5 minutes after immersion. Sonicated and sham cells were tested for caping at 37°C immediately after at 15 minute irradiation at different temperatures and intensities. Figure 20 demonstrates that for the non-irradiated shams, capping of membrane-bound Ag-Ab complexes was reduced from 90% at 37°C to 60% at 41°C, to 20% for cells pretreated at 42°C. There was no significant difference in the percentage of capping between

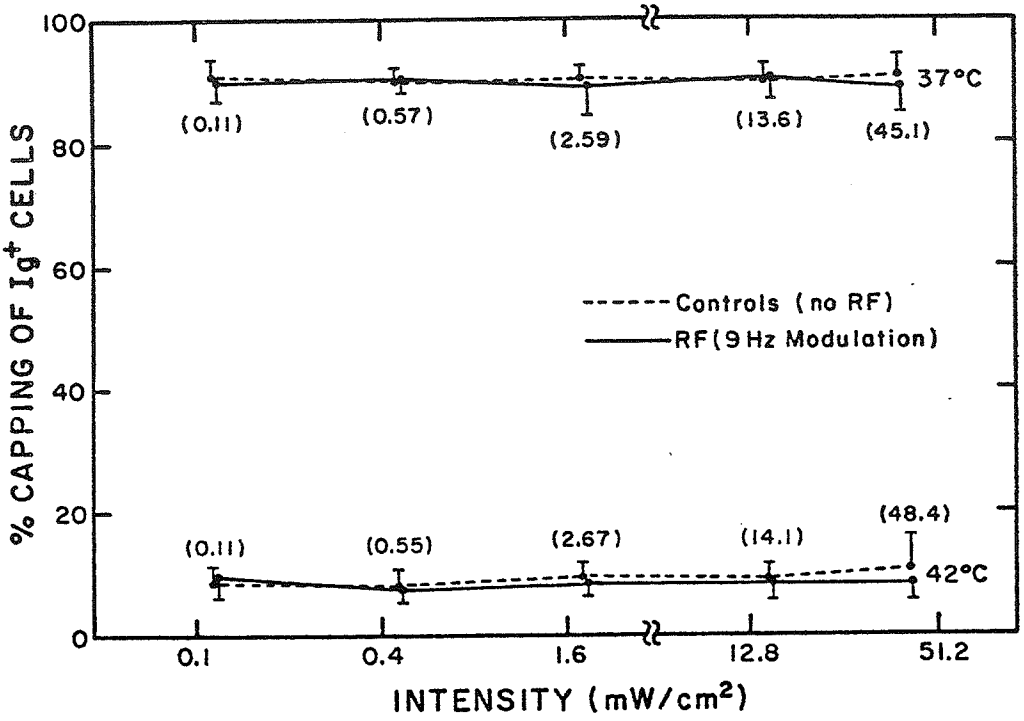

Figure 16: Percentage of capping of Ig-positive B cells pre-exposed for 30 minutes to 37°C or 42°C water bath (---) and 9 Hz amplitude modulated radio frequency (RF) radiation (————). Horizontal scale not linear. Error bars represent one SD.

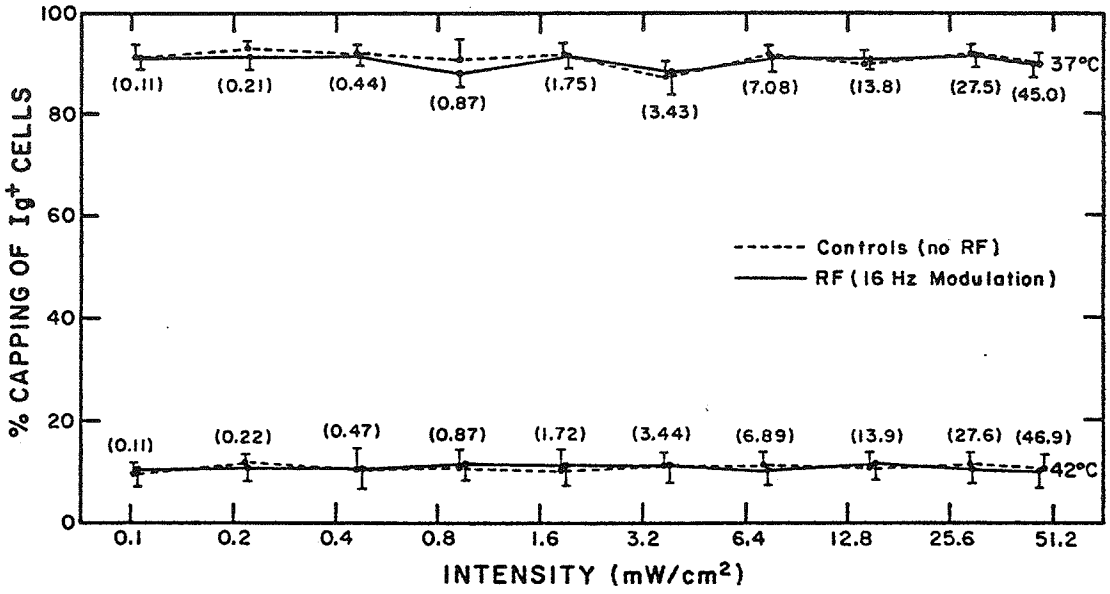

Figure 17: Percentage of capping of Ig-positive B cells pre-exposed for 30 minutes to 37°C or 42°C water bath (---) and 16 Hz amplitude modulated RF radiation (———). Horizontal scale not linear. Error bars represent one SD.

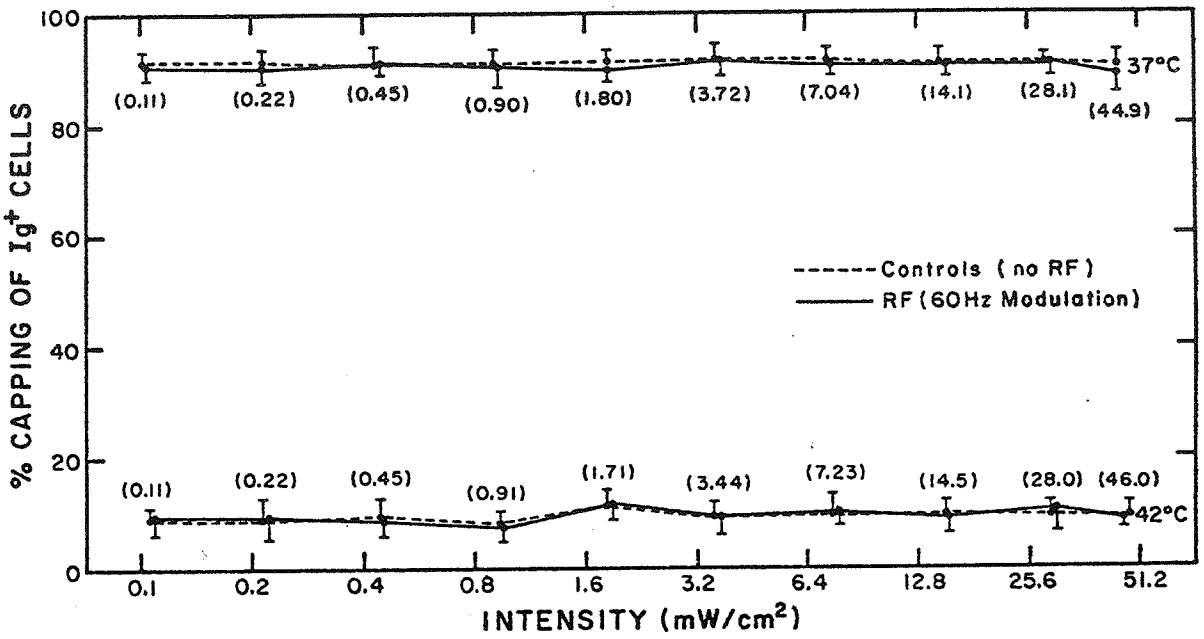

Figure 18: Percentage of capping of Ig-positive B cells pre-exposed for 30 minutes to 37°C or 42°C water bath (---) and 60 Hz amplitude modulated RF radiation (———). Horizontal scale not linear. Error bars represent one SD.

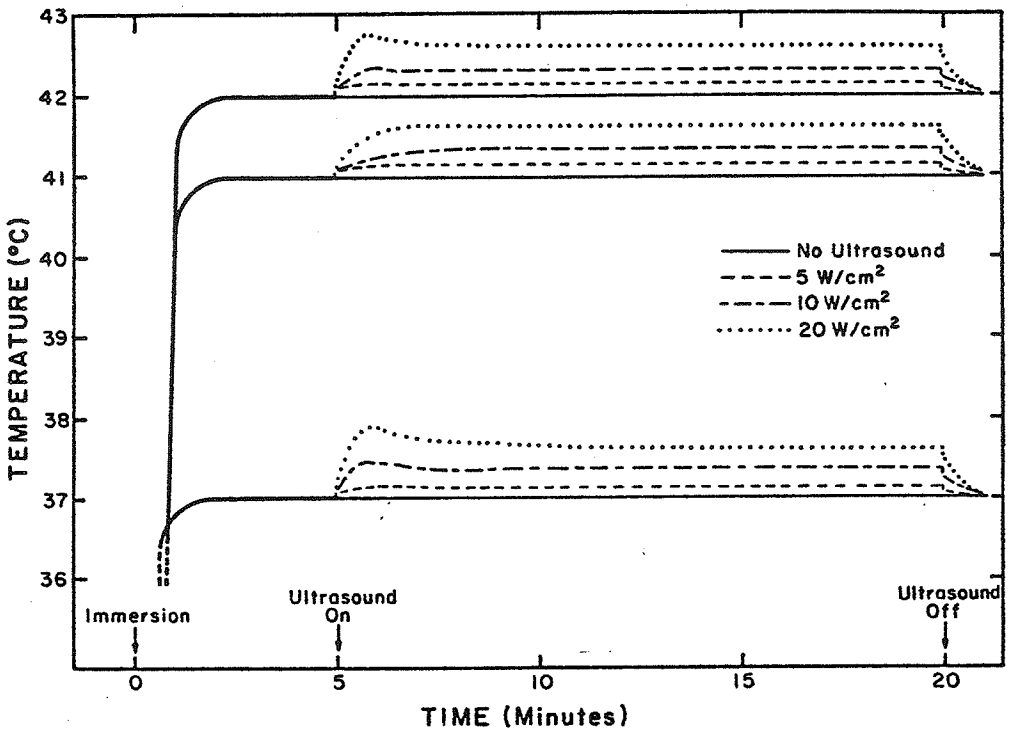

Figure 19: Temperature variation in sonicated and control cell suspensions. A rapid 0.1°C temperature rise (fall) component in the sonicated cell suspensions was observed at the onset (shut-off) of the ultrasound beam. This artifact was due to visco-elasticity at the boundary of the fine wire thermocouple which was used.

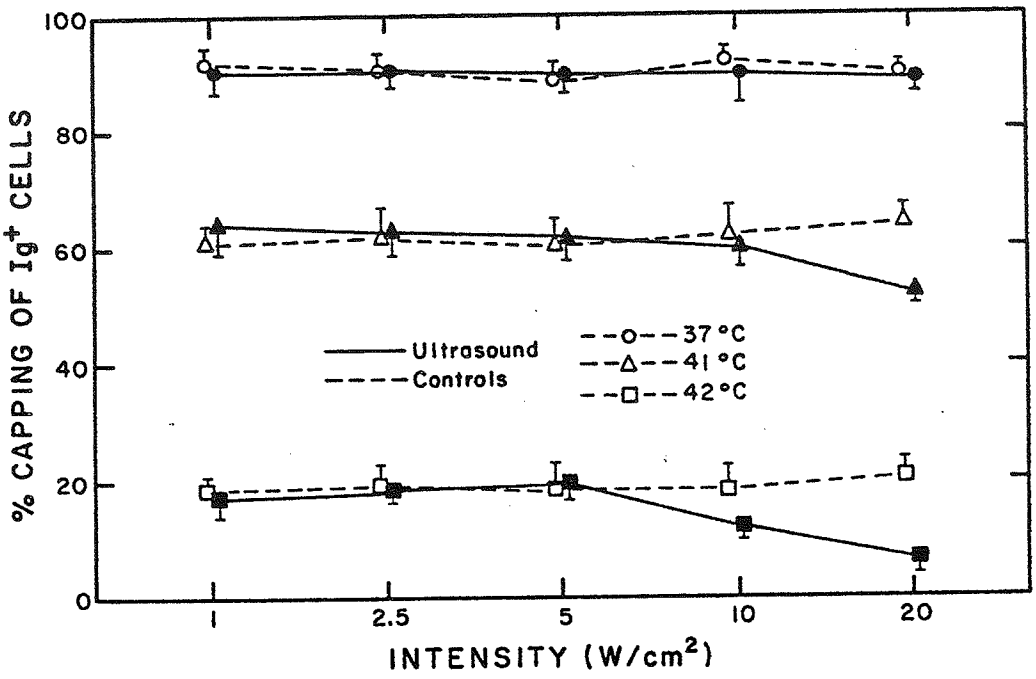

Figure 20: Percentage of capping of Ig-positive B cells pre-exposed for 20 min to 37°C, 41°C, or 42°C hyperthermia (- - -) and 15 min to ultrasound (———). Horizontal scale not linear. Error bars represent one SD.

sonicated and sham cells when the temperature of the water-bath was 37°C ($p>0.6$, Student's t-test). However, a further and significant reduction of capping was observed in cells exposed to 20 W/cm^2 when the temperature of the water bath was 41°C or 42°C ($p<0.05$, Student's t-test). This reduction of capping could have been caused by the 0.5°C temperature-rise which was observed in cell suspensions exposed to 20 W/cm^2 ultrasound (Figure 19). To test this possibility, control cell suspensions were heated for 20 minutes at different temperatures between 37°C and 43°C, while other suspensions were heated at the same temperatures during the first 5 minutes, and for the remaining 15 minutes the temperature was raised by 0.5°C, thus simulating the ultrasound-induced temperature-rise in suspensions exposed to 20 W/cm^2. Immediately after heat treatment, all samples were tested for capping at 37°C. Figure 21 demonstrates that for the control cells (solid curve), capping was gradually inhibited at temperatures above 37°C. A further reduction in capping was observed on cells which underwent a 0.5°C rise (Figure 21, dashed curve). This reduction in capping was comparable to the one obtained in cells exposed to 20 W/cm^2 ultrasound. No significant reduction in capping was observed when the temperature was 37°C ($p > 0.8$), Student's t-test), because at that temperature capping was not very sensitive to temperature increments of the order of 0.5°C, as can be seen from the slope of the solid curve in Figure 21. Reduction of capping was most significant at 41°C and 42°C ($p < 0.05$, Student's t-test) where sensitivity to small temperature increments was greatest (Figure 21). These observations strongly support the idea that the

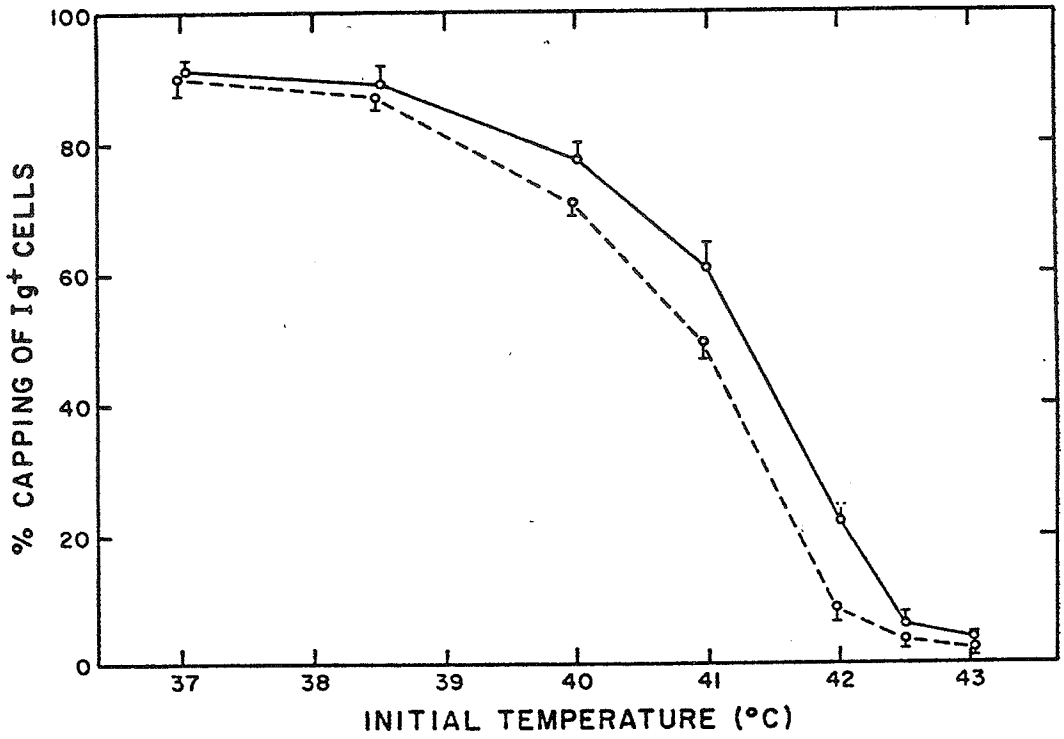

Figure 21: Percentage of capping of Ig-positive B cells pre-heated for 20 min at temperatures indicated on the horizontal axis (———), and cells pre-heated for 5 min at the indicated temperatures followed by 15 min incubation at 0.5°C above the indicated (initial) temperatures (- - -). Error bars represent one SD.

reduction of capping on cells exposed to 20 W/cm^2 ultrasound was thermally induced.

E2. RECOVERY FROM ULTRASOUND AND HEAT TREATMENT

To determine whether ultrasound and heat treatment caused reversible or irreversible reduction of capping, cells were exposed for 15 minutes to 20 W/cm^2 ultrasound, at 37°C, 41°C, and 42°C. Immediately after irradiation, the cell suspensions were transferred to 37°C to allow for recovery. After different intervals of time, samples were tested for capping. The results demonstrated that for the non-irradiated controls, cells which were heated at 41°C recovered almost completely the ability to cap antigen-antibody complexes within an hour (85% capping), while two and a half hours were required for partial recovery (67% capping) when non-irradiated cells were heated at 42°C (Figure 22). Partial recovery of capping was also observed on irradiated cells. The shift between the CONTROL curves (Figure 22, dashed curves), and the ULTRASOUND curves (Figure 22, solid curves) which was observed at 41°C and 42°C, was believed to be due to the 0.5°C temperature-rise in the sonicated cell suspensions. These observations are in agreement with the previous studies on the inhibition and recovery of capping on heat treated B-lymphocytes (Figures 5 and 6).

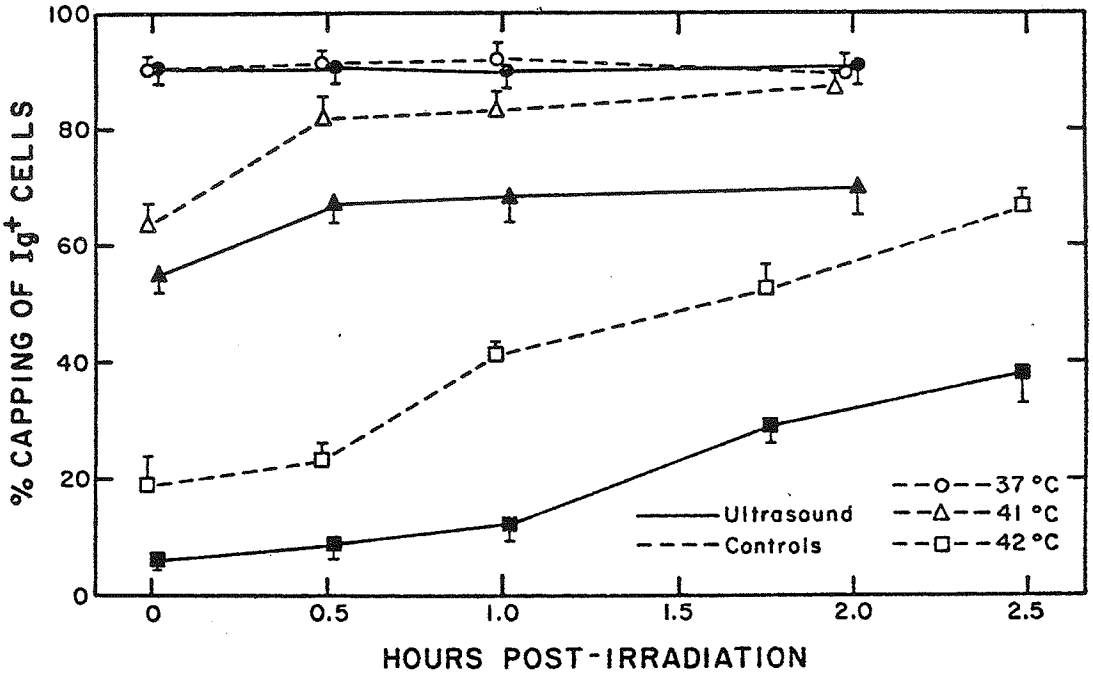

Figure 22: Recovery of Ig-positive B cells from exposure to heat (- - -) and 20 W/cm^2 ultrasound (———), as measured by the ability to cap Ag-Ab complexes. Error bars represent one SD.

V. DISCUSSION

A. HYPERTHERMIC INHIBITION OF CAPPING

There is increasing evidence that hyperthermia can have a beneficial effect on host defense mechanisms. Better response to bacterial or viral infections in different animal species (19-22), and enhanced mitogenic responses of human lymphocytes (23-25), have been reported to occur as a result of hyperthermic treatment. However, other studies indicate that hyperthermia can have adverse effects on such immune functions as the cytolytic activity of killer T-lymphocytes (31-33). Some reports have indicated that local heating of tumors may be beneficial, whereas whole-body hyperthermia may result in significant immunosuppression. Shah and Dickson (35,36) observed that local heating of VX2 tumor bearing rabbits was followed by tumor regression and a marked increase in cell-mediated immunity, whereas total body hyperthermia led to temporary restraint of tumor growth followed by a return to an exponential increase in tumor volume.

This study indicates that hyperthermia inhibits capping of Ag-Ab complexes on the surface of normal mouse B-lymphocytes (Figures 3,4). When the cells were heated in the presence of antibody, the percentage of capping dropped gradually from more than 90% at 37°C, to less than 10% at 42°C (Figure 4c). Similar results were obtained when the unbound antibody was washed out before heating the cells, ruling out the possibility that inhibition of capping was due to an inefficiency in binding of antibodies to Ig receptors at hyperthermic temperatures (Figure

4a). In support of this idea was the fact that heat pretreatment in the absence of antibody, followed by incubation with antibody at 37°C, resulted in a similar inhibition of capping (figure 4b).

Cells that were preheated for 30 minutes at 41°C or 42°C recovered the ability to cap antigen-antibody complexes within two hours. However, no such recovery was observed when cells were preheated at 43°C (Figure 5). This threshold for irreversible inhibition of capping occurs at a temperature where others have noted a transition in the thermal response of mammalian cells as measured by a change in slope of heat survival curves (109). A similar transition temperature, this time at 43.5°C, has been noted in experiments to determine the thermal inactivation energy for granulocyte-monocyte stem cell proliferation. Moreover, the same researchers noted that above 43.5°C the survival curve no longer exhibited a shoulder region implying a loss in ability to repair sublethal heat-induced damage (110). It is interesting to speculate that perhaps the same metabolic, cytoskeletal, and/or membrane function which is necessary for capping in B-lymphocytes, and which is irreversibly inactivated above 43°C, may also be involved in the control of cell proliferation and repair of heat-induced sublethal damage.

The mechanisms which are responsible for capping have not been completely determined. Capping is inhibited at 4°C, or in the presence of metabolic inhibitors (97). There is increasing evidence that the cytoskeletal system is actively involved in the capping process. Colchicine, a microtubular inhibitor, can

enhance capping (97). Cytochalasin-B, a drug which impairs microfilament function (111), inhibits capping reversibly (97,112). Recently it has been suggested that heat treatment of tumor cells may enhance antibody-complement cytotoxicity reactions by disrupting the cytoskeletal system and preventing capping and subsequent shedding of Ag-Ab complexes (27,28). Similarily, it is possible that heat treatment of B cells inhibits Ig-anti Ig capping by disrupting the cytoskeletal microfilament system directly, or inhibiting a process necessary for its function. The results presented in this report demonstrate that the processes involved in inhibition of capping by cytochalasin-B were fully reversible and much more rapid than recovery from heat pretreatment (Figure 7). Thus hyperthermia may inhibit a slowly recovering metabolic process necessary for microfilament function, whereas recovery from cytochalasin-B may be simply microfilament repolymerization or reorganization which has faster kinetics and is fully reversible even at the high doses used in this study.

B. HYPERTHERMIC ENHANCEMENT OF ANTIBODY-COMPLEMENT CYTOTOXICITY

Recent studies have indicated that in vitro hyperthermia may enhance tumor cell immunogenicity in vivo (113), as well as sensitivity to cytolysis by immunological effectors (27,28,35,114,115). Jasiewicz and Dickson (114) reported that heating of synchronized cultures of rat adenocarcinoma cells markedly enhanced sensitivity to lysis by Ab-C. Similar results have been reported in other cell lines such as Moloney virus lymphoma cells (115), human colon tumor cells (27), and

virus-transformed hamster PARA-7 cells (28).

In this study, it was found that heat treated B-lymphocytes were more sensitive to the cytotoxic activity of Ab-C than cells maintained at 37°C. The enhanced sensitivity to Ab-C lysis was observed during or immediately following heat treatment (Figures 8-11). Ab-C cytotoxicity returned to normal levels by 2.5 hours post exposure at 42°C hyperthermia, but no recovery was observed when cells were preheated at 43°C (Figures 11-12).

Several mechanisms may explain the heat-induced sensitization of cells to Ab-C lysis. Mondovi et al. (113) suggested that their observation of increased immunogenicity of Ehrlich ascites cells in Swiss mice was due to an antigenic change in the cell membrane after heating. Similarly, Jasiewicz and Dickson (114) proposed that exposure to heat may produce some cell surface modification facilitating interaction of antibody with cell antigens. Thus the apparent increased immunogenicity in vivo (113) and increased sensitivity to immune reactions in vitro (27, 28, 35, 114, 115) of heated cells may represent an increase or more favorable exposure of antigens on the cell surface. However, studies on antibody adsorption do not suggest that heated cells provide more antibody binding sites because both heated and non-heated human colon tumor cells adsorbed equivalent amounts of specific anti-tumor cell antibodies (27). Other studies have indicated that the relative efficiency of Ab-C cytotoxicity may reflect the ability of the target cell to repair complement damage to the cell membrane (116). Thus hyperthermic enhancement of Ab-C cytotoxicity may be the result of inactivation by heat of these repair enzymes,

so that fewer lytic events would be required for membrane damage and cell death; hence lower concentrations of antibody or complement could mediate cell death.

The data presented in this report provides evidence of a direct correlation between heat-induced enhancement of Ab-C cytotoxicity and inhibition of capping on the surface of B-lymphocytes. It was found that both events (enhancement of Ab-C cytotoxicity and inhibition of capping) occur immediately after 42°C or 43°C hyperthermia, and that they return to normal levels with comparable kinetics after heating at 42°C (Figure 12). Cells heated at 43°C were still sensitive to Ab-C cytotoxicity and did not recover the ability to cap Ag-Ab complexes even 2.5 hours post-exposure to heat. These observations suggest that the same cellular components which are reversibly affected by 42°C hyperthermia, and irreversibly affected by heat treatment at 43°C, may be directly responsible for both inhibition of capping and enhancement of Ab-C cytotoxicity. However, a more plausible explanation may be that the heat affected cellular components are primarily responsible for inhibition of capping and subsequent endocytosis of Ag-Ab complexes, thus leaving more binding sites for complement acitivity. Cell death could then be mediated by lower concentrations of complement. In support of this idea, it was found that at least 30 minutes of incubation with complement were necessary for significant Ab-C lysis at 42°C (Figure 10). By that time, non-heated cells would have capped and removed all Ag-Ab complexes, since these processes are completed within 10 - 12 minutes at normal temperatures. With no binding sites

for complement activity, no complement-mediated cytolysis can take place. This agrees with the observations that with the dilutions of anti-Ig used, no Ab-C lysis occurs at 37°C, while Ab-C cytotoxicity increases with duration of incubation with complement at 42°C (Figure 10).

C. EFFECTS OF MICROWAVES AND HYPERTHERMIA ON CAPPING

Many of the microwave effects on immune reactions have been attributed to a thermal action of the microwave field on different components of the immune system. The existence of non-thermal effects is still controversial. To determine whether or not exposure to microwaves had non-thermal effects on capping, it was necessary to have a good temperature control in the irradiated suspensions, since the earlier part in this study demonstrated that capping is an extremely temperature sensitive process above 37°C. The capping assay was performed during or immediately after exposure to microwaves at different intensities and temperatures. The results demonstrated that for the contol cells, the percentage of capping dropped from 90% at 37 C, to less than 5% for cells that were preheated at 42.5 C (Figure 14). There was no significant difference in the percentage of capping between control and irradiated cells at any of the intensities and temperatures used, as long as both control and microwave preparations were kept at the same temperature (Figures 14,15). No synergism between field specific and hyperthermia effects on capping was observed.

D. EFFECTS OF AMPLITUDE MODULATED RADIOFREQUENCY AND HYPERTHERMIA ON CAPPING

Cellular membranes have been suggested as a site of interaction between radiofrequency (RF) radiation and biological systems. One of the most commonly reported effects of low-level RF radiation has been the release of calicum ions from brain tissue during irradiation (47, 117-120). The effect has been reported to occur at a narrow power density window (0.83 mW/cm^2, and at a modulation frequency window (16 Hz) within the range of frequencies associated with the electroencephalogram. Bawin and her associates (117) have attributed the efflux of calcium ions to a field-induced cooperative interaction taking place between adjacent cation binding sites at the outer surface of the plasma membranes. Several theoretical models based on cooperative phenomena and long-range coherence have been proposed to explain the interaction of oscillating fields with biological membranes (43-45).

It is not yet known whether amplitude-modulated RF fields induce a change in intracellular calcium concentrations. If such an effect does exist, a change in intracellular calcium concentrations might alter various metabolic and cellular functions (121). One particular event which is altered by a change of intracellular calcium concentration is the redistribution and capping of Ag-Ab complexes on the surface of B-lymphocytes, following the binding of antibody molecules to surface immunoglobulins. Schreiner and Unanue (97) reported that the introduction of a calcium ionophore into the plasma membrane of B-lymphocytes completely suppresses capping. If the ionophore-mediated calcium influx occurs after cap formation,

the cap is completely disrupted by a metabolically active process which involves the lymphocyte's cytoskeletal system. These observations and other considerations led Schreiner and Unanue to suggest a model for capping whereby a calcium dependent bond between antigen receptors and calcium responsive cytoplasmic microfilaments effects the transport of the receptors through the plane of the membrane without affecting other components of the membrane.

If intracellular calcium levels are shifted by application of an amplitude modulated RF field, the change might affect the capping of membrane bound Ag-Ab complexes. To test this hypothesis, a series of experiments was conducted to see if there is any effects of amplitude modulated RF fields on capping. The 16 Hz modulation frequency was chosen because the reported effects on calcium efflux from brain tissue occurred at 16 Hz (47, 117-120). Two other modulation frequencies were also tested because there is no a priori reason why B-lymphocyte membranes should react at the same frequencies as those of neurons. A wide range of power densities was investigated, including the range over which the calcium efflux effects were reported, in order to reduce the possibility of missing any power density window. the irradiations were done during heat treatment at 37°C or 42°C.

The results did not demonstrate any effect of amplitude modulated RF radiation on capping (Figures 16-18). At the modulation frequencies and power densities used, no significant difference in the percentage of capping was found between the controls and cellsthat were pre-exposed to amplitude modulated

RF radiation, as long as both preparations were kept at the same temperatures. The percentage of capping was reduced from about 90% at 37°C to about 10% at 42°C, in agreement with results obtained previously in this study.

Although no evidence of amplitude modulated field-specific effects on capping was found, these results cannot be regarded as definitive. Much additional work is necessary to determine the existence or nonexistence of frequency and power density window effects on capping.

E. EFFECTS OF ULTRASOUND AND HYPERTHERMIA ON CAPPING

Several field-specific mechanisms have been proposed to explain some of the reported biological effects of ultrasound (49-55). These mechanisms include cavitation (49-53), acoustic streaming and microstreaming (54), and radiation force effects (55). However, ultrasound bioeffects may also be caused by thermal heating of the sonicated tissues. Marmor et al. (122) observed tumor regression after localized ultrasound heating and suggested that such effect might be related to the stimulation of patient antitumor immunity. Similar observations and suggestions were made earlier when hyperthermia was not induced by ultrasound (18).

In this study, the combined effects of ultrasound and hyperthermia were analysed immediately after treatment. As before, a good temperature control in the sonicated cell suspensions was necessary. The temperature was controlled within acceptable limits (0.1°C) at intensities up to 5 W/cm^2. However, at the highest intensity used (20 W/cm^2), a 0.5°C

temperature-rise was sustained in the irradiated suspensions. This temperature-rise was sufficient for significant reduction of capping on the surface of sonicated cells with respect to the controls, when the controls were kept at 41°C or 42°C. No significant difference in the percentage of capping between sonicated and control cells was observed at lower intensities at 41°C or 42°C, or at any intensity when the temperature was 37°C (Figure 20). The reason why no significant reduction of capping was observed on cells exposed to 20 W/cm^2 at 37°C was that capping was less sensitive to small temperature increments at 37°C than at hyperthermic temperatures, as demonstrated in Figure 21. Partial to total recovery from inhibition of capping was observed within 2.5 hours post-exposure to 20 W/cm^2 ultrasound and/or hyperthermia. The difference in the kinetics of recovery curves between sonicated and control cells (Figure 22) was due to the temperature-rise which was observed in the sonicated preparations. These results demonstrate that the observed ultrasound effects on capping are thermally induced.

F. SUMMARY

In conclusion, it has been demonstrated that capping of Ag-Ab complexes was significantly reduced on the surface of heat-treated mouse B-lymphocytes. Total inhibition of capping was observed at and above 42°C. The ability to cap was recovered within two hours post hyperthermia, as long as the temperature did not exceed 42°C.

A strong correlation was found between hyperthermic enhancement of Ab-C cytotoxicity and hyperthermic inhibition of

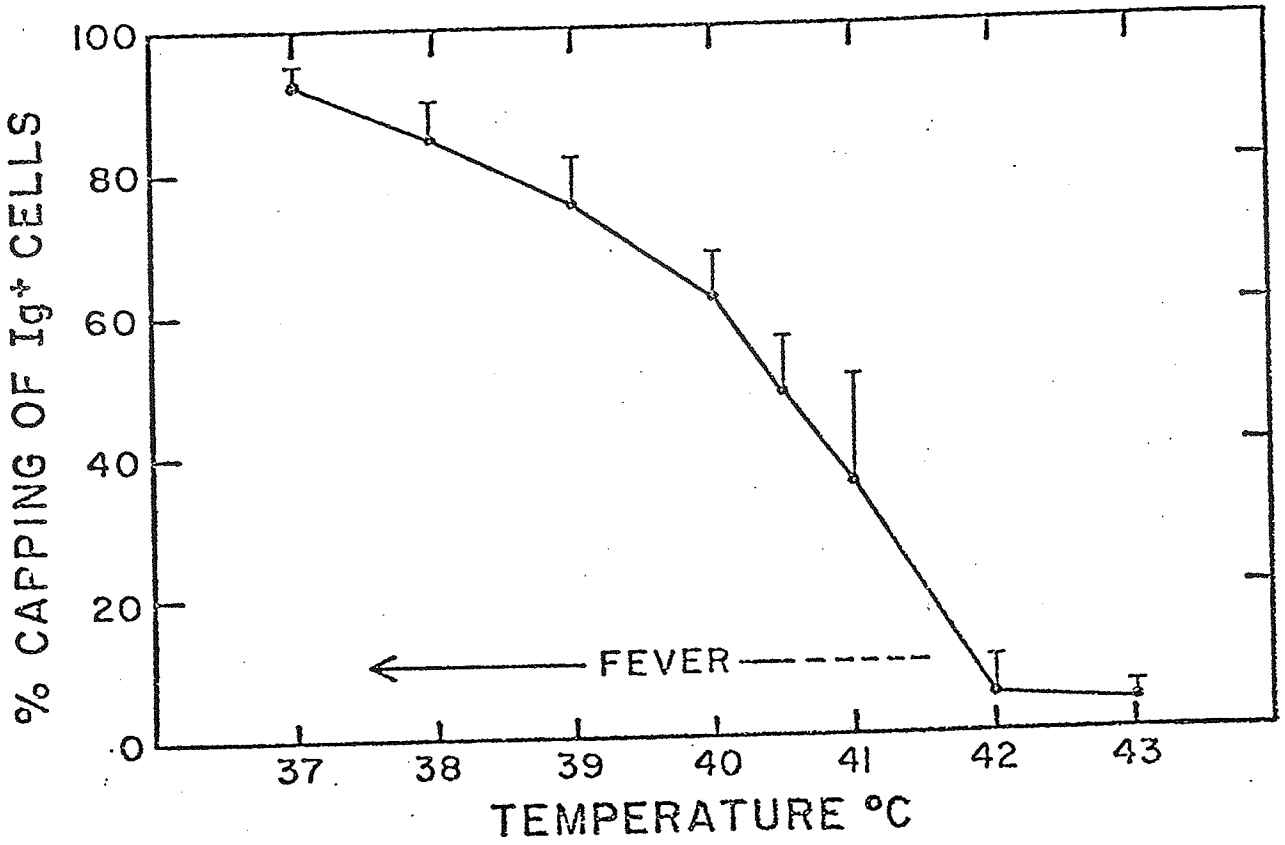

Figure 23: Inhibition of capping of Ig-anti Ig complexes at temperatures associated with fever. Points represent mean ± SD of all assays done during this study.

capping, suggesting that the increased sensitivity to Ab-C lysis resulted from the availability of more binding sites for complement activity. Further studies are needed to more fully test this hypothesis and, in particular, if it applies to cancer cells.

Figure 23 shows the sensitivity of capping to heat pretreatment at temperatures between 37°C and 43°C. At any of these temperatures, even a 1°C increase in temperature may result in significant reduction of capping. From this obsevation, it appears that a good temperature control is essential during irradiation experiments, specially if non-thermal effects are to be investigated. With a carefully designed temperature control system, no significant non-thermal effects on capping were observed following exposure to microwaves, amplitude modulated radiofrequency, or ultrasound. Reduction of capping was observed on cells exposed to 20 W/cm^2 ultrasound at 41°C or 42°C where capping was most sensitive to small temperature variations. However, this significant reduction of capping was explainable on the basis of thermal effects only.

The mechanisms responsible for the hyperthermic inhibition of capping are not knowr. There is evidence that the cytoskeletal system is involved in the capping process. Thus, it is possible that heat treatment of B-lymphocytes inhibits capping by disrupting the cytoskeletal microfilament system directly, or by inhibiting a process necessary for its function. This hypothesis was tested by comparing the rates of recovery of B-cells from heat treatment to the rates of recovery from

cytochalasin-B, a microfilament inhibitor. Recovery from cytochalasin-B was fully reversible and more rapid than recovery from heat pretreatment, which suggested that hyperthermia may inhibit a slowly recovering process necessary for microfilament function, whereas recovery from cytochalasin-B may be simply microfilament repolymerization or reorganization which has faster kinetics and is fully reversible.

In vivo hyperthermic inhibition of capping may have significant immunological consequences which are yet to be determined. It has been suggested that capping of Ag-Ab complexes may be involved in triggering B-cell differentiation (97). Consequently, hyperthermic inhibition of capping might depress B-cell proliferation. On the other hand, inhibition of antigen-Ig capping may facilitate the cooperation of B-cells with other components of the immune system, particularly macrophages and helper T-cells, by slowing the removal of B-cell surface Ig by endocytosis of capped Ag-Ig complexes.

Figure 23 shows that significant inhibition of capping by in vitro hyperthermia occurs over the temperature range associated with fever. Only further research will determine the role fever (or hyperthermia) plays in regulation of immune responses. An understanding of this role will help provide a rational basis for the design of therapeutic hyperthermia protocols.

Finally, the relevance of the observations made in this study and their applicability to cancer cells are particularly important. Further research in this direction is definitely essential to fully determine whether hyperthermia enhances the

immunogenicity of cancer cells by inhibiting capping and subsequent shedding of membrane bound Ag-Ab complexes.

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Inhibition of capping of antigen-antibody complexes on the surface of B-lymphocytes by hyperthermia. Submitted for publication.

Hyperthermic enhancement of antibody-complement cytotoxicity against normal mouse B-lymphocytes and its relation to capping. Submitted for publication.

Effects of microwaves and hyperthermia on capping of antigen-antibody complexes on the surface of normal mouse B-lymphocytes. Submitted for publication.

Immunological effects of amplitude modulated radio frequency radiation: B-lymphocyte capping. Submitted for publication.

Immunological effects of ultrasound: B-lymphocyte capping. Submitted for publication.

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