

Effect of Hyperthermia on the Immune Response of Normal Rabbits¹

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ABSTRACT

Sequential skin responses to dinitrochlorobenzene challenge and repeat assays of serum antibody titer after two injections of bovine serum albumin were used as functional indices of cellular and humoral immunocompetence following hyperthermia in normal adult New Zealand White rabbits. The animals were subjected to different degrees of local hyperthermia by watercuff or radio-frequency heating of the normal thigh muscles maintained at 42° for 1 hr on 3 consecutive days or 47–50° for 30 min, respectively, or to total body hyperthermia (42° for 1 hr on three occasions) in a humidified incubator.

No alteration occurred in the response of heated rabbits to dinitrochlorobenzene challenge over a 3-month period. The humoral immune response to bovine serum albumin was significantly depressed ($p < 0.02$) in the treated animals, and the reduction was independent of method and degree of heating. The results suggest that the B-lymphocytes are more susceptible to hyperthermic damage than is the T-cell population.

INTRODUCTION

A considerable and increasing body of data denotes that hyperthermia (temperatures in excess of 40°) has a selective destructive effect on malignant tumors in both animals and humans (29–31). It is suggested, based on strong circumstantial evidence, that the beneficial effect of hyperthermia in the tumor-bearing host involves advantageous participation of the immune system (6, 8, 10, 13, 18, 21, 22, 24, 25, 34). There is little evidence, however, as to whether such an immune response is specifically related to tumor destruction or results from stimulation of the immune system by heat. The role of the immune system assumes further importance in relation to the therapeutic potential of hyperthermia when it is recalled that the 2 major potentiators of heat, irradiation and cytotoxic drugs (29, 31), are immunosuppressive agents (2). In this and the following paper (32), we present evidence indicating that, although both local and total-body hyperthermia depress the humoral immune response to foreign antigen in normal rabbits, this effect is of little moment in the VX2 tumor-bearing host treated by curative local hyperthermia. Effective local heating evokes a marked cellular and humoral response to both nonspecific and tumor-associated antigen and is accompanied by cure of the host. Total-body heating, on the other hand, abrogates both arms of the immune response in tumor-bearing animals and is associated with rapid death of the host (32).

The rabbit was chosen as the model system for study

since previous work in this laboratory has shown that rabbits can withstand repeated total-body hyperthermia at 42° (10). Smaller laboratory animals, e.g., rodents, tolerate body temperatures in the region of 42° poorly (6, 9). In addition to being suitable for the skin tests used, the rabbit readily tolerates repeated blood sampling and the procedure is technically easy.

It has become apparent from recent work that a majority of human solid cancers (4, 7, 14, 28) and some animal tumors, including the rabbit VX2 carcinoma (8, 11, 12), are not heat sensitive at 42°. However, 70% of VX2 carcinomas given a single heat treatment at 47–50° for 30 min regressed completely with cure of the host (11, 12). This report, therefore, describes the effect of heating normal rabbit thigh muscles to temperatures up to 50° on the cellular and humoral response of the host.

MATERIALS AND METHODS

Male New Zealand White rabbits weighing 2.3 to 2.5 kg were obtained from Ranch Rabbits, Crowley Down, Sussex, England. The rabbits were fed an *ad libitum* Beta diet (Cooper Nutrition Products Ltd., Stepfield, Witham, Essex, England) and were housed in individual metal cages.

Immunity Studies

The effect of hyperthermia on the cell-mediated immunocompetence of normal rabbits was assessed by skin testing with DNCB² (Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England). The humoral response was studied by the ability of the animal to develop antibody against BSA (BDH Chemicals Ltd., Poole, Dorset, England).

The experimental protocol for monitoring host immunocompetence was as follows. Rabbits were sensitized to BSA (50 mg/kg body weight) on Day -7. On Day 0, the animals were sensitized to DNCB (20 mg/kg body weight) applied in acetone on the skin of the back. Seventeen days later the first challenge with DNCB (1 mg/kg body weight) was given on the ear. On Day 21 the BSA injection was repeated as above.

Cellular Response: DNCB Test. Initially, for standardization of the test, a group of normal rabbits was sensitized with DNCB at 2 or 20 mg/kg body weight. The DNCB dissolved in 0.1 ml acetone was spread with a glass rod within an area of approximately 10 sq cm of the shaved skin of the back, about 10 cm below the neck of the rabbit. The acetone was then evaporated with a hair dryer. Six or 17 days later, the rabbits were challenged on the ear with one-twentieth of the sensitizing DNCB dose (0.1 or 1 mg/kg body weight). Ear thickness (mm) was first measured at 6 different places along the ear with a Mitutoyo engineer's

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² The abbreviations used are: DNCB, 1-chloro-2,4-dinitrobenzene; BSA, bovine serum albumin; RF, radiofrequency; PHA, phytohemagglutinin.

micrometer (Mitutoyo Co., Tokyo, Japan), and one-half the required challenge dose of DNCB in 0.1 ml acetone was applied on either side of the ear (stock DNCB solutions; 1 or 10 mg/ml). Ear thickness was remeasured after 24, 48, 72, and 96 hr and expressed as the mean increase in thickness above base-line values.

Humoral Response: Anti-BSA Antibody. BSA was dissolved in 0.9% NaCl solution and mixed with an equal volume of complete Freund's adjuvant to give a concentration of 150 mg albumin per ml. Rabbits were given injections of this mixture in the right hind leg muscle at a dose of 50 mg albumin per kg body weight. The animals were bled weekly from the ear veins for 4 weeks. The secondary response to BSA was obtained by repeating the above injection 28 days after the primary injection. For the rabbits treated by hyperthermia, the second BSA injection was given 30 min before the heat treatment was commenced. The rabbits were bled weekly for the following 10 to 12 weeks, and the sera were stored at -20° until analysis.

Anti-BSA antibody titers in the sera samples were determined by passive hemagglutination testing. Albumin was coupled to sheep RBC in the presence of glutaraldehyde, and antibody titers were determined as described by Onkelinx *et al.* (27).

Heating Procedure

The normal hind leg muscle of the rabbit was treated by local hyperthermia on Day 21, or the total body temperature of the animal was elevated to 42° for 1 hr on Days 21, 22, and 23.

The rabbits were anesthetized with either Sagatal, 0.6 ml/kg i.v. (Sagatal veterinary solution containing 60 mg pentobarbitone sodium per ml; May & Baker Ltd., Dagenham, England), or with Hypnorm, 0.5 ml/kg i.m. (Hypnorm, Janssen Pharmaceutica; Fentanyl base, 0.2 mg/ml, and Fluanisone, 10 mg/ml, from Crown Chemical Co. Ltd., Lamberhurst, Kent, England). For local heating the left hind leg of the rabbit was shaved with clippers and rendered free of hair by a 5-min application of hair remover cream. The temperature-monitoring probes (thermistor and thermocouple) were inserted in the thigh muscles, and the leg was heated to the required temperature with watercuff or with a RF machine.

For watercuff heating the shaved leg of the rabbit was encased in a plastic cuff through which hot water at $50-52^{\circ}$ was circulated. The thigh temperature was maintained at 42° for 1 hr. This heating procedure was repeated after 24 and 48 hr. With the RF heating method, the rabbit thigh was placed between two 5-cm-diameter circular flat metal electrodes and heated with a RF generator operating at a crystal-controlled frequency of 13.56 MHz. The temperature of the thigh muscles was maintained at $47-50^{\circ}$ for 30 min as determined by 2 or more implanted sensors. Details of the heating techniques were as described previously for local hyperthermia of the VX2 carcinoma in the limb of rabbits (11, 12).

For total body hyperthermia the temperature of the anesthetized rabbit was elevated to 42° in a standard laboratory incubator maintained at $46-48^{\circ}$ with moist air. During the heating, rectal and intraabdominal temperatures were mea-

sured; the intraabdominal thermistor needle was introduced under the liver in a right paramedian position. Details of the temperature-measuring equipment are given in Ref. 10. The central body or "core" temperature of the rabbit was raised to 42° within 45 min, and this temperature was maintained for a further 1 hr. Total-body heating was repeated on each of 2 successive days at 24-hr intervals.

RESULTS

The untreated rabbits and those subjected to local RF heating at $47-50^{\circ}$ for 30 min gained body weight with time. Following local or total body hyperthermia at 42° for 1 hr on 3 occasions at 24-hr intervals, there was loss of body weight in all rabbits during the next 2 weeks. RF heating appeared less disturbing to the animals than did heating at 42° for a more prolonged period of time.

Skin Test: DNCB Response. Only rabbits that were sensitized with DNCB at 20 mg/kg body weight and challenged 17 days later with DNCB, 1 mg/kg body weight, gave a positive response to the challenge. The positive reaction became apparent after 6-8 hr, and the rabbit ear then exhibited gradually increasing erythema and induration with maximal response at 24 hr after the DNCB challenge. One hundred % of the animals responded to this regimen, and the test was therefore standardized to these conditions, with 1 mg/kg also being used for challenge doses subsequent to the first.

Chart 1 shows the effect of hyperthermia on the response to DNCB challenge. There was a wide spread in the responses of rabbits studied sequentially over a period of 100 days, and there was also considerable variation in the response of individual animals to successive challenges (Chart 1a). Following hyperthermia, in 9 of 10 rabbits the response to DNCB achieved and maintained a higher level of intensity than that resulting from initial challenge (100%), but the marked quantitative disparity in the response profiles remained (Chart 1, b to d).

The results for the 4 groups, a to d, in Chart 1 were compared by the Mann-Whitney *U* test (2-tailed). For all possible comparisons (control *versus* local heat at 42° , control *versus* local heat at $47-50^{\circ}$, control *versus* total-body heat, local heat at 42° *versus* local heat at $47-50^{\circ}$, local heat at 42° *versus* total-body heat, local heat at $47-50^{\circ}$ *versus* total-body heat) at intervals of 30, 50, 60, 80, 90, and 100 days, no significant differences were found ($p > 0.05$ all cases).

Antibody Responses to BSA. Chart 2 details the effect of local (Chart 2, b and c) and total-body (Chart 2d) hyperthermia on the secondary antibody response to BSA in normal rabbits. In the 6 control animals, there was wide variation in the ability to react to BSA, as indicated by the response patterns depicting the ratio of antibody titers that developed after 2 injections of the antigen (Chart 2a). The ratio also varied in each rabbit over a 12-week period. The distributions of the ratios of secondary to primary response to BSA in the 3 heated groups at each of the 10 weekly intervals after heating (Chart 2, b to d) were compared by the Mann-Whitney *U* test (2-tailed), and no significant differences were found ($p > 0.05$ all cases). The 3 heated groups (11 animals) were therefore combined and compared with the

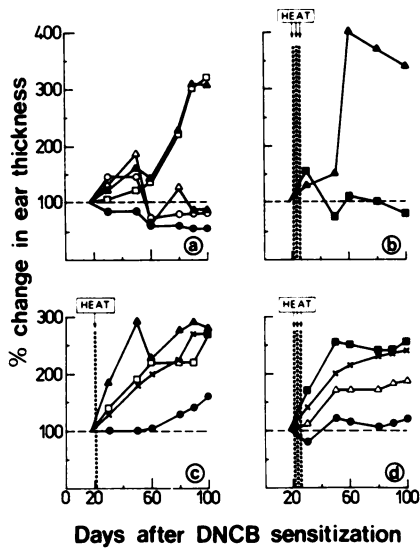


Chart 1. Effect of hyperthermia on the DNCB response of normal rabbits. Response to the first DNCB challenge at 17 days was taken as 100% value (---). Change in ear thickness on subsequent DNCB challenge was expressed as percentage of change compared to the initial value of 100% for each rabbit. After the first DNCB challenge, the rabbits were left untreated (a) or were subjected to hyperthermia 4 days later (b to d). The left hind leg was heated at 42° for 1 hr by watercuff on Days 21, 22, and 23 (b), or the leg muscle was heated on 1 occasion by RF heating at 47-50° for 30 min (c). Total-body temperature of rabbits was elevated to 42° for 1 hr on 3 successive days as in b in a laboratory incubator with moist air (d). Because of the variation encountered in the response of different rabbits to DNCB and to BSA, the results could not be expressed as a mean \pm S.D. at each time point. Each animal was used as its own control, therefore, and in this chart and Chart 2 each set of symbols refers to sequential determinations on an individual rabbit (15 animals in Chart 1, 17 animals in Chart 2).

control group of rabbits (Chart 2a) at each of the 10 weekly intervals. At the first week no significant difference was found, but at Weeks 2, 3, 4, and 5 the distributions of the heated group were significantly lower than those in the control group ($p < 0.02$; Mann-Whitney *U* test, 2-tailed). At subsequent weeks the distributions of the heated group were larger than those of the control group, but the difference was not statistically significant ($p > 0.05$).

DISCUSSION

It is usually assumed that elevated temperature has a beneficial effect on the immune system since infectious diseases are accompanied by leukocytosis, and the pyrexia is often followed by development of a high antibody titer against the specific causative agent. It must be borne in mind, however, that fever temperatures in excess of 41° are rare (16) and that the physiological response to externally applied heat differs radically from that obtaining in clinical fever, when the body thermostat continues to operate (3). In 1938 Neymann (26) reviewed the data accumulated during the heyday of the use of physically induced hyperpyrexia for the treatment of nonmalignant conditions such as syphilis, arthritis, and asthma. At body temperatures of 39.7-42.0° maintained for 6 to 10 hr, there was a fairly constant stimulation of the hemopoietic system confined almost exclusively to the polymorphonuclear leukocytes; there was a concomitant destruction of lymphocytes. These effects became more pronounced with increasing degrees of heating, but the blood picture returned to normal within 24

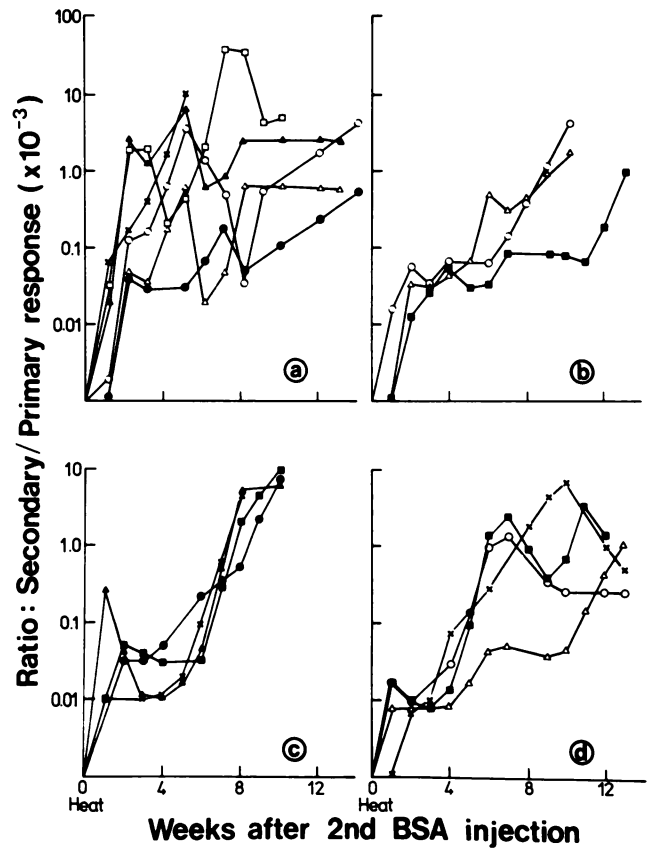


Chart 2. Effect of hyperthermia on the secondary response to BSA in normal rabbits. The rabbits were given BSA injections as described in the text. When the primary response to BSA had reached a plateau at 28 days after the first injection, the second BSA injection was given, and the rabbits were left untreated (a) or treated by hyperthermia (b to d) as described in Chart 1. The time interval between the second BSA injection and heat treatment was 30 min. The ability of the rabbit to respond to BSA has been expressed as a ratio, the ratio of antibody titer present at different times after the second injection to the maximum titer developed after the first injection.

48 hr after treatment. Serum levels of complement, opsonin, and agglutinins showed little change following hyperthermia. At a body temperature in the region of 42.5°, however, organic damage occurred, and the changes in blood chemistry and serology were no longer readily reversible (26).

In 1937, Doan *et al.* (15) described a postfebrile leukocytosis in normal rabbits heated above 41° for 50 min to 24 hr by total-body hyperthermia. As in the case of humans, the majority of cells constituting the leukocytosis were polymorphonuclear neutrophils and there was an accompanying lymphopenia. Histological examination of the lymph nodes and bone marrow revealed degeneration and fragmentation of lymphocytes. The destruction of lymphocytic elements was in direct proportion to the height and duration of the temperature; prolonged heating (>5 hr) led to hypoplasia of the nodes, and this was irreversible after 24 hr above 41° (15). More recently, Williams and Galt (35) reported changes indicative of a depressed immunocompetence in rats following whole-body heating at 41° for 2 to 3 hr. Degenerative histological changes were seen in many lymphoid tissues, there was a decrease in the concentration of circulating lymphocytes, and the ability of these cells to

transform after PHA stimulation was depressed for at least 3 days after heating (35). Fabricius *et al.* (17), on the other hand, found an increase in the ability of peripheral human blood lymphocytes to respond to PHA after total-body heating at 40° for 1 hr; there was also a marked increase in the number of colony-forming peripheral lymphocytes during the hyperthermia. The disparity between the results of Williams and Galt (35) and those of Fabricius *et al.* (17) may be attributable to the distinctive hosts involved or to differences in the degree of heating which, as indicated, can be critical in both humans (26) and animals (15). Lymphocyte reactivity to PHA, like other *in vitro* immunological assays, is being subjected to increasingly critical scrutiny as regards its significance *in vivo* (19, 23). There are insufficient data available on humans or rats to evaluate the extent to which the results of other workers thus far discussed can be interpreted in terms of altered host immunocompetence.

For the present study, host responses to DNCB and to BSA were selected as functional indices of cellular and humoral immunocompetence on the basis of results obtained in VX2 tumor-bearing animals. Host reactivity to both these agents correlated with stage of the disease and with tumor response to hyperthermia (32). The cellular response was not affected by either of the different degrees of heating applied to the normal muscle (Chart 1, *b* and *c*) or by fractionated total-body hyperthermia at the maximum temperature tolerated by the rabbit (Chart 1*d*). The anamnestic response to BSA, however, was significantly reduced for 3 weeks from the second week following hyperthermia, and the depression was independent of the degree and method of heating (Chart 2, *b* to *d*). From the evidence of other workers (15, 26), total-body heating probably produces its effect by a generalized damage to the lymphoid tissues; local heating may act on the lymphocytes circulating in the volume of treated tissue, as postulated for the mechanism of action of local irradiation on host immune response (33). In contrast to irradiation (33), the B-lymphocyte population appears to be more susceptible to heating than the T-cells. However, in view of the importance of T-cell-B-cell cooperation for B-lymphocyte function (5) and the possible participation of macrophages in this reaction (1), there may be an indirect component to the damaging effect of heat on B-lymphocytes, as well as a direct destructive action.

In the tumor-bearing rabbit any such deleterious effect of heat on the lymphocytes is amply counterbalanced by the marked immune response (both cellular and humoral) generated following tumor destruction by local hyperthermia (32). This marked response is abrogated by total-body heating (32). In the tumor-bearing rabbit, therefore, local and total-body heating have opposing effects on the immune system which, within the limitations of the current experimental protocol, were not apparent in studies on the normal host. Such results may be a reflection of the alterations in host biology and biochemistry that accompany the malignant state, as described almost a quarter of a century ago by Greenstein (20).

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