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Effect of Hyperthermia on the Immunocompetence of VX2 Tumor-bearing Rabbits[1]

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ABSTRACT

Nonspecific immunocompetence in VX2 tumor-bearing rabbits was assessed by skin response to dinitrochlorobenzene and development of antibody titer to bovine serum albumin. Skin reactivity to a 3 M KCl extract of the VX2 and antitumor antibody titer were used to monitor host immunocompetence against the tumor.

Rabbits bearing a 15- to 20-ml i.m. VX2 tumor in the hind limb were treated by local and total-body heating after established metastases were present in regional and aortic lymph nodes and lungs.

Following local heating of the VX2 (intratumor temperature, 47-50°/30 min) achieved by radiofrequency current at 13.56 MHz, there was tumor regression and host cure in 9 of 13 (70%) rabbits. Tumor regression was accompanied by a marked and sustained increase in skin reactivity to both tumor extract and dinitrochlorobenzene, and there was a 100-fold increase in serum levels of antitumor and anti-bovine serum albumin antibody. The animals are alive 2 years after heating and are immune to inoculation of 30×10^6 VX2 cells; 1×10^6 tumor cells led to a 100% death rate in 72 ± 7 (S.D.) days in untreated rabbits. In the 4 rabbits that did not respond to heating, unrestrained tumor growth was accompanied by a progressive decrease in host response to skin tests and antibody titers of 1:10 or less, findings similar to those in untreated tumor-bearing hosts.

Eight rabbits were subjected to total-body hyperthermia (42°/60 min on 3 successive days) 7 days after radiofrequency treatment. In 7 of the animals (88%), temporary restraint of tumor growth was followed by return to exponential increase in tumor volume and rapid death. This was accompanied by abrogation of the enhanced cellular and humoral immune responsiveness that followed local heating; in the single animal that was cured there was a sustained increase in skin response to dinitrochlorobenzene and tumor extract and in serum levels of antitumor and anti-bovine serum albumin antibody.

Necrotic material removed from regressing VX2 carcinomas up to 17 days after radiofrequency heating produced tumors on inoculation into rabbits.

It is concluded that the immune response generated following curative local heating of the VX2 carcinoma is involved in regression of the primary tumor as well as in the destruction of metastases. The abrogation of such enhanced immunocompetence by total-body heating supports the concept that in this animal tumor system whole-body hyperthermia is hazardous for the host.

INTRODUCTION

Recently, there has been a revival of interest in the treatment of cancer by hyperthermia (35, 48). Participation of the immune system in the response of tumors to heat has been proposed by several workers (5-7, 9-11, 16, 29-31, 41, 44, 45). Firm evidence in support of this postulate is lacking, however.

It has been suggested that local tumor heating and total-body hyperthermia may have different effects on the host's immune system (8). Dickson and Muckle compared total-body heating with local tumor heating in the rabbit VX2 system. In both cases the intratumor temperature was maintained at 42.6° for 1 hr. Local heating cured 50% of the animals, while with total-body heating only 7% were cured. It was postulated that local hyperthermia stimulated an antitumor immune response whereas total-body hyperthermia suppressed the immune system of VX2 tumor-bearing rabbits (8). In the Lewis lung carcinoma system in mice, Yerushalmi (49) found that metastases in the lungs were at an advanced stage following whole-body heating at 41.9°; on the other hand, there was a delay in the appearance of metastases in animals that received local hyperthermic treatment to the primary tumor in the muscles of the hind leg. Yerushalmi supported the proposal of Dickson and Muckle (8) that the elevation of whole-body temperature may depress the immune system of the host, leading to a more rapid growth of metastases (49).

In this paper we report data on the response of cellular and humoral components of the immune system following local and total-body hyperthermia in VX2 tumor-bearing rabbits. The strain of VX2 carcinoma used in this work was not sensitive to 42°, in contrast to the VX2 tumor previously studied in this laboratory (8, 30). However, a single local heat treatment applied at 47-50° for 30 min cured 70% of the treated rabbits (9, 10). This communication therefore describes the effect of curative local heating (47-50°/30 min) and total-body hyperthermia at 42° on the immunocompetence of VX2 tumor-bearing rabbits.

MATERIALS AND METHODS

The VX2 carcinoma arose as the result of spontaneous transformation of a virus-induced skin papilloma in a domestic rabbit some 40 years ago (20). Antiviral antibody has not been detected in the blood of tumor-bearing rabbits since 1945; the detailed history of the tumor is given in Ref. 42. For the present work VX2 tumor was disaggregated by

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an enzymatic method, and the cells were preserved in a liquid nitrogen storage tank at −196° as described previously (30, 38). When required, the ampuls containing VX2 cells were rapidly thawed in a water bath at 37°, and the cells were resuspended in Waymouth's medium (MB 752/1) containing 1% BSA[2] (Miles Laboratories Ltd., Stoke Poges, England; albumin, bovine, Fraction V). The tumor cells were washed 2 times with albumin-free Waymouth's medium and resuspended in the medium at 2×10^6 cells/ml. Male New Zealand White rabbits weighing 2.3 to 2.5 kg were inoculated with 1×10^6 viable tumor cells (as assessed by trypan blue dye exclusion) in 0.5 ml medium, 1 cm deep into the thigh muscle of the left hind limb.

VX2 tumor volumes were calculated from caliper measurements made in the anteroposterior, lateral, and vertical planes of the leg. Allowance was made for each animal's normal tissues on the basis of measurements made on the limb before inoculation of tumor cells (30).

Immune Response Assay

The effect of hyperthermia on the immunocompetence of tumor-bearing rabbits was assessed by specific and nonspecific reactions. Cell-mediated immune response directed against tumor antigens was measured by the skin response to tumor extract. Serum antitumor antibody levels were estimated with a passive hemagglutination test. The effect of thermotherapy on the ability of the rabbit to respond to foreign antigens was determined by skin testing with DNCB, as a measure of cellular response, and the antibody response to BSA.

Tumor-specific Immune Response

Cellular Response: Skin Testing with Tumor Extract. VX2 tumor protein was solubilized with 3 M KCl. The method used was that of Meltzer *et al.* (25) modified for tumor slices. VX2 slices (5 g) were washed with ice-cold 0.9% NaCl solution and homogenized in a Polytron homogenizer for 10 min (rheostat setting 7) in 50 ml 3 M KCl in PBS, pH 7.2, in the cold room. The cell homogenate was stirred for 20 hr at 4°, and the suspension was centrifuged at 40,000 × *g* for 1 hr. The supernatant was dialyzed against 20 volumes of deionized water for 1 hr and then against 20 volumes of PBS for 24 hr with 3 changes of PBS. The fine gelatinous precipitate that formed was separated from the extract by centrifugation at 40,000 × *g* for 30 min. The solubilized antigens in the supernatant were concentrated by ultrafiltration to approximately one-tenth the original volume, and the proteins were precipitated by adding an equal volume of 4 M $(NH_4)_2SO_4$. The precipitate was left in an ice bath for 1 hr and then centrifuged at 40,000 × *g* for 30 min. The pellet was redissolved in PBS (5 ml) and dialyzed against PBS for 4 hr with 2 further changes of PBS. Protein concentration was determined by the method of Lowry *et al.* (22), and the preparation was stored at −70°.

For skin testing, the 3 M KCl extract of the VX2 tumor (75 μg of protein) in 0.1 ml of 0.9% NaCl solution was injected i.d. in the rabbit ear. The skin reaction was measured at maximal size 24 hr after injection. The area of skin swelling and erythema was assessed by squaring the average radius of the reaction (24, 26).

Humoral Response: Antitumor Antibody. The antitumor antibody titers were determined by the sheep RBC hemagglutination test. The method of Onkelinx *et al.* (33) was used, the RBC being sensitized with 3 M KCl tumor extract in the presence of 0.2 ml of 2.5% 17 mM glutaraldehyde.

Nonspecific Immune Response

Cellular Response: Skin Testing with DNCB. The method used for sensitizing, challenging, and measuring the skin response to DNCB in tumor-bearing rabbits was as for normal rabbits (39).

Humoral Response: Anti-BSA Antibody. Primary and secondary antibody response to BSA in VX2 tumor-bearing rabbits was monitored as described for normal rabbits (39). The second BSA injection was given to the animals about 30 min before the tumor was treated with RF heating.

Hyperthermic Treatment of the VX2 Tumor

The VX2 tumor was treated on Day 21 after cell inoculation. At this time, the primary tumor volume was 15 to 20 ml, and metastases were present in the lymph nodes and lungs. The tumor-bearing rabbit was anesthetized with Sagatal or Hypnorm as described for normal rabbits (39).

For local tumor heating with RF currents, the tumor-bearing hind limb was shaved and 5-cm-diameter circular-paddle electrodes were applied on either side of the tumor. With an RF generator operating at 13.56 MHz, the tumor temperature was gradually increased to 47–50° over a period of 10 to 15 min. With large tumors treated by RF heating, there was often a spread of 2–3° in intratumor temperature as measured with multiple probes. The heating temperature was taken as the lowest temperature of the quoted range (9, 10), in this case 47°. The tumor was maintained at this temperature for a further 30 min. Intratumor temperatures during the treatment were monitored with thermistor and thermocouple needle probes connected to a 12-channel, direct-reading Light electric thermometer or Doric digital meter. Details of the RF generator circuitry, temperature-monitoring equipment, and heating procedure have been published elsewhere (9, 10).

Elevation of total-body temperature to 42° in tumor-bearing rabbits was achieved with a laboratory incubator as described for normal rabbits (39). The animals were treated at 42° on 3 occasions; each treatment lasted for 1 hr and the time interval between successive treatments was 24 hr.

Immunization Experiments

Attempts were made to render rabbits immune to the VX2 by inoculation of tumor slices heated *in vitro* at 48.5° for 30 min or tumor slices subjected to 15,000 rads irradiation (6 animals in each case). The treated tumor was finely diced with a scalpel blade, and 0.5 g material was mixed with 0.5 ml Freund's complete adjuvant was injected i.m. via a trocar into each hind leg of the rabbit. The injection was

[2] The abbreviations used are: BSA, bovine serum albumin; DNCB, 1-chloro-2,4-dinitrobenzene; PBS, phosphate-buffered saline (145 mM NaCl-2.5 mM $NaH_2PO_4 \cdot 2H_2O$-7.5 mM Na_2HPO_4); i.d., intradermally; RF, radiofrequency.

repeated twice at 10-day intervals. After a further 10 days the rabbits were given an i.m. challenge of 1×10^6 VX2 cells.

In 10 rabbits the VX2 (15 to 20 ml) was removed from its primary site in the leg muscle by wide surgical excision, and the animal returned to its cage after wound suture.

In a further 12 hosts necrotic tumor material was removed at 7 or 17 days after RF heating (47–50°/30 min), and 1 g material was inoculated i.m. into the rear limb of 12 normal animals.

Experimental Design

The schedule used in monitoring the response of the immune system in tumor-bearing rabbits to hyperthermia is detailed in Chart 1. For BSA and DNCB treatment, the protocol was as detailed for normal rabbits (39); the timing of these injections, of challenge with tumor extract, and of local and total-body heating was in relation to tumor cell inoculation (Day 0).

RESULTS

Local Tumor Heating

Chart 2 shows the effect of local RF heating on primary tumor volume in 7 of the 13 treated rabbits. From an initial transplant of 1×10^6 VX2 cells into the left hind leg muscles of the rabbit, the tumor became palpable by 3 weeks and increased in volume exponentially until about the 65th day after cell inoculation. Untreated rabbits died at 72 ± 7 (S.D.) days with metastases in the regional, iliac, and paraaortic lymph nodes and lungs. Heating was applied on Day 21 (tumor volume, 15 to 20 ml) at the beginning of the logarithmic phase of tumor growth. Intratumor temperature was maintained at 47–50° for 30 min. Nine of 13 rabbits treated were cured; the tumor regressed completely within 60 to 80 days after heating. Rabbits that were cured by heat treatment gained weight with time, whereas those that failed to respond to treatment did not. All animals that died had metastases in lungs and lymph nodes at autopsy. The 9 cured rabbits are alive, with no signs of tumor, 2 years after treatment, and they are immune to challenge i.m. with up to 30×10^6 VX2 cells. Heating the normal muscle of the right leg in tumor-bearing rabbits at 47–50° for 30 min did not affect the primary tumor or survival time of the host.

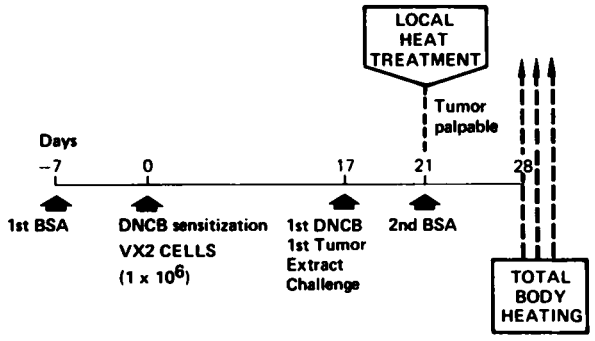

Chart 1. Experimental protocol for monitoring immunocompetence in VX2 tumor-bearing rabbits treated by hyperthermia. Rabbits were given the first BSA injection (50 mg/kg) in the right hind leg muscle on Day −7. On Day 0 the animals were sensitized to DNCB (20 mg/kg) and VX2 tumor cells (1×10^6) were injected into the left hind leg muscle. The first challenge with DNCB (1 mg/kg) and tumor extract (75 μg protein) was given on Day 17. On Day 21 albumin injection was repeated as above, and the i.m. tumor (volume, 15 to 20 ml) was treated by local RF heating (47–50° for 30 min). Some animals were further treated by total-body hyperthermia (42° for 1 hr) on Days 28, 29, and 30. Because the VX2 tumor was not heat sensitive at 42° and the host did not tolerate body temperatures in excess of 42° (8), the effects of local and total-body heating could not be directly compared. The effect of total-body hyperthermia on the immune response generated after curative local hyperthermia was therefore examined.

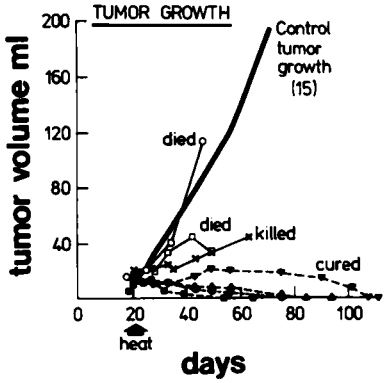

Chart 2. Changes in VX2 tumor volume following curative RF heating. Twenty-one days after tumor cell inoculation, the tumor (15 to 20 ml) was treated by a single RF heat treatment at 47–50° for 30 min. Tumor volumes for the growth curve were obtained from caliper measurements. The number in parentheses for the control tumor growth curve denotes the number of tumors (animals) left untreated. One rabbit repeatedly chewed the flesh off its left foot after heating and had to be killed at 64 days.

Effect of Local Hyperthermia on the Immune Response

Cellular Response: Skin Tests. Chart 3 illustrates the response of the VX2 tumor-bearing rabbits to skin tests with 3 M KCl extract of VX2 carcinoma. In the untreated rabbits the response changed from positive to negative or remained negative as the primary tumor increased in volume and the animals died. Following curative RF heating, tumor regression was accompanied by a marked increase in the skin response to challenge with tumor extract. In the unsuccessfully treated rabbits the response decreased with time as in untreated rabbits. Three of the 4 cured rabbits illustrated continued to react strongly against the tumor extract after the primary tumor had completely regressed. The response to a 3 M KCl extract of normal rabbit liver (75 μg protein), tested on the same ear of the hosts as the VX2 extract, was always negative.

Chart 4 shows the effect of curative local RF heating on the DNCB response in VX2 tumor-bearing rabbits. In untreated rabbits the response to each of the DNCB challenges subsequent to the first at 17 days (Chart 1) decreased as the primary tumor volume increased, and the animals died. In successfully treated rabbits the DNCB response increased as the tumor regressed, and the animals were cured. The animals that failed to respond to RF heating showed a decreasing response to DNCB challenge, comparable to that of untreated animals, as the primary tumor volume continued to increase with time.

Humoral Response: Antitumor Antibody. Chart 5 compares the antitumor antibody levels in untreated and in heat-treated tumor-bearing rabbits. In the untreated rabbits the antibody titers did not increase above 1/10 during the

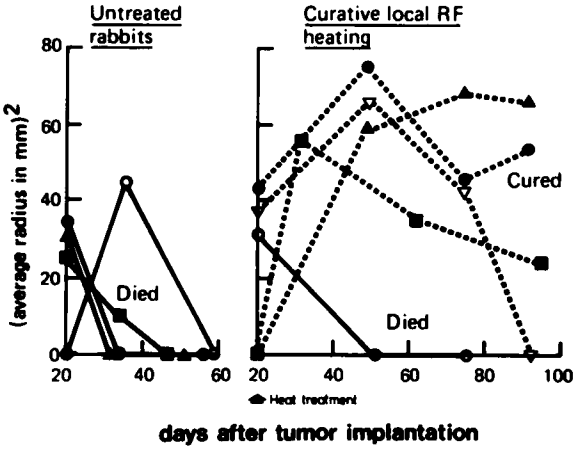

Chart 3. Effect of curative RF heating on the delayed cutaneous hypersensitivity reaction to tumor protein. The 3 M KCl extract of VX2 tumor (75 μg protein per 0.1 ml 0.9% NaCl solution) was injected i.d. in the rabbit ear. Twenty-four hr after the injection, a positive reaction was indicated by erythema and a bright red ring at the site of injection. The average radius (mm) of 3 diameters of the red ring was calculated, and the response to challenge was expressed as sq mm. Because of considerable quantitative differences in the response of the rabbits to the various immunological tests, the results could not be expressed in a meaningful manner as means ± S.D. Each rabbit acted as its own control, therefore, and each set of symbols in the charts refers to sequential results in an individual animal.

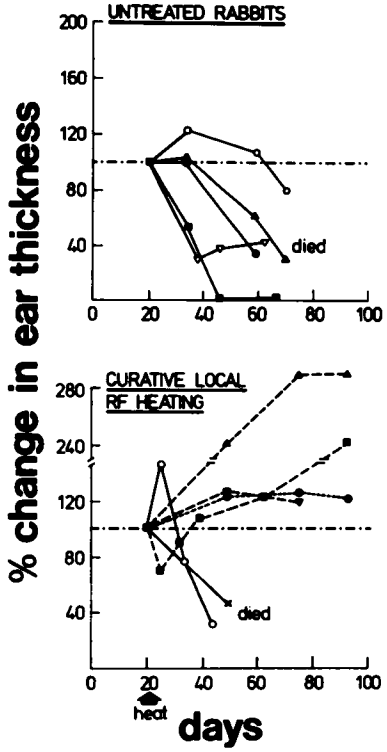

Chart 4. Effect of curative local RF heating on the DNCB response in VX2 tumor-bearing rabbits. The response to DNCB was measured as change in ear thickness in mm, 24 hr after DNCB was applied in acetone on the ear. The response to the first DNCB challenge was taken as 100% value. Tumor-bearing rabbits were left untreated or the primary tumor was treated by local RF heating. Intratumor temperature was maintained at 47-50° for 30 min. The response to subsequent DNCB challenges at 10- to 15-day intervals was compared with the first DNCB response, and the percentage of change obtained is plotted against the time in days after VX2 tumor cell inoculation in the rabbits.

entire period of tumor growth. Following RF heating the antitumor antibody titers increased in about 80% of the rabbits. Titers continued to increase in animals that responded to the treatment and were cured, and a plateau level for antibody titers in the region of 1/1000 was achieved in some cases. The increase in antitumor antibody titers was delayed in rabbits that showed a slower than usual regression of the primary tumor following heat treatment. In rabbits that failed to respond to the treatment, the antibody levels began to decrease within about 10 days after the heating.

Anti-BSA Antibody

Chart 6 depicts the antibody response to BSA in untreated and in RF-treated rabbits. In tumor-bearing rabbits as in normal rabbits (39), the antibody response to the second injection of BSA was always greater than the response to the first injection. The ability to respond to BSA was 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 in each animal (secondary response/primary response). Two weeks after the second BSA injection, the BSA antibody levels in untreated rabbits achieved a ratio of up to 100/1, but this decreased to less than 10/1 as the tumor progressed, and the animals died. The secondary/primary antibody ratio to BSA in untreated tumor-bearing rabbits usually achieved a lower peak than in normal rabbits (100/1 *versus* 1000/1), and in the normal rabbits the high antibody levels were maintained with time (39). Following heat treatment the secondary response to BSA in the cured rabbits increased markedly after the first 4 weeks as the tumor regressed. The pattern of response in rabbits that failed to respond to treatment was similar to that in untreated rabbits; *i.e.*, after an initial increase, the ratio decreased with progress of the disease until the animals died.

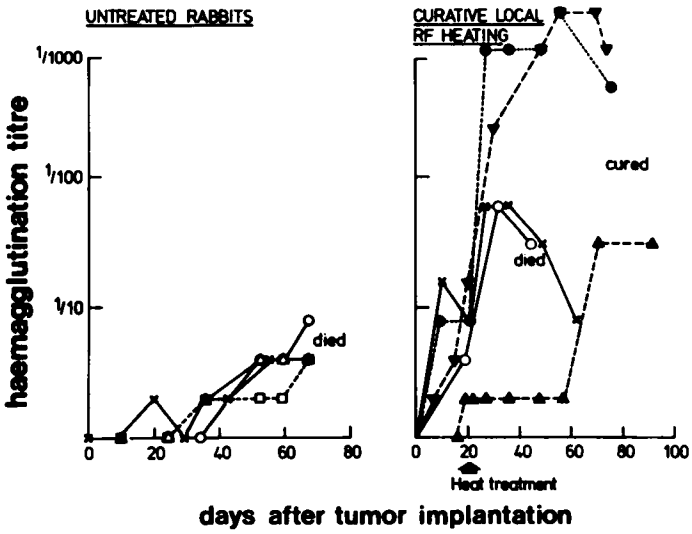

Chart 5. Effect of curative RF heating on antitumor antibody levels in VX2 tumor-bearing rabbits. The antitumor antibody titers in rabbit serum were determined by passive hemagglutination with the use of sheep RBC sensitized with 3 M KCl-solubilized VX2 tumor protein.

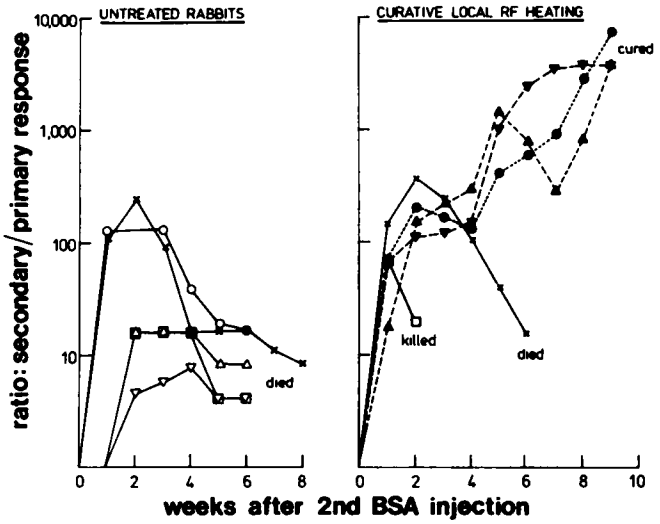

Chart 6. Effect of curative hyperthermia on the anamnestic response to BSA in VX2 tumor-bearing rabbits. The BSA injections were given as denoted in Chart 1. Anti-BSA antibody titers in serum from untreated or RF-treated rabbits were determined by sheep RBC hemagglutination. The results are expressed as the ratio of anti-BSA antibody titer present at weekly intervals after the second BSA injection to the maximum (plateau) titer developed after the first injection.

Local Tumor Heating followed by Total-Body Hyperthermia

Chart 7 details the changes in tumor volume observed in 6 of the 8 rabbits that were treated by local RF heating followed 7 days later with total-body hyperthermia. The protocol for immunological monitoring was as depicted in Chart 1, and local RF heating was performed at 21 days as for the animals illustrated in Chart 2. On Days 28, 29, and 30, these tumor-bearing rabbits were further treated by total-body hyperthermia (Chart 1). In 5 of 8 animals treated in this manner, tumor growth was restrained temporarily, followed by return to an exponential rate of growth and death of the rabbits within 30 days of starting treatment. In a sixth rabbit tumor growth remained in abeyance for almost 80 days after heating; there was then a rapid increase in tumor volume and the animal was killed because of its cachectic state. The primary tumor in 2 rabbits regressed completely; however, one of these rabbits died with metastases in the iliac lymph node and lungs. At 4 weeks after curative RF heating alone (Chart 2, Day 50), only 1 of 10 rabbits had a primary tumor with a volume greater than 40 ml, whereas 5 of the 8 rabbits treated by local followed by total-body hyperthermia had tumors ≥ 40 ml at 50 days.

Effect of Local plus Total-Body Hyperthermia on the Immune Response

The markedly decreased survival rate of rabbits treated by local RF heating plus total-body hyperthermia was accompanied by abrogation of the stimulation of cell-mediated and humoral immune response that followed local RF heating. This is illustrated in Chart 7 for the response to DNCB. Following the local RF heating, the response to DNCB increased, as already described. The response then decreased sharply following the application of total-body heating. The animals died with a negative skin reaction to tumor protein and low antitumor and anti-BSA titers, the results being comparable to the response in untreated tumor-bearing rabbits. In the 1 rabbit that was cured after this heating procedure, the DNCB response increased and remained elevated, as after curative local heating. The cured rabbit also gave a positive skin response to VX2 tumor extract, and the antitumor and anti-BSA antibody titers in the serum were similar to those that occurred in animals cured by RF heating alone (Charts 3, 5, and 6).

Table 1 compares the response of normal and heat-treated tumor-bearing rabbits to a range of DNCB challenge doses. At 2 weeks after RF heating, the response to the standard 1-mg/kg DNCB challenge dose was similar to that of animals in the untreated group ($p > 0.05$; Student's t test). The response in the rabbits given total-body heating after the local heating, however, was very significantly reduced ($p < 0.001$), in keeping with the abrogation of the augmented DNCB reaction after local heating (Chart 7). At 8 weeks after treatment, the response of rabbits given RF heating remained unaltered compared to the response at 2 weeks, while in rabbits surviving after local plus total-body hyperthermia, reactivity to DNCB had increased 2-fold to a level comparable to that in the control group.

At the DNCB challenge dose dilutions tested, very low responses were obtained in some rabbits, especially the local plus total-body heating group. The number of results is too small for statistical evaluation, but the only survivor

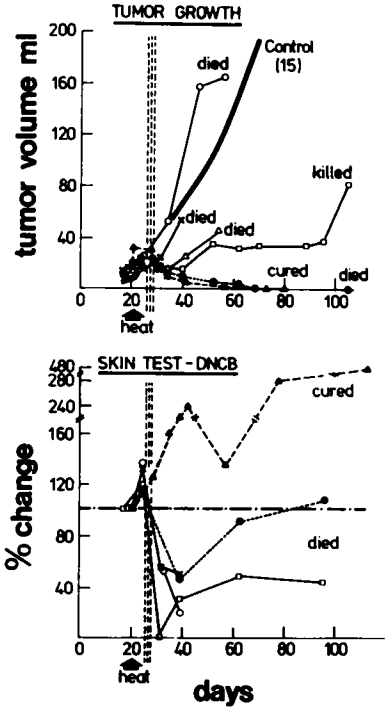

Chart 7. Effect of curative RF heating followed by total-body hyperthermia on tumor growth and DNCB response in rabbits. Rabbits were treated by RF heating on Day 21 as described in Chart 2, and the animals were further treated by total-body hyperthermia 7 days later (*vertical broken lines*). Body temperature of the rabbits was elevated to 42° for 1 hr on 3 consecutive days at 24-hr intervals, beginning on Day 28 after VX2 cell inoculation (Chart 1). Response to DNCB challenges was measured as described in Chart 4.

Table 1

Skin response of VX2 tumor-bearing rabbits after hyperthermia to different concentrations of DNCB

The rabbits were sensitized to DNCB at 20 mg/kg body weight. Following local or local plus total-body heat treatment, the animals were tested on the ear with the standard challenge dose of 1 mg DNCB per kg. The other ear was simultaneously tested with a dose of 0.5 or 0.25 mg/kg at 2 weeks after heating and with a dose of 0.125 mg/kg at 8 weeks after heating. The figures represent the mean change in ear thickness (cm × 10^3) measured at 24 hr after the DNCB challenge.

		Response 2 wk after local RF heating				Response 8 wk after local RF heating		
		DNCB test dose				DNCB test dose		
Rabbit	Treatment	1.0 mg/kg	0.5 mg/kg	0.25 mg/kg	Primary tumor volume (ml)	1.0 mg/kg	0.125 mg/kg	Primary tumor volume (ml)
Normal								
A202	None	58	36			39	22	
A203		81		30		70	30	
A204		60	34			63	17	
A205		86		15		74	33	
Tumor-bearing								
A216	Local RF heating	30	15		Regressed	31	8	Regressed
A218		50	17		Regressed	56	21	Regressed
A221		48		6	9	43	19	Regressed
A225		20		1	40	Died		
A227		74	25		29	Died		
Tumor-bearing								
A213	Local RF heating + total-body heating	27	11		3	55	9	Regressed
A219		16	4		16	25	3	31
A220		17	5		54	Died		
A223		31		10	9	59	3	Regressed
A229		39		15	53	Died		

in this group (A213) had a high level of response to DNCB at all doses comparable to that of rabbits cured by RF heating (A216, A218, A221).

Immunization Experiments

All animals inoculated with tumor that had been heated or irradiated *in vitro* or removed after curative RF heating produced tumors at the site of injection and died within 15 weeks. Following surgical excision of the primary VX2, all rabbits died in 3 to 6 weeks.

DISCUSSION

The immunocompetence of the untreated VX2 tumor-bearing rabbits and of the rabbits after hyperthermia, as assessed by skin tests with DNCB and tumor extract, antitumor antibody levels, and the anamnestic response to BSA, correlated well with survival of the animals. With increasing host tumor burden, the immunological responses became progressively less; after curative heating, the change in skin reactivity to antigen and the increasing titer of antibodies was marked enough to be used as an index of successful treatment. Several workers have reported impaired hypersensitivity reactions to DNCB and tumor extracts in patients with advanced cancer; in patients studied sequentially, those who failed to respond to DNCB or tumor extracts had, in general, a poor prognosis compared to patients who responded well (1, 14, 17, 21, 28). In cancer patients after surgery (13) or undergoing chemotherapy (18), prognosis was found to be related to the level of general immunocompetence and the level of specific antitumor immunity; the more vigorous the state of competence the better is the prognosis. Less information is available on animal tumor systems. Delayed cutaneous hypersensitivity reactions have been obtained with syngeneic animals rendered immune by tumor inoculation (26, 32) or surgical removal of the tumor (3, 47); few sequential studies have been reported for animals bearing progressively growing tumors.

In contrast to the correlation between the *in vivo* functional assays and clinical stage of disease, *in vitro* testing proved to be of little value in assessing host immunocompetence in the rabbits. The cytotoxicity of host rabbit lymphocytes and serum for cultured VX2 cells at various ratios of effector to target cell was tested by microcytotoxicity assay (36, 37), and the response of lymphocytes to PHA at a range of concentrations was also examined. No significant alteration occurred in the reactivity of host lymphocytes with advancement of disease or following cure of the animals.

Involvement of an immune response in the tumor regression that occurs after curative hyperthermia has been suspected since the work of W. B. Coley in the early years of this century. Coley obtained some remarkable cures of advanced cancer following inoculation of pyrogenic bacterial toxins. Some of the patients developed immunity to the toxins, and Coley surmised that perhaps the immune system was also involved in the tumor regression (see Ref. 31). In more recent years considerable circumstantial evidence has accumulated implicating an immune component in the host reaction to hyperthermia. This includes reports that in several different laboratory animals [rabbits (30), hamsters (16), rats (7, 11)] successful heat treatment of a tumor can

lead to regression of tumor at other anatomical sites [the so-called "abscopal" antitumor effect (16)] and to cure of the host. Analogous clinical findings are evident in patients treated by regional hyperthermic perfusion for tumors of the limb. In cases of multiple localization of tumors, destruction of the limb tumor resulted in disappearance of the others (29), and heat treatment of primary cancers has strikingly reduced the incidence of metastases in tumors notoriously prone to rapid spread to distant sites, e.g., sarcoma and melanoma (29, 41). *In vitro*, Ehrlich ascites cells exposed to 42.5° for 3 hr were more effective in immunizing Swiss mice against the tumor than are control tumor cells (27), and at 42° cell-specific antiserum showed increased binding to rat breast cancer cells and potentiated the destructive effect of the heat on the cells by a factor of 4 (19). These findings support the putative connection between hyperthermia and an immune reaction and focus attention on the cell membrane of the heated tumor cells as a possible link in the response.

Strauss treated cancer in animals and humans by electrocoagulation in a series of investigations spanning 50 years. Strauss believed that, following electrical heating of the tumors, the necrotic cancer cells were absorbed and acted as an antigen increasing the immunity of the tumor-bearing host with subsequent destruction of distant metastases (44, 45). In a series of 276 rabbits bearing bilaterally implanted Brown-Pearce carcinomas, electrocoagulation of the tumor in 1 testis was followed by regression of the contralateral testicular tumor and of abdominal secondaries if these were present. Conversely, heating of the metastatic tumor in the abdomen led to disappearance of tumors in the testes. Following electrocoagulation of the tumor, an inconsistent rise in antitumor antibody titers occurred. The titers increased to about 1/100 from 2 to 3 weeks after treatment and remained at this level for the following 3 to 4 weeks while the tumor was being resorbed. The antibody titers then decreased to zero during the next 3 to 4 months, despite the fact that all the cured rabbits were immune to challenge with Brown-Pearce carcinoma. The sera (0.2 ml undiluted) from immune rabbits caused lysis of Brown-Pearce tumor cells growing in Petri dishes within 3 days (44, 45).

Strauss further observed that, if the Brown-Pearce tumor was transplanted 7 days after electrocoagulation (7-day *in situ*-treated tumor), no growth occurred in the testis of normal rabbits although the injected material contained viable-looking cancer cells; the animals subsequently resisted repeated challenge with fresh carcinoma. The 7-day *in situ* material, when injected into tumor-bearing rabbits, cured the animals, which also became immune to challenge with Brown-Pearce carcinoma. When sera from these immune rabbits were inoculated into normal rabbits, the normal animals rejected tumor challenges. Furthermore, when the sera from immune rabbits were inoculated into tumor-bearing rabbits, the tumors regressed with cure of the animals (44, 45).

The results as described by Strauss are almost totally bereft of details. Tumor size, the time of treatment, the tumor temperature achieved during electrocoagulation, and the methodology and controls used for immune studies are not reported. The Brown-Pearce tumor has several controversial features (43). Spontaneous regression of the tumor is a frequent occurrence, and apparent recovery of the host may take place even after extensive metastases have developed. It has also been reported that the metastatic growths in rabbits appeared within 4 to 6 weeks during cold weather, while regression of the primary without metastases was the rule during hot weather (43). It is therefore difficult to evaluate the role of electrocoagulation and the part (if any) played by the immune system in the cure of tumor-bearing rabbits as reported by Strauss.

In the current work attempts to immunize normal rabbits with 7- and 17-day *in situ* RF-heated VX2 tumor resulted in tumor formation. The only method found to produce immunity in the rabbit to the VX2 was curative heating of the tumor in the host.

Stehlin *et al.* (40, 41) have reported that hyperthermic perfusion of tumor-bearing limbs in patients with melanoma increased the cytotoxic effect of the patient's lymphocytes and plasma against the melanoma cells *in vitro*. After application of a proximal tourniquet, the limb was perfused with heated blood to a temperature of 38.8–40°. The alkylating agent melphalan (L-phenylalanine mustard) was then added to the venous line, and perfusion was continued for up to 2.5 hr. The cytotoxic activity of plasma plus lymphocytes from patients against autologous melanoma cells increased to a peak level (80 to 85% inhibition) approximately 2 weeks after treatment, chiefly due to an increase in activity of the plasma, and then decreased to base-line level (10% inhibition) by 10 weeks after treatment.

The work of Stehlin *et al.* (40, 41) is difficult to assess, since no details of the preparation or purity of lymphocyte populations, the use of control target cells, what constituted tumor cell "inhibition," or what percentage of patients showed the response are reported. No patients were treated by hyperthermic perfusion alone; hence the part played by tumor heating at 40° in the observed increase in activity of lymphocytes and plasma is unknown. However, addition of heat to the melphalan perfusion has produced an increase in the 5-year survival rate for patients with Stage IIIA melanoma (in-transit metastasis without microscopic involvement of the regional lymph nodes) from 22 to 77%, which is claimed to be due to immunostimulation by the heated tumor (40, 41).

Szmigielski et al. (46) reported that local heating of the Guérin epithelioma in Wistar rats caused stimulation of immune reactions against the tumor. The tumor was treated by microwave heating at 43 ± 0.5°. The nonspecific immune reactions studied were antibody response to albumin, lymphocyte stimulation with mitogens, and serum lysozyme levels as a measure of macrophage activity. The tumor-specific reactions examined were cytotoxicity of spleen cells and peritoneal macrophages to cultured tumor cells as measured by ^{51}Cr release assay.

At 21 days after VX2 implantation in the leg (the time of local heating), the host rabbits have always been found to have metastases in the iliac and aortic lymph nodes and in the lungs (9). Stimulation of the immune system was detected 7 to 10 days after local heating (Charts 3, 5, and 7). Although 30% of the 8 rabbits given local heating as recorded in Chart 7 would not be expected to benefit from

the treatment, only 1 animal survived following local plus total-body heating; the others died with progressively enlarging primary tumors and metastases. Since the primary VX2 contained viable tumor cells until at least 17 days after local heating, as evidenced by transplantation of *in situ*-heated material, it is concluded that the immune response was involved in regression of the primary tumor after hyperthermia as well as in cure of the metastases. This conclusion is in accord with recent results of Marmor *et al.* (23) with the EMT6 sarcoma in mice. After curative RF heating at 43–44°, survival of cells from the treated tumor, as determined by plating efficiency, was 10 to 80%. These workers concluded that direct cell killing by the heat could not account for the tumor cures and that delayed mechanisms, either intratumor or involving host response, must play a major part in tumor eradication (23). In previous work reported from this laboratory, the effects of local and total-body hyperthermia were compared with the use of a strain of VX2 that was heat sensitive at 42° (8). For a similar degree of heating of the primary VX2 in the limb (42.6° for 60 min), the tumor regressed 3 times more rapidly after local compared to total-body heating ($p < 0.001$ for the difference in slope of the regression lines for tumor volume). Animal survival rate after total-body heating was 7 *versus* 50% for local heating, and it was suggested that depression of the immune response by total-body heating may have accounted for the difference in survival rate (8). In the light of the present results, the difference in the rate of volume decrease with the 2 methods of heating may represent the contribution of the immune response to tumor regression after curative local heating.

Gee *et al.* (15) recently reported that, in patients with advanced cancers, lymphocyte response to phytohemagglutinin was depressed during whole-body hyperthermia at 41.8° for up to 4 hr. The response subsequently recovered and increased to a peak 3 to 4 days after heat treatment, returning to the pretreatment value within 1 week. Antibody-dependent cytotoxicity against a human tumor cell line was depressed in both mononuclear cells and neutrophils obtained from cancer patients treated by total-body hyperthermia. Gee *et al.* concluded that whole-body hyperthermia treatment of cancer patients did not produce the long-term immunosuppression characteristic of radiotherapy and chemotherapy (15). DeHoratius *et al.* (4) recorded a significant decrease in C3 complement activity and antibody-dependent cell-mediated cytotoxicity in 3 cancer patients after total-body hyperthermia at 42° for 2 hr. All 3 patients, 2 with malignant melanoma and the other with adenocarcinoma of the stomach, had a temporary objective remission following treatment.

To date, treatment of cancer in humans by local hyperthermia (29, 41) has proved more effective than total-body heating (4, 12, 34). Because of the paucity of data available, however, it cannot be assessed whether host immunocompetence is implicated in this differential response in a way similar to that detailed for the rabbit VX2 system or whether the results in humans reflect the response to heat of particular tumor types (melanoma, sarcoma) in the limbs of relatively fit patients as opposed to the response of terminally ill patients with disseminated disease.

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