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Cell-mediated Immunity against Allogeneic Tumor after *in vitro* Depletion of Histocompatibility Reactive Cells[1] (38552)

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The "hot-pulse suicide" technique was first applied to lymphoid cells by Dutton and Mishell (1). The objective of this technique is to kill replicating cells *in vitro* by having them incorporate radioactive components such as thymidine, thereby eliminating the population proliferating in response to a specific antigen. Unidirectional mixed leukocyte cultures (MLC) "pulsed" with tritiated thymidine (^{3}H-thy) of high specific radioactivity, for 3–36 hr 1 day after the initiation of the cultures were markedly inhibited in reactivity towards the stimulating cell but retained the capacity to respond to a second unrelated allogenic stimulus (2, 3). Similarly, MLC responses to a particular set of H-LA antigens can be suppressed by treatment with the thymidine analog 5-bromodeoxyuridine (BUDR) followed by exposure to light in the visible or near visible region (4). This treatment does not significantly alter the capacity of the remaining lymphocytes to respond to cells of other H-LA specificity (4). Using the popliteal lymph node graft versus host (GVH) assay Rich *et al.* (5) demonstrated that Lewis rat spleen cells stimulated in mixed lymphocyte culture with F_1 hybrid spleen cells in the presence of BUDR and then treated with light did not induce local GVH response in F_1 recipients. Merino *et al.* (6) reported that Lewis rat spleen cells cultured with irradiated BN rat spleen cells and subjected to BUDR-light treatment are unable to elicit local GVH reaction under the kidney capsule of $LBNF_1$ rats but they could still elicit GVH reaction in $(L/BuF)F_1$ kidney. These findings suggested that GVH reactions which complicate bone marrow and lymphoid tissue transplantation could be controlled by selectively destroying the clones responding to histocompatibility antigens. Since the remaining cells retained their capacity to respond to other cellular antigens, it was possible to consider the application of the "hot pulse suicide" technique to sorting out cellular immune reactions to histocompatibility antigens from those directed against tumor specific antigens.

In the experiments presented in this paper we have attempted to test the capacity of a ^{3}H-thy "hot pulse" technique to remove lymphocytes reacting to histocompatibility antigens leaving those mediating tumor specific immunity.

Materials and Methods. Rats. ACI, Lewis, Brown Norway (BN) and Lewis × BN hybrids ($LBNF_1$) were obtained from Microbiological Associates (Walkersville, MD). Female animals were used at 3–4 mo of age.

Tumor. The polyoma virus-free fibrosarcoma designated Lewis PYD14 adapted to growth in tissue culture was maintained in RPMI-1640 media (Associated Biomedic Systems, Buffalo, NY) to which 20% fetal calf serum (FCS); Grand Island Biological Co., Grand Island, NY) 100 units penicillin/ml and 100 μg streptomycin/ml were added. Cells were injected into syngeneic Lewis rats and this transplanted line used for tumor immunization.

Immunization. Inbred BN rats were given an implant of Lewis PYD14 polyoma tumor measuring 1 mm^3. Then after rejection the rats were subsequently injected two to three times with 5×10^7 tumor cells in 0.5 ml media without FCS. Two weeks after the final injection, rats were sacrificed by exsanguination and spleen cells used in the experiment.

Preparation of cell suspensions. Spleen cell suspensions were prepared by gently pressing

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	$BN + LBNF_1$[a] culture started ↓	^{3}H-thy hot pulse added ↓	Washed and ACI* or $LBNF_1$* cells added ↓	↓	↓	^{14}C-thy added ↓	Cultures harvested ↓
Days	1	2	3	4	5	6	7

[a] Irradiated cells.

FIG. 1. Experimental procedure of the "Hot Pulse" MLC experiments.

the tissue through a 100-mesh stainless steel screen into cold medium RPMI-1640 with penicillin and streptomycin. Cells were washed two times and cell viability determined by trypan blue exclusion. Cells were counted in a standard hemocytometer chamber.

Mixed leukocyte cultures (MLC). All cultures were started (day 1) in glass culture tubes in a total volume of 1 ml RPMI-1640 + 10% inactivated FCS + antibiotics and 1 mM glutamine and incubated in an air-CO_2 atmosphere. Oneway MLC cultures were set up using equal numbers (5×10^6) of responding (BN) and stimulating ($LBNF_1$) cells. Stimulating cells were previously irradiated with 3000 rads. DNA synthesis was measured by adding 1 μCi/ml of ^{14}C-thymidine (NEN, Boston, MA) to each culture on day 6 and harvesting cultures 16 hr later. Cells were washed with cold phosphate buffered saline (pH 7.4), precipitated twice with cold 5% trichloracetic acid, and solubilized with Soluene 100 (Packard Instrument Company, Inc., Downers Grove, IL). Radioactivity was assayed in a liquid scintillation spectrometer with a counting solution containing PPO, POPOP and toluene. Window settings were selected such that counts from residual ^{3}H-thy were reduced to background levels avoiding corrections for ^{14}C readings. The results are presented as means of quadruplicate cultures.

Tritiated thymidine hot-pulse technique. Five microcurie ^{3}H-thy of high specific activity (50 C/m*M*) (ICN Pharmaceuticals, Inc., Irvine, CA) was added to the cultures after 36 hr of incubation. The pulse was terminated 16 hr later by spinning down the cells, washing three times and resuspending in fresh media. Then 5×10^5 stimulating ($LBNF_1$) or third party (ACI) cells were added. The procedure is outlined in Fig. 1.

Tumor neutralization test. Culture cells to be used in a tumor neutralization test following the hot pulse procedure were pooled on day 3. The test dose of 10^5 tumor cells was added to remaining cells from the original mixture of 10^7 BN cells from immune or normal animals and 10^7 irradiated stimulating cells. The total suspension was injected subcutaneously in the interscapular region of the $LBNF_1$ rats. Rats were palpated for tumor growth for 60 days.

TABLE I. THE EFFECT OF ^{3}H-THYMIDINE "HOT PULSE" ON THE MLC REACTION OF BN AND IRRADIATED (L/BN)F_1 OR ACI SPLEEN CELLS.

Cultured spleen cells[a]	Hot pulse[b]	Stimulating cells[c]	Stimulation index (SI)
BN + BN[d]	−	BN[d]	1.0
BN	−	F_1[d]	2.4
BN	+	F_1[d]	2.8
BN + F_1[d]	+	F_1[d]	1.3
BN	−	ACI[d]	3.7
BN	+	ACI[d]	4.2
BN + F_1[d]	+	ACI[d]	3.5

[a] 5×10^6 BN spleen cells were mixed with 5×10^6 irradiated stimulating cells on day 1 of the cultures where indicated.

[b] ^{3}H-Thymidine was added at 36 h of incubation and cultures washed 16 hr later.

[c] 5×10^6 irradiated responding cells were added to the cultures on day 3 of incubation.

[d] Irradiated cells.

Results. The mixed lymphocyte reactivity of BN spleen cells stimulated by irradiated allogenic and semiallogeneic cells was measured by incorporation of ^{14}C thymidine. The DNA synthesis in 5×10^6 BN spleen cells initially mixed on day 1 with 5×10^6 irradiated BN cells, then washed in the same way as "hot pulsed" cultures to which 5×10^6 irradiated BN cells were added on day 3 is considered as a background level (stimulation index 1.0). Stimulation indices (SI) are presented in Table I as the ratio of the cpm in

cultures containing 5×10^6 BN spleen cells and 5×10^6 stimulating cells to cpm in cultures of 5×10^6 BN spleen cells and 5×70^6 irradiated BN spleen cells. When irradiated cells from $LBNF_1$ rats were added as stimulating cells to the BN spleen cells cultured for 2 days and then washed, a stimulation of DNA synthesis in responding cells was observed (SI = 2.4). Exposure of BN spleen cells to ^{3}H-thy of high specific activity for 16 hr followed by consecutive washings did not alter the capacity of these cells to respond to allogeneic stimuli. However, when ^{3}H-thy of high specific activity was added to the cultures containing BN spleen cells plus irradiated $LBNF_1$ stimulating cells, the BN cells lost their capacity to respond to repeated stimulus with cells from the same origin as measured by subsequent ^{14}C-thymidine incorporation (SI = 1.3). The same procedure, however, did not significantly alter the response of BN spleen cells to a third party allogeneic stimulus. BN cells "hot pulsed" with stimulator $LBNF_1$ cells present respond equally well to stimulation with ACI spleen cells (SI = 3.5) as non- "hot pulsed" BN spleen cells (SI = 3.7). Thus, removal of one population of cells does not significantly alter the ability of the remaining BN spleen cells to respond to other stimuli.

We next tested the ability of spleen cells obtained from animals immunized against PYD14 tumor cells and depleted of histocompatibility reactive cells by hot pulsing to prevent the appearance of the tumor in $LBNF_1$ rats.

In preliminary experiments we were able to demonstrate that tumor cells obtained from Lewis rats produce growing tumors in $LBNF_1$ rats. Although the appearance of palpable tumor was slightly slower in $LBNF_1$ hybrids than in parental strain (Lewis), no significant differences in tumor producing dose was found (data not shown). When 10^5 tumor cells were injected in the interscapular region of the $LBNF_1$ rats, tumor regularly became palpable 12–19 days after the inoculation. No spontaneous regression of the palpable tumors were observed in F_1 hybrids. As seen in Table II, the inoculation of the same dose of tumor cells mixed with cultured spleen cells from nonimmunized BN or $LBNF_1$ rats (groups I, II and VII) produced palpable tumors in 14 out of 15 $LBNF_1$ rats. However, tumor appeared later in the animals injected with normal spleen cells and PYD-14 tumor cells than in the animals injected with tumor cells only. The maximal delay in the appearance of a tumor was 12 days, i.e., in some animals of group I and group II tumor was first palpable one month after the injection.

TABLE II. TUMOR CELL INHIBITING ACTIVITY OF IMMUNE SPLEEN CELLS AFTER SELECTIVE DESTRUCTION BY HOT PULSE ^{3}H-THYMIDINE DURING A BLASTOGENIC RESPONSE TO TRANSPLANTATION ANTIGENS.

Group no.	Cultured spleen cells[a]	Hot pulse[b]	Animals with palpable tumors[c] Animals inoculated
I	$BN_{(n)} + BN_{(n)}$	−	7/8
II	$BN_{(n)} + F^*_1$	−	4/4
III	$BN_{(i)} + BN_{(n)}$	−	0/8
IV	$BN_{(i)} + F^*_1$	−	0/4
V	$BN_{(i)} + F^*_1$	+	0/5
VI	$BN_{(i)} + BN^*_{(n)}$	+	0/3
VII	$F^*_1 + F^*_1$	+	3/3

[a] 10^7 responding (BN) cells from immune (i) or normal animal (n) and/or 10^7 irradiated (*) stimulating cells were cultured.

[b] ^{3}H-Thymidine was added at 36 hr of incubation and cultures washed 16 hr later.

[c] 10^5 polyoma tumor cells were added to each culture; the cells were resuspended in 0.5 ml and injected sc in interscapular region of $(L/BN)F_1$ rats. Rats were palpated for tumor growth for 60 days.

Spleen cells from BN rats immunized against Lewis PYD-14 were able to prevent tumor growth in $LBNF_1$ rats when administered in a ratio of 100:1. The same cells after 2 days of culture with irradiated BN or $LBNF_1$ spleen cells were still able to prevent the appearance of a tumor (groups III and IV). ^{3}H-thymidine of high specific activity when given in the absence of stimulator cells did not abolish the tumor-inhibiting activity of spleen cells from immune BN rats (group VI). Finally, a "hot pulse" of the ^{3}H-thy in the presence of stimulator cells previously shown to abolish MLC reactivity toward allogeneic cells did not interfere with the antitumor activity of the spleen cells from the immune animals (group V).

Discussion. Spleen cells from BN rats respond in one-way MLC with $LBNF_1$ irradiated spleen cells. Studying MLC reactivity of various rat strains and their hybrids Wilson (7) found BN and $LBNF_1$ cultures least reactive. Similarly, lymph node cells from Lewis rats respond better than lymph node cells from BN rats, when stimulated with $LBNF_1$ cells in MLC (8). This could explain the relatively low degree of stimulation in our MLC cultures (SI = 2.4 before and 2.8 after hot pulse). "Hot pulse" deletion of BN lymphocytes responding to $LBNF_1$ leukocyte antigens however did not significantly alter the ability of the remaining cells to respond to ACI histocompatibility antigens (SI = 3.7 before and 3.5 after hot pulse).

It had previously been shown that cells harvested from tissue cultures maintained their capacity to initiate GVH reactions (5) and that "Tolerance by Suicide" techniques abolished this GVH reactivity. Thus, suppression of GVH reactivity was apparently accomplished by selective killing of cells synthesizing DNA (5, 6). We have now demonstrated that a significant degree of immunity against tumor specific antigens is maintained in immune lymphocyte cultures deleted of immunologic reactivity to histocompatibility antigens by exposure to ^{3}H-thymidine "hot pulse" treatment.

This finding represents an encouraging step in the efforts to prepare a lymphoid cell preparation adequate for clinical use in adoptive immunotherapy of a neoplastic disease by immunizing across histocompatibility barriers.

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