Brown Fat: Its Possible Role in Immunosuppression During Hibernation[1] (34305)

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It has been shown that immunological capacity of hibernating animals may be drastically curtailed (1–4) and that this depression can be manifested in *in vitro* systems (5). The decrease in immunological competence, furthermore, was not due simply to depletion of the general lymphoid cell populations (5, 6), although a selective destruction of a minority cell type would not necessarily have been detected by the gross morphological criteria employed. Since brown fat is abundant in animals capable of hibernation, and since its hypertrophy during hibernation has been associated with several physiological changes, it seemed appropriate to test the possible effects of brown fat on antibody production *in vitro*.

Materials and Methods. Ground squirrels (*Citellus tridecimlineatus*) were captured in the vicinity of Madison, Wis. between April and September. Beginning in late July, animals were induced to hibernation at 4° (7). Squirrels hibernated from 5 to 15 days at a time, and usually resumed sleep after only a few hours of activity. Body temperature of hibernating animals was between 5–6° and heart beat and respiratory rate was greatly depressed. As control animals, only nonhibernating animals obtained prior to the end of October were used, since even nonhibernating animals will show many of the physiological changes associated with hibernation during the winter months (8).

Only brown fat from the axillary region was used. Blood samples were obtained by heart puncture. Brown fat tissue was homogenized in a manual Pyrex grinder using either Eagle's minimum essential medium, or standard tissue culture medium (see below). The homogenate was centrifuged at 17,300*g* for 20 min, and the top lipid pellicle was separated from the supernatant clear fluid. The pellicle was emulsified in medium and stored until use. Chloroform extracts were prepared by the method of Zöllner and Wolfarm (9). Golden hamster (*Mesocricetus auratus*) spleens were dissected in saline, cut to 1.5 mm^2 dimensions (2–3 $\times$ 10^6 cells) and placed either alone or in combination with other tissues into the filter well of a Millipore filter assembly (10). Culture fragments contained from 2 $\times$ 10^6 to 3 $\times$ 10^6 nucleated cells. Sheep red blood cells (SRBC) were added as a drop (0.01 ml) of a 1% suspension in saline. For transfilter experiments, brown fat tissue was held in place with a plasma clot (11). The culture medium consisted of Eagle's basal medium supplemented with 10% agamma horse serum (Hyland), 5% 9-day chick embryo extract, 1% glutamine and 50 μg/ml of penicillin, streptomycin, erythromycin, and mycostatin. Cultures were incubated at 37° in a water-saturated atmosphere of 5% CO_2–95% O_2. Medium was changed at 3-day intervals, and fresh SRBC were added each time. Detailed description of these methods has been given elsewhere (10).

Cultures were scored daily for agglutination and were considered to be positive when SRBC could not be dispersed readily upon agitation. To determine number of antibody-forming cells, cultures were teased in saline and the resultant suspension was diluted for counting and assay. The Cunningham and Szenberg slide method for enumerating direct

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TABLE I. Effect of Hibernating Squirrel Brown Fat on *in Vitro* Induction of Antisheep Red Blood Cell Agglutinins by Hamster Spleen Explants.

Conditions[a]	No. of expts.	No. of positive aggl./total	% Positive cultures
+ SRBC	11	94/100	94.0
+ SRBC			
NBF fragments in combination	6	38/56	67.9
NBF lipid	4	30/40	75.0
HBF fragments in combination	6	4/55	7.3
HBF lipid	9	5/90	5.6
— SRBC	3	0/29	0.0

[a] NBF = nonhibernating squirrel brown fat; and HBF = hibernating squirrel brown fat.

plaque-forming cells (PFC) was employed (12).

Hamster serum was tested for natural antibodies to SRBC, and none were detected by standard hemagglutination and hemolysin assay procedures. Background plaque-forming cells varied in the range of 1–10 plaque-forming cells/10^6 total nucleated spleen cells.

Results. As shown in Table I brown fat from hibernating squirrels when combined with hamster spleen explants caused a reduction in agglutinin production. Normal (nonhibernating) squirrel brown fat was not able to produce an equivalent effect, although a partial reduction was observed. In additional experiments it has been shown that (a) brown fat from hibernating animals exerts a similar effect when separated from the spleen explant by a Millipore filter barrier (0.45 μ); (b) white fat does not shown an immunosuppressive effect; (c) the duration of response in those cultures which do respond in the presence of hibernating brown fat is reduced from the standard 8–15 days to 2–6 days; (d) lipid extracts obtained from "nonhibernating" animals in the winter months may be inhibitory; (e) other tissues from hibernating animals do not show a strong suppressive effect; and (f) no immunosuppressive activity was found in the serum of hibernating animals. While initial experiments tested only the agglutinin response of cultures, later experiments were assayed for both agglutination and hemolysin response. As shown in Table II, the effect on PFC paralleled the observed effect on agglutination production; in other words, the number of detectable antibody-forming cells was severely reduced by hibernating brown fat.

When the lipid extract was tested (Table I and II), it contained demonstrable suppressive activity. This activity could be detected

TABLE II. Effect of Hibernating Squirrel Brown Fat on *in Vitro* Induction of Antisheep Red Blood Cell Hemolysin-Forming Cells by Hamster Spleen Explants.[a]

Conditions	No. of expts.	No. of cultures	No. of PFC/Culture (mean)
+ SRBC	5	43	1697
+ SRBC			
NBF fragments in combination	1	9	470
NBF lipid	1	10	1115
HBF fragments in combination	1	9	23
HBF lipid (1 pt/20)	5	50	6.6
— SRBC	3	29	3

[a] PFC = plaque-forming cells; NBF = nonhibernating squirrel brown fat; and HBF = hibernating squirrel brown fat.

TABLE III. Effect of Concentration of Hibernating Squirrel Brown Fat Lipid Layer on Anti-sheep Red Blood Cell Antibody Induction *in Vitro*.

Conditions	No. of expts.	No. of cultures	No. of positive agglutination	% Positive agglutination	No. of PFC/culture (mean)
+ SRBC	5	43	40[a]	93.0	1697[b]
+ HBF lipid					
1/20	5	50	0	0	6.6
1/50	4	39	19	48.7	384
1/100	1	9	5	55.5	430
1/200	2	19	13	68.4	457
1/500	1	10	8	80	930
1/1000	1	10	9	90	1070
+ NBF lipid					
1/20	1	10	7[a]	70	1115[b]
− SRBC	3	29	0[a]	0	3[b]

[a] Included in Table I.

[b] Included in Table II.

at a concentration of 1/200 (Table III), suggesting that the extract had considerable potency. The clear supernatant had no detectable effect.

Histologically, all spleen cultures appeared healthy and lymphoid at the periphery. A central necrotic area usually appeared in the center of the explant, but no differences between normal and brown fat-treated cultures was apparent from gross inspection.

In an attempt to rule out a general, nonspecific inhibitor effect on differentiation, the lipid fraction of hibernating squirrel brown fat was tested on two embryonic differentiating systems, the 13-day mouse embryonic thymus rudiment (13) and the 13-day mouse salivary gland rudiment (14). The same lipid preparation which caused an almost complete inhibition on hamster antibody formation *in vitro* did not cause any recognizable block to mouse thymus lymphoid differentiation or to mouse submandibular gland branching. Differentiating hamster tissues were not studied, however.

Discussion. While several effects of brown fat from hibernating animals have been reported in the literature (8, 15–18), the present findings of an effect of hibernating brown fat and its lipid component on the immune response appears to represent a new, yet unexplored function of brown fat not obviously related to its effect on arousal or hibernation. While the present demonstration is on an *in vitro* system, it seems reasonable to suggest that the observed depression of immune response *in vivo* might well be caused by brown fat as well. The facts that brown fat extracts are effective, that the effect can be transmitted readily across a filter barrier, and that the effect can be observed at low dilutions of the extract support this suggestion. Again, however, it should be emphasized that immunosuppressive activity has not been found in hibernating serum.

In more recent experiments we obtained an active preparation from chloroform extracts of hibernating brown fat. A more refined chemical characterization of the active principle in the brown fat extract is being attempted. It would be attractive to consider such material as a potential immunosuppressive agent; however, it should be pointed out that to date it has been impossible to obtain a suppressive effect on antibody formation of mouse spleen explants, and that the general applicability of these findings to nonhibernating species needs to be established.

Summary. Brown fat from hibernating ground squirrels can depress the primary immune response of hamster spleen fragments in tissue culture. The immunosuppressive activity is preserved in brown fat homogenates

and appears to be associated with the crude lipid layer.

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