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Acute starvation in mice reduces the number of T cells and suppresses the development of T-cell-mediated immunity

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SUMMARY

Experiments were performed to determine the effect of starvation on T-cell mediated host defences. In mice starved for 72 hr, the number of thymocytes fell by 98%, spleen cells by 82% and peripheral blood cells by 44%. By 7 days after the end of starvation, values had returned to within 50% of baseline. The percentage of L3T4 and Lyt-2 antigen-bearing cells fell in the thymus, but the percentage of Thy-1.2-positive cells did not change. Starvation decreased the percentage of lymphocytes in peripheral blood but increased the percentage of granulocytes. During starvation, the cellularity in thymuses, spleens and peripheral blood was preserved in adrenalectomized mice compared to normal or sham-adrenalectomized mice. Confirming previous results of ours, starved mice were resistant to i.v. challenge with *Listeria monocytogenes* immediately after starvation. However, when starved mice were immunized with a sublethal dose of *Listeria* immediately after starvation and challenged 3–4 weeks later, they were less resistant to *Listeria* than fed, immunized mice. Similarly, spleen cells of starved, immunized mice had a reduced capacity to transfer immunity passively to non-immune mice. Increasing the immunizing dose of *Listeria* in starved mice increased the level of immunity that developed. These data indicate that starved mice have a marked reduction in T-cell cellularity, possibly related to corticosteroid production during the stress of starvation. Although starved mice were relatively resistant to *Listeria* immediately after starvation, they had a reduced capacity to develop T-cell mediated immunity to *Listeria*. This deficiency could be partly overcome by increasing the immunizing dose of *Listeria*.

INTRODUCTION

Chronic malnutrition affects adversely a variety of host defence mechanisms, particularly cell-mediated immune responses, and is believed to contribute to the morbidity and mortality of infectious diseases. A voluminous literature has documented the suppressive effects of many types of malnutrition on immune processes in both experimental animals and humans (Gross & Newborne, 1980; Stinnet, 1983; Watson & McMurray, 1979). In contrast, acute short-term nutritional deprivation has been less intensively studied, and there are few data on its effect on the number and function of immune cells. In previous studies, we reported that mice starved for 48–72 hr were paradoxically resistant to *Listeria monocytogenes* infection (Wing & Young, 1980). Protection could not be transferred with serum or lymphocytes of starved animals. The increased resistance was eliminated by sublethal radiation, and was presumed to be mediated by a bone marrow-derived cell, possibly of the granulocyte–macrophage lineage (Wing & Barczynski, 1984; Wing, Barczynski & Boehmer, 1983a). Additional studies indicated that parameters of macrophage production, such as colony-stimulating factors and the number of macrophage progenitor cells, were not markedly altered by starvation (Wing, Barczynski & Sherbondy, 1986).

One aspect of host defences not studied extensively in our model was T-cell immunity. It is known that malnutrition adversely affects T-cell numbers and function (Stinnet, 1983). Since T cells are essential for the development of immune responses to *Listeria* in mice, we postulated that the induction of specific immune responses would be diminished in starved mice. In the present series of experiments, we measured the quantitative and qualitative changes in T-cell populations and the changes in functional capacities of T cells during and following starvation.

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MATERIALS AND METHODS

Mice

Female C57Bl/6 mice were purchased from either Jackson Laboratories, Bar Harbor, ME, or Charles River Breeding Laboratories Inc., Wilmington, MA. CFW outbred mice were

purchased from Charles River Breeding Laboratories. Mice were used between 8 and 16 weeks of age. They were housed in well ventilated rooms that were kept between 21° and 22° and maintained on an alternating 12-hr light/dark cycle. The normal diet consisted of rodent laboratory chow (Ralston Purina Co. no. 5001, St Louis, MO) that contained by weight not less than 23% protein and not more than 4·5% crude fat, 6·0% fibre, 8·0% ash and 2·5% minerals. For starvation, mice were placed in clean cages without food for 48–72 hr. Water was given *ad libitum*. Starvation for 72 hr results in a 25% weight loss but no mortality (Wing & Young, 1980).

Adrenalectomized mice

For some experiments, C57Bl/6 mice were either adrenalectomized or had sham operations at Charles River Breeding Laboratories. Animals were delivered 1 week after surgery and were used within 1–2 weeks. Adrenalectomized mice in these experiments were given 0·9% NaCl solution in place of water to drink. Corticosteroid replacement was unnecessary, and no significant mortality occurred during starvation. To insure that adrenalectomy was effective, corticosterone levels were determined (kindly performed by Dr William Sonntag, Wake Forest University, NC). Sham-operated mice had a mean concentration of 190 ± 99 ng/ml serum, whereas adrenalectomized mice had 13 ± 12 ng/ml ($P < 0{\cdot}001$).

Antibodies

The following rat monoclonal antibodies were obtained from Becton-Dickinson Immunocytometry (Mountain View, CA): anti-Thy-1.2 (30-H12), anti-L3T4 (GK 1.5), and anti-Lyt-2 (53-6.7). The second antibody used to label the rat IgG was fluorescein-labelled goat anti-rat IgG [Fc fragment, gamma-chain specific, $F(ab')_2$; Cooper Biomedical, Malvern, PA].

Quantification of thymic, splenic and blood cellularity

To determine the number of cells in thymuses and spleens, mice were killed by cervical dislocation, and organs removed and forced through a stainless steel mesh screen to form a single-cell suspension. Cell counts were determined with a haemacytometer on appropriate dilutions of cell suspensions. Viability was estimated by exclusion of trypan blue. For peripheral blood counts, mice were bled from the retro-orbital venous plexus. Cell numbers were determined with a Coulter counter. Slides of peripheral blood were stained with Wright's stain and differential counts were performed on 200 cells/slide.

Determination of T-lymphocyte surface membrane antigens

For phenotyping, thymus cells were suspended in RPMI-1640 (MA Bioproducts, Walkersville, MD) plus 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (medium). Spleen cells were first suspended in 0·9% ammonium chloride for 3 min to lyse erythrocytes. Additional medium was then added, and cells were centrifuged at 180 ***g*** for 10 min, resuspended in medium, and counted. Both types of cells were then suspended in phosphate-buffered saline (pH 7·2) plus 0·5% fetal calf serum (PBS + FCS). To label cells, dilutions of monoclonal antibody were added to 4×10^5 cells in 400 λ PBS + FCS in polypropylene tubes. The tubes were vortexed and incubated on ice for 30 min. The cells were washed, reconstituted in 200 λ of PBS + FCS, and then incubated with 50 λ of a 1:2000 dilution of fluorescein-labelled goat anti-rat IgG for 30 min at 4°. After being washed three times, the cells were resuspended in PBS and analysed for fluorescence on an Ortho Spectrum III flow cytometer (Ortho Diagnostic System, Westwood, MA).

L. monocytogenes

The isolate of *L. monocytogenes* and its preparation have been described previously (Wing & Kresefsky-Friedman, 1980). The 50% lethal dose for C57Bl/6 and CFW mice was approximately 1×10^5 organisms i.v. Mice were inoculated in a lateral tail vein with a suspension of bacteria in 0·9% NaCl. The number of viable *Listeria* in each inoculum was determined by subculturing dilutions on tryptic soy agar. Mice were immunized by inoculating 1×10^4 or 1×10^5 *Listeria* i.v. and were used 3–4 weeks later.

Preparation of spleen cells for passive transfer

To prepare spleen cells, immunized mice were killed by cervical dislocation. Spleens were removed by sterile technique and then forced through a wire mesh into medium. The cells were then placed on a nylon-wool column to enrich for T cells (Weinblatt, Vogel & Rosenstreich, 1981). This procedure increased the percentage of T cells from a mean (± 1 SD) of 40% (± 13) to 75% (± 13). The cells were washed once in medium, counted and brought to an appropriate concentration of viable cells—as determined by trypan blue exclusion.

Passive transfer of immunity

To passively transfer immunity, non-immune mice were injected i.v. with 1×10^7 viable spleen cells from immune mice. One hour later, the animals were challenged with 2×10^5 *Listeria* i.v. Host resistance was estimated by determining bacterial counts in livers and spleens at various times during infection by methods described previously (Wing & Kresefsky-Friedman, 1980).

Statistical analysis

Statistical analysis was performed using the Student's *t*-test, χ^2 analysis or one-way analysis of variance with grouping of means by Fisher's least-significant difference method (Snedecor & Cochran, 1980).

RESULTS

Effect of starvation on resistance to Listeria

Initial experiments were performed to confirm previous reports from this laboratory that starved mice were protected against listerial infection compared to fed mice (Wing & Barczynski, 1984; Wing *et al.*, 1983b; Wing & Young, 1980). Mice were fed or starved for 72 hr and then inoculated with 1×10^5 *Listeria*. The number of bacteria in the spleens of starved mice was less than that of fed mice at 48 and 96 hr ($P < 0{\cdot}05$) (Fig. 1). Similarly, the number of *Listeria* in livers of starved mice was less at 96 hr ($P < 0{\cdot}05$).

Effect of starvation on the cellularity in thymuses and spleens

The next studies were designed to determine the effect of acute starvation on the cellularity of the thymus and spleen. The number of cells in these organs was determined in C57Bl/6 mice that were starved for 72 hr and then refed. During the period of starvation, the number of cells in thymuses fell to approximately

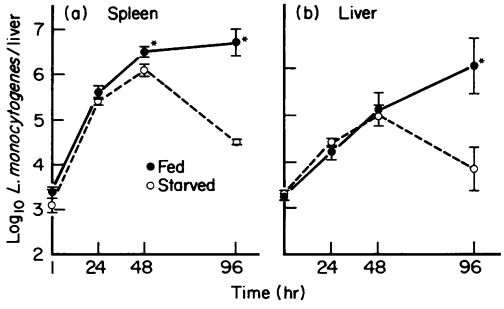

Figure 1. Number of *Listeria* in spleens (a) and livers (b) of starved and fed mice. C57Bl/6 mice were fed or starved for 72 hr and then inoculated with 10^5 *Listeria*. The number of bacteria per organ is expressed as the mean (log 10) ± 1 SEM for five mice. An asterisk signifies $P < 0{\cdot}05$ by Student's *t*-test.

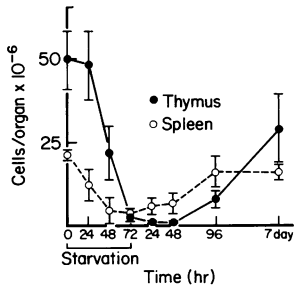

Figure 2. Cellularity of thymuses and spleens during and after starvation. C57Bl/6 mice were starved for 72 hr and then refed. Organs were removed and the number of cells determined by microscopy. In each experiment three mice were killed at the specified time period for determination of cellularity. Each point represents the mean ± 1 SEM of three separate experiments.

5% of baseline (Fig. 2). The numbers continued to fall during the first 2 days of refeeding to approximately 2% of baseline and then began to return towards normal. By 7 days, thymic cellularity had recovered to more than 50% of baseline values. Changes in the spleen were similar although not as marked. Numbers fell during the period of starvation to 18% of baseline at the end of starvation and thereafter returned towards normal.

Changes in lymphocyte cell membrane surface markers during starvation

The percentages of Thy-1.2, Lyt-2 and L3T4 antigen-bearing cells in thymuses and spleens were determined by fluorescent flow cytometry at various times during and after starvation. The percentage of Thy-1.2-positive cells in thymuses was constant during starvation (Fig. 3a). L3T4-positive cells fell slightly to a nadir by 2 days after starvation, whereas Lyt-2-positive cells fell to less than 50% of baseline at this time. Both percentages returned to baseline by 4 days after starvation. By contrast, the percentage of Thy1.2-positive cells in spleens rose slightly during starvation, but fell afterwards (Fig. 3b). The percentage of L3T4- and Lyt-2-positive cells did not change appreciably in spleens.

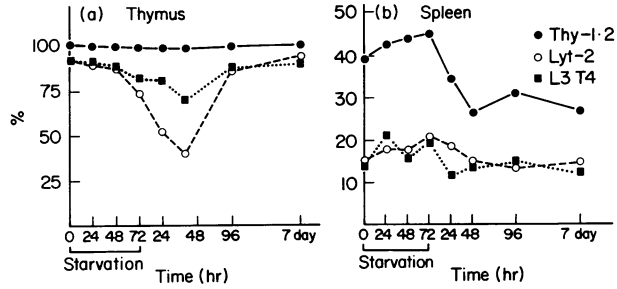

Figure 3. Cell membrane surface antigens on thymic (a) and splenic (b) cells. Cell membrane surface antigens were determined at various times during and after starvation by fluorescent flow cytometry. Results represent mean ± SEM of three separate experiments.

Effect of adrenalectomy on changes in cellularity during starvation

Since corticosteroids are known to be lympholytic in rodent species (Dougherty & White, 1945), experiments were performed to determine if the changes in cellularity noted during starvation were secondary to hormonal secretion by the adrenal glands. Normal, sham-operated or adrenalectomized mice were compared. Mice were either fed or starved for 48 hr and refed for 24 hr. At that time, the animals were bled to determine peripheral blood leucocyte counts and then killed to measure cellularity in thymuses and spleens. Starvation produced the reduction in thymus and spleen cellularity in normal and sham-operated mice observed previously (Table 1). In addition, peripheral blood counts were measured and were found to decrease significantly during starvation. Values obtained from sham-operated mice did not differ significantly from normal mice. The numbers of cells in thymuses, spleens, and peripheral blood of adrenalectomized fed mice were significantly greater than the numbers in organs of normal-fed or sham-operated fed mice. Starvation of adrenalectomized mice decreased the thymic cellularity and peripheral blood counts but not the splenic cellularity. However, the numbers in adrenalectomized starved mice were still greater in all conditions than normal or sham-operated mice.

Qualitative effect of starvation on peripheral blood leucocytes

Differential counts were performed on peripheral blood to determine whether starvation affected the percentage of blood lymphocytes. Starvation decreased the percentage of lymphocytes in starved and sham-operated mice, but not in adrenalectomized mice (Table 2). The percentage of monocytes did not change significantly during starvation. The percentage of granulocytes rose in normal and sham-operated mice but did not change in adrenalectomized mice.

Effect of starvation on the development of immunity to L. monocytogenes

Because starvation decreased the number of tissue T lymphocytes, we postulated that the capacity of mice to develop immunity to an intracellular pathogen would be impaired at the time of or immediately following starvation. Experiments were performed in which CFW mice were starved for 72 hours and then immunized with a sublethal dose of *L. monocytogenes*

Table 1. Cell number/organ in normal, sham-operated, and adrenalectomized mice

			Sham-operated		Adrenalectomized	
	Normal fed	Normal starved	Fed	Starved	Fed	Starved
Thymus $\times 10^7$	11·29 ±1·37	1·239±0·461*	12·12 ±2·521	1·264±0·383*	35·04 ±7·843†	24.40 ±3·853†
Spleen $\times 10^7$	8·362±2·131	1·990±0·280*	7·071±1·54	2·005±0·544*	12·683±2·921†	12·250±2·420†
Peripheral blood $\times 10^6$	8·672±1·648	4·484±1·877*	7·596±0·568	3·261±0·268*	16·101±3·72 †	9·190±2·014

Organ and peripheral blood counts were determined after mice were starved for 48 hr and refed for 24 hr. Values represent the mean ± 1 SD. From four to seven mice were used for each condition. Values for each organ were analysed by one-way analysis of variance (ANOVA) and grouped by Fisher's least significant difference method.

* Values significantly lower ($P<0·05$) than normal fed values.

† Values significantly greater ($P<0·05$) than normal fed values.

Table 2. Peripheral blood differential counts*

			Sham-operated		Adrenalectomized	
	Normal fed	Normal starved	Fed	Starved	Fed	Starved
Lymphocytes	83·6±4·7	71 ±9·4†	86 ±2·1	69·3±8·3†	89·0±4	86·3±7·1
Monocytes	3·2±2·4	3·5±1·0	2·2+0·4	2·5±1·7	1·2±0·8	1·8±1·0
Granulocytes	11·2±5·3	25·5±10·2‡	11·8±2·0	29·5±6·6‡	10·0±4·1	11·8±6·6

* Differential counts were performed on peripheral blood that was obtained in the experiment described in Table 1. Values are expressed as a mean percentage ± 1 SD.

† Indicates values significantly lower and ‡values higher ($P<0·05$) than normal fed values by ANOVA.

Table 3. Development of immunity in fed and starved mice*

Organ	Day 1	Day 2
Spleens		
Fed (10^4)	5·061±0·592	5·127±0·406
Starved (10^4)	6·220±0·474†	7·125±0·277†
Starved (10^5)	5·209±0·760	6·508±1·14†
Livers		
Fed (10^4)	6·007±0·561	6·705±0·366
Starved (10^4)	6·212±0·169	8·022±0·567†
Starved (10^5)	5·878±0·195	7·295±0·378

* Fed and starved (72 hr) CFW mice were immunized by injecting either 1×10^4 or 1×10^5 *L. monocytogenes*. Four weeks later, mice were challenged with 1×10^7 bacteria. Liver and spleen bacterial counts were determined at 1 and 2 days. Values for each period were analysed by one-way analysis of variance.

† Indicates values significantly greater ($P<0·05$) than normal fed values by ANOVA.

(1×10^4 or 1×10^5). Four weeks later, mice were challenged with a lethal dose (1×10^7) of bacteria. Spleen and liver counts were determined at various times during infection as measures of immunity (Table 3 shows results from a representative experiment). One day after challenge, organ counts were not significantly different between the groups. By 2 days, immune-fed mice had lower numbers of bacteria in spleens than starved mice immunized with an equivalent (1×10^4) or even a 10-fold dose (1×10^5) of *Listeria*. Similar results were noted in livers. By Day 4, 5/12 fed, 11/12 starved (1×10^4), and 8/12 starved (1×10^5) mice had died [$P<0·01$ by χ^2 analysis for fed versus starved (1×10^4)].

Attempts to passively transfer immunity with spleen cells from fed and starved immunized mice

Experiments were performed next to determine whether or not the defect in the development of immunity in starved mice resulted from impaired T-cell function, as defined by the capacity to transfer immunity passively. Column-purified spleen cells from C57Bl/6 unimmunized mice, immunized mice, and starved mice immunized with two doses (1×10^4 and 1×10^5) of *Listeria* were prepared 3 weeks after immunization. Purified spleen cells (1×10^7/mouse) were injected i.v. into unimmunized C57Bl/6 mice. The mice were then challenged with 2×10^5 *Listeria* and spleen and liver counts were determined (Table 4). Mice that received spleen cells from immunized fed mice had lower organ bacterial counts than mice that received spleen cells from either unimmunized or starved immunized mice. Differences were greatest 2 days after challenge with *Listeria*. Spleen cells from starved mice immunized with 1×10^5 *Listeria* protected better than spleen cells from starved mice immunized with 1×10^4. Similar results were noted in two other experiments.

Table 4. Capacity of fed and starved immunized mice to transfer protection to *L. monocytogenes**

Donor cell from	Organs	Day 1	Day 2	Day 4
Unimmunized mice	Spleen	5·756±0·255†	6·748±0·301†	7·287±0·064†
	Liver	5·128±0·239†	6·247±0·494†	7·600±0·260†
Immunized mice	Spleen	5·324±0·086	4·510±0·835	3·733±1·032
(1×10^4)	Liver	4·776±0·095	3·817±0·455	4·365±1·347
Starved immunized mice	Spleen	6·640±0·270†	5·982±0·498†	6·382±1·203†
(1×10^4)	Liver	4·944±0·059	5·196±0·363†	6·425±0·935†
Starved immunized mice	Spleen	6·437±0·068†	5·933±0·268†	4·486±1·116
(1×10^5)	Liver	4·904±0·093	5·239±0·567†	5·231±1·331

* Fed and starved (72 hr) C57Bl/6 mice were immunized with *L. monocytogenes*. Three weeks later, mice were killed and spleen cells prepared by passing over nylon-wool columns. Normal, unimmunized mice were injected with 10^7 cells from either normal unimmunized mice, fed immunized mice, or starved immunized mice. One hour later, mice were challenged with 2×10^5 *Listeria* i.v. Spleen and liver bacterial counts were determined at various times during infection.

† Indicates values significantly greater ($P < 0{\cdot}05$) than immunized mice by ANOVA.

DISCUSSION

In the experiments reported here, starvation had a striking lympholytic effect in mice. Thymic cellularity fell dramatically to less than 5% of baseline values after 72 hr of starvation. The percentage of Lyt-2 cells fell more than L3T4 cells in the thymus, but the decreases in both cell types were overshadowed by the overall drop in cellularity. Spleen and peripheral blood cellularity also decreased, but to a lesser extent. Other types of cells in these populations, such as monocytes, were presumably resistant to the lytic effect of starvation. Previous investigators have noted that a variety of types of malnutrition can result in reduced lymphoid tissue. Reduction in the size of lymphoid tissue has been documented in chronic protein-calorie malnutrition (Gross & Newborne, 1980; Watson & McMurray, 1979), chronic-calorie restriction (Weindruch & Suffin, 1980), as well as in acute starvation (Frank, Kumagai & Dougherty, 1953; White & Dougherty, 1947; Wing & Young, 1980). In severe chronic malnutrition, these changes have been associated with reduced function of cell-mediated immunity and increased susceptibility to certain types of infection.

Our studies on the mechanism of lympholysis suggest that the adrenal glands help to regulate lymphocyte cellularity. Fed adrenalectomized mice had greater numbers of cells in thymus, spleen, and peripheral blood than normal or sham-operated mice, suggesting that the number of lymphocytes in these tissues is under continuous suppressive influences from the adrenal gland. In addition, the lympholytic effect of starvation was mostly eliminated in adrenalectomized mice, although there was a slight reduction in thymic cellularity and peripheral blood counts. One conclusion to draw from these findings is that corticosteroid secretion increases during the stress of starvation, and that this results in marked lympholysis. In support of this possibility is the known lympholytic effect of corticosteroids in mice (Dougherty & White, 1945). This includes corticosterone, the major corticosteroid produced in mice (Spackman & Riley, 1978). It is also known that malnutrition is associated with increased secretion of corticosterone (Stinet, 1983; Watson, Chein & Chung, 1983; Winick & Nobel, 1966). Our results with adrenalectomized mice are consistent with previous data showing that adrenalectomy tended to reverse the lympholytic effects of food deprivation (Frank *et al.*, 1953; White & Dougherty, 1947). Although the major lympholytic mechanism appears to reside in the adrenal glands, other as yet unknown factors may contribute to the decrease in cellularity in thymic and peripheral blood cellularity.

Data on lymphocyte function during acute starvation is limited to a few studies using human subjects. In one of these, healthy volunteers were fasted for 3 days. Mild reductions in lymphocyte proliferation to phytohaemagglutinin M (PHA) and pokeweed mitogen (PWM), but not concanavalin A (Con A) were noted (Neuvonen & Salo, 1984). When subjects were fasted for 10 days, similar findings were noted: depression in lymphocyte responses to PHA and PPD but not to Con A (Palmblad *et al.*, 1977). No changes were observed in the number of circulating lymphocytes or monocytes. We also reported changes in certain parameters of immunity in 15 obese subjects undergoing a 14-day fast (Wing *et al.*, 1983b). Lymphocyte blastogenic responses to PHA, but not to PWM, were significantly decreased. The number of circulating lymphocytes was slightly, but not significantly, decreased.

In this study we next asked whether the decrease in the number of T lymphocytes affected the development of cell-mediated host defences against intracellular pathogens. We reported earlier that delayed hypersensitivity responses to *Listeria* antigen were decreased in starved mice (Wing & Young, 1980). In the present experiments mice were starved, immunized with *Listeria* and then challenged 4 weeks later with a lethal dose of *Listeria*. Fed immunized mice manifested a greater degree of resistance than starved mice immunized with the same dose of *Listeria*. One explanation for this reduced immunity is that the starved mice were exposed to less antigen because of their increased resistance immediately after starvation. To equalize antigenic doses, a second group of starved mice were immunized with 10 times the usual immunizing dose. Immunity in these mice was greater than starved mice immunized with the lower

dose, but not as great as fed mice. These experiments indicate that the impaired development of immunity in mice immediately following starvation can be partly reversed by increasing the dose of antigen.

To determine whether the defect in immunity was related to T cells, experiments were performed in which immunity was passively transferred with spleen cells. These cells were purified and enriched for T cells by passage over nylon-wool columns. Column-purified spleen cells from starved, immunized mice had a reduced capacity to transfer immunity compared to fed immunized mice, confirming that T-cell immunity was defective. The reduced immunity was due in part to decreased exposure to antigen. When starved mice were immunized with the higher dose of *Listeria,* they developed specific immunity that approached that of fed mice. The apparent defect in T-cell function, therefore, could be partially overcome by increasing the immunizing concentration of antigen.

Taken together, our studies indicate that the stress of acute starvation causes marked lympholysis, probably from increased secretion of corticosterone. As a result, starved mice have an impaired capacity to develop T-cell immune responses to intracellular pathogens.

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