

# Hyperthermia and the Generation and Activity of Murine Influenza-Immune Cytotoxic T Cells In Vitro

ARNO MÜLLBACHER

*Department of Microbiology, John Curtin School of Medical Research, Australian National University, Canberra A.C.T. 2601, Australia*

Received 4 June 1984/Accepted 28 August 1984

**The rate of generation of murine secondary influenza virus-immune cytotoxic T cells in vitro is enhanced under limiting dilution conditions at hyperthermal temperatures (39 versus 37°C). Increased mean values of cytotoxic activity were observed in the presence as well as absence of exogenous helper factors. Elevated cytotoxic activity at 39°C was observed after day 3 to day 5 of culture. The number of autoreactive cytotoxic cells observed was not greater at 39°C than at 37°C. Elevated temperature did not influence target cell lysis or release of isotopes from killed target cells. The results are discussed with a view to the role of fever in augmenting the cellular immune response responsible for the host defense against primary viral infection.**

The molecular basis for fever in humans and animals is well understood (6). Interleukin I, the predominant inducer of leukocyte pyrogen, the agent responsible for the induction of the febrile condition, is also an important factor in the induction of the immune response. Interleukin I is thought to be involved in the activation of T cells to produce lymphokines (8).

There still exists, however, considerable controversy in regard to the beneficial or deleterious effects of the accompanying hyperthermia in a febrile response to host defense mechanisms in general and in immune responses in particular (11, 12, 18).

A number of studies on primary in vitro immune responses in rodents under hyperthermal conditions demonstrated enhancement of both T-cell-dependent and -independent antibody production (10, 20). One group (10) also demonstrated that the augmenting effect occurred early during induction of T helper cells, and little evidence for a direct effect on B cells has been obtained. There is evidence in the literature that hyperthermia enhances the proliferative response of mitogen-stimulated lymphocytes (3, 19), amplifies macrophage-mediated cytotoxicity but not macrophage-mediated bactericidal effects (1, 19), and increases antibody-complement cytotoxicity (22). This contrasts, however, with natural killer cell activity, which has been reported to decrease during hyperthermal conditions (4, 17).

On the other hand, little is known of the effect of elevated temperatures on the generation of virus-immune cytotoxic T cells (Tc cells). Tc cells are of first importance in recovery from certain primary viral and bacterial infections (5) and have also been shown to be involved in recovery by mice from lethal influenza virus infection (14). The induction of influenza-immune Tc cells requires at least two T cell subpopulations: Tc cell precursors and a T helper cell population responsible for the augmentation of the response (2). T helper cells can be replaced, in in vitro cultures, by concanavalin A-activated spleen cell supernatants (CoS) (23).

It was therefore of interest to investigate the effect of hyperthermia on the generation and activity of influenza-immune Tc cells in bulk and limiting dilution conditions and in the presence or absence of exogenous help.

## MATERIALS AND METHODS

**Animals.** Female BALB/c mice were bred at the Animal Breeding Establishment of the John Curtin School of Medical Research and used at >8 weeks of age.

**Virus and immunization.** A/WSN influenza virus (H1N1) was grown in embryonated eggs and titrated for hemagglutination as described previously (7). The virus used throughout the experiments had a titer of  $1.024 \times 10^3$  hemagglutinin units per ml. Mice were immunized with 0.2 ml of a 1:5 dilution of virus stock in normal saline intravenously and used 2 to 8 weeks postimmunization.

**Preparation of CoS.** Mouse CoS was prepared as described by Woolnough and Lafferty (23).

**Target cells.** The tumor cell line P815 was grown in Eagle minimal essential medium F15 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% fetal calf serum and was labeled with  $^{51}\text{Cr}$  and infected as described in detail previously (2).

**Generation of secondary influenza-immune Tc cells (bulk cultures).** Published methods were used for the generation of influenza-immune Tc cells (16). In brief,  $10^7$  responder spleen cells from A/WSN influenza-primed mice were erythrocyte depleted by hypotonic shock treatment and cocultured with  $5 \times 10^6$  A/WSN-infected normal spleen stimulator cells in F15 supplemented with 5% fetal calf serum plus  $10^{-4}$  M 2-mercaptoethanol for various times in a Forma scientific gas incubator (Marietta, Ohio) in a humidified 5%  $\text{CO}_2$  atmosphere at temperatures of  $37 \pm 0.2^\circ\text{C}$  or  $39 \pm 0.2^\circ\text{C}$ . The stimulator cells were infected by incubating  $8 \times 10^7$  spleen cells in 1 ml of F15 with 0.25 ml of virus for 1 h. Cells were washed and pelleted twice and irradiated with 2,000 rad from a  $^{60}\text{Co}$  source.

**Microcultures and limiting dilution cultures.** Responder and stimulator cells were prepared as described above for bulk cultures. Various numbers of responder cells were distributed in 0.1-ml portions in 96-well microtiter plates (catalog no. 76-013-05, Linbro, Flow Laboratories, McLean, Va.) and cocultured with  $10^5$  stimulator cells in a final volume of 0.2 ml. Culture conditions were as for bulk cultures. In certain instances, as indicated below, culture

TABLE 1. Kinetics of generation of influenza Tc cells at 37 and 39°C

Day of harvest	Temp (°C)	% Recovery of culture <sup>a</sup>	% Specific lysis of <sup>51</sup> Cr-labeled P815 targets <sup>b</sup>	
			Uninfected	A/WSN-infected
3	37	100	4.1 (1.1)	49.6 (4.2)
	39	90	2.0 (0.2)	55.7 (1.4)
4	37	72	2.2 (1.0)	64.9 (2.5)
	39	42	1.8 (0.5)	76.3 (1.0)
5	37	66	1.4 (0.3)	77.9 (1.9)
	39	26	1.8 (0.7)	63.4 (4.5)
6	37	52	1.4 (0.8)	68.1 (1.0)
	39	26	0.3 (0.5)	41.2 (2.1)
7	37	30	2.9 (1.2)	40.2 (1.5)
	39	28	-0.8 (0.9)	27.2 (3.6)

<sup>a</sup> Yield of viable cells harvested after 3 to 7 days in culture is expressed as a percentage of the number of viable responder cells initially set up.

<sup>b</sup> Mean percent specific <sup>51</sup>Cr release from targets over 8 h. Spontaneous release for uninfected and for infected targets was never greater than 15%. Means of triplicates are given with the standard error of the mean in parentheses. Effectors were titrated in threefold dilution steps from 10 to 0.3% of initial responder cells set up in culture, thus incorporating both yield and cytotoxic potency per harvested cell in the evaluation of Tc cell responses. Values given here are for 1.1% of culture and are below plateau levels of lysis. Actual killer/target ratios can be calculated as follows. Responder cells were set up at 10<sup>7</sup> cells per culture in 5 ml. For day 4, the yield at 39°C was 42%, i.e., 4.2 × 10<sup>6</sup> cells. Of these, 1.1% (i.e., 4.7 × 10<sup>4</sup> cells) were added to each assay containing 2 × 10<sup>4</sup> target cells, at a killer/target cell ratio of 2.35:1.

medium was supplemented with 3% (vol/vol) 10-fold-concentrated CoS.

**Cytotoxic assays.** The cytotoxic assays for bulk cultures have been described by Müllbacher and Blanden (16). For microcultures and limiting dilution cultures, the trays were spun for 1 min at 1,000 rpm, the supernatant was thrown off, and the cell pellets were suspended in 0.1 ml of F15 plus 5% fetal calf serum. Target cells, either infected or uninfected, were added in 0.1-ml portions at 10<sup>5</sup> cells per ml and incubated at 37°C for 8 h. Samples (0.1 ml) of individual well supernatants were removed, and their radioactivities were measured in a gamma counter. Spontaneous release was estimated by culturing target cells in the absence of effectors. Total releasable <sup>51</sup>Cr was estimated by lysing target cells with 1% Triton-X solution. Percent specific lysis was calculated by the following formula: Percent specific lysis = [(experimental release - medium release)/(maximum release - medium release)] × 100.

RESULTS

**Kinetics of generation of influenza-immune Tc cells at elevated temperatures in microcultures and bulk cultures.** Memory spleen cells from single BALB/c animals were cultured at 10<sup>4</sup> cells per well plus 10<sup>5</sup> infected, irradiated stimulator cells in microculture wells or at 10<sup>7</sup> cells per well plus 5 × 10<sup>6</sup> infected, irradiated stimulator cells for bulk cultures. Microcultures were supplemented with CoS to a final concentration of 3%. Duplicate sets of plates were incubated at either 37 or 39°C. Individual wells from bulk cultures and plates from microcultures were assayed for Tc cell activity on infected and uninfected P815 targets. Half the wells (48) in the microtiter plate received 10<sup>4</sup> <sup>51</sup>Cr-labeled, uninfected cells and the other half received A/WSN-infected P815 target cells. Effector cells from bulk cultures were titrated and assayed as described above.

Table 1 illustrates results from bulk cultures in one representative experiment. Slightly higher values of lytic activity were observed at days 3 and 4 with effectors from 39°C cultures. On days 5, 6, and 7, cultures maintained at 37°C gave slightly higher values of target cell lysis. However, cell recovery was appreciably lower at 39°C than at 37°C. On day 7, a marked decline in activity was observed at both temperatures, probably reflecting exhaustion of nutrients. This decline was already apparent in the day 6 39°C culture.

A markedly different picture emerges from the microcultures (Fig. 1). At days 3, 4, and 5, plates incubated at 39°C

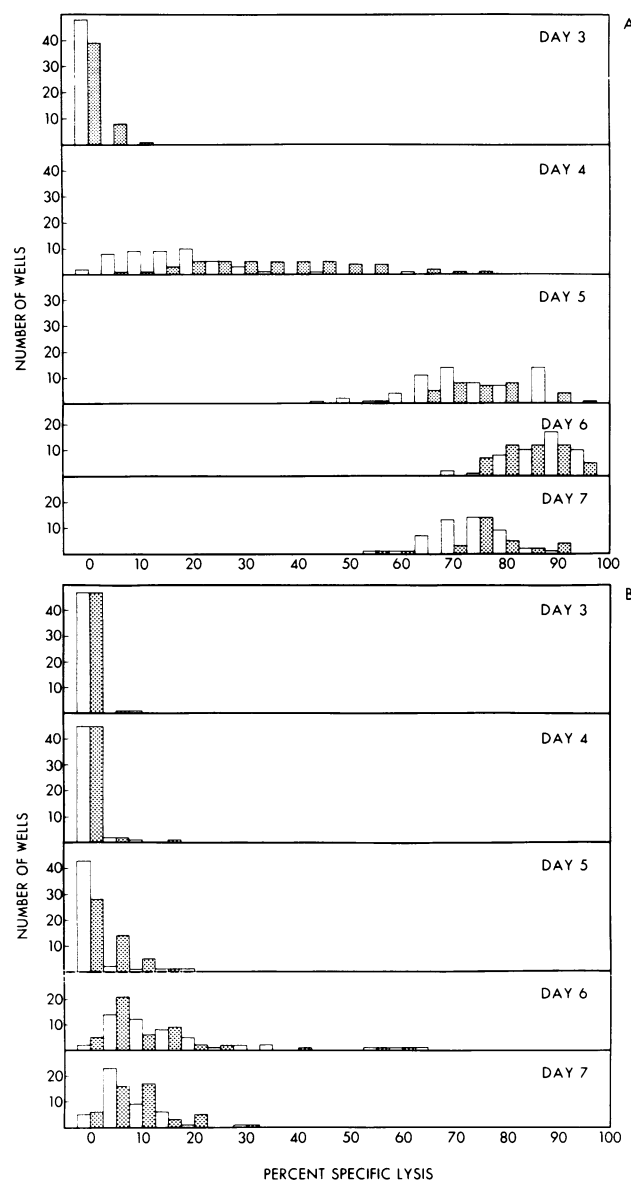


FIG. 1. Kinetics of generation of influenza-immune Tc cells in microcultures. An aliquot of 10<sup>4</sup> memory spleen cells was cocultured with 10<sup>5</sup> influenza-infected, irradiated spleen stimulator cells supplemented with 3% CoS for 3 to 5 days at 37°C (open bars) or 39°C (shaded bars), and individual wells were tested for cytotoxicity on infected (A) or uninfected (B) P815 target cells. Forty-eight wells were assayed per target and temperature. On day 7, 30 wells (39°C) were assayed with infected targets, and 45 wells (37°C) were assayed with uninfected targets. Spontaneous release for uninfected and infected P815 was never greater than 15%.

showed an increased number of wells with higher values of specific target cell lysis than those at 37°C. This difference disappeared at day 6, and lysis values decreased slightly again at day 7 at both temperatures, probably also due to nutrient exhaustion in the cultures. The generation of effectors lysing uninfected targets does not seem to be as modulated by temperature as that of the virus-specific effectors (Fig. 1B). Only on day 5 was a slight difference observed.

**Limiting dilution analysis of memory Tc cells at 37 and 39°C in the presence or absence of exogenous help.** It was of interest to investigate whether hyperthermia acts preferentially on Tc cells or T helper cells or whether it influences both T cell subpopulations. Graded numbers of memory influenza spleen cells obtained from a single animal were cocultured with a fixed number of infected, irradiated stimulator cells in duplicate microtiter wells (1 cell concentration per plate).

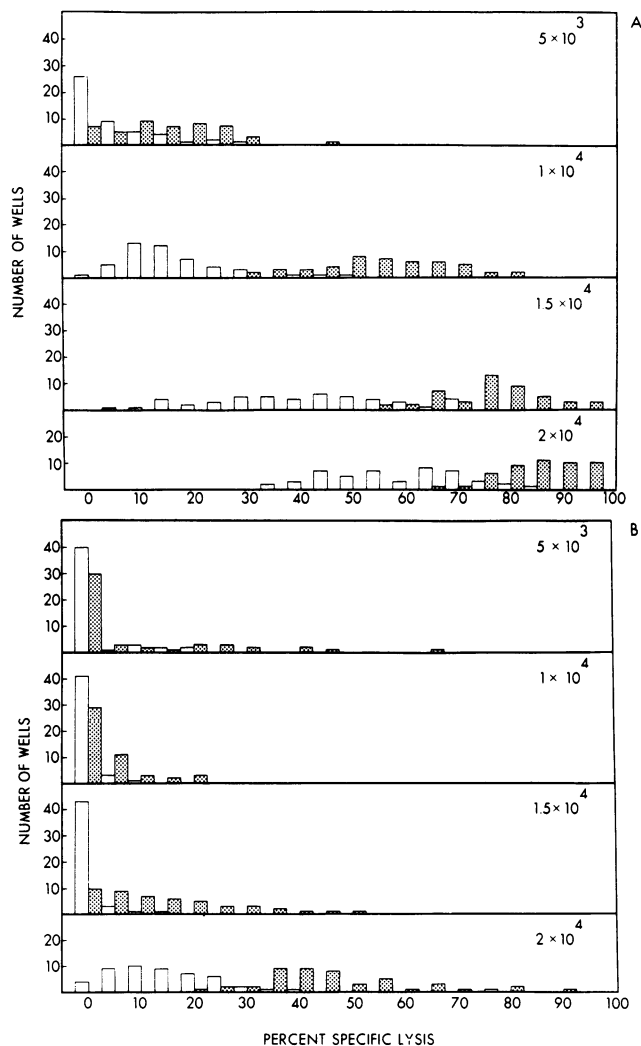


FIG. 2. Limiting dilution analyses of influenza-immune Tc cells at 37 and 39°C. Graded numbers of memory spleen cells were cocultured for 4 days with  $10^5$  irradiated influenza-infected stimulator cells in the presence (A) or absence (B) of exogenous helper factors (CoS) at 37°C (open bars) or 39°C (shaded bars). Individual wells were assayed for cytotoxicity against influenza-infected P815 targets for 8 h. Spontaneous release was 17.6%. Forty-eight wells were assayed for each temperature and culture condition.

TABLE 2. Target cell lysis by influenza-immune Tc cells at 37 and 39°C

Assay temp	Killer/target cell ratio	% Specific lysis of $^{51}\text{Cr}$ -labeled P815 targets <sup>a</sup>	
		Uninfected	A/WSN-infected
37°C	10	3.3 (0.2)	86.5 (4.0)
	3	1.5 (0.3)	78.8 (2.9)
	1	0.7 (0.0)	46.6 (5.0)
39°C	10	6.6 (0.4)	86.6 (2.1)
	3	2.7 (0.3)	76.3 (2.1)
	1	-0.7 (0.2)	53.4 (0.6)

<sup>a</sup> Mean percent specific  $^{51}\text{Cr}$  release from targets over 8 h. Spontaneous release was 9.6 and 12.1% for uninfected and infected targets, respectively, at 37°C, and 13.5 and 13.9% at 39°C. Means of triplicates are given with the standard error of the mean in parentheses.

Half of the wells of each plate (48 wells) were supplemented with CoS to a final concentration of 3% (vol/vol). One set of plates was incubated at 37°C and the other was incubated at 39°C for 4 days, after which the Tc cell activity for lysis of A/WSN-infected P815 target cells of each individual well was estimated (Fig. 2). Cytotoxic activity in all cases was higher in the plates incubated at 39°C, irrespective of the presence or absence of exogenous help. In the absence of CoS, a decline in the number of positive wells and also in strength of lytic activity was observed at  $10^4$  responder cells per well as compared with  $5 \times 10^3$  responders per well, a phenomenon which was also observed by others (15) and which may reflect the presence of suppressor cells.

**Target cell killing at hyperthermal temperatures.** The results in Fig. 1 and 2 clearly show that hyperthermia influences the generation of influenza-immune Tc cells. The question of whether the killing event per se is also enhanced at elevated temperatures was investigated. Secondary influenza-immune Tc cells, generated in bulk cultures at 37°C, were tested on infected and uninfected P815 target cells at 37 or 39°C for 8 h (Table 2). No significant difference in the specific lysis was observed between the assays incubated at the two temperatures. Only a slight increase in the spontaneous  $^{51}\text{Cr}$  release was observed from infected and uninfected targets at 39°C.

## DISCUSSION

This report formally demonstrates that hyperthermia influences the potency of the response of cytotoxic T cells to influenza virus *in vitro*. Under microculture conditions, a Tc cell response was observed with substantially higher mean values of lytic activity per well in plates incubated at 39°C than in plates incubated at 37°C (Fig. 1A). The difference was most marked at days 3, 4, and 5 and thus seems to indicate that the rate of generation of effector cells was accelerated and not necessarily that the number of detectable precursors had increased or that antigenic recognition was augmented at induction. The mere fact that all the wells became positive for cytotoxicity at days 5 and 6 in cultures incubated at 37°C does support the finding that elevated temperature decreases cell cycle time (3). In the experiment shown in Fig. 1, the higher mean values of lysis of wells incubated at 39°C most likely reflected the increased clone size of cytotoxic cells as all cultures were supplemented with exogenous helper factors derived from concanavalin A-activated spleen cells. It was also apparent from the limiting dilution experiment in Fig. 2 that cytotoxicity was, on the average, substantially higher in wells incubated at 39°C than

in those incubated at 37°C, irrespective of whether helper factor(s) was added or not. It cannot, however, be ascertained whether T cells providing help were also affected by higher temperatures from the experiments shown here. Of some interest is the phenomenon seen in the limiting dilution experiment without help (Fig. 2B), in which reduction in positive wells was observed at a cell concentration of 10<sup>4</sup> cells per well as compared with a concentration of 5 × 10<sup>3</sup> cells per well. This was seen irrespective of the incubation temperature. If, as has been suggested by others (9, 15), this was due to the action of suppressor cells, the overall balance of activity of suppressor, helper, and Tc cells seems not to be affected by hyperthermia.

The lysis of uninfected P815 targets was not augmented by an incubation temperature of 39°C (Fig. 1B), except possibly on day 5. This increased lysis of uninfected targets from 39°C 5-day cultures, however, was not observed in repeat experiments. Thus, elevated temperatures did not necessarily cause an increase in cytotoxic cells with autolytic activity. Autoreactivity has been demonstrated in limiting dilution assays measuring precursor frequencies of influenza-specific Tc cells (13), and fever has been postulated to be one cause of an increase in breakdown of self-tolerance (21) and accompanying autoaggression by the immune system. Finally, influenza-immune Tc cells were not more effective in target cell lysis at 39°C than at 37°C. This in part supports the conclusion reached from the kinetic study, namely, that antigen recognition per se was not augmented by hyperthermia.

In conclusion, hyperthermia increased the rate of generation of Tc cells to influenza virus and possibly also augmented generation of help. Therefore, fever early in an infection may prove to be beneficial by specifically enhancing the cellular immune response required to successfully combat a primary viral infection.

#### ACKNOWLEDGMENTS

I thank J. P. Mundy for excellent technical assistance, A. Kesson for critically reading the manuscript, and L. Hardy for typing the manuscript.

#### LITERATURE CITED

1. **Andreesen, R., J. Osterholz, A. Schulz, and G. W. Lohr.** 1983. Enhancement of spontaneous and lymphokine activated human macrophage cytotoxicity by hyperthermia. *Blut* **47**:225-229.
2. **Ashman, R. B., and A. Müllbacher.** 1979. A T-helper cell for anti-viral cytotoxic T cell responses. *J. Exp. Med.* **150**:1277-1282.
3. **Ashman, R. B., and A. J. Nahmias.** 1977. Enhancement of human lymphocyte responses to phytomitogens *in vitro* by incubation and elevated temperatures. *Clin. Exp. Immunol.* **29**:464-467.
4. **Azokar, J., E. J. Yunis, and M. Essex.** 1982. Sensitivity of human natural killer cells to hyperthermia. *Lancet* **i**:16-17.
5. **Blanden, R. V.** 1974. T cell response to viral and bacterial infection. *Transplant Rev.* **19**:56-88.
6. **Dinarello, C. A., and S. M. Wolff.** 1982. Molecular basis of fever in humans. *Am. J. Med.* **72**:799-819.
7. **Fazekas de St. Groth, S., and R. G. Webster.** 1966. Disquisition on original antigenic sin. I. Evidence in man. *J. Exp. Med.* **124**:331-338.
8. **Gillis, S., and S. B. Mizel.** 1981. T cell lymphoma model for the analysis of interleukin 1 mediated T-cell activation. *Proc. Natl. Acad. Sci. U.S.A.* **78**:1133-1137.
9. **Goronzy, J., U. Schaefer, K. Eichmann, and M. M. Simon.** 1981. Quantitative studies on T cell diversity. II. Determination of the frequencies and Lyt phenotypes of two types of precursor cells for alloreactive cytotoxic T cells in polyclonally and specifically activated splenic T cells. *J. Exp. Med.* **153**:857-870.
10. **Jampel, H. D., G. W. Duff, R. K. Gershon, E. Atkins, and S. K. Durum.** 1983. Fever and immunoregulation. Hyperthermia augments the primary *in vitro* humoral immune response. *J. Exp. Med.* **157**:1229-1238.
11. **Klustersky, J., and E. H. Kass.** 1970. Is suppression of fever or hypothermia useful in experimental and clinical infectious diseases? *J. Infect. Dis.* **121**:81-86.
12. **Kluger, M. J., D. H. Ringler, and M. R. Anver.** 1975. Fever and survival. *Science* **188**:166-168.
13. **Komatsu, Y., Y. Nawa, A. R. Bellamy, and J. Marbrook.** 1978. Clones of cytotoxic lymphocytes can recognise uninfected cells in a primary response against influenza virus. *Nature (London)* **274**:802-804.
14. **Lin, Y. L., and B. A. Askonas.** 1981. Biological properties of an influenza A virus specific T killer cell clone; inhibition of virus replication *in vivo* and induction of delayed type hypersensitivity reactions. *J. Exp. Med.* **154**:225-235.
15. **Mak, N. K., C. Sweet, G. L. Ada, and G. A. Tannock.** 1984. The sensitization of mice with a wild-type and cold-adapted variant of influenza A virus. *Immunology* **51**:407-416.
16. **Müllbacher, A., and R. V. Blanden.** 1979. Cross-reactivity patterns of murine cytotoxic T lymphocytes. *Cell. Immunol.* **43**:70-81.
17. **Onsrud, M.** 1983. Effects of hyperthermia on human natural killer cells. *Acta Pathol. Microbiol. Immunol. Scand.* **91**:1-8.
18. **Roberts, N. J., Jr.** 1979. Temperature and host defense. *Microbiol. Rev.* **43**:241-259.
19. **Roberts, N. J., Jr., and R. T. Steigbigel.** 1977. Hyperthermia and human leukocyte functions: effects on response of lymphocytes to mitogen and antigen and bactericidal capacity of monocytes and neutrophils. *Infect. Immun.* **18**:673-679.
20. **Saririan, K., and D. A. Nickerson.** 1982. Enhancement of murine *in vitro* antibody formation by hyperthermia. *Cell. Immunol.* **74**:306-312.
21. **Sohnle, P. G., and S. R. Gambert.** 1982. Thermoneutrality: a evolutionary advantage against aging. *Lancet* **i**:1099-1100.
22. **Sultan, M. F., W. A. Tompkins, and C. A. Cain.** 1983. Hyperthermic enhancement of antibody-complement cytotoxicity against normal mouse B lymphocytes and its relation to capping. *Radiat. Res.* **96**:251-260.
23. **Woolnough, J. A., and K. J. Lafferty.** 1979. Generation of homogeneous populations of alloreactive T cells *in vitro*. *Aust. J. Exp. Biol. Med. Sci.* **57**:127-132.