Effect of Hyperthermia on Murine Cell Surface Histocompatibility Antigens

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ABSTRACT

The effect of heat on the density of cell surface histocompatibility antigens was examined. Antigen density and distribution were determined by radioimmunoassay and flow cytometry after the binding of radiiodinated or fluoresceinated monoclonal antibody (anti-H-2Kk and anti-H-2Kb) to murine lymphoma cells in suspension cultures.

Antibody binding was unaffectected by temperatures between 37°C and 41°C following a 30-min heat exposure. At 42°C, some inhibition of binding was measurable. However, at 43°C, antibody binding was reduced by 30 to 50%, and a further 15 to 20% reduction was observed at 45°C. Flow cytometry showed that all cells were equally affected. There was no indication of the selection of a specific cell population. The temperature-dependent decrease in antibody binding was due to a decrease in receptor number and not to changes in the affinity. Measurement of the diffusion coefficient of the lipid probe N,N-dioctadecyl indocarboyanine iodide showed that heat did not affect significantly the fluidity of the membrane lipids. Hyperthermic temperatures, therefore, have a direct effect on these membrane proteins.

INTRODUCTION

The effects of hyperthermia (41-45°C) on mammalian cells are currently under intensive study, because heat appears to be a useful form of anticancer therapy. It is frequently suggested that heat has special effects on the body’s immune system and that heat-treated cells have unusual antigenic properties. For example, Mondovi et al. (16) compared the ability of heat- or radiation-killed Ehrlich ascites cells to immunize syngeneic mice against subsequent challenges by live cells. They found heat-killed cells to be far more efficient. This observation, however, could not be repeated by other workers (22). In the opposite direction, heat-treated T-lymphocytes were found to be less able to mount an attack on several tumor cell lines than were unheated controls (6, 8, 10, 13, 24).

This is clearly an important area of study. The existing data, however, neither relate hyperthermia unequivocally to immune function nor, of course, do they permit identification of a specific immune mechanism that might be involved in effecting tumor cures in response to hyperthermia. For this reason, we have initiated a study to examine the effects of hyperthermia on surface characteristics of mammalian cell membranes. It would seem that only at the molecular level can immune responses be established with any degree of precision.

Gene products of the major histocompatibility complex play an important role in most cell-mediated immune responses, including tumor cell cytolyis by the T-lymphocytes (11). In this paper, we report on effects of elevated temperatures on histocompatibility antigens of murine lymphoid cells.

MATERIALS AND METHODS

Cells and Culture Conditions. EL-4 (H-2Kb) and RDM-4 (H-2Kk) murine lymphoma cell lines (derived from C57BL/6N and AKR mice, respectively) were grown in RPMI 1640 supplemented with 15% fetal calf serum and antibiotics. Incubation was at 37°C in a humidified atmosphere of a mixture of 95% air and 5% CO₂.

Preparation of Fluorescent Antibodies. Monoclonal anti-H-2Kk antibody was purified from the supernatants of hybridoma cell line 11-4.1 (17). Monoclonal anti-H-2Kb was purified from the supernatants of cell line 20.8.4 (18). Pure preparations of the monoclonal antibody 11-4.1 were also obtained commercially from the Becton Dickinson Monoclonal Center, Mountain View, CA. Fluoresceination with FITC was as described by Wofsky et al. (25). The fluorescein-protein ratios of the fluoresceinated antibody have been given for the experiment as needed.

Radioiodination of Protein A. Radioiodination of Protein A was by the chloramine-T method as modified by Mehdi and Nussey (14). Specific radioactivities of 10 to 15 µCi/µg were obtained.

Heat Treatment. RDM-4 cells (5 x 10⁶/ml) in RPMI 1640 medium were incubated in stoppered tubes in water baths at 37, 41, 42, 43, and 45°C in an atmosphere of 95% air and 5% CO₂ for 30 min unless stated otherwise. EL-4 cells which do not bind the anti-H-2Kk antibody were included as controls. The temperature was controlled within 0.1°C. There was no significant change in the pH of the medium during heating. Tubes were transferred to an ice-water bath at 4°C for the binding of fluoresceinated antibody and for radioimmunoassay.

Radioimmunoassay of Antibody Binding. After heat treatment, tubes were centrifuged at 600 x g for 3 min in a Beckman Microfuge 12, and the cell (5 x 10⁶) pellets were resuspended in 50 µl of PBS, pH 7.4. Tubes were kept on ice, and 20 µg of the appropriate monoclonal antibody in 20 µl of PBS were added to each tube. The tubes were incubated for 30 min, and unbound antibody was removed by centrifugation and resuspension in 1 ml of ice-cold PBS. After 3 washes, the cells were resuspended in 0.5 ml of PBS, 1²⁵I-Protein A (approximately 100,000 cpm) in 20 µl of PBS was added, and the tubes were incubated for a further 1 h. The cells were washed again 3 times by centrifugation and resuspension in ice-cold PBS. Finally, the supernatant was carefully removed without disturbing the cell pellet, and the tips of the tubes containing the cell pellets were excised. Radioactivity was measured using a Beckman Autogamma 8000 counter.

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7 The abbreviations used are: FITC, fluorescein isothiocyanate; FACS, fluorescein-activated cell sorter; PBS, phosphate-buffered saline; Dil, N,N-dioctadecyl indocarboyanine iodide.
Binding of Fluoresceinated Antibody. Each heat-treated cell sample was centrifuged at 600 x g for 3 min, and the pellet was resuspended in 50 μl of PBS. The tubes were kept on ice, and the quantity of fluoresceinated monoclonal anti-H-2Kk antibody indicated was added in 20 μl of PBS. Monoclonal anti-H-2Kk which does not interact with RDM-4 cells was used as a control as described for the individual experiment. After incubation for a further 20 min, cells were washed 3 times by centrifugation and resuspension in 1 ml of ice-cold PBS, pH 7.4. The cells were fixed in 100 μl of an ice-cold solution of 1% p-formaldehyde in PBS. After 30 min at 4°C, the cells were washed 3 times with PBS and resuspended in 1 ml of the same buffer. Flow cytometric fluorescence measurements were performed using a Becton Dickinson FACScan analyzer (19) with 485-nm excitation and 520- to 560-nm emission. The photo-multiplier voltage was set at 550 V, and fluorescence was measured using logarithmic amplification. Data acquisition was triggered with the electronic volume pulse. Histogram data were stored and processed using the Becton Dickinson FACS Consort 20 data system (FACS systems; Becton Dickinson, Sunnyvale, CA). Fluorescence readings were calibrated using the FACS analyzer calibration particles with known FITC content (Becton Dickinson). Small debris was excluded by setting a scatter threshold. Fluorescence intensity was calculated from the major peak of brightly labeled cells in a sample would lead to an erroneous estimation of the quantity of antibody specifically and reversibly bound to the cell surface, it is important to select a time-temperature combination that would avoid their presence (see below).

RESULTS

The time-temperature relationship of the specific binding of monoclonal anti-H-2Kk to RDM-4 cells is shown in Chart 1. Maximum binding was observed at 37°C. At 41°C, no significant change was observed for approximately 90 min, but a 10 to 15% decrease was observed after heating for 180 min (not shown). At 42°C and above, there was a significant time- and temperature-dependent decrease in specific antibody binding. All cells appeared to be equally affected, and there was no indication of selective heat effects on a specific cell population. After 45 min of incubation at 43°C or 30 min of incubation at 45°C, a second well-identified peak of brightly labeled cells was observed (Chart 2b). This peak nonspecifically trapped both the specific and irrelevant antibodies to the same extent. After 120 min at 45°C, there was a conspicuous absence of cells specifically binding the anti-H-2Kk antibody. The single peak of brightly labeled cells also trapped both antibodies nonspecifically (Chart 2c). Nonspecific trapping of ligand by dead and moribund cells with deranged permeability properties has been thoroughly investigated by other groups (4, 9). Since the presence of such cells in a sample would lead to an erroneous estimation of the quantity of antibody specifically and reversibly bound to the cell surface, it is important to select a time-temperature combination that would avoid their presence (see below).

The temperature-dependent decrease in antibody binding could either be due to an actual decrease in the number of available receptors or to an alteration in the affinity of the receptor for the antibody. To examine these possibilities, cells heated at various temperatures were incubated with different concentrations of the FITC-labeled antibody. Chart 3 shows that receptor saturation took place at lower antibody concentrations for cells heated at 43 and 45°C compared to cells at 37°C. Scatchard (20) plots of antibody binding data showed that the affinity properties of the receptors remained unaltered. Affinity constants obtained from computer-derived linear portions of the curve gave Kd values of 2.12 x 10^-9 M^-1, 2.23 x 10^-9 M^-1, and 2.65 x 10^-9 M^-1 at 37, 43, and 45°C, respectively. The number of available binding sites decreased (approximately 105,000, 70,000, and 50,000, respectively, at 37, 43, and 45°C with increasing temperature (Chart 4). Data analysis using double reciprocal plots (not shown) gave similar values. The upward convexity of the Scatchard plots indicates receptor dimerization or aggregation which is not unexpected of a bivalent ligand, such as an antibody molecule (3).

As mentioned above, for meaningful comparisons to be made over the entire temperature range under examination, it is important to choose a heating period during which cells showing altered or deranged permeability properties are either absent or negligible. Chart 5 shows that, for the cell lines used in this study, a 30-min heat treatment gave similar results whether antibody binding was measured by flow cytometry or by radioimmunoassay. It is quite clear that a dramatic reduction in antibody binding takes place between 37 and 45°C. The results discussed above suggest that the decrease in antibody binding is not due to changes in the affinity of the receptor for the antibody, but to a decrease in the number of available receptors at elevated temperatures. In previous papers, it was shown that the binding...
The experimental procedure was the same as for Chart 1, except that cells were incubated for 30 min at 37 (°A), 43 (°C), and 45° (°B). The different concentrations of FITC-labeled antibody added to each tube are indicated.

Chart 5. The experimental procedure was the same as for the experiment in Chart 1, except that RDM-4 (and EL-4, control, which do not bind anti-H-2Kk antibody, not shown) cells were heated for 30 min at the temperatures indicated. A, effect of heat on the binding of FITC-labeled monoclonal anti-H-2-Kk measured by flow cytometry; B, measurement of antibody binding by radioimmunoassay. Points, mean of quadruplicate samples; bars, S.D.

DISCUSSION

The exposure of murine lymphoid cells to hyperthermic temperatures resulted in a decrease in the binding of monoclonal antibodies to the cell surface histocompatibility antigens. This decrease was both time and temperature dependent. If cells were heated for a prolonged period at the higher temperatures (e.g., 45 min at 43°, or more than 30 min at 45°), a population of cells that nonspecifically accumulate large amounts of both specific and irrelevant antibody molecules appeared. This population increased with time. Live RDM-4 cells did not bind (nor nonspecifically adsorb) the irrelevant antibody (H-2Kb). After heating the lymphoid cells at 45° for 120 min or more, flow cytometry showed that there was a conspicuous absence of live cells. This observation showed that the choice of the method used for examining the effect of heat on the binding of antibody (or ligand) to a specific cell line is of critical importance. As pointed out by Juliano and Behar-Bannelier (9) and Bohn and Manske (4), the presence of a few cells of deranged permeability properties within an otherwise viable population may constitute a dangerous source of error, since nonspecific trapping of antibody by severely damaged cells with impaired membrane barrier functions takes place according to diffusion gradients and not by specific reversible binding to receptors. Flow cytometry permits the exclusion of these cells from analysis, and computations can be made of the average number of antibody or ligand molecules bound per live cell. Although good correlations were obtained between the temperature-dependent decrease in antibody binding by the 2 methods (Chart 5), care had to be taken in choosing the incubation times for radioimmunoassay. As discussed above, flow cytometry does not require the precautions needed for radioimmunoassays and was therefore the method of choice for these studies.

Specific binding of monoclonal anti-H-2Kk to RDM-4 cells showed saturation of receptor sites at all temperatures between 37° and 45°. The maximum time-temperature-dependent decrease in receptor density was 50 to 60%. It is noteworthy that,
although the number of available receptors decreased with increasing temperatures, Scatchard analysis as well as double reciprocal plots (not shown) of the antibody binding data showed that the affinity properties of the receptors did not seem to be altered in a major way. In this respect, the data are similar to those obtained for another membrane protein, the insulin receptor (5). The upward convexity of the Scatchard plots indicated positive cooperative interactions and the possibility that receptor dimerization took place at all the temperatures examined (3). As expected, positive cooperativity became most striking at 37°C where receptor occupancy was greatest.

The binding of ligand (hormone or antibody) may also depend on the physical state of the membrane lipids. For example, the binding of the polypeptide hormone thyrotropin to its receptors on human thyroid membranes showed a temperature dependence with a temperature transition at 29°C. Above this temperature, hormone binding increased with temperature (2, 15). An analysis of the fluidity of thyroid membrane lipids showed a similar increase, with a transition at 29°C. Hence, hormone binding increased in proportion to the fluidity of the membrane lipids. On the other hand, it is also known that the availability and binding of a lipid hapten to its antibody are decreased or abolished in fluid membranes (1).

In order to examine the possibility that heat may alter the "fluidity" of membrane lipids, we measured the rate of diffusion of the fluorescent lipid analogue DII. Our results, confirming data obtained earlier (7, 12), showed that, over the temperature range examined, fluidity did not change significantly. We therefore conclude that the decreased density of H-2Kk antigens after hyperthermia reflects direct action of heat on this protein molecule.

A number of laboratories have examined the effect of hyperthermic temperatures on the activity of both killer lymphoid cells as well as target tumor cells (6, 8, 10, 13, 24). Both T-lymphocytes (6, 8, 10, 13, 24) and natural killer cells (10) were found to lose cytotoxic activity following brief exposures to hyperthermic temperatures (44°C for 10 min). However, recovery of T-lymphocytes from this heat-induced functional impairment took place within 2 to 3 hr in the absence of protein synthesis (8).

Since the nature of the effector T-cell receptor is not understood, changes in the density of histocompatibility antigens described in this paper may not be important in the lytic activity of these killer cells. However, these antigens play a central role in the recognition of the target cell, leading finally to its immune cytolysis (11). Existing work does not show that heat treatment of the target cell affects its susceptibility to cytolysis (6, 8, 10, 24). It is clear that further work is needed before any correlations can be drawn between the heat-induced decrease in the number of histocompatibility antigens on the target cell surface and their susceptibility to immune cytolysis by mechanisms other than T-cell-mediated killing.

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REFERENCES

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