

# Temperature Shifts Induce the Selective Loss of Alveolar-Macrophage Plasma Membrane Components

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**ABSTRACT** A shift in the incubation temperature of rabbit alveolar macrophages ( $0^{\circ}\text{C} \rightarrow 37^{\circ}\text{C} \rightarrow 0^{\circ}\text{C}$ ) resulted in a 40–60% reduction in the ability of cells to bind  $\alpha$ macroglobulin- $^{125}\text{I}$ -trypsin complexes ( $\alpha\text{M}\cdot^{125}\text{I}\text{-T}$ ). The reduction in binding activity did not reflect a disruption of cell integrity since the levels of intracellular components (lactate dehydrogenase,  $\beta$ -*N*-acetylhexosaminidase) or other plasma membrane components (alkaline phosphodiesterase) were unaltered. Analysis of receptor-ligand interaction indicated that the temperature shift effected a decline in receptor number rather than an alteration in ligand-receptor affinity. Studies indicated that a temperature shift resulted in the loss of unoccupied receptors, and that ligand bound to receptors was not lost. However, after ligand internalization, receptors were removed by the temperature shift.

The rate of receptor loss was maximal when cells were incubated at temperatures  $>24^{\circ}\text{C}$ . Receptor loss was not prevented by treatment of cells with colchicine, cytochalasin B, or *N*-ethylmaleimide, but was prevented by treatment with the cross-linking agent paraformaldehyde.

Data indicate that the reduction in  $\alpha\text{M}\cdot^{125}\text{I}\text{-T}$  binding activity resulted from shedding of receptors into the media since media obtained from temperature-shifted cells contained material that competed with cell-bound receptors for  $\alpha\text{M}\cdot^{125}\text{I}\text{-T}$ . Additionally, binding of  $\alpha\text{M}\cdot^{125}\text{I}\text{-T}$  was diminished on membrane fragments obtained from temperature-shifted cells.

Incubation with Triton X-100, of cells whose receptors were occupied with  $\alpha\text{M}\cdot^{125}\text{I}\text{-T}$ , led to the extraction of 40% of cell-bound activity. However, no radioactivity was extracted from cells labeled with  $\alpha\text{M}\cdot^{125}\text{I}\text{-T}$  after a temperature shift. Measurement of ligand accumulation by control and temperature-shifted cells incubated at  $20^{\circ}\text{C}$  indicated that control cells exhibited a subpopulation of receptors capable of binding ligand but only slowly internalizing it. This subpopulation was not present on temperature-shifted cells. These results indicate that surface receptors for  $\alpha$ macroglobulin-protease complexes are heterogeneous and that the temperature shift resulted in the selective loss of membrane components.

Mammalian plasma membrane components are continuously being turned over. While one of the major routes for removal of membrane proteins involves internalization and catabolism, evidence exists demonstrating that membrane proteins may be released into the media. For some classes of membrane proteins, "exfoliation" has been hypothesized to constitute the major route by which the concentration of that protein in the membrane is lowered. However, the exact mechanism by which membrane proteins become exfoliated, or the degree to which exfoliation is a pathway for membrane protein turnover, is, in most instances, unclear. Not only is there evidence indicating spontaneous shedding of membrane components but data indicate that release of membrane components may be induced.

For example, the literature on lymphocytes is replete with references to the phenomenon of "ligand induced shedding" (cf. review, reference 2). Addition of ligand, either lectin or divalent antibody, to lymphocytes results in the shedding or release of ligand-bearing membrane components. Whether ligand-induced shedding is involved in modulation of lymphocyte function is unresolved.

In previous studies, we reported on the metabolism of alveolar macrophage receptors for  $\alpha$ macroglobulin-protease complexes (6, 7, 8). During the course of those studies, we observed that cells could precipitously lose up to 50% of ligand-binding activity merely as a result of altering the temperature of the incubation media. In this communication we report that a

temperature shift results in the loss of receptor activity. Of particular interest is the observation that temperature shifts resulted in a selective loss of membrane components. Further studies demonstrate that, based on a number of criteria, the population of  $\alpha M \cdot P$  receptors is heterogeneous.

## MATERIALS AND METHODS

### Cells

Rabbit alveolar macrophages were obtained from New Zealand white rabbits using the technique of Myrvik et al. (11). Cells were washed by centrifugation with Hank's balanced salt solution (HBSS) and, if necessary, erythrocytes were removed by incubation with cold 0.15 M  $\text{NH}_4\text{Cl}$  for 5 min. The cells were washed and resuspended in either cold ( $0^\circ\text{C}$ ) Eagle's Minimal Media EMEM or HBSS, both of which contained 1 mg/ml bovine serum albumin (BSA).

### Preparation of $\alpha M$ and $\alpha M \cdot$ Protease Complexes

Rabbit macroglobulin (a mixture of  $\alpha_1$ macroglobulin and  $\alpha_2$ macroglobulin) was prepared from freshly drawn rabbit plasma (7). Purified  $\alpha$ macroglobulin was converted to the protease complex by addition of either trypsin or  $^{125}\text{I}$ -trypsin (prepared as previously described [7]). The resultant  $\alpha$ macroglobulin-trypsin complex ( $\alpha M \cdot T$ ) or  $\alpha$ macroglobulin- $^{125}\text{I}$ -trypsin complex ( $\alpha M \cdot ^{125}\text{I-T}$ ) was purified by molecular sieve chromatography (7). The specific activity of  $\alpha M \cdot ^{125}\text{I-T}$  ranged from 20–60 cpm/fm.

### Assay of $\alpha M \cdot T$ Binding to Cells

To assay cell-bound receptors, cells were incubated in Sarstedt microfuge tubes at a final concentration of  $1-2 \times 10^6$  cells/tube in a total volume of 1 ml. To measure total binding,  $\alpha M \cdot ^{125}\text{I-T}$  ( $10^{-9}$  M) was added to one set of tubes. To measure nonspecific binding an identical amount of labeled complex was added to another set of tubes in the presence of a large excess of unlabeled  $\alpha M \cdot T$  ( $10^{-7}$  M). After 60 min at  $0^\circ\text{C}$ , the tubes were centrifuged for 15 s in a Brinkmann 3700 centrifuge (Brinkmann Instruments, Inc., Westbury, NY). The supernatant was removed by aspiration, and the cell pellet was resuspended and washed twice with cold HBSS (without albumin). After a final wash, the cell pellet was dissolved in 1% SDS and samples were taken for radioactivity and protein determination. Generally, nonspecifically bound radioactivity constituted <20% of total bound radioactivity.

### Measurement of Extracellular "Receptor" Activity

Medium to be tested for "receptor" activity was centrifuged at 10,000  $g$  for 15 min to remove any possible cellular debris. Up to 800  $\mu\text{l}$  of the supernatant was placed in microfuge tubes. To the tubes was then added a trace amount of  $\alpha M \cdot ^{125}\text{I-T}$ ,  $\sim 1-4 \times 10^{-11}$  M. This quantity of  $\alpha M \cdot ^{125}\text{I-T}$  was capable of occupying only 10–20% of the normal quantity of surface receptors. After a 60-min incubation at  $0^\circ\text{C}$ , 100  $\mu\text{l}$  of a cell suspension ( $10^7$  cells  $\cdot$  ml $^{-1}$ ) was added to each tube and the incubation allowed to proceed for an additional 60 min. Nonspecific binding was determined by incubation of cells with  $\alpha M \cdot ^{125}\text{I-T}$  and  $\alpha M \cdot T$  simultaneously. Cell-bound radioactivity was determined as described above.

### Assay of $\alpha M \cdot ^{125}\text{I-T}$ Binding to Subcellular Fractions

Cells that had been maintained at  $0^\circ\text{C}$ , or subjected to a temperature shift, were washed and suspended in buffer A (0.1 M NaCl, 1 mM CaCl $_2$ , 0.02 M Tris-HCl pH 7.2) at a density of  $\approx 1 \times 10^7$  cells/ml. Cells were homogenized with a polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY; 2 min at setting seven). The homogenized cells were centrifuged at 200  $g$  for 10 min. Albumin and  $\alpha M \cdot ^{125}\text{I-T}$  (final concentration 1 mg/ml,  $10^{-9}$  M respectively) were added to the supernatant. To determine nonspecific binding,  $\alpha M \cdot T$  ( $10^{-7}$  M) was added to an identical set of tubes. After a 90-min incubation at  $0^\circ\text{C}$  the sample was centrifuged at 10,000  $g$  for 20 min and the pellets were washed twice by centrifugation using albumin-free buffer A. The pellet was resuspended in 1% SDS and samples taken for protein and radioactivity determinations. The results are expressed as specifically bound radioactivity per microgram of protein.

### Additional Assays

$\beta$ -D-N-acetyl hexosaminidase, acid phosphatase, and  $\beta$ -galactosidase were

assayed as described previously (5). Lactate dehydrogenase was determined using the procedure of Bergmeyer (1). Alkaline phosphodiesterase was assayed using the procedure of Edelson and Erbs (3). Protein determinations were performed according to the procedure of Lowry et al. (10)

## RESULTS

The last step in the isolation of rabbit alveolar macrophages involved incubating cells at  $0^\circ\text{C}$ . Cells maintained at  $0^\circ\text{C}$  for even extensive periods of time (i.e. up to 24 h) were capable of binding 0.7 fm  $\alpha M \cdot ^{125}\text{I-T}/\mu\text{g}$  cell protein when binding was assayed at  $0^\circ\text{C}$  (Table I). When cells were exposed to a temperature shift there was a marked reduction in the ability to bind  $\alpha M \cdot T$ . The temperature shift had no effect on the levels of the cytosol enzyme lactate dehydrogenase, the lysosomal enzymes  $\beta$ -N-acetyl hexosaminidase or acid phosphatase, or the plasma membrane enzyme alkaline phosphodiesterase. Analysis of the kinetic parameters of alkaline phosphodiesterase in control and temperature-shifted cells have demonstrated identical  $V_{\text{max}}$  and  $K_m$  (0.2 mM), indicating no change in substrate affinity or activity. The decrease in  $\alpha M \cdot T$  binding activity was observed regardless of whether binding activity was normalized to cell number or micrograms of protein. These results indicate that the temperature shift induced decrease in  $\alpha M \cdot T$  binding activity did not result from a general disruption of cell integrity. Although the absolute level of  $\alpha M \cdot T$  bound to cells varied with different cell preparations, subjecting cells to a temperature shift invariably resulted in a 40–60% reduction in binding activity.

The results in Fig. 1 delineate the kinetics of loss of binding activity. In cells that were maintained at  $0^\circ\text{C}$  binding activity remained stable, but incubation at  $37^\circ\text{C}$  led to a time-dependent reduction in binding activity. At 2 min after the temperature shift there appears to be a transitory increase in receptor number. At 15 min after the temperature shift, there was a 45% decrease in binding activity which persisted for at least 60 min. Subsequently, receptor number started to increase slowly (data not shown). The reduction of receptor activity appeared independent of the rate of the temperature shift; cells shifted from  $0^\circ\text{C}$  to  $37^\circ\text{C}$  in 20 s exhibited the same final reduction in binding ability as cells in which the shift in temperature took 15 min. The possibility was considered that the loss of receptors might be ascribed to an abrupt removal of serum. While the

TABLE I  
Effect of a Temperature Shift on Levels of Macrophage Enzymes and  $\alpha M \cdot T$  Binding Activity

Sample	Specific Activity*	
	Control $0^\circ\text{C}$	Temperature Shift $0^\circ\text{C} \rightarrow 37^\circ\text{C} \rightarrow 0^\circ\text{C}$
Lactate dehydrogenase‡	0.578 (0.006)	0.542 (0.005)
Acid phosphatase‡	19.4 (0.4)	20.0 (0.7)
$\beta$ -D-N-Acetyl hexosaminidase‡	203.0 (21.0)	237.0 (14.0)
Alkaline phosphodiesterase‡	0.127 (0.005)	0.129 (0.005)
$\alpha M \cdot ^{125}\text{I-T}$ Binding§	0.71 (0.04)	0.42 (0.01)

Cells were incubated at  $0^\circ\text{C}$  in HBSS. An aliquot of cells was taken and incubated at  $37^\circ\text{C}$  for 30 min and then returned to  $0^\circ\text{C}$ . The cells were washed twice with cold HBSS. Samples were taken from cells maintained at  $0^\circ\text{C}$  and cells subjected to the temperature shift for determination of protein, enzyme activities, and specific binding of  $\alpha M \cdot ^{125}\text{I-T}$ .

\* M + SEM.

‡ Sp act expressed in mU/mg cell protein.

§ Sp act expressed in fmol  $\alpha M \cdot T/\mu\text{g}$  cell protein.

protein concentration of alveolar fluid can only be estimated at best, the temperature-induced loss of  $\alpha M \cdot T$  binding activity occurred regardless of whether the cells were maintained in BSA (1–20 mg/ml) or 40% rabbit serum during the temperature shift (the rabbit serum was heat-inactivated and depleted of  $\alpha M$  or  $\alpha M \cdot P$  complexes by passage over an affinity column consisting of guinea pig anti-rabbit  $\alpha M$ ).

The results in Fig. 2 demonstrate that the extent of receptor loss exhibited a strong dependency on the temperature to which cells were shifted. Incubation of cells at temperatures above 24°C led to a marked decrease in binding activity, whereas the reduction of binding activity was much lower below 24°C. The reduced loss of binding activity at temperatures below 24°C most probably reflects an alteration in rate of receptor loss. The rate of receptor loss from cells incubated at temperatures below 20°C was minimal. Cells incubated at 20°C for up to 3 h showed little diminution in surface receptor number.

### Characteristics of the Temperature Shift Induced Loss of Receptor Activity

The data in Fig. 3 demonstrate the relationship between concentration of  $\alpha M \cdot ^{125}I\text{-T}$  and binding of the labeled probe to control (0°C incubated cells) and temperature shifted cells. The slow rate of dissociation of surface receptor  $\alpha M \cdot ^{125}I\text{-T}$  complexes precludes traditional analyses of receptor-ligand interactions; i.e., Scatchard plots (7). However, in both control and temperature-shifted cells, the concentration required for half maximal saturation was similar,  $2 \times 10^{-9}$  M. Even at high concentrations of  $\alpha M \cdot ^{125}I\text{-T}$ , temperature-shifted cells bound 40% less ligand than control cells. These results indicate that ligand-receptor affinity was unaltered by the temperature shift, suggesting that the reduction in binding activity resulted from

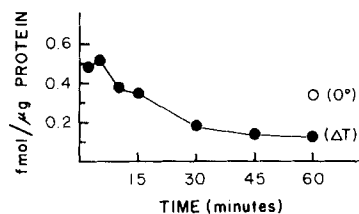


FIGURE 1 Loss of binding activity after a temperature shift. Cells incubated at 0°C were washed and incubated at 37°C in EMEM. At specified times cells were washed with cold media and incubated at 0°C. Binding of  $\alpha M \cdot ^{125}I\text{-T}$  to cells was determined as described in Experimental Procedures. The data was plotted as specific binding,  $\text{fmol} \cdot \mu\text{g protein}^{-1}$ .

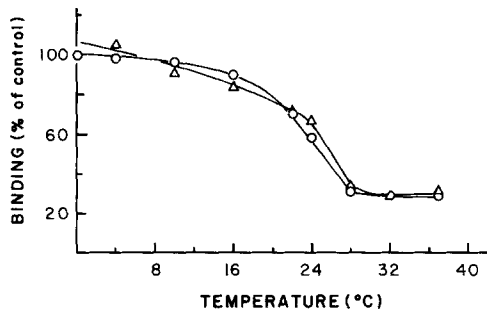


FIGURE 2 Effect of different temperatures on the loss of  $\alpha M \cdot ^{125}I\text{-T}$  binding activity. Cells that had been incubated at 0°C were incubated at specified temperatures for 60 min. Cells were then washed with cold media, incubated at 0°C, and specific binding was determined. Data were plotted as the ratio of  $\alpha M \cdot ^{125}I\text{-T}$  bound to temperature shifted cells relative to  $\alpha M \cdot ^{125}I\text{-T}$  bound to cells maintained at 0°C. The symbols indicate results from two separate experiments.

a decline in the number of surface receptors.

The data in Table II detail some of the characteristics of the temperature shift-induced loss of binding activity. While 40–60% of  $\alpha M \cdot T$  binding activity was lost due to a temperature shift, a second immediate temperature shift did not result in a further decrease in binding ability. The temperature shift induced loss of receptors was independent of the incubation media. Similar decreases in binding activity were observed with cells incubated in Eagles Minimal Media or in HBSS. Previously we demonstrated that binding of  $\alpha M \cdot ^{125}I\text{-T}$  to cells was dependent on divalent cations (7). However the tempera-

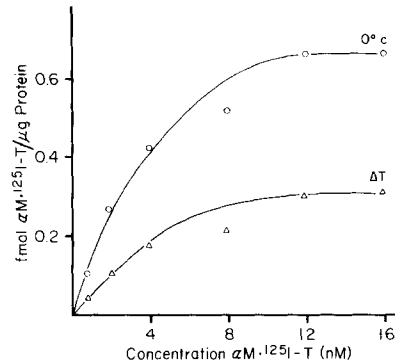


FIGURE 3 Binding of  $\alpha M \cdot ^{125}I\text{-T}$  to both control and temperature shifted cells as a function of  $\alpha M \cdot ^{125}I\text{-T}$  concentration. Cells were subjected to a temperature shift (0°C → 37°C [30 min] → 0°C). Binding of  $\alpha M \cdot ^{125}I\text{-T}$  to both temperature shifted cells and to cells maintained at 0°C (control cells) was measured as a function of  $\alpha M \cdot ^{125}I\text{-T}$  concentration. The data was expressed as specific binding ( $\text{fmol} \alpha M \cdot ^{125}I\text{-T} \cdot \mu\text{g cell protein}^{-1}$ ). Control (○). Temperature shift (Δ).

TABLE II

Effect of Different Treatments or Pharmacological Agents on the Temperature Shift-induced Loss of  $\alpha M \cdot ^{125}I\text{-T}$  Binding Activity

Treatment	Decrease in binding activity %
0°C incubation	0
ΔT (30 min)	47
ΔT (15 s)	52
ΔT + second ΔT	51
ΔT (HBSS – glucose)	44
ΔT (Ca <sup>2+</sup> , Mg <sup>2+</sup> -free HBSS + 5 mM EDTA)	47
Colchicine 10 <sup>-7</sup> M	49
10 <sup>-6</sup> M	46
10 <sup>-5</sup> M	53
Cytochalasin B 10 <sup>-8</sup> M	44
10 <sup>-6</sup> M	56
10 <sup>-5</sup> M	63
N-Ethylmaleimide (0.1 mM)	47
Paraformaldehyde (1%)	14

Cells were isolated by lung lavage and were washed and maintained at 0°C in EMEM. All binding studies were performed at 0°C in EMEM. Where indicated, cells were preincubated with cytochalasin B or colchicine for 60 min at 0°C, and the drugs were present during the temperature shift (ΔT, 30 min at 37°C). Neither colchicine nor cytochalasin B had any effect on binding of  $\alpha M \cdot ^{125}I\text{-T}$  to control cells (data not shown). Where indicated, cells were incubated with 0.1 mM *N*-ethylmaleimide in HBSS for 15 min at 0°C or 1% paraformaldehyde in HBSS for 30 min at 0°C. Cells were then extensively washed with EMEM, incubated at 37°C for 60 min, returned to 0°C and specific binding determined. In cells maintained at 0°C throughout, *N*-ethylmaleimide had no effect on binding, whereas paraformaldehyde treatment reduced binding activity by 10–30% (data not shown). The data is expressed as percent reduction in specific binding activity ( $\text{fmol} \alpha M \cdot ^{125}I\text{-T} / \mu\text{g cell protein}$ ).

ture-induced loss of binding activity occurred even in the presence of EDTA and thus was divalent cation independent.

The data in Table II also demonstrate the effect of pharmacological agents, or chemical treatment, on the temperature shift-induced loss of surface receptors. Pretreatment of cells with either colchicine or cytochalasin B did not prevent receptor loss. Pretreatment of cells with *N*-ethylmaleimide, which inhibits internalization of receptor-bound  $\alpha M \cdot T$  complexes (8), also did not prevent loss of sites. Fixation of cells with paraformaldehyde, an irreversible cross-linking agent, did reduce the loss of sites. However, paraformaldehyde treatment also resulted in a 10–30% reduction in receptor activity in control cells (data not shown).

Further studies demonstrated that temperature shifts induced only the loss of unoccupied receptors but not of receptor-ligand complexes. The data in Table III demonstrate that although the temperature shift induced a loss of receptor binding activity, it did not effect a reduction in receptor-bound  $\alpha M \cdot ^{125}I\text{-T}$ . The data also demonstrate that receptor-bound  $\alpha M \cdot ^{125}I\text{-T}$  was internalized as evidenced by the fact that cell-bound radioactivity was EDTA resistant (8). However, after internalization of ligand, there was a temperature shift-induced reduction in surface receptor number. This conclusion is based on the observation that even though  $\alpha M \cdot T$ -occupied receptors were not decreased, there was a reduction in ligand binding activity. This result is consistent with the rapid internalization of ligand-receptor complexes (6) followed by the reappearance of unoccupied receptors which are subject to loss by the temperature shift.

#### Evidence that the Temperature Shift Promotes Loss of Surface Receptors

The above results suggest that the decline in receptor activity did not result from an alteration in ligand-receptor affinity but rather from a reduction in receptor number. To determine if receptors were released into the medium, we devised an assay for soluble receptors. The assay was predicated on the view that extracellular receptors would compete with cell-bound receptors for ligand when ligand was present in subsaturating amounts. The results in Fig. 4 are from a typical experiment and indicate that media obtained from cells during a temperature shift could prevent the binding of  $\alpha M \cdot ^{125}I\text{-T}$  to cells,

TABLE III  
Effect of Receptor Occupancy on the Temperature-induced Loss of Receptors

Sample	Specific activity <i>fmol</i> $\alpha M \cdot T$ / $\mu g$ protein
0°C	0.38
$\Delta T$	0.13
$\alpha M T \Delta T_{30}$	0.16
$\alpha M \cdot ^{125}I\text{-T}^*$	0.39
$\alpha M \cdot ^{125}I\text{-T}^\dagger$	0.37

Cells were incubated at 0°C in HBSS. Aliquots of cells were taken for measurement of receptor activity both before and after a temperature shift. A sample of cells was incubated with  $\alpha M \cdot ^{125}I\text{-T}$  at 0°C for 60 min, washed with HBSS and incubated at 37°C for 15 min and the samples were washed with either HBSS (surface and intracellular radioactivity) or washed with  $Ca^{2+}$ ,  $Mg^{2+}$ -free HBSS containing 5 mM EDTA (intracellular radioactivity). The amount of cell-associated radioactivity was then determined. A final aliquot of cells was incubated at 0°C with  $\alpha M \cdot T$ , washed with HBSS and incubated at 37°C for 30 min. The cells were then placed at 0°C and specific binding was measured.

\* 0°C → 37°C, 15 min; washed with HBSS.

† 0°C → 37°C, 15 min; washed with  $Ca^{2+}$ ,  $Mg^{2+}$ -free HBSS containing 5 mM EDTA

whereas media obtained from cells maintained at 0°C, or from cells incubated at 37°C after the temperature shift, were unable to prevent binding. That inhibition of binding activity resulted from a competition for ligand binding was demonstrated by the following experiments. Incubation of cells with conditioned media (that is, media from a temperature shift) followed by washing did not prevent the subsequent binding of ligand to cells. This result indicates that conditioned media did not alter surface receptors per se. Addition of  $\alpha M \cdot ^{125}I\text{-T}$  to cells, such that saturating concentrations of ligand were approached, resulted in increased cell binding. This result demonstrates again that cell-bound receptors were unaltered by incubation with conditioned media. Addition of conditioned media to cells whose receptors were occupied with  $\alpha M \cdot ^{125}I\text{-T}$  did not result in a loss of cell-bound radioactivity, indicating that conditioned media did not alter preformed cell receptor-ligand complexes. These results suggest that the inhibitory effect of conditioned media resulted from the presence of factors that competed with cell-surface receptors for binding of  $\alpha M \cdot ^{125}I\text{-T}$ .

The inhibitory activity in conditioned media was nondialyzable indicating that it was macromolecular. After centrifugation at 100,000 g (60 min) it was only partially sedimentable. The inhibitory activity was heat sensitive, complete activity being lost after heating at 50°C for 2 min. Although the inhibitory activity could be concentrated, we were unable to concentrate the material to the point at which complete inhibition of binding activity occurred; at most, we only inhibited 80% of binding activity. Whether the resistant fraction indicates heterogeneity in the probe or in the receptor remains to be determined. Although these results are consistent with the presence of soluble receptors, we have been unable to define a stable soluble  $\alpha M \cdot ^{125}I\text{-T}$  receptor complex. Possible reasons for this result will be discussed below.

Due to our inability to quantify extracellular receptors, or to define a stable soluble receptor-ligand complex, the above data, at best only suggest that receptors are shed into the medium. Thus the possibility still exists that the temperature shift-induced decline in receptor number reflects alterations in either cell shape or volume. This possibility was excluded by the observation that isolated membranes from temperature-shifted cells reflected the decrease in ligand binding. As demonstrated elsewhere (6) the characteristics of  $\alpha M \cdot ^{125}I\text{-T}$  binding to membranes in cell homogenates was almost identical to the binding of ligand to receptors on intact cells. The data in Table IV demonstrate that temperature shifted cells exhibit reductions

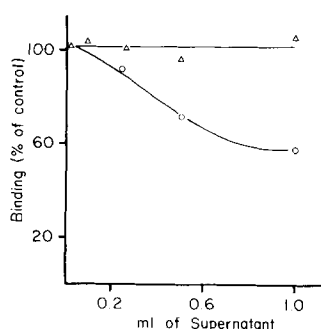


FIGURE 4 Effect of media from cells exposed to a temperature shift on the binding of  $\alpha M \cdot ^{125}I\text{-T}$  to cells. Cells ( $10^7$  cells/ml) incubated in HBSS with 5 mM glucose were subjected to a temperature shift. The cells were centrifuged at 200 g for 10 min and the supernatant was removed. The supernatant was centrifuged at 10,000 g (30 min) to remove

any cellular debris. Media obtained from temperature shifted cells that had been washed after the temperature shift and incubated for 60 min at 37°C was treated similarly. The ability of these preparations to inhibit the binding of  $\alpha M \cdot ^{125}I\text{-T}$  was determined as described in Materials and Methods. Media from temperature shift (O). Media from 0°C incubated cells ( $\Delta$ ).

TABLE IV

Effect of a Temperature Shift on the Binding of  $\alpha M \cdot ^{125}I$ -T to Whole Cells and Membrane Fragments

Experiment	Sample	Specific activity	
		$fmol/\mu g$ protein $0^\circ C$	$\Delta T$
1	Cells	0.33	0.11
	Membranes	1.05	0.69
2	Cells	0.17	0.14
	Membranes	1.5	0.33
3	Cells	0.07	0.04
	Membranes	0.22	0.13

Preparations of cells were divided into aliquots. One aliquot was incubated and maintained at  $0^\circ C$  while the other aliquot was subjected to a temperature shift. Samples of both aliquots were taken for the measurement of specific binding. The remainder of the aliquots were homogenized and binding of  $\alpha M \cdot ^{125}I$ -T to membrane fragments was determined as described in Materials and Methods.

in both membrane-bound and cell-bound ligand. These results strongly suggest that the reduction in binding activity did not result from an alteration in cell shape but, in fact, from a decline in receptor number.

#### Evidence that the Temperature Shift Results in the Loss of a Specific Class of $\alpha M \cdot P$ Receptors

The above data indicate that a fraction of receptors was lost from cells after a temperature shift. The fact that not all receptors were capable of being shed indicates heterogeneity among receptors. Further studies suggest that surface receptors, by other criteria, may be heterogeneous and that temperature shifts result in a selective loss of one class of receptor. Extraction of cells, bearing  $\alpha M \cdot ^{125}I$ -T bound to surface receptors, with Triton X-100 led to a loss in cell-associated ligand (Fig. 5). The data demonstrate that 45% of the initially bound radiolabel was extracted by low concentrations of Triton X-100, whereas the remaining radiolabel was resistant to even high concentrations of Triton. That the Triton-resistant radiolabel was accessible to the external media was demonstrated by the observation that it was completely released with 1 mM EDTA, an agent demonstrated to dissociate surface-receptor bound but not internalized ligand complexes (8).

The loss in radioactivity did not result from removal of  $^{125}I$ -T from the  $\alpha M \cdot ^{125}I$ -T complex<sup>1</sup>. Triton-extracted radioactivity could be immunoprecipitated by guinea pig antisera directed against rabbit  $\alpha M$ . Additionally, the extracted radioactivity exhibited a velocity sedimentation pattern in sucrose gradients identical to  $\alpha M \cdot ^{125}I$ -T that had never been exposed to Triton X-100. If cells were incubated with  $\alpha M \cdot ^{125}I$ -T subsequent to a temperature shift, the majority of cell-bound ligand was resistant to Triton extraction, although it could still be removed with 1 mM EDTA. These results suggest that with respect to Triton extractability, surface receptors are heterogeneous and that the temperature shift resulted in the selective loss of a particular class of surface receptors.

<sup>1</sup> Earlier studies suggested that proteases were bound to  $\alpha M$  by non-covalent bonds. However, recent studies indicate that radiolabeled plasmin (4) or trypsin (Kaplan and Keogh, unpublished observations) remain associated with  $\alpha M$  subunits even after being boiled in SDS and mercaptoethanol. This observation indicates a covalent association between  $\alpha M$  and, at least, serine-dependent proteases.

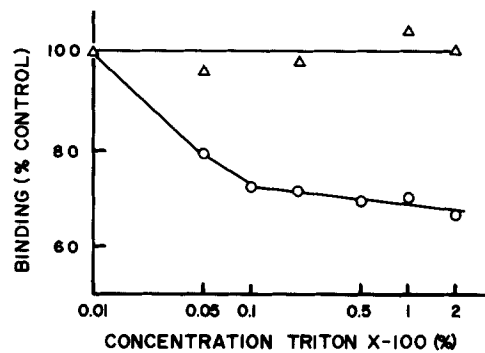


FIGURE 5 Effect of the temperature shift on the ability of Triton X-100 to extract radioactivity from control and temperature shifted cells. Cells that had been maintained at  $0^\circ C$  or subjected to a temperature shift, were incubated with  $\alpha M \cdot ^{125}I$ -T for 60 min. at  $0^\circ C$ . Cells were then washed twice with cold HBSS and incubated in different concentrations of Triton X-100 (in buffer A) for 60 min at  $0^\circ C$ . At the end of the incubation the sample was centrifuged for 5 min in a microfuge ( $\approx 5,000 g$ ). The supernatant was removed and the pellet washed twice with the appropriate concentration of Triton X-100. The data is plotted as the ratio of radioactivity pelletable in Triton-treated extracts relative to the amount of radioactivity pelletable in cells not extracted with Triton. Radioactivity bound to control cells (O). Radioactivity bound to temperature shifted cells ( $\Delta$ ).

#### Ligand Accumulation by Control and Temperature-shifted Cells Incubated at $20^\circ C$

The above results indicate that surface receptors exhibit heterogeneous behavior with respect to extraction by Triton X-100. We therefore asked if there were differences in the rate of ligand accumulation between the control and temperature-shifted cells. Since our previous results demonstrated that the rate of receptor loss was substantially reduced in cells incubated at temperatures below  $24^\circ C$  we examined the rate of ligand accumulation by control and temperature shifted cells incubated at  $20^\circ C$ . The data in Fig. 6 demonstrate that both control and temperature-shifted cells at  $20^\circ C$  accumulate radiolabeled ligand above the level bound to cells incubated at  $0^\circ C$ .

While the amounts of ligand accumulated by control and temperature-shifted cells were different, the rate of uptake (the slope of the accumulation curve) was almost identical. The difference between the two curves could be related to a difference in initial ligand binding activity (i.e. the y-intercept). This difference correlates well with the difference in receptor binding activity seen in control and temperature shifted cells incubated at  $0^\circ C$ . Further studies indicated that the twofold difference in ligand binding activity between control and temperature-shifted cells was maintained for up to 2 h at  $20^\circ C$  regardless of whether or not cells were exposed to ligand (data not shown). Under the present experimental conditions there was no detectable ligand catabolism by either cell population incubated at  $20^\circ C$ . These results suggest that although control cells exhibit twice the ligand-binding capacity of temperature-shifted cells the rate of ligand uptake was the same in both populations.

Further evidence demonstrating that control and temperature shifted cells exhibit the same rate of ligand uptake is based on the observation that both cell populations exhibit identical amounts of cell-associated radioactivity after washing with  $Ca^{2+}$ ,  $Mg^{2+}$ -free HBSS containing 5 mM EDTA (Fig. 7). This procedure removes cell surface bound but not internalized

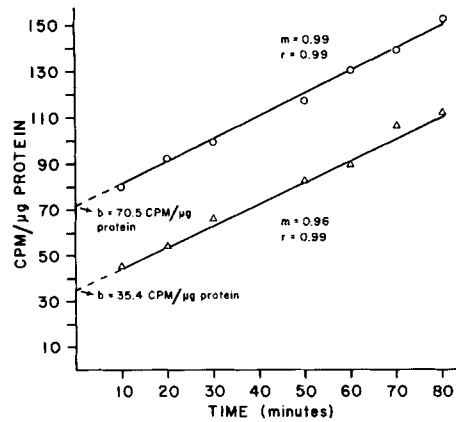


FIGURE 6 Ligand uptake by control and temperature shifted cells incubated at 20°C. An aliquot of cells was subjected to a temperature shift (0°C → 37°C → 0°C). The number of surface receptors on an aliquot of control cells and temperature shifted cells was determined at 0°C. Both temperature shifted and control cells were then incubated at 20°C with  $\alpha M \cdot ^{125}I$ -T and at specified times the amount of specifically bound cell-associated radioactivity determined. The data were analyzed using the formula for a straight line,  $y = mx + b$ , and by linear regression analysis. The symbols denote the slope (m), the y-intercept (b), and the correlation coefficient (r). Radioactivity bound to control cells (O). Radioactivity bound to temperature shifted cells ( $\Delta$ ).

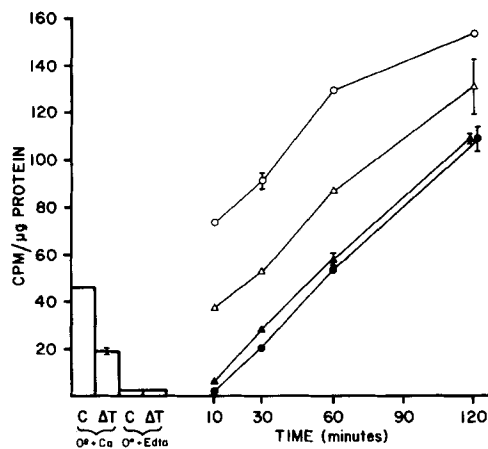


FIGURE 7 Intracellular ligand accumulation by control and temperature shifted cells incubated at 20°C. The protocol for this experiment was identical to that in Fig. 6 with the exception that, at each specified time point, aliquots of cells were washed with either HBSS or  $Ca^{2+}$ ,  $Mg^{2+}$  free HBSS containing 5 mM EDTA. The bars denote radioactivity bound to control (C) and temperature shifted cells that had been incubated with ligand at 0°C and washed with either HBSS (total cell bound radioactivity) or  $Ca^{2+}$ ,  $Mg^{2+}$  free HBSS containing 5 mM EDTA (intracellular radioactivity). The error bars denote the SEM.

Radioactivity associated with control cells (O) and temperature shifted cells ( $\Delta$ ) at 20°C after washing with HBSS. Radioactivity associated with control cells (●) and temperature shifted cells ( $\blacktriangle$ ) at 20°C after washing with  $Ca^{2+}$ ,  $Mg^{2+}$  free HBSS containing 5 mM EDTA.

ligand (8). This result suggests that only a portion of the receptors on control cells internalize ligand (or are reused) at rates comparable to the bulk of receptors on temperature-shifted cells. This conclusion is reinforced by the results of the experiment presented in Fig. 8 in which the rate of internalization was measured directly in both cell populations. Control and temperature-shifted cells were incubated at 0°C with  $\alpha M \cdot$

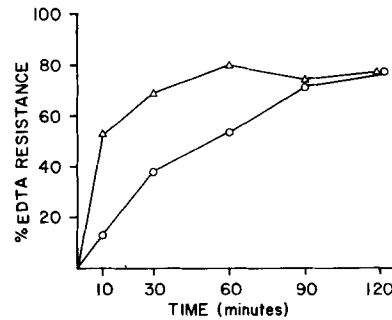


FIGURE 8 Rate of ligand internalization at 20°C by control and temperature shifted cells. Control and temperature shifted cells were incubated at 0°C with  $\alpha M \cdot ^{125}I$ -T for 60 min. The cells were washed with HBSS to remove nonbound ligand, and then incubated at 20°C. At specified time points aliquots of cells were removed and washed with either HBSS or  $Ca^{2+}$ ,  $Mg^{2+}$  free HBSS containing 5 mM EDTA and the amount of specifically bound cell associated radioactivity determined. At the start of the experiment specific binding to control and temperature shifted cells were respectively 45.0 and 19.5 cpm/ $\mu g$  protein. EDTA resistance is the ratio of cell bound radioactivity associated with cells washed with  $Ca^{2+}$ ,  $Mg^{2+}$  free HBSS containing 5 mM EDTA divided by specifically bound radioactivity associated with cells washed with HBSS. EDTA resistance of cell associated ligand in control (O) and temperature shifted cells ( $\Delta$ ).

$^{125}I$ -T for 60 min to allow surface receptors to be saturated with ligand, and the cells were then washed with HBSS to remove nonbound ligand. The cells were incubated at 20°C and at selected times aliquots were removed and washed with either HBSS or  $Ca^{2+}$ ,  $Mg^{2+}$  free HBSS containing 5 mM EDTA. This procedure allows for a determination of the rate of internalization. As demonstrated by the data, the bulk of cell-bound ligand in temperature-shifted cells became EDTA resistant within 10 min whereas the rate of ligand internalization by control cells was much slower. These results are consistent with the view that control cells exhibit a subpopulation of receptors which, while capable of binding ligand at 20°C, exhibit a slow rate of ligand internalization. This population of receptors is lost as a result of the temperature shift. These results support the view that surface receptors exhibit heterogeneity.

## DISCUSSION

Alteration in the incubation temperature of rabbit alveolar macrophages led to modulation in the number of surface receptors. The data demonstrate that shifting cells (incubated at 0°C) to higher temperatures, led to a marked reduction in the number of surface receptors. Our studies suggest that the reduction in receptor activity resulted from a loss of surface receptors. While we can find evidence for released receptors, we have been unable to rigorously define the relationship between loss of surface receptors and their appearance in the medium. As of yet we have been unable to identify a soluble receptor-ligand complex. We suggest that this may be in part ascribed to an alteration in ligand-receptor affinity. Although  $\alpha M \cdot ^{125}I$ -T receptor complexes on the surface of intact cells exhibited a slow rate of dissociation (7), ligand-receptor complexes on membrane fragments exhibited a much faster, although variable, rate of dissociation. Whether the rate of dissociation reflected the action of hydrolytic enzymes, of which macrophages are an extremely rich source, or some other alteration in ligand affinity, has not yet been determined. However, alterations in ligand affinity leading to an increased rate of dissociation would preclude demonstration of soluble receptor-ligand complexes by the methodology used.

Two other lines of evidence indicated that the temperature shift resulted in a loss of receptors. (a) The temperature shift

did not alter receptor-ligand affinity, and (b) membranes obtained from temperature-shifted cells demonstrated a reduction in binding activity. While the data are consistent with a loss of receptors the mechanism behind the loss of receptors is currently unknown. Experiments using pharmacological agents indicated that neither microtubules nor microfilaments were involved. As a working hypothesis, we suggest that the loss of receptors may result from a temperature-induced redistribution in membrane lipids that leads to aggregation and exfoliation of classes of membrane components. It remains to be determined whether the temperature shift-induced loss of sites is totally the result of an experimental manipulation, or in fact, an exacerbation of a normal physiological process.

Examination of the literature indicates that a temperature shift-induced loss of membrane components is not restricted to alveolar macrophages and may be observed in a variety of cell types. Gergely and co-workers (12) demonstrated that temperature shifts induced the shedding of Fc and C<sub>3</sub>b receptors from normal human peripheral lymphocytes, as well as from leukemic cells. Recently Koch and Smith (9) suggested that P<sub>815</sub> cells, a murine mastocytoma, spontaneously shed large amounts of membrane in the form of vesicles, a process they referred to as "exfoliation." However, analysis of their data (cf. legends to Fig. 1 and Fig. 2 in reference 9) suggests that the majority of membrane release occurred within 60 min of shifting cells from 4°C to 37°C. Thus it appears that rather than spontaneously shedding membrane, the appearance of released membrane components may have resulted from a temperature shift.

While the above observations indicate that temperature shifts may induce membrane loss in a number of cell types, evidence exists suggesting such loss may be selective; i.e. only a subset of all membrane proteins may be lost. Koch and Smith (9) determined that P<sub>815</sub> cells "exfoliated" 50% of their H<sub>2</sub> antigen but only 5% of <sup>125</sup>I-labeled membrane proteins. We observed a loss of receptors for αM·T complexes but no reduction in the level of the plasma membrane ectoenzyme alkaline phosphodiesterase. Sarmay et al. (12) observed different patterns of C<sub>3</sub>b and Fc receptor shedding in lymphocytes from different chronic leukemic patients.

It should be stressed that only a fraction of the total receptor population was lost due to the temperature shift, and that a second immediate temperature shift did not effect a further reduction in ligand binding. These results indicate heterogeneity in the population of receptors. Further studies demonstrate that, with respect to other parameters, the population of surface receptors is heterogeneous. Our data indicate that 40% of surface receptor-bound αM·<sup>125</sup>I-T was extracted with low concentrations of Triton X-100, whereas the remainder resisted extraction with even higher (2%) concentrations of Triton X-100. The receptors lost, as a result of the temperature shift, appear to be almost exclusively those that were extractable with low concentrations of Triton X-100. Analogous results have recently been reported by Schecter and Bothuen (13). They demonstrated that 75% of nerve-growth-factor receptors on a permanent line of nerve-growth-factor responsive cells could be extracted with low concentrations of Triton X-100. The remaining 25% of the receptor population was resistant to extraction. The hypothesis was advanced that the resistance of receptors to Triton extraction resulted from some interaction, as of yet undefined, between receptor and cytoskeletal elements.

Further evidence for receptor heterogeneity arose out of an examination of the rate of ligand uptake in control and tem-

perature-shifted cells incubated at 20°C. Since the rate of receptor loss was severely reduced at temperatures below 24°C we initially thought that evaluation of ligand uptake at 20°C by control and temperature shifted cells would provide a critical test of the recycling hypothesis. Control cells would have twice the number of surface receptors as temperature-shifted cells and if surface receptors are reused then control cells should exhibit a twofold increase in ligand uptake. As demonstrated by the data in Figs. 6 and 7 the rate of ligand uptake was the same for both cell populations. The explanation for this result appears to lie in the fact that control cells incubated at 20°C have a subpopulation of receptors which, while capable of binding ligand, exhibit a slower rate of internalization and thus reutilization. That these receptors can internalize ligand, albeit at a much slower rate than receptors on temperature-shifted cells, is suggested by the observation that given enough time comparable percentages of receptor bound ligand became EDTA resistant on control and temperature-shifted cells. We hypothesize that the population of slowly internalizing receptors is the same as the population of receptors that are lost from cells as a result of the temperature shift. One possible explanation for receptor heterogeneity is that receptors may be localized in different lipid domains and thus receptors would be topologically heterogeneous.

Of interest was the observation that at 37°C only unoccupied receptors and not receptor-ligand complexes were released from cells (Table III). The half-time for internalization of ligand at 37°C was 2 min (8), whereas the loss of receptors takes ~15–30 min. It is possible that ligand bound to receptors may be internalized and the non-occupied receptors are susceptible to a temperature shift.

While the mechanisms behind the temperature-induced loss of membrane components remain to be understood, two points strike our attention. (a) It is a routine feature of many studies investigating membrane fluidity or receptor metabolism to incubate cells at 0°C before incubation at 37°C, the objective being to occupy receptors with ligand such that ligand-receptor complexes can be studied independent of the time required to form the complex. Our studies indicate that in many instances the results obtained may reflect not the normal fate of a receptor-ligand complex but an altered metabolism due to a temperature shift. Certainly we feel that studies of ligand-induced shedding need to be reevaluated. (b) The ability of temperature shifts to release selective classes of membrane components in a manner which does not destroy cell integrity suggests that this might be a useful procedure to obtain material for isolation or analysis of membrane components. For example, immunological analysis by monoclonal antibody techniques of material released as a result of a temperature shift may allow not only for identification of receptors but also provide insight into the topological relationship of membrane components.

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