

Washing Macrophage Suspensions Inhibits Protein Synthesis (39734)¹FRANK ULRICH^{2, 3}*Surgical Research Unit, Veterans Administration Hospital, Boston, Massachusetts 02130, and Department of Surgery, Tufts Medical School, Boston, Massachusetts 02111*

I reported recently that treatment of rabbit alveolar macrophages with trypsin causes inhibition of protein synthesis (1). Indirect evidence indicated that the inhibition was due to changes in metabolism resulting from the action of the enzyme at the cell surface. During these studies it was necessary to wash control and enzyme-treated macrophages before pulse-labeling them with radioactive leucine. When control cells which had been washed were compared with control cells which had not been washed, it was found that the washed control cells incorporated significantly less radioactive leucine into protein than the unwashed control cells. A more detailed study of this observation is the subject of this report.

Materials and methods. With the following exceptions, all of the materials and methods used have been described (1, 2). Most of the experiments were done at least twice and duplicate values in all of the experiments varied by less than 5% from the mean and usually were between 1 and 3%. Macrophages were washed by gently suspending 5×10^8 cells with a glass rod in 10 ml of ice-cold KRP⁴ and centrifuging at 4° for 5 min at 250g_{max}.

Macrophage monolayers were prepared in 35 × 10-mm Falcon plastic tissue culture dishes. A 1-ml macrophage suspension in KRP (10⁷ cells) was added to the dish and put on a slide-warmer at 37°. After 45 min, the medium and any nonadherent cells were removed by suction. When monolayers

were prepared from unwashed or from three-times-washed cell suspensions, the monolayers were rinsed twice with 1-ml amounts of KRP before the addition of radioactive leucine. In other experiments designed to study the effects of washing the monolayers, control monolayers were rinsed twice with KRP and washed monolayers were rinsed six times with KRP before the addition of radioactive leucine.

KRP (1 ml) + 5 mM glucose containing 0.025 μCi of [¹⁴C]leucine (L-[1-¹⁴C]leucine; 312 mCi/mmmole) was pipetted on to the monolayers after first suctioning off the wash fluid. The monolayers were kept at 37° and incubation was stopped by suctioning off the radioactive medium and immediately rinsing the monolayer twice with 1 ml. amounts of ice-cold 10 mM leucine in 0.9% NaCl. The monolayers were scraped with a rubber policeman into 1 ml. of 10 mM leucine in 0.9% NaCl. A 0.5 ml. aliquot of the cell suspension was mixed with 0.5 ml. 10% TCA-10 mM leucine, kept at 4° overnight, and then heated for 15 min. at 90°. The precipitated protein was filtered on 25 mm diameter 0.45 μm Millipore filters using a Millipore sampling manifold and washed four times with 1 ml. amounts of 5% TCA-5 mM leucine. The filters were air dried and their radioactivity determined as described previously (1).

Results. Effects of repeated washing. When macrophages were washed repeatedly with KRP, the incorporation of [¹⁴C]leucine decreased progressively as the number of washings was increased (Table I, experiment 1). The relatively small loss of cell protein did not reflect the much larger decrease in leucine incorporation.

Repeated washing could have leached compounds from the macrophage essential for optimum protein synthesis. If this did occur, resuspending cells in the same medium instead of fresh medium after each

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⁴ Abbreviations used are: KRP, Krebs-Ringer phosphate buffer, pH 7.4, without Ca²⁺; MEM, Eagle's minimum essential medium for suspension cultures.

centrifugation ("pseudowashing") might minimize or abolish the inhibition of leucine incorporation. The second experiment in Table I shows that three pseudowashes reduced leucine incorporation as effectively as three washes in which the cells were resuspended with fresh KRP after each centrifugation.

That cell destruction could not account for the inhibition of protein synthesis is shown by the experiment in Table II. Macrophages were washed three times with KRP and incubated at 37° for 30 min with either opsonized heat-killed ³H-labeled *E. coli* (2) or [¹⁴C]leucine. The washed cells exhibited a 35% reduction of [¹⁴C]leucine incorporation, but no significant alteration in protein or DNA content. Since phagocytosis of *E. coli*⁵ also remained unchanged after washing, it is unlikely that a significant reduction in cell viability caused the reduction in leucine incorporation.

The differences in specific activity between Tables I and II may be due to differences in the cell suspensions which were isolated from different rabbits on different days. The data for each experiment, irrespective of whether more than one experiment is presented in a table, were obtained from different cell preparations. Specific activities like those in experiment 2 of Table I were observed more frequently than the higher specific activities. Despite these differences in specific activities, the effects of washing on the percentage of inhibition were consistently similar in different experiments. For example, in Table I, in spite of a sixfold difference in control specific activities between experiments 1 and 2, cells washed three times showed a 59% inhibition of [¹⁴C]leucine incorporation in experiment 1 and a 53% inhibition in experiment 2.

Effects of washing followed by incubation in MEM on inhibition of protein synthesis. When ascites cells are incubated in media deficient in essential nutrients, a breakdown of polyribosomes together with a decrease in the synthesizing activity of the polyribo-

TABLE I. EFFECTS OF WASHING OR PSEUDOWASHING ON PROTEIN SYNTHESIS BY MACROPHAGES.^a

Number of washings	Cell protein (mg)	Specific activity (cpm/mg of protein)	Change in specific activity, (%)
Experiment 1			
0	9.3	5266	—
1	9.0	3845	-27
2	8.7	2543	-52
3	8.8	2141	-59
4	8.7	1518	-71
6	8.2	989	-81
Experiment 2			
Unwashed	10.4	902	—
Pseudowashed 3 ×	10.4	375	-58
Washed 3 ×	9.9	420	-53

^a In both experiments after washing, macrophages were resuspended in KRP containing 5 mM glucose and pulse-labeled for 30 min at 37° with [¹⁴C]leucine. In experiment 2, pseudowashed cells were *not* resuspended in fresh KRP after each centrifugation. Regularly washed cells were resuspended with fresh KRP after each centrifugation. Incubations were done in duplicate in these and all other experiments.

TABLE II. EFFECTS OF WASHING ON PHAGOCYTOSIS OF ³H-LABELED *E. coli* AND ON PROTEIN SYNTHESIS BY MACROPHAGES.^a

Number of washings	Cell protein (mg)	Cell DNA (μg.)	<i>E. coli</i> uptake (%)	¹⁴ C specific activity (cpm/mg of protein)
0	5.5	120	39.8	13,128
3	5.6	127	38.3	8,584

^a Unwashed or 3×-washed macrophages were incubated at 37° for 30 min in KRP-5 mM glucose with either [¹⁴C]leucine or ³H-labeled *E. coli*.

somes occurs (3). These effects are reversible. Since washing of the macrophages could have resulted in a similar metabolic defect, experiments were performed to determine whether the inhibition of protein synthesis was reversible. Macrophages were washed and then incubated for several hours in Eagle's MEM supplemented with 20% calf serum. After incubation the cells were centrifuged, resuspended with fresh MEM (leucine omitted), and pulsed for 30 min with [¹⁴C]leucine. The results (Table III) show a striking increase in protein synthesis after incubation in MEM, both in the washed and in the control cells. In the first experiment, where the cells were washed three times, 22 hours of incubation reduced

⁵ The number of *E. coli* added to the macrophage suspensions was such that their uptake was not limited by their numbers.

TABLE III. EFFECTS OF WASHING FOLLOWED BY INCUBATION IN MEM ON PROTEIN SYNTHESIS BY MACROPHAGES.^a

Number of washings	Incubation in MEM (hr)	Cell protein (mg)	Specific activity (cpm/mg of protein)	Change in specific activity (%)
Experiment 1				
0	0	7.3	1514	
3	0	6.2	409	-73
0	22	4.9	6109	
3	22	3.0	2599	-57
Experiment 2				
0	0	5.8	735	
1	0	5.6	571	-22
0	5	5.5	2683	
1	5	5.1	2637	0
0	22	4.7	3006	
1	22	4.5	2954	0

^a Unwashed or washed macrophages were incubated at 37° in MEM supplemented with 20% calf serum. After incubation, the cells were centrifuged, resuspended with fresh MEM (leucine and serum omitted), and pulsed for 30 min at 37° with [¹⁴C]leucine.

the inhibition from 73 to 57%. In the second experiment, where the cells were washed only once, both 5 and 22 hr of incubation reduced an inhibition of 22% to zero. Some of the marked loss of cell protein after 22 hr of incubation may be due to cellular breakdown since, under the conditions of these experiments, the macrophages do not divide and grow.

The three- to fourfold increase in leucine incorporation by unwashed cells after incubation in MEM may be due to the method used for isolating the cells. The macrophages are washed out of the lungs with large volumes of isotonic saline, and after the lung washings are pooled and centrifuged, the pelleted cells are washed once with KRP before being used. Therefore the cells have already been washed before the start of the experiment.

The progressive increase in protein synthesis following incubation in MEM suggested synthesis of a factor which had been removed during washing. To test this, three-times-washed cells were incubated in MEM containing either 10 μ g/ml of cycloheximide or 10 μ g/ml of actinomycin D. At the end of the incubation, the cells were washed to remove the inhibitor and then pulsed with [¹⁴C]leucine. With the concentrations used, cycloheximide inhibited [¹⁴C]leucine incor-

poration 90–95% and actinomycin inhibited [¹⁴C]uridine incorporation 79%. The results indicated that both cycloheximide and actinomycin slowed but did not prevent the stimulation of protein synthesis.

Effects of washing on protein synthesis by macrophage monolayers. In order to determine whether protein synthesis was also inhibited in macrophage monolayers, two types of experiments were performed, typical examples of which are presented in Fig. 1. When macrophages were washed *before* establishing monolayers, washing resulted in a small but significant inhibition of protein synthesis (Fig. 1A). When macrophages were washed *after* monolayers were established, protein synthesis was not significantly different in the washed and unwashed cells (Fig. 1B). Washing monolayers is probably a milder procedure than washing cells in suspension since cells washed in suspension are subjected to both centrifugation and mechanical separation during the washing procedure. The much smaller inhibition of protein synthesis in macrophages which are washed before being plated as monolayers may be due partly to recovery of the cells while they are attaching to the plastic surface.

Effects of washing on protein breakdown. The decreased incorporation of radioactive leucine into protein after washing could have been due to an increase in the intracellular pool of [¹²C]leucine by dilution of the labeled leucine with cold leucine as a result of increased protein breakdown. To test this, a large batch of macrophages was

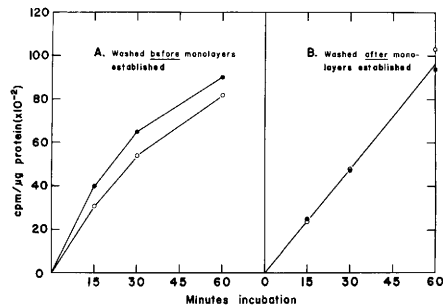


FIG. 1. Effects of washing on incorporation of [¹⁴C]leucine into proteins of macrophage monolayers. Each point on the chart is the mean of duplicate incubations. For details, see text. Closed circles, controls; open circles, washed cells.

pulse-labeled for 2 hr with [³H]leucine. Then half of the cells were washed three times, and the unwashed and washed macrophages were chased for 0.5, 1, or 2 hr with cold leucine (see Table IV and refs. 1 for details). The results (Table IV) showed that, despite a small decrease in the specific activity of the intracellular proteins during the chase, the specific activities were similar in the control and the washed cells.⁶ However, if the difference in the total amount of radioactive protein between 0 and 120 min is calculated, the controls show a loss of 29,404 cpm while the washed cells show a loss of 42,795 cpm. Since this suggested a faster rate of degradation of newly synthesized protein by the washed cells, these results did not rule out the possibility that the decrease in [³H]leucine incorporation by the washed cells was due to an increase in the intracellular pool of [¹²C]leucine. To test this, cells were incubated with [³H]leucine + increasing amounts of [¹²C]leucine. If washing had increased the [¹²C]leucine pool, pulse-labeling the cells with [³H]leucine + [¹²C]leucine should have decreased the difference in [³H]leucine incorporation into the proteins of washed and unwashed cells. The results (Table V) demonstrate that addition of 0.1–3 mM [¹²C]leucine, although markedly decreasing the specific activities of the cell proteins, did not significantly change the percentage of inhibition. These data indicate that washing probably did not increase the [¹²C]leucine pool. It should be pointed out that even though the specific activities with 1–3 mM [¹²C]leucine were relatively low, the differences between duplicates of control and washed cells were always significant. For example, in experiment 2 (Table V), with 2 mM [¹²C]leucine, duplicate specific activities were 572 and 587 for the controls and 463 and 483 for the washed cells.

⁶ To make certain that the macrophages washed after the 2-hr pulse still showed inhibition of protein synthesis, the same batch of cells used for the pulse chase was incubated for 2 hr at 37° without [³H]leucine. Half of the cells were then washed three times and, finally, both washed and unwashed cells were pulse-labeled for 30 min with [³H]leucine. The results (data not presented) showed a 63% inhibition of protein synthesis by the washed cells.

TABLE IV. EFFECTS OF WASHING ON ³H-LABELED PROTEIN CONTENT OF MACROPHAGES AFTER A 2-HR [³H]LEUCINE PULSE FOLLOWED BY A COLD LEUCINE CHASE.^a

Cold leucine chase (min)	Intracellular protein (mg)	Specific activity of intracellular protein (cpm/mg of protein)
Control		
0	4.9	30,256
30	4.8	27,430
60	4.8	26,007
120	4.6	25,837
Washed		
0	4.6	32,828
30	4.5	27,958
60	4.5	26,634
120	4.3	25,166

^a Macrophages (18×10^6) were incubated for 2 hr at 37° in KRP-5 mM glucose which contained 18 μ c of [³H]leucine. Incubation was stopped by adding cold leucine (10 mM final concentration). The suspension was centrifuged and the cells were resuspended with KRP-5 mM glucose-20 mM leucine. One-half of the suspension was kept at 4° and the other half was washed three times at 4° with KRP-5 mM glucose-20 mM leucine. After washing, aliquots of 10^8 macrophages in the above medium were incubated at 37° for different times and incubation was stopped by centrifuging the suspensions at 4°. The rest of the procedures are the same as described under Materials and Methods and in ref. 1.

Net leucine transport and ribosome-polyribosome profiles. The data in Table VI show that inhibition of [³H]leucine incorporation into protein is accompanied by a decrease in the amount of free intracellular [³H]leucine indicating that net leucine transport is depressed in washed macrophages. That decreased leucine transport is probably not the only cause of the inhibition of protein synthesis is demonstrated in the ribosome-polyribosome profiles in Fig. 2. Although washing macrophages caused little, if any, change in the profiles, washing did result in decreased [³H]leucine incorporation in the polyribosome region. This suggests a decrease in the translational activity of the polyribosomes from washed cells.

Discussion. The inhibitory effects of washing macrophage suspensions may be due to mechanical sloughing of part of the cell surface resulting from repeated centrifugation and resuspension. Washing might also leach one or more factors from the cytoplasm which are essential for optimum protein synthesis. Evidence was presented

TABLE V. EFFECTS OF INCUBATING WASHED ALVEOLAR MACROPHAGES WITH INCREASING CONCENTRATIONS OF [¹²C]LEUCINE ON THE INCORPORATION OF [³H]-LEUCINE INTO PROTEIN.^a

Incubation (min)	± Washing	[¹² C]Leucine added (mM)	Specific activity (cpm/mg of protein)	Change in specific activity (%)
Experiment 1				
30	-	0	46,823	
	+		22,178	-53
30	-	0.1	3,151	
	+		1,322	-58
30	-	0.5	842	
	+		436	-48
30	-	1.0	549	
	+		303	-45
Experiment 2				
30	-	0	12,148	
	+		9,722	-20
30	-	1.0	324	
	+		248	-23
120	-	0	20,579	
	+		17,259	-16
120	-	1.0	1,105	
	+		906	-18
120	-	2.0	579	
	+		473	-18
Experiment 3				
120	-	0	23,939	
	+		16,872	-30
120	-	1.5	629	
	+		496	-21
120	-	3.0	388	
	+		279	-28

^a The cells were washed three times in experiments 1 and 2 and four times in experiment 3. In experiments 1, 2, and 3 unwashed cells contained 1.6, 4.7, and 3.2 mg of protein, respectively; washed cells contained 1.7, 4.8, and 3.0 mg of protein, respectively. Total number of cells per incubation was between 4.5 and 8.2×10^7 .

recently which suggested that the inhibition of protein synthesis after trypsin treatment was due to changes in metabolism resulting from the action of the enzyme at the cell surface (1). Washing could be considered the mechanical counterpart of the chemical action of trypsin on the cell surface. In this connection it is of interest that both trypsinized (1) and washed macrophages show a decrease in net leucine transport and a decrease in the translational activity of the polyribosomes which is not accompanied by disaggregation of polyribosomes. These two metabolic changes may be partly or completely responsible for the inhibition of protein synthesis by washed macrophages.

If one assumes that the effects of washing are due to changes of the cell surface, the stimulation of protein synthesis which occurs after incubation in MEM may result from the resynthesis of a cell surface factor essential for optimum protein synthesis which is removed by washing. The stimulation of protein synthesis in the control unwashed macrophages may be due to the method of cell collection which involves repeated irrigation of the lungs with large volumes of saline followed by centrifugation and one cycle of washing with KRP. This single wash may have been sufficient to alter the cell surface so as to reduce the level of protein synthesis.

If the inhibition of protein synthesis is due to changes at the cell surface, the mechanism of this inhibition may be related to a

TABLE VI. EFFECTS OF WASHING ON ACCUMULATION OF [³H]LEUCINE BY ALVEOLAR MACROPHAGES.^a

Incubation (min)	± Washing	[³ H]Leucine incorporation into protein (cpm)	[³ H]Free leucine (cpm)	Total [³ H]leucine in cells (cpm)
Experiment 1				
20	-	5,482	17,093	22,575
20	+	4,243	15,788	20,031
40	-	17,981	16,365	34,346
40	+	11,401	13,656	25,057
60	-	26,591	15,950	42,541
60	+	16,894	13,900	30,794
Experiment 2				
30	-	29,998	10,647	40,645
30	+	21,636	10,156	31,792
60	-	52,743	9,008	61,751
60	+	35,617	9,134	44,751

^a In both experiments, unwashed and three-times-washed macrophages were suspended in 2 ml of KRP-5 mM glucose and pulse-labeled with 1 μ Ci of [³H]leucine at 37° for different times. Incubation was stopped by adding cold leucine to a concentration of 10 mM, rapidly cooling the suspension to 0-4°, and then centrifuging at 4° to pellet the cells. The cells were rapidly washed with approximately 100 vol of ice-cold 10 mM leucine-KRP, re-centrifuged, and the supernatant fluid was removed. The cell pellets were suspended with 5% TCA and processed as described under Materials and Methods. In experiment 1 approximately 2×10^7 macrophages were used (control cells, 1.8 mg of protein; washed cells, 1.6 mg of protein). In experiment 2 approximately 7×10^7 macrophages were used (control cells, 4.3 mg of protein; washed cells, 4.2 mg of protein).

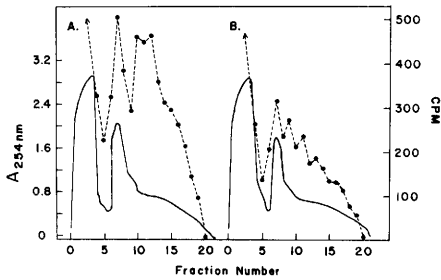


FIG. 2. Effects of washing on ribosome-polyribosome profiles of rabbit alveolar macrophages. Approximately 7.5×10^6 control cells (A) and 7.5×10^6 cells washed three times (B) were pulsed with $15 \mu\text{Ci}$ of [^3H]leucine for 3 min at 37° . The reaction was stopped with cycloheximide followed by rapid cooling to 0° . After centrifugation, the cells were disrupted with 1% Triton X-100-0.5% sodium desoxycholate. Following removal of nuclei by centrifugation, 1.5-ml aliquots of the postnuclear supernates were layered on top of 10 ml of linear 15-40% (w/v) sucrose gradients which were centrifuged at $272,700g$ for 1 hr at 0° . Fractions (0.6 ml) were collected for radioactive measurements. For details, see ref. 1. Abscissa, fraction number; left ordinate, $A_{254 \text{ nm}}$ (solid lines); right ordinate, cpm (interrupted lines). In (A) the radioactivity in the first three fractions was 4131, 3876, and 2318 cpm, respectively. In (B) the radioactivity in the first three fractions was 2775, 2957, and 1885 cpm, respectively.

number of seemingly unrelated observations. The first of these is a recent report that inhibition of protein synthesis occurs when alveolar macrophages ingest latex particles (4). One interpretation of this finding, which has been confirmed in this laboratory (unpublished observations), is that during phagocytosis part of the cell coat is interiorized and thus lost from the cell surface. If there are receptors on the plasma membrane which could regulate protein synthesis, e.g., RNA, physiologic removal of these receptors by endocytosis could interfere with the normal regulation of protein synthesis.

The second observation is that very small

amounts of RNA have been found in the surface membranes of a variety of normal and malignant cells (5-7) including peritoneal macrophages (8). In mouse L cells the surface membranes, which can incorporate radioactive amino acids into hot TCA-insoluble material, contain RNA organized in ribosome-like particles (8). Therefore, removal of part or all of this RNA during washing could adversely affect protein synthesis.

Summary. When suspensions of rabbit alveolar macrophages are washed repeatedly at 4° by centrifuging and resuspending the cells, incorporation of [^{14}C] or [^3H]leucine into protein is inhibited, the inhibition increasing with increasing number of washings. Although washing decreases the protein content of macrophages, it does not change the DNA content or the phagocytosis of ^3H -labeled *E. coli*. Washing results in a depression of net leucine transport and a reduction in the translational activity of the polyribosomes, but it does not appear to change the intracellular leucine pool. Macrophage monolayers which are washed do not show inhibition of protein synthesis.

1. Ulrich, F., *Exp. Cell Res.* **101**, 267 (1976).
2. Ulrich, F., *Amer. J. Physiol.* **220**, 958 (1971).
3. van Venrooij, W. J. W., Henshaw, E. C., and Hirsch, C. A., *J. Biol. Chem.* **245**, 5947 (1970).
4. Massaro, D., Kelleher, K., Massaro, G., and Yeager, H., Jr., *Amer. J. Physiol.* **218**, 1533 (1970).
5. Weiss, L., and Mayhew, E., *J. Cell. Physiol.* **68**, 345 (1966).
6. Weiss, L., and Mayhew, E., *J. Cell. Physiol.* **69**, 281 (1967).
7. Glick, M. C., and Warren, L., *Proc. Natl. Acad. Sci. USA* **63**, 563 (1969).
8. Weiss, L., in "Biological Properties of the Mammalian Surface Membrane" (L. A. Manson, ed.), pp. 73-77. Wistar Institute Press, Philadelphia (1968).

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