The Effect of Tobacco Smoke on Protein Synthesis in Macrophages[1] (37217)

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Exposure to cigarette smoke has been shown to elicit a number of metabolic changes in macrophages both *in vivo* and *in vitro* (1–8). Yeager (9) has recently reported that the acute exposure of *M. bovis* induced rabbit alveolar macrophages to aqueous extracts of tobacco smoke severely reduced the rate of incorporation of ^{3}H-leucine into protein, while other workers have presented electron microscopic evidence of increased protein synthesis in alveolar macrophages lavaged from human smokers' lungs (10).

These two contradictory reports, together with our own observations of increased ^{3}H-RNA synthesis in rat and murine macrophages following acute exposure to tobacco smoke (11), prompted us to reexamine protein synthesis in macrophages in response to a number of tobacco smoke regimes.

Materials and Methods. Alveolar macrophages were obtained from outbred New Zealand white rabbits by the method of Myrvik, Leabe and Fariss (12). Peritoneal macrophages were collected from the peritoneal cavity of C57 black inbred mice in chilled culture medium (13). Macrophages were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% calf serum (CAS), in 35 mm plastic petri dishes (Falcon Plastics, USA) containing stainless steel squares (13). Cultures were maintained in a humidified incubator in an atmosphere of 5% CO_2 for 48–72 hr before experimentation. Primary cultures of fibroblasts were obtained from whole C57 black mouse embryos by trypsinization, grown in Dulbecco's modification of Eagle's minimal medium (DEM) with 10% CAS, and stored in liquid nitrogen. After thawing, secondary cultures of fibroblasts were grown to confluency in DEM + 10% CAS (48-72 hr), and then utilized immediately in experiments or transferred to medium containing 2.5% CAS where maintenance *in vitro* for longer periods was required.

The radiotracer technique for the determination of ^{3}H-protein synthesis was similar to that previously employed to investigate ^{3}H-RNA synthesis (13). Cell cultures on steel squares were transferred to prewarmed medium containing ^{3}H-leucine, at a final concentration of 1.7 μCi/ml for fibroblast cultures, 3.3 μCi/ml for peritoneal and 6.6 μCi/ml for alveolar macrophage cultures. After 40 min labeling at 37°, the cultures were washed sequentially in phosphate buffered saline (PB S) containing 0.05 *M* leucine, PBS, 5% trichloroacetic acid (TCA) containing 0.05 *M* leucine, 5% TCA and finally ethanol:acetone, 3:1. After a final wash in acetone and air drying, they were placed on aluminum planchets and their radioactivity was measured in Nuclear-Chicago gas-flow detector (Model D-47), using a geiger counter gas (98% argon + 2% propane). Each sample was counted to within 1% error. The technique for measuring ^{3}H-RNA synthesis differed from the above in that ^{3}H-uridine (0.85 μCi/ml for all cell types) was substituted for ^{3}H-leucine, and uridine (0.05 *M*) substituted for leucine in the washing solutions.

Cell cultures were exposed to whole cigarette smoke or its vapor phase in a vertical, Perspex chamber as previously described (11). The chamber is designed to expose stainless-steel squares in culture dishes from which medium has been removed to fresh smoke (or its vapor phase) from one ciga-

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rette mixed with air in the ratio of 1:7 for 2 sec, followed by 58 sec exposure to fresh air (1 puff). "Half puff" indicates a 2 sec exposure to smoke/air, 1:14; "2 puffs" indicates 2 × 2 sec exposures to a 1:7 mixture, interspersed with 2 × 58 sec exposures to fresh air.

Aqueous extracts of cigarette smoke were obtained by drawing 35 ml of air through a lighted cigarette into a syringe containing 8.0 ml of culture medium. The syringe was stoppered and shaken manually untill all visible smoke was dissolved in the medium.

^{3}H-5-Uridine (sp act 5.0 Ci/mmole) and uniformly labeled ^{3}H-L-leucine (sp act 250 mCi/mmole) were obtained from the Radiochemical Centre, Amersham, Bucks, U.K. Uridine and L-leucine were from Sigma Chemical Co. (St. Louis, MO). The cigarettes, representative of king-sized filters smoked in Australia, were supplied by the Australian Tobacco Research Foundation.

Results. In Table I, the acute effects of cigarette smoke on the incorporation of ^{3}H-leucine into protein (^{3}H-protein synthesis) are compared for rabbit alveolar macrophages, murine peritoneal macrophages and murine fibroblasts, 30 min after exposure. Macrophage cultures exhibit a decrease in both cell viability and ^{3}H-protein synthesis, while fibroblast cultures show depressed ^{3}H-protein synthesis without a decrease in cell viability.

In Table II, the rates of ^{3}H-protein synthesis in cultures of murine fibroblasts and peritoneal macrophages are shown 30 min and 24 hr after exposure to whole smoke or its vapor phase. Preparations of fibroblasts show a significant depression of ^{3}H-protein synthesis 30 min after exposure to whole smoke or its vapor phase. However, 24 hr later, cultures exposed to whole smoke show greater depression of ^{3}H-protein synthesis than those exposed to the vapor phase.

In cultures of murine peritoneal macrophages, both cell viability and ^{3}H-protein synthesis decrease 30 min after exposure to either whole smoke or its vapor phase. After 24 hr, no further cell death had occurred, and ^{3}H-protein synthetic rates in exposed cultures did not differ significantly from those of control cultures.

The results of exposure of cultures of murine peritoneal macrophages and fibroblasts to aqueous extracts of tobacco smoke are shown in Fig. 1. Macrophages show a gradual linear decrease in the rate of ^{3}H-protein synthesis in the presence of increasing amounts of tobacco smoke extract. The results obtained with fibroblast preparations were ex-

TABLE I. The Acute Effects of Tobacco Smoke Exposure on ^{3}H-Protein Synthesis in Macrophages and Fibroblasts.[a]

	Viable cells ($\times 10^{-5}$)		cpm/10^6 cells			
			Control		Smoked	
Cell types	Control	Smoked	$\bar{x}$	SD	$\bar{x}$	SD
Rabbit alveolar macrophages	1.50	1.20	1853	320	1022	180
					$p < .001$[b]	
Murine peritoneal macrophages	1.61	1.22	2750	195	2150	230
					$.01 > p > .001$[b]	
Murine embryonic fibroblasts	0.48	0.48	4620	320	2615	195
					$p < .001$[b]	

[a] All determinations were carried out 30 min after the exposure of stainless-steel squares containing cells in monolayer to 2 × 2 sec puffs of fresh tobacco smoke. Viability counts were performed by trypan blue exclusion, and represent the number of cells per stainless-steel square. Fibroblasts were maintained in DEM + 10% CAS for 72 hr before exposure, while macrophages were maintained in MEM + 10% CAS for 48–72 hr. The results represent the mean of a minimum of 4 replicates for each determination.

[b] The significance of the differences between smoked and control was determined by Student's *t* test.

TABLE II. Comparison of ^{3}H-Protein Synthesis in Cultures of Murine Peritoneal Macrophages and Fibroblasts 30 min and 24 hr After Exposure to Fresh Tobacco Smoke or Its Vapor Phase.[a]

	Fibroblasts			Peritoneal macrophages		
	Viable cells	cpm/10^6 cells		Viable cells	cpm/10^6 cells	
Treatment	($\times 10^{-5}$)	$\bar{x}$	SD	($\times 10^{-5}$)	$\bar{x}$	SD
Control + 30 min	1.40	5272	120	1.10	4485	541
Smoke + 30 min	1.30	3153	132	0.93	2508	476
		$p < .001$			$.01 > p > .001$	
Vapor phase + 30 min	1.35	3708	122	0.87	3203	346
		$p < .001$			$.02 > p > .01$	
Control + 24 hr	1.20	8597	642	1.11	1029	159
Smoke + 24 hr	1.05	5778	215	0.91	1349	849
		$p < .001$			ns	
Vapor phase + 24 hr	1.12	7513	573	0.86	1151	185
		$.05 > p > .02$[b]			ns[b]	

[a] Cultures were exposed to 2 × 2 sec puffs of fresh tobacco smoke or its vapor phase (obtained with the insertion of a Millipore prefilter No. AP 2004700 over the inlet) as outlined above. Thirty minutes later, half of the cultures were examined for viability and ^{3}H-protein synthesis. The remaining cultures were maintained for 24 hr before assay. The results represent the mean of a minimum of 4 replicates at each sample time.

[b] The significance of the differences between test and control cultures was determined by Student's *t* test.

tremely variable. However, stimulation in rate of ^{3}H-protein synthesis always appeared at one of the intermediate concentrations used.

In Fig. 2, cultures of murine peritoneal macrophages and fibroblasts were exposed to three different levels of fresh tobacco smoke on 4 consecutive days, prior to the determination of the rates of ^{3}H-protein and ^{3}H-RNA synthesis on the fifth day. Fibroblast cultures show little change in synthetic rate, with a moderate depression in cell viabilities at high smoke exposure. In preparations of macrophages, the low exposure (½ puff) produced large increases in both ^{3}H-RNA and ^{3}H-protein synthesis. At the higher exposures (1 and 2 puffs), cell viabilities and differences between control and test synthetic rates progressively decreased.

Discussion. The results presented here are part of a long-term study of the effects of tobacco smoke exposure on the immune system of the murine lung. Our previous data have shown that cells of the immune series show greater susceptibility to the acute toxic effects of tobacco smoke than fibroblastic and epithelioid cells (11, 14).

In the murine system, it is difficult to obtain sufficient alveolar macrophages by lavage for *in vitro* experiments involving ^{3}H-protein synthesis, and consequently peritoneal macrophages have been utilized. Previous work has shown that these cells react to acute tobacco smoke exposure in a similar way to alveolar macrophages with respect to ^{3}H-RNA synthesis and viability in culture (11). The data of Table I also suggest that rabbit alveolar macrophages show similar effects to murine peritoneal macrophages *in vitro* with respect to ^{3}H-protein synthesis after tobacco smoke exposure.

The data of Tables I and II and Fig. 1 support those of Yeager (9), showing that acute exposure to the vapor phase of tobacco smoke, or whole smoke (including aqueous extracts thereof) leads to a depression in the rate of macrophage ^{3}H-protein synthesis in short-term experiments. Table II illustrates that surviving cells "recover" by 24 hr after exposure, and ^{3}H-protein synthetic rates from smoked cultures do not differ from controls. These results contrast with our previous data on ^{3}H-RNA synthesis, which showed a marked stimulation of ^{3}H-RNA synthesis in

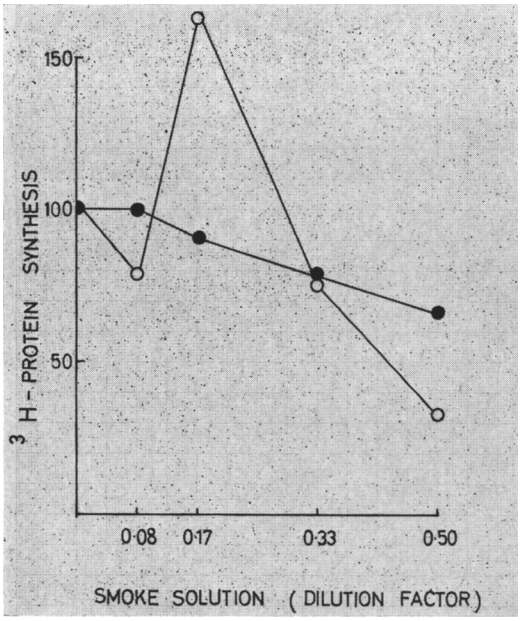

FIG. 1. The effect of varying dosages of aqueous tobacco smoke extract on ^{3}H-protein synthesis in murine macrophages and fibroblasts. Cultures of macrophages (●) and fibroblasts (○) were maintained in MEM + 10% CAS and DEM + 10% CAS, respectively, for 48–72 hr. At the commencement of the experiment, fresh medium containing "smoke solution" (see Materials and Methods) at the dilutions shown was added to the cells. Thirty minutes later, ^{3}H-leucine was added to replicate culture dishes. After a further 40 min, stainless-steel squares containing cells were processed (see Materials and Methods) and viability counts performed. A small depression (10–20%) in cell viability was seen at the higher concentrations. The rate of ^{3}H-protein synthesis for both cell types was determined as cpm/10^6 viable cells, and plotted as a percentage of that seen in control cultures for each dilution of smoke solution employed. A minimum of 4 replicates were employed for each determination shown.

macrophages 30 min after exposure to cigarette smoke, and a decrease in most cultures after 24 hr (11). Fibroblast preparations show a similar depression in ^{3}H-protein synthesis up to 30 min after exposure, but do not show the same degree of "recovery" at 24 hr. These cells show no change in ^{3}H-RNA synthesis 30 min after smoke exposure, and decreased ^{3}H-RNA synthesis 24 hr later (11).

Electron microscopic examination of alveolar macrophages lavaged from human smokers' lungs has provided evidence of highly active protein synthesis (10). Thus the experiments illustrated in Fig. 2 could reflect the *in vivo* situation in the smoker's lung more closely than the short-term experiments outlined above. Unlike fibroblast preparations, which show no significant alterations in either ^{3}H-protein or ^{3}H-RNA synthesis, the macrophages show a marked dose-dependent response, with both ^{3}H-RNA and ^{3}H-protein synthesis greatly increased at low exposures. Consequently, it appears that long-term exposure of macrophages to tobacco smoke leads to the production of highly metabolically active cells.

These data show that macrophages have

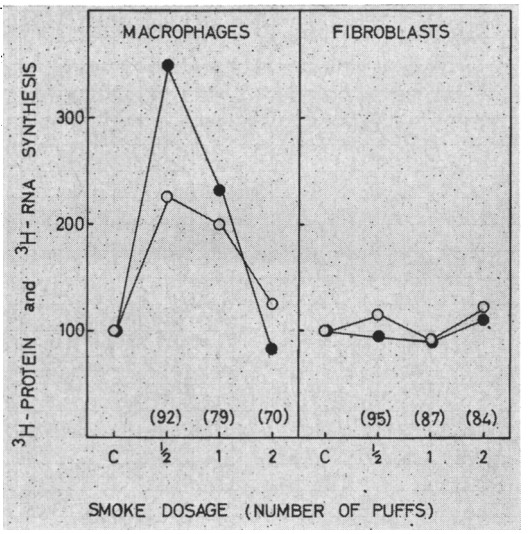

FIG. 2. The effect of long-term tobacco smoke exposure on ^{3}H-protein and ^{3}H-RNA synthesis in cultures of murine macrophages and fibroblasts. Cultures of macrophages were maintained in MEM + 10% CAS for 48 hr, and cultures of fibroblasts for 72 hr in DEM + 10% CAS, prior to changing to DEM + 2½% CAS at the commencement of the experiment. On each of 4 consecutive days cultures received ½, 1 or 2 puffs of fresh tobacco smoke. On the fifth day, viability counts were performed, and rates of ^{3}H-protein (○) and ^{3}H-RNA synthesis (●) were determined and plotted as a percentage of control rates. Cell viability counts (expressed as a percentage of that in control cultures) are shown in parenthesis for each exposure level. A minimum of 4 replicates were employed for each determination shown.

the capacity to undergo significant changes in their basal metabolism in response to a toxic environment. How such changes may affect their important role in the immune response in the smoker's lung will be the subject of further research.

Summary. The results of short-term experiments suggest that exposure to cigarette smoke severely inhibits protein synthesis in macrophages. However, when macrophages are exposed to low levels of cigarette smoke for longer periods, both protein and RNA synthesis show marked increases. These data support electron microscopic evidence of active protein synthesis in alveolar macrophages from human smokers' lungs, and suggest that macrophages have the capacity to adapt to toxic changes in their environment.

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