THE EFFECT OF REAEROSOLIZED FLY ASH FROM AN ATMOSPHERIC FLUIDIZED BED COMBUSTOR ON MURINE ALVEOLAR MACROPHAGES

by

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ABSTRACT

Male $B6CF_1$ mice were exposed to reaerosolized fly ash. The fly ash was collected from the final filter of an atmospheric pressure fluidized-bed coal combustor and reaerosolized from a liquid nitrogen suspension. In four short experiments, (30-36 hrs; 6 hrs/day for 5-6 days) the fly ash was a composite of both start-up and steady-state material. In another experiment of longer duration, (100 hrs; 6 hrs/day over a 4-week period), the fly ash used consisted of material from only steady-state operation of the combustor. Fly ash collected during steady-state operation is about 10 times less mutagenic in the Ames Salmonella test than is fly ash collected during start-up operation of the combustor.

Mice exposed to fly ash in the short experiments showed a 2-4 fold increase in the number of alveolar macrophages 4 days after exposure and these macrophages were full of engulfed particles when exqmined by light microscopy. In in vitro tests 4-26 days after this exposure macrophages from mice were unable to kill a challenge dose of Staphylococcus aureus as effectively as those from control mice. There was impairment in both phagocytic ability and in intracellular killing.

Macrophages from mice exposed for 100 hrs to fly ash collected during steady-state operation were similarly full of engulfed particles when examined 4 days after exposure. However, the ability of these macrophages to kill S. aureus was unimpaired at this time and six months after exposure. These early observations suggest that the presence of mutagenic material on fly ash impairs the ability of macrophages to phatocytize and kill a challenge dose of S. aureus.

INTRODUCTION

Fluidized bed combustion of coal represents an emerging technology with great promise for meeting Environmental Protection Agency performance standards. The Argonne National Laboratory Division of Chemical Engineering operates an experimental bench-scale atmospheric fluidized bed combustor (AFBC) from which the staff of the Division of Biological and Medical Research have the opportunity to evaluate the toxic potential of both the particulate material and the total effluent stream.

We plan to study the effects on animals of exposure to whole, diluted, aged effluent from the AFBC but this report deals only with exposure to particulate material (fly ash). We have focused particular attention on alveolar macrophage (AM) function. The macrophage serves as the primary means of foreign particle removal (Green, 1970) including inhaled pathogens (Goldstein, 1977). Any compromise in their functional integrity could represent a serious health hazard.

The literature on the effects of fly ash *per se* on AM's is scant, although a large body of information exists relative to other environmental toxicants (Goldstein, 1977; Gardner and Graham, 1977). Alarie et al., 1975 reported that long-term exposure of monkeys and guinea pigs did not result in pathological changes in the lungs although fly ash was observed in the AM's by light microscopy. They did not test the functional integrity of these cells. Aranyi et al., 1977 demonstrated cytotoxicity *in vitro* to fly ash from conventional coal combustion which was dependent on particle size. Particles < 2 μm in diameter were more toxic than larger particles. These investigators also found that fly ash coated with heavy metals was much more cytotoxic than was uncoated fly ash.

More recently, Wehner et al. (1979) reported a rather low acute and subchronic toxicity of both nickel enriched fly ash and fly ash in the hamster lung. Fly ash was observed in AM's 2 mo after completion of the subchronic exposure. Functional tests on the AM's were not performed.

We report here AM function following short-term exposure to fly ash collected during start-up operation of the AFBC and AM function after longer exposure to steady-state collected fly ash.

MATERIALS AND METHODS

The fly ash used in these experiments was collected from the final filter of the ANL AFBC (Kirchner et al., 1979b). We had two samples of fly ash available for use. The first sample was a composite of both start-up and steady-state material and it was used in four short experiments, (30-36; 6 hrs/day for 5-6 days) designed to test our exposure facility and to determine acute effects. The second sample was collected during steady-state operation of the combustor only. It was used in an experiment of longer duration (100 hrs; 6 hrs/day over a 4 week period), designed to determine long-term effects of fly ash exposure. The two fly ash examples were similar in all respects except that the sample collected during steady-state operation was about 10 times less mutagenic in the Ames Salmonella test than that containing start-up material (Kirchner et al., 1979a).

The experimental mice were male B6CF$_1$ hybrids (C_{57}Bl6J ♀ x BalbcJ ♂/Anl), approximately 150 days of age. They were exposed to reaerosolized fly ash using the liquid nitrogen system and exposure chambers described by Kirchner et al., 1979a. The exposure system is shown in Figure 1. The system first disaggragates fly ash with an air grinding device followed by further disaggregation by sonication in liquid nitrogen reduced to its freezing point (-210° C). The fly ash is maintained in suspension by stirring and the fly ash-liquid nitrogen mixture is sprayed under pressure into an evaporation chamber where it is mixed with air. The liquid nitrogen rapidly evaporates leaving the particles airborne. Essentially no change occurs in the physical and chemical properties of the fly ash during reaerosolization (Kirchner et al., 1979a). The total particle concentration in this system is 1.3 x 10^8/l of which about 3% reaches the exposure chamber. In the chamber, greater than 95% of the particles are less than 3 μm in diameter and thus well within the respirable range.

At selected times after the completion of the exposures AM's were obtained from the mice using the method described by Fisher et al., 1978. Twenty to thirty

exposed and control mice were used at each time point. The cells were pooled and washed twice in RPMI 1640 medium (Microbiological Associated, Bethesda, MD) containing 10% fetal calf serum, 1% freshly thawed L-glutamine and 100 units/ml of penicillin and 100 µg/ml of streptomycin. The AM's were resuspended in complete RPMI medium and adjusted to a concentration of approximately 1 x 10^6/ml. Viability was determined by trypan blue exclusion and was always greater than 95%. The cells were seeded into 60 x 15 mm plastic petri dishes and allowed to attach for 3 hrs at 37 C in 5% CO_2. The non-adherant population was decanted and the remaining adherant cells were refed with complete medium. Following overnight incubation, the adherant population of AM's was harvested in saline and their ability to phagocytize and kill a challenge dose of Staphylococcus aureus determined. Cytocentrifuge preparations were also made and these were stained with Wright-Giemsa for light microscopy.

The assay used to test the functional integrity of AM's was a modification of that described by Tan et al., 1971. Its biggest advantage is that it differentiates defective phagocytosis from impaired intracellular killing. The test uses lysostaphin (Schindler and Schuhardt, 1964, 1965) a muralytic enzyme which does not enter intact macrophages and which rapidly and specifically eliminates extracellular S. aureus. Briefly, the AM's were incubated with a logarithemic phase S. aureus (ATTC 12600) culture for 2 hrs at 37 C in the presence of fetal calf serum in a shaking water bath. The ratio of S. aureus to AM's was approximately 10:1. Following the 2 hr incubation a sample was withdrawn from control (no AM's) and from tubes containing AM's from fly ash exposed and unexposed mice. These samples were serially diluted in distilled water (to disrupt the macrophages) and the number of viable bacteria determined by plate count. The difference between the count without AM's and with AM's is the total number of bacteria killed. The remaining AM's were centrifuged at 500 xg for 5 min to remove bacteria not associated with cells. The AM pellet was resuspended in saline and incubated with 20 units/ml

of lysostaphin for 20 min. The lysostaphin kills extracellular S. aureus. Trypsin (2.5%) was then added to inactivate the lysostaphin and the incubation continued for an additional 10 min. The AM's were then lysed in distilled water, serially diluted and plate counts made. These counts represent the number of S. aureus ingested but not killed.

RESULTS

Four days after the completion of exposure to fly ash containing start-up material 2-4 times as many AM's were obtained from exposed mice than from unexposed mice. Light microscopic examination of these AM's showed them to be full of engulfed particles (Fig. 2b). We examined slides prepared at each time point and determined the number of macrophages containing particles. The number of macrophages with particles declined as the period from the time of exposure increased (Table 1) but interestingly, 47% of the cells counted still contained particles 26 days after exposure.

Next, we re-examined the slides in an effort to quantitate the number of particles per macrophage and these results are shown in Table 2. Not only did the number of AM's with particles decline with time but the number of particles per macrophage declined over the observation period.

Table 3 shows the functional ability of macrophages from mice exposed to fly ash containing start-up material. At all times after exposure, AM's from exposed mice were unable to eliminate a challenge dose of S. aureus as effectively as those from control mice. Four days after exposure this defect appeared to result from impaired intracellular killing because the percentage of unkilled phagocytized bacteria was high. Impaired intracellular killing is believed to be due to interference with cellular lysosomal enzymes (Vassallo et al., 1973, Goldstein, 1977). Twelve and 26 days after exposure phagocytic ability was still impaired in AM's from exposed mice.

Macrophages were obtained from mice exposed for 100 hrs to fly ash collected during steady-state operation of the AFBC. Four days after completion of the exposure the AM's were full of engulfed particles and appeared similar to those shown in Figure 2b. However, the ability of these AM's to phagocytize and kill S. aureus did not differ from those obtained from unexposed mice. Macrophages tested 6 mo after completion of the 100 hr exposure were also able to kill S. aureus as effectively as those from unexposed mice.

DISCUSSION

Cells which eliminate at least 90% of the initial inoculum are considered to phagocytize and kill normally (Tan et al., 1971). When insignificant reduction in the number of S. aureus is observed the defect may be either in the ability to phagocytize or in the ability to kill the bacteria intracellularly. With poor phagocytic ability one observes a low number of surviving bacteria after the addition of lysostaphin. Conversely, a high number of surviving bacteria after the addition of lysostaphin suggests impaired intracellular killing.

In the short-term experiments reported here, AM's from control mice exhibited unimpaired phagocytosis and killing 4-26 days after the end of the exposure. Macrophages from exposed mice showed impairment in these functions at all time intervals. By 12 and 26 days after exposure repair was evident in the intracellular killing ability of these cells indicating a return of lysosomal enzyme function. The phagocytic capability remained impaired, suggesting damage to the cell membrane (Goldstein, 1977).

In contrast to the effects on AM's following exposure to more mutagenic fly ash for one week we found no impairment in AM function following much longer exposure (100 hrs) to less mutagenic fly ash. Heavy metal analysis of both samples of fly ash as well as surface analysis and scanning electron microscopy showed that the samples were essentially identical (Kirchner et al., 1979a). Preliminary analysis did reveal a higher hydrocarbon content in the mutgenic fly ash. Therefore

our results, while preliminary in nature, suggest that the effect we observed in the short-term exposures was caused by mutagenic hydrocarbons adsorbed on fly ash particles. This hypothesis is supported by the work of Fisher et al., 1979, who found that short-term exposure of mice to mutagenic stack-collected fly ash resulted in decreased AM function. Long-term exposure to less mutagenic fly ash had no effect.

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TABLE 1

Macrophages Containing Start-Up Fly Ash Particles After Exposure[a]

Days after Exposure	Macrophages with particles/ number counted[b]	Percent with particles
2	188/212	89
4	182/215	84
8	172/228	75
12	162/219	73
16	136/264	52
26	126/268	47

[a]Mice were exposed to reaerosolized fly ash for 6 hr/day for 6 days.

[b]Macrophages were obtained by lavage, pelleted and stained with Wright-Giemsa.

TABLE 2

Number of Fly Ash Particles in Macrophages After Exposure

Days after Exposure	Number of macrophages counted							
	No particles		1-10 particles		10-20 particles		> 20 particles	
2	9	(7.7%)	27	(23.3%)	21	(18.1%)	59	(50.9%)
4	13	(10.4%)	29	(23.3%)	25	(20.1%)	57	(45.8%)
8	27	(23.5%)	56	(48.7%)	8	(7.0%)	24	(20.9%)
12	35	(28.9%)	49	(40.4%)	17	(14.0%)	20	(16.5%)
16	64	(56.1%)	23	(20.2%)	12	(10.5%)	15	(13.2%)
26	78	(58.6%)	32	(24.0%)	12	(9.0%)	11	(8.2%)

[a]Mice were exposed to reaerosolized fly ash for 6 hr/day for 6 days.

[b]Macrophages were obtained by lavage, pelleted and stained with Wright-Giemsa.

TABLE 3

Alveolar Macrophage Function After Exposure to Fly Ash

Days after exposure[a]	*S. aureus* killed[b]		*S. aureus* Phagocytized but not killed	
2	Control	50%	Control	47%
	Exposed	25%	Exposed	71%
4	Control	95%	Control	4%
	Exposed	75%	Exposed	20%
8	Control	99%	Control	0.06%
	Exposed	81%	Exposed	NT[†]
12	Control	90%	Control	0.5%
	Exposed	80%	Exposed	2%
26	Control	99%	Control	< 0.1%
	Exposed	71%	Exposed	1%

[a] Mice were exposed to reaerosolized fly ash for 30-36 hrs for 6 hr/day.

[b] Results are expressed as the percent of a challenge dose of bacteria killed or phagocytized.

[†] NT = Not tested.

FIGURE LEGENDS

Figure 1. Reaerosolization system and exposure chambers.

Figure 2a. Alveolar macrophages from an unexposed mouse.

2b. Alveolar macrophages from a mouse exposed to reaerosolized fly ash 4 days after the end of exposure.

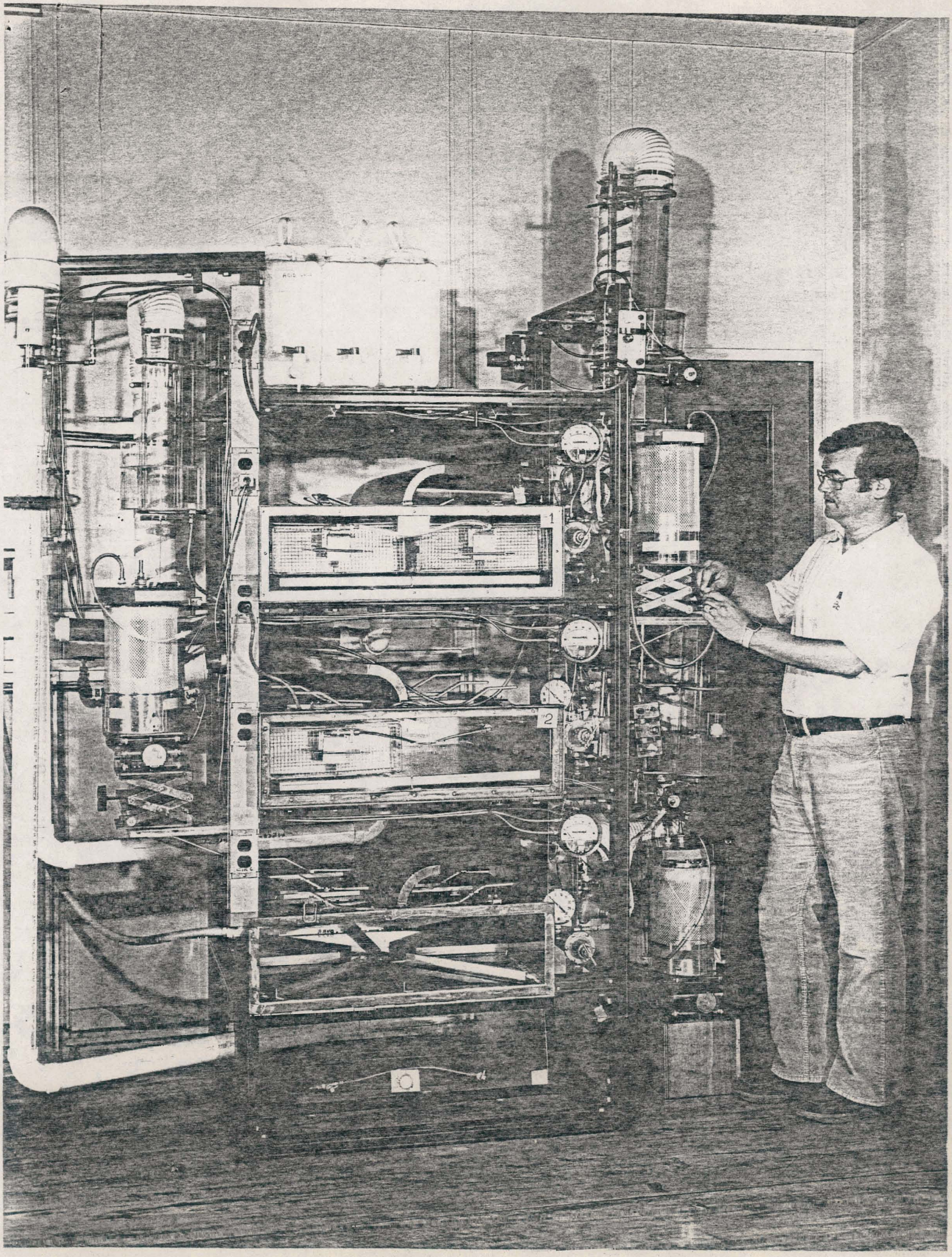
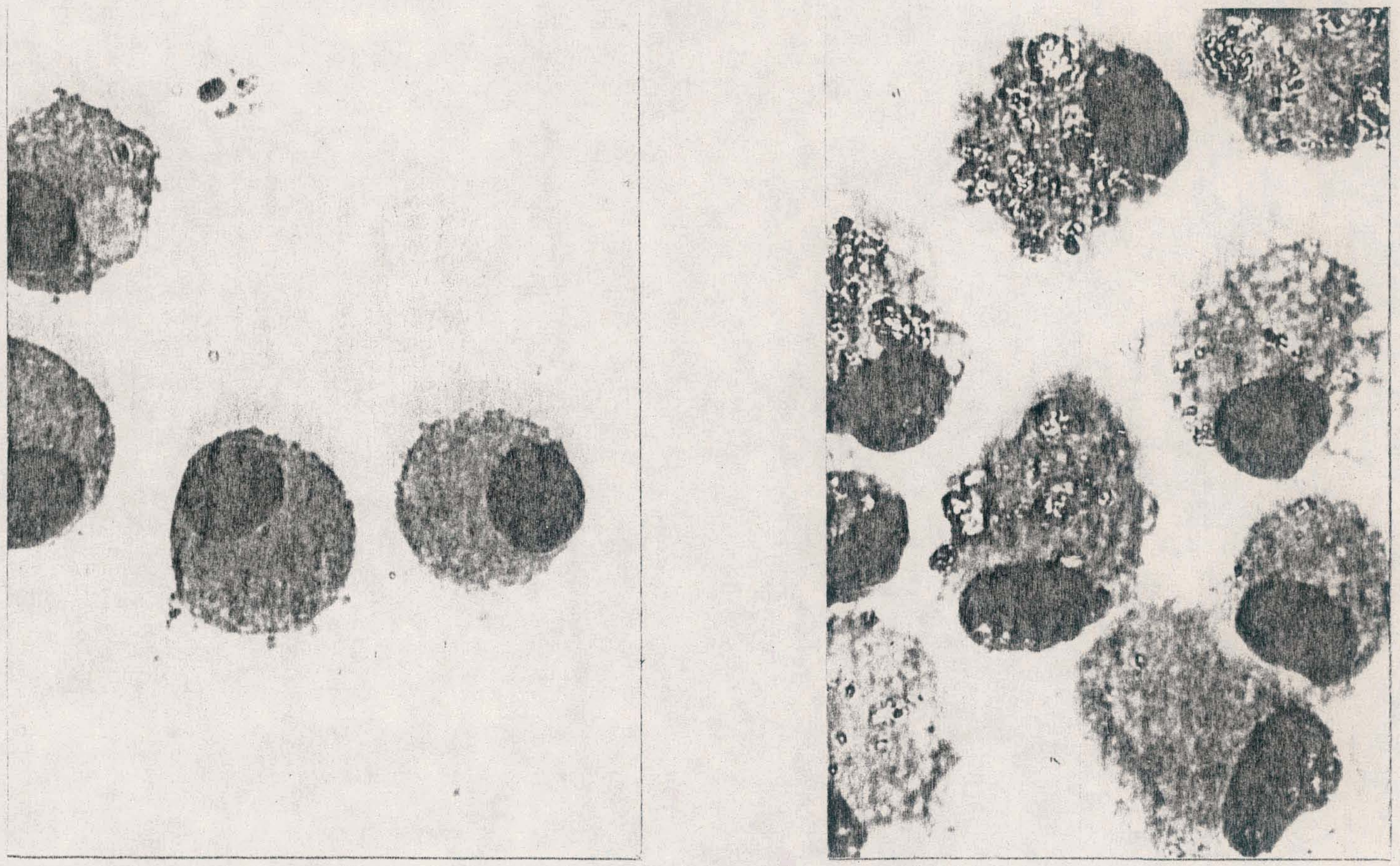


Fig 2



a　　　　　　　　b