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Bioassay for hamster macrophage chemotaxis: application to study particle-lung interactions

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Abstract. Attraction of lung macrophages to particle deposition sites has been demonstrated in different animal species. We reported a threefold increase of the number of macrophages to occur within 40 min after polystyrene particle deposition in hamster airways [Geiser et al. (1994) Am. J. Respir. Cell Mol. Biol. **160:** 594–603]. Complement-derived chemotactic activity is one of the mechanisms postulated for macrophage recruitment. It was the aim of this study to test whether complement-derived chemotactic activity is involved in the rapid recruitment of macrophages to the site of deposited polystyrene particles in hamster airways. We first developed an in vitro cell migration assay for hamster macrophages to assess complement-derived chemotaxis. Second, the bronchoalveolar lavage fluids (BALF) of four hamsters that had inhaled aerosols of polystyrene microspheres were tested for chemotactic activity by this bioassay and compared with BALF of four sham-exposed hamsters. Chemotactic response of macrophages was found toward complement-activated hamster serum, whereas macrophage migration was not increased toward BALF of particle and sham-exposed hamsters. In contrast, macrophage migration to BALF of both groups was reduced by 1.6-fold. Thus, the stimulus for macrophage recruitment to the site of deposited polystyrene particles in hamster airways could not be demonstrated using this bioassay.

Key words. Bioassay; chemotaxis; complement; hamster; lung; macrophages; polystyrene particles.

Macrophages of the lung are crucial defenders against inhaled and deposited particles [1–3]. To perform this task, they have to localize deposited material, migrate to it and phagocytose it. Macrophages have been reported to accumulate at sites of asbestos deposition in rats [4–6], which was explained by chemotaxis [7, 8]. Asbestos fibres and polystyrene particles were shown in vitro to activate complement proteins by the alternative pathway [9–12]. Moreover, inhaled asbestos fibres were reported to activate complement proteins in vivo [13], and the resulting C5a was shown to attract macrophages to the sites of deposited particles [14]. This complement-derived chemotactic activity was demonstrated in rats, mice [15] and baboons [16]. Macrophages of other species like hamsters and guinea pigs, however, did not exhibit complement-dependent chemotaxis in vitro [15, 17, 18], but macrophage accumulation at sites of deposited particles was demonstrated in situ in hamster airways [18, 19]. The rapid recruitment of macrophages to the site of particle deposition in hamster airways was reflected by a threefold increase of macrophage number reported to occur within 40 min after the inhalation of polystyrene particles [19]. Twenty-four hours later the number of macrophages was still up to twofold higher than in airways of control hamsters. To the best of our knowledge, the mechanisms by which these macrophages were attracted remained unknown, as do the effects of complement activation on particle clearance in human lungs [20]. It was the aim of this study to test whether complement-derived chemotactic activ-

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Table 1. Physiological and inhalation data, and protein recovery in BALF*.

Hamster No.	Body weight (g)	$N_{dep} \times 10^5$	Time from beginning of inhalation until BAL (min)	Protein recovery in BALF (mg)
Control group				
1	189	-	-	1.0
2	132	-	-	1.5
3	153	-	-	1.4
4	138	-	-	1.2
Mean	153	-	-	1.3
SD	25.6	-	-	0.2
PSP group				
5	139	7.8	41	2.0
6	136	9.4	44	4.2
7	160	7.4	37	1.7
8	135	5.7	39	1.9
Mean	142.5	7.5	40	2.5
SD	11.8	1.5	3	1.2

*The hamsters were exposed to either room air (control) or aerosols of PSP. The total number of deposited microspheres (N_{dep}) was derived from the continuously monitored flow rates and aerosol particle number concentrations.

Table 2. Number, viability and morphology of BAL cells used for the bioassay*.

Hamster No.	Body weight (g)	Cells recovered by BAL $\times 10^6$	Cell viability (%)	Macrophages (%)†
9	158	4.1	>95	>90
10	127	3.9	>95	
11	131	5.2	>95	>90
12	123	3.8	>95	
13	163	4.6	>95	
14	138	5.4	>95	
Mean over all	140	4.5		
SD	16,7	0.7		

*Untreated hamsters were used as source to recover BAL macrophages for the bioassay. The total number of cells recovered by BAL was assessed using a Neubauer counting chamber. Viability was tested by trypan blue dye exclusion.
†For differential cell counting, the BAL cells of the hamsters nos. 9 and 10 as well as those of nos. 11–14 were pooled. For both groups, 400 cells were counted.

ity is involved in the rapid recruitment of macrophages to the site of deposited polystyrene particles (PSP) in hamster airways. We first developed an in vitro cell migration assay for hamster macrophages to assess complement-derived chemotactic activity. Second, bronchoalveolar lavage fluids of hamsters that had inhaled aerosols of polystyrene microspheres that deposit in airways were tested for chemotactic activity by this bioassay.

Materials and methods

Experimental design. The in vitro cell migration assay was established for hamster macrophages, recovered by bronchoalveolar lavage (BAL), using serum in which complement had been activated by zymosan. This bioassay was used to test BAL fluids (BALF) of four hamsters that had inhaled aerosols of polystyrene particles (PSP) for chemotactic activity and compare them to those of four sham-exposed hamsters (control).

Animals. Fourteen male Syrian golden hamsters (FUME LakIbm:FUME; Biological Research Laboratory, Füllinsdorf, Switzerland), weighing 119–189 g, were used for the entire study, including the development of the bioassay (tables 1, 2). The animals were fasted 12 h prior to the experiment. Otherwise, they had access to food and water ad libitum. For particle inhalation and subsequent lung fixation, complete analgesia and anaesthesia were achieved with intraperitoneal (i.p.) injections of ketamine-hydrochloride followed by barbiturate [19].

Particle inhalation. For the inhalation, 6-μm polystyrene microspheres (PSP; Polybead, Polysciences, Eppelheim, Germany), which deposit almost exclusively in airways, were chosen, since those particles had been previously reported to attract macrophages to the deposition site in hamster airways [19]. Aerosols were generated by jet nebulization of particle suspensions. An on-line open flow system with pneumotachography and laser light scattering photometry was used to moni-

tor inhaled and exhaled air volumes and particles breath by breath [21]. The spontaneously breathing hamsters inhaled the aerosol for 20 min through an intratracheal cannula (PSP-group; $n = 4$). The total number of deposited microspheres, derived from the continuously monitored aerosol flow rates and the aerosol particle number concentrations ($N_{dep} = N_{inhaled} - N_{exhaled}$), ranged from 5.7×10^5 to 9.4×10^5 particles (table 1). Control animals were exposed to room air ($n = 4$).

Serum preparation for the bioassay. The pooled fresh serum of two to three untreated hamsters was divided into three samples of equal size and prepared as follows: The first sample, zymosan-activated serum (ZAS), was mixed with zymosan (Zymosan A, Sigma, Buchs, Switzerland, 25 mg/ml), a well-known complement activator, and incubated at 37 °C for 45 min [7, 13, 22]. To prevent further activation, complement in ZAS was subsequently heat-inactivated (56 °C, 30 min) [13]. Finally, the zymosan particles were removed from ZAS by centrifugation (8000*g*, 20 min). The second sample, heat-inactivated serum (HIS), was first heat-inactivated and thereafter prepared as described for ZAS. The third sample, normal serum (NS), was left untreated.

In an initial set of experiments, serial dilutions of all sera in tissue culture medium were tested for chemotactic activity. From these experiments serum concentrations that gave maximal chemotactic response were derived. In further experiments NS and HIS were used diluted to 1:8, ZAS to 1:128.

Bronchoalveolar lavage (BAL), macrophage and protein recovery. The lungs of either untreated, room air- or PSP-exposed hamsters were lavaged with 10×5 ml divalent cations free Dulbecco's phosphate-buffered saline (PBS; Amimed, Allschwil, Switzerland) [23]. Macrophages used for the cell migration assay were recovered by BAL from untreated hamsters. To obtain BALF for chemotactic activity testing, BAL yielded from PSP and sham-exposed hamsters was performed 40 min after the beginning of the inhalation (table 1). This was in accordance with the time point when the number of macrophages was found to be increased in hamster airways after PSP inhalation [19].

Cells were separated from BALF by centrifugation (300*g*, 10 min). The pellet was resuspended in 2 ml of PBS, and the total number of cells was assessed in a Neubauer counting chamber, using trypan blue dye exclusion for viability (table 2). For the bioassay, the cells from two to four untreated hamsters were pooled and diluted to 1×10^6 cells/ml in tissue culture medium (RPMI 1640, Amimed, Muttenz; 10 mM Hepes, Sigma, Buchs; 1% bovine serum albumin, Sigma, Buchs, all Switzerland). An additional sample of cells was smeared on a glass slide, fixed with methanol and stained with May-Grünwald-Giemsa for differential cell counting.

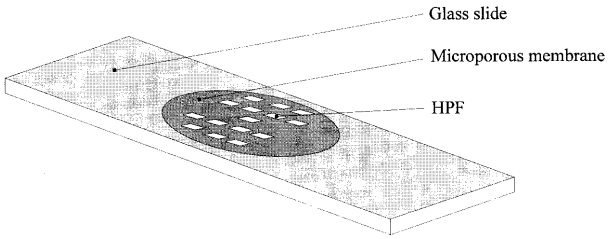

Figure 1. Schematic drawing of a microporous membrane (diameter, 6.5 mm) mounted on a glass slide and the localization of the 15 predetermined high-power fields (HPF, area per field, 0.01 mm^2).

For protein recovery from BALF, the procedure described by Warheit et al. [13] was essentially followed. Briefly, low speed (300*g*, 10 min) and high speed (80,000*g*, 120 min) centrifugation was used to obtain cell and particulate lipid-free lavage fluids. The resulting supernatants were concentrated to a volume of about 1.5 ml by a centrifugal concentrator (*Ω* Macrosept microconcentrator 3 kD, Skan, Basel, Switzerland) at 5000*g* for 10 h. The samples were kept at 5 °C during the whole procedure that took less than 20 h. Subsequently, the concentrates were lyophilized in a rotovac (Speed-Vac, Savant Instruments, Henry Sarasin, Basel, Switzerland) and then stored frozen at −75 °C.

For chemotaxis experiments, the lyophilized samples were resuspended in tissue culture medium to protein concentrations of 125, 63, 31 and 16 μg/ml, determined by measuring the absorbency maximum shift of Coomassie Brilliant Blue G-250 upon protein binding. (Bio-Rad protein assay, Bio-Rad Laboratories, Glattbrugg, Switzerland). Serial dilution of pure ovalbumin (Servan, Heidelberg, Germany) was used as standard.

Cell migration (chemotaxis) assay. Macrophage chemotactic activity was tested in an in vitro cell migration assay [8, 24, 25]. In the present study, Transwell chambers (Costar/Nuclepore, Tecnomara, Wallisellen, Switzerland) were used which consist of two compartments with a microporous polycarbonate membrane (5-μm pores) in between. In the upper compartment 1×10^5 macrophages, suspended in 100 μl of tissue culture medium, were pipetted. The lower compartment contained 600 μl of the diluted test sample. The chambers were incubated at 37 °C, 5% CO_2 and 95% relative humidity for 3.5 h. Thereafter, cell migration was stopped by fixation of the microporous membranes with ethanol. The membranes were then stained with hematoxilin-eosin and mounted on glass slides with their lower side turned up. The number of macrophages that had migrated to the opposite (lower) side of the microporous membrane was assessed on 15 predetermined high-power fields (HPF, area per field 0.01 mm^2) using an inverted light microscope (Axiovert 10, Carl Zeiss,

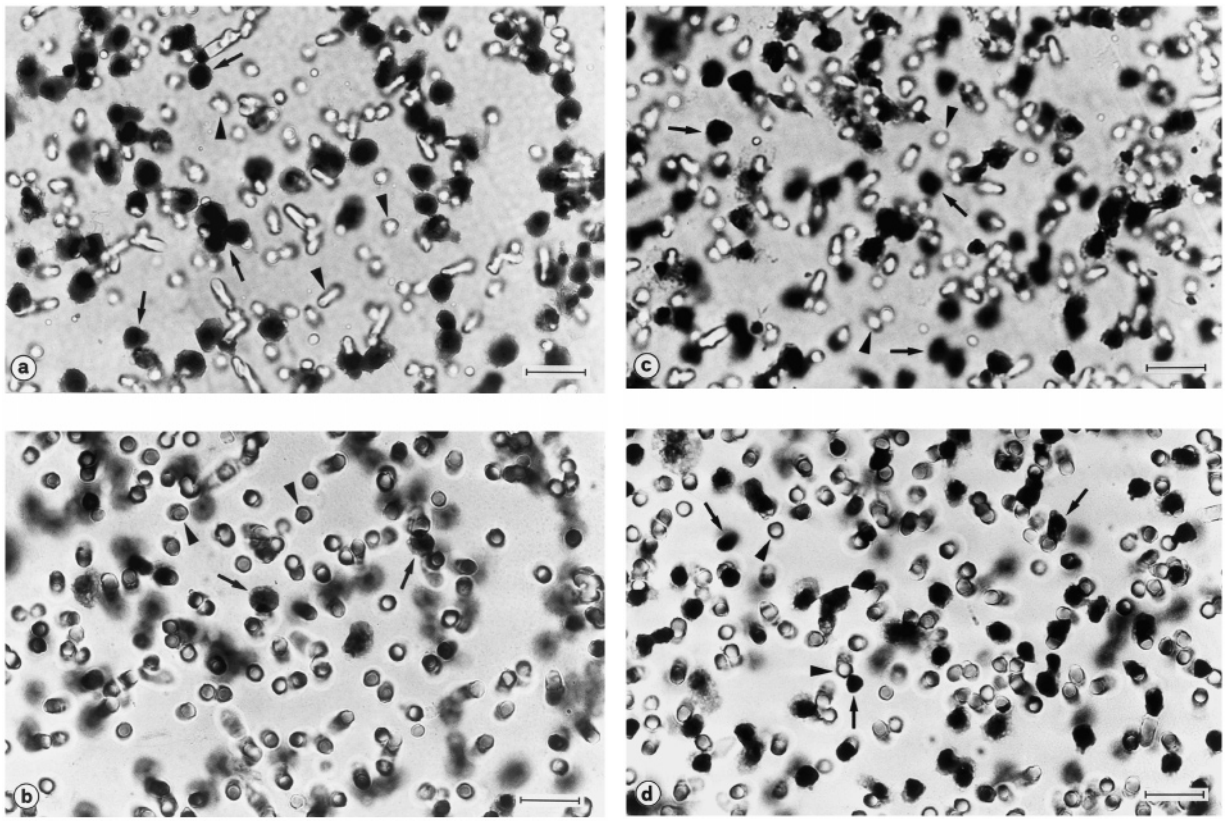

Figure 2. Light micrographs of microporous membranes showing pores (arrowheads) and macrophages (arrows). In (*a*) and (*c*) the membrane sides that faced the macrophage containing chamber are in focus, whereas in (*b*) and (*d*) those that faced the test sample are viewed. Note that more macrophages migrated through the pores toward ZAS (*d*) than toward tissue culture medium (*b*). Hematoxilin-eosin staining; internal scale bar, 20-μm.

Zurich, Switzerland) with a 100× oil immersion objective lens (figs 1, 2).

Analysis of macrophage chemotactic activity. To standardize the bioassay, serial dilutions of pretreated sera (e.g. ZAS) were tested for chemotactic activity. To distinguish chemotactic movements from enhanced random migration, checkerboard analysis (CA), where both compartments of the chamber contained ZAS at equal dilution (e.g. 1:128), was performed [26, 27].

Proteins recovered from BALF were tested for chemotactic activity at concentrations of 125, 63, 31 and 16 μg/ml. As negative controls tissue culture medium (M), fresh 'normal' serum (NS 1:8) and heat-inactivated serum (HIS 1:8) from untreated hamsters were used, as positive control zymosan activated serum (ZAS 1:128).

Statistics. The group means were compared by Wilcoxon–Mann–Whitney rank sum test [28]. Probability was tested at $P < 0.05$ and $P < 0.1$.

Results

BAL cells. As shown in table 2, the total number of cells recovered by BAL ranged from 3.8×10^6 to 5.4×10^6. The procedure did not affect cell viability. The trypan blue dye exclusion test showed more than 95% of the cells to be viable. As revealed by differential cell counting, and as expected of healthy animals, the majority of BAL cells were macrophages. According to an earlier stereological study about the efficiency of macrophage recovery by BAL in hamsters, 95% of BAL macrophages are expectantly from the alveolar region and about 5% from the airways [23].

Protein recovery by BAL. As demonstrated in table 1, significantly ($P < 0.05$) higher amounts of proteins were isolated from BALF of PSP-exposed hamsters than from control animals. No specific relation was found between N_{dep} or body mass and total amounts of proteins in BALF.

Chemotactic response of macrophages to ZAS. ZAS was observed to induce a concentration-dependent increase of macrophage chemotactic response and reach a plateau at dilution of 1:128 to 1:512 (figs 2, 3). The chemotactic response to ZAS 1:32 to 1:2048 was found to be significantly ($P < 0.05$) increased as compared with the negative controls (HIS, NS and M), whereas inhibition of macrophage migration was detected at

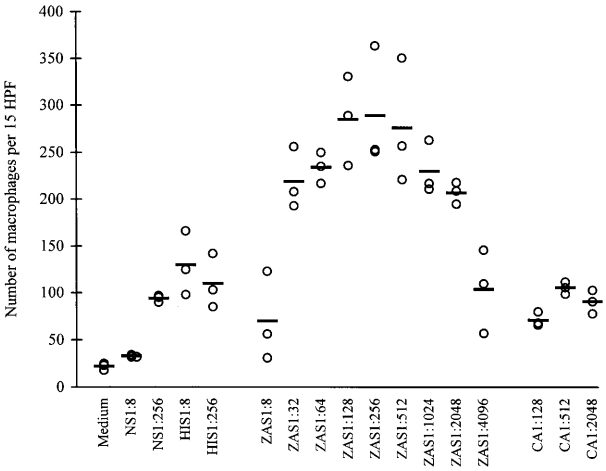

Figure 3. Chemotactic response of macrophages to tissue culture medium, NS and HIS; and serial dilution of ZAS. In checkerboard analysis (CA), ZAS was given to both compartments of the Transwell chamber. The data of one experiment are presented as total number of macrophages per 15 HPF from a single microporous membrane (circles) and as mean values of three membranes (bars). The mean values of ZAS 1:32–1:2048 were found to be significantly ($P < 0.05$) increased as compared with HIS, NS and M. In CA macrophage chemotactic activity was observed to decrease significantly ($P < 0.05$).

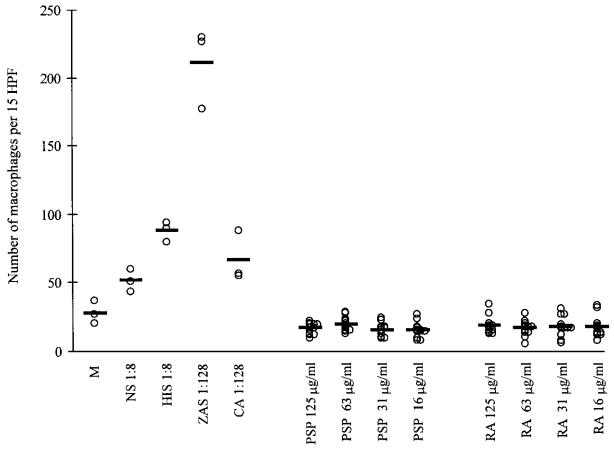

Figure 4. Chemotactic response of macrophages to BALF proteins of hamsters exposed to either polystyrene aerosols (PSP; $n = 4$) or room air (RA; $n = 4$). Data of one experiment are given as total number of macrophages per 15 HPF from a single microporous membrane (circles) and as mean values (bars) of the cell counts of 12 membranes (3 membranes per animal). As negative control M, NS and HIS were used, as positive control ZAS (1:128). CA was performed to distinguish chemotaxis from random migration.

high concentrations of ZAS (1:8). When ZAS was given to both filter sides (checkerboard analysis), macrophage chemotactic response was observed to decrease significantly ($P < 0.05$).

Macrophage chemotactic activity in BALF. Neither proteins recovered from BALF of PSP nor those of sham-exposed hamsters were found to increase macrophage migration at any of the tested concentrations (fig. 4). No difference was found between BALF proteins of PSP-exposed and control hamsters. Comparing all BALF samples to tissue culture medium revealed a trend ($P < 0.1$) for decreased macrophage migration toward BALF proteins.

Discussion

In the first part of this study, a bioassay for hamster macrophages to assess complement-derived chemotactic activity was established. Hamster macrophages were found to exhibit a concentration-dependent chemotactic response to ZAS which was significantly reduced by heat inactivation of the serum prior to mixing with zymosan. When equal concentrations of ZAS were placed in the upper and lower compartments of the chamber (checkerboard analysis), the migration activity was found to be significantly lower, indicating that the migration toward ZAS was due to chemotaxis and not to stimulated random migration [26].

In contrast to our study, Warheit et al. [15, 18] reported that hamster macrophages do not exhibit chemotactic response to ZAS. Even though we used the same microporous membrane system, our protocol contained variations that might explain the different result: (i) We activated hamster serum with higher concentrations of zymosan; (ii) macrophages were used at a lower concentration; and (iii) ZAS was diluted further. The relation between zymosan concentration and chemotactic activity was described for rat macrophages, where zymosan was found to be a very potent chemotactic activator at 10 mg/ml [8]. The authors used the same zymosan concentration for their studies in hamsters [15, 18]. However, the optimal zymosan concentration may vary between species. In our study, hamster macrophages reacted best toward zymosan concentrations of 25 mg/ml (data not shown). Moreover, cell migration assays were reported to lose sensitivity at higher macrophage concentrations [7]. Hence, a bioassay using macrophages at concentrations of 1.5×10^6 cells/ml [15, 18] might be less sensitive than one using macrophages at 1×10^6 cells/ml as in our study. In the experiments performed by Warheit et al. [15, 18], hamster ZAS was not diluted further than to 1:100, even though chemotactic activity increased at this dilution. We tested serial dilution of ZAS up to 1:4096 and found chemotactic activity to increase and reach a plateau at 1:128 to 1:512. Therefore, chemotactic activity might not have been detected at the lower dilution used by others [15, 18].

We found that the chemotactic response to serum that had been heat-inactivated prior to zymosan incubation (HIS 1:8) was significantly increased over that to normal fresh serum (NS 1:8). No such effect was found in rats [8]. A possible explanation might be that our serum was not completely heat-inactivated. Indeed, when we tested the temperature stability of our water bath, we found it to range from 53 to 58 °C.

In the second part of this study we wanted to test whether complement-derived chemotactic activity is involved in the recruitment of macrophages in hamster airways reported to appear within 40 min after the inhalation of 6-μm polystyrene microspheres which deposit almost exclusively in airways [19]. We therefore tested BALF of hamsters that had inhaled PSP of the same size and under equivalent conditions as in the study mentioned above for chemotactic activity by this bioassay. There was no increased chemotactic response observed to BALF of PSP-exposed hamsters in this study. Among possible explanations are that (i) the specificity and/or the sensitivity of our bioassay was not appropriate to detect the stimulus for macrophage recruitment that occurred in vivo; (ii) chemotactic factors were lost or inactivated by the protein isolation and concentration procedure; (iii) BALF contained components that inhibited chemotaxis in the bioassay; or (iv) BAL macrophages did not respond to the stimulus.

The cell migration assay used in this study was designed for complement-derived chemotaxis, especially for C5a-dependent directed migration. Its specificity for other chemotactic factors was not tested. The bioassay might not have been sensitive enough to detect very low concentrated, locally acting chemoattractants either complement-derived or secreted by epithelial cells or macrophages [29, 30]. In addition, we have to consider that such factors were further diluted by the BAL procedure, during which proteins and mediators are recovered not only from airways but also from the much larger alveolar compartment. The lavage of the airways only would result in a more specific yield of components from this compartment. The use of small volumes of liquid for lavage is often called 'airway lavage'. This procedure may be applicable to lavage the extrapulmonary trachea and main stem bronchi. However, to lavage the intrapulmonary airways with this technique, as required for this study, is not feasible, since the accurate airway volume of this lung and the exact distribution pattern of the fluid would have to be known. Particularly in rodents, which have very asymmetrical airway branching patterns, even very small amounts of fluid may be distributed to the alveoli that appear after a few airway generations.

Loss of complement-derived chemotactic factors by the protein concentration procedure is not very likely, since the recovery of 9- to 12-kD proteins, the molecular weight range of C5a, is close to 100% with the 3-kD-cutoff filters used in this study. However, other chemotactic factors might not have been preserved by BALF treatment: in a first step, lipid soluble components were separated. This procedure causes loss of SP-A, a chemotaxis-activating surfactant protein [31]. By the subsequent protein concentration molecules smaller than 3 kD were lost, which should be less important. There are no reports of such small chemotactic factors except for the N-formyl peptides [17] synthesized by bacteria [32], which are not important for the clearance of inorganic particles. That complement-derived chemotactic factors were inactivated during sample preparation including lyophilization is not very likely, since no such effect has been reported by other authors who use this procedure routinely [8, 25].

The existence of inhibitors of complement-derived chemotaxis in vitro was demonstrated in human BALF and serum [20]. The role of one of them, the chemotactic factor inactivator (CFI) was ascribed to modulation of C5a-directed inflammation. Whether such a factor was active in our bioassay resulting in the decreased macrophage migration toward all samples containing BALF proteins remains to be determined.

We cannot rule out the possibility that BAL macrophages, which consist of approximately 95% alveolar macrophages, did not respond to a chemotactic factor airway macrophages would respond to. Phenotypically and functionally distinct macrophage populations were isolated from the inner surface of the lung [33, 34]. However, it is difficult to attribute the differences to airway or alveolar macrophage populations. In one study airway macrophages were suggested to be less active than those from the alveoli [33]. So far there is no evidence for airway macrophages to respond differently or to other stimuli than alveolar macrophages. The higher amount of proteins found in BALF of PSP-exposed hamsters might not be relevant, since there was no specific relation found between the amount of proteins and total number of deposited particles and/or body mass. In addition, substantially more proteins were recovered from one animal only.

In summary, this in vitro study revealed that (i) hamster macrophages recovered by BAL exhibited a concentration-dependent chemotactic response to autologous complement-activated serum, and (ii) a similar chemotactic activity was not detected in BALF of hamsters which had inhaled PSP by this bioassay.

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