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Cell-mediated immunity to soluble and particulate inhaled antigens

J. O. HILL* & R. BURRELL *Department of Microbiology, West Virginia University Medical Center, Morgantown, WV 26506, USA*

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SUMMARY

In order to determine the influence of an antigen's physical properties on the development of cell-mediated immunity (CMI) in the lung following aerosol immunization, human serum albumin (HSA) was prepared in either a soluble or a particulate form, the latter being coupled to respirable, carboxylated latex beads. Antigen was administered via an aerosol to groups of guinea-pigs, twice weekly for up to 4 weeks. Additional groups of animals served as unexposed and unconjugated latex controls. Lymphoid cells for CMI assays were isolated from the lung by bronchopulmonary lavage and from blood for use in mitogen- and antigen-induced lymphocyte transformation assays, as well as indirect macrophage migration inhibition tests.

Particulate HSA-exposed animals yielded the highest numbers of free lung cells containing predominantly macrophages, with up to 33% lymphocytes. These were followed by the latex control, soluble HSA and unexposed control groups, respectively.

Only the animals exposed to particulate HSA had evidence of antigen reactivation in the lung cell populations as measured by lymphocyte stimulation assays. In contrast, a response to polyclonal mitogens was found only in animals exposed to antigen in a soluble form. Data from macrophage depletion experiments suggest that the antigenicity of inhaled antigens may be due to the types and numbers of cells responding to the stimulus, and the subsequent role the alveolar macrophage may play in the modulation of cellular immunity.

INTRODUCTION

There exists a wealth of information concerning cell-mediated immune (CMI) responses to peripherally injected antigens, but knowledge concerning this type of response to inhaled antigens is scant. Moreover, the information available concerning the relative roles of the two major components of pulmonary effector cell populations, lymphocytes and macrophages, is even less. Kaltreider (1976a, b) has contributed greatly to what is known and has recently summarized gaps in our knowledge. One such gap concerned the characteristics of the alveolar macrophage with respect to its role in the afferent limb of the immune response. Before we can consider this important aspect, attention must be paid to the physical state of the antigen inhaled, i.e. whether soluble or particulate, since macrophage processing is greatly affected by particle size.

In a previous paper (Burrell & Hill, 1976), we described studies which compared pulmonary and systemic CMI responses to soluble and particulate antigens. It was found that although the CMI-responsive cells produced in the lung increased following immunization with either antigen, only the particulate antigen (a cellular suspension of *Thermoactinomyces vulgaris*) was also able to induce a systemic

* Present address: Inhalation Toxicology Research Institute, Lovelace Biomedical and Environmental Research Institute, P.O. Box 5890, Albuquerque, NM 87115, USA.

Correspondence: Dr R. Burrell, Department of Microbiology, West Virginia University Medical Centre, Morgantown, WV 26506, USA.

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CMI response following aerosol immunization. This led to an hypothesis of pulmonary compartmentalization where a 'barrier' was postulated that must be overcome before systemic responses can be induced following respiratory immunization.

Since the above study was made with antigens that differed chemically as well as physically, the results could not be explained on the basis of physical characteristics alone. The present study elected to prepare a single antigen in two physical forms, soluble and particulate, to determine differences in the immune response following inhalation. It is the purpose of this paper to determine the influence of an inhaled antigen's physical characteristics on the development of cell-mediated immune responses in the lung.

MATERIALS AND METHODS

Animals. Outbred English White strain guinea-pigs (Hilltop Laboratories, Scottdale, Pennsylvania) were used in this study. Each weighed 350–450 g when the project began.

Immunochemicals. Human serum albumin (HSA) (4X crystalline, Nutritional Biochemicals Corporation, Cleveland, Ohio) was the specific antigen used in these investigations. One mg/ml of stock solutions of HSA as well as of the mitogens, phytohaemagglutinin (PHA-P, Difco Laboratories, Detroit, Michigan) and concanavalin A (Con A, Pharmacia Fine Chemicals, Piscataway, New Jersey) were prepared in RPMI-1640 (Grand Island Biological Company, Grand Island, New York) supplemented with 9·53 g/l HEPES buffer, 0·62 g/l sodium bicarbonate, penicillin (100 units/ml) and streptomycin (100 μg/ml). Stock solutions were stored at −20°C. Working solutions were prepared at least every 2 weeks. Carboxylate-modified uniform latex particles (0·926 ± 0·0045 μm diameter) were obtained from Dow Chemical Company (Indianapolis, Indiana).

Antigen preparation. HSA was prepared in two forms for aerosol administration. 'Soluble HSA' was a 200 μg/ml solution of protein in 0·02 M phosphate-buffered saline (PBS), pH 7·2. 'Particulate HSA' was prepared by conjugating HSA to the functionally modified (carboxylated) latex beads as follows. The carboxylated latex reagent was cleaned with ion exchange resins as per the supplier's instructions prior to use. To 0·1 ml of cleaned, washed, packed latex was added 1·5 ml of aqueous HSA (2·5 mg/ml). After a 10 min equilibration period, the latex-protein suspension was acidified with 0·75 ml of 0·1 M phosphate buffer, pH. 5·6, followed 10 min later by the addition of 4 ml of 1% aqueous ECDI (1-ethyl-3 dimethyl aminopropyl) carbodiimide hydrochloride, (Calbiochem, San Diego, California). A control tube containing latex, protein, buffer and 8 ml of water (no ECDI) was also set up in parallel. The reactions were allowed to proceed with continuous, gentle agitation for 24 hr at room temperature. The following day the suspensions were dialysed against distilled water to remove unreacted ECDI and reaction by-products.

After 24 hr of dialysis, samples were filtered through 0·22 μm filters and Lowry protein assays performed on a latex free aliquot. The amount of protein conjugated to the beads was determined by subtracting post-reaction free protein concentrations from the concentration of protein present in a filtered aliquot of the control (no ECDI) suspension. Under these conditions 0·1 g of latex (1 ml of 10% stock of 0·1 ml of packed latex) reacted with approximately 1·5 mg of HSA. After centrifugation the supernate containing unbound protein was removed and the 'particulate HSA' dispersed gently, but thoroughly, to a concentration of 200 μg protein/ml in 0·02 M PBS, pH 7·2. This yielded approximately 7·5 ml of a suspension containing $2{\cdot}7 \times 10^{10}$ latex particles per ml and resulted in a monodispersed suspension as verified microscopically.

Aerosol exposure protocol. Age-matched guinea-pigs were divided into five groups: Group 1—untreated controls; Group 2—received aerosol exposures of unconjugated latex; group 3—received aerosol exposures of soluble HSA; Group 4—received aerosol exposures of particulate HSA.

Twenty minutes before aerosols began, the animals were lightly anaesthetized with 30 mg/kg of sodium pentobarbital. Each animal was exposed in two aerosols, 1 hr each, per week, 3 days apart. At least four animals from each group were killed weekly, for immunological work up, 3 days after the last exposure.

Aerosols were produced with a Devilbiss No. 40 nebulizer (DeVilbiss Company, Somerset, Pennsylvania) fitted to a pump operating at a pressure of 2 psi, effecting a flow rate of 13 l/min into the exposure chamber. Droplets with a mass median diameter of 2·3 μm are produced with this device (Larson, Young & Walker, 1976). The exposure chamber was a $1{\cdot}2 \times 10^4$ cm^3 cuboidal lucite chamber which could accommodate the heads of four guinea-pigs simultaneously. A closed system was effected by supporting the animals' heads in ports fitted with rubberized collars.

Cell-mediated immunity assays. Lymphoid cells for CMI assays were isolated from the lung and peripheral blood of the experimental animals. Thirty minutes prior to being killed, the animals received 10 mg of diphenhydramine hydrochloride intraperitoneally to help prevent bronchospasm. Twenty minutes later, the animals were anaesthetized as described above and exsanguinated. Peripheral blood lymphocytes were isolated from heparinized blood on Ficoll–Paque gradients (Pharmacia Fine Chemicals, Piscataway, New Jersey). Free lung cells were isolated by *in situ* pulmonary lavage by a modification of the method of Myrvik, Leake & Farriss (1961). For these studies a total of 100 ml of pre-warmed (37°C), heparinized saline was used to lavage the lung, with 10–12 ml aliquots per lavage. The cells were pelleted at 100 *g* for 8 min, washed three times in RPMI-1640, counted and stained for viability with trypan blue. Lymphocytes were quantified by staining (Kazmierowski, Pauci & Reynolds, 1976).

Pulmonary lymphocytes, when cell yields allowed, were concentrated and semi-purified by plastic adsorption and the removal of adherent cells (macrophages) as described by Bloom & Bennett (1971). To assure that any changes in lymphocyte

reactivity were due only to the depletion of macrophages, pulmonary lymphoid cell populations were adsorbed to the point where significant lymphocyte loss was not a factor. Effecting a lymphocyte concentration of greater than 50% by plastic adsorption techniques resulted in significant lymphocyte as well as macrophage depletion. Concentrated as well as unadsorbed pulmonary lymphoid cell suspensions were adjusted to 5×10^6/ml lymphocytes in RPMI-1640 plus 40% heat-inactivated, autologous plasma. Peripheral blood lymphocytes were removed from the gradients and similarly washed, counted, stained and resuspended to a count of 5×10^6/ml in the same medium. This permitted about twenty-five tests per cell suspension.

Lymphocyte suspensions were tested *in vitro* for mitogen and antigen reactivity. Aliquots of 0·1 ml, containing 5×10^5 lymphocytes, were added to the wells of sterile microtitre plates (Linbro Scientific Inc., Hamden, Connecticut.) To these microcultures was added 0·1 ml of RPMI-1640 alone or medium containing 1 or 2 μg of mitogen, or 20, 10 or 5 μg of antigen. The addition of mitogen or antigen in 0·1 ml of RPMI-1640 effected a final concentration of 20% autologous plasma in this microculture system.

Triplicate cultures supplemented with mitogen were terminated after 4 days while antigen containing lymphocyte cultures were terminated at 6 days, these being the optimum culture periods under the conditions described. During the last 18 hr in culture, cells were pulsed with 0·01 μCi of ^{14}C thymidine (thymidine-^{14}C, 6·7 Ci/mmol, New England Nuclear, Boston, Massachusetts). Cells were harvested onto fibre glass filter paper discs using a multiwell harvester. Filter paper discs were placed in 5 ml disposable scintillation vials and dried overnight at 37°C. The following day samples were counted in a scintillation counter. Results are expressed as a stimulation index, calculated as: mean cpm with stimulant/mean cpm without stimulant. Control cultures exhibited activities of approximately 30 cpm. Macrophage depletion did not consistently or significantly affect control levels of isotope incorporation.

Indirect macrophage migration inhibition (MMI) assays were performed using cultured adsorbed or unadsorbed pulmonary and peripheral blood lymphocyte suspensions as the source of antigen-induced migration inhibitory factor (MIF) release. One ml of RPMI-1640 containing 10 μg of soluble HSA was added to 1·0 ml of the cell suspension. After 48 hr, cells were pelleted and the supernatant fraction was assayed for MIF activity with normal pulmonary macrophages as target cells. MMI greater than 20% was considered significant in this study. At least four capillary tubes were set up per animal.

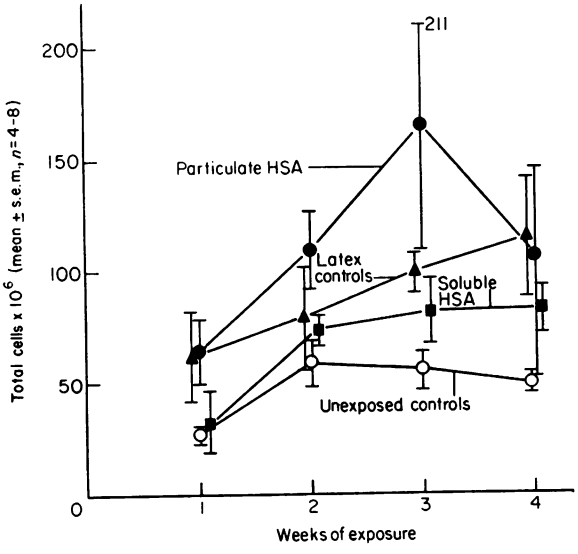

FIG. 1. Total cell yields obtained from guinea-pig lung by bronchopulmonary lavage. (●——●) Particulate HSA; (▲——▲) latex controls; (■——■) soluble HSA; and (○——○) unexposed controls.

RESULTS

Yield and type of pulmonary lavage cells

The objectives of this project required the isolation of pulmonary lymphoid cells from the lungs of guinea-pigs in sufficient numbers for manipulation and study. Previous experience had shown that the isolation of lung cells from guinea-pigs is fraught with complications. Most of the problems stemmed from non-specific bronchoconstriction which trapped cells and lavage fluid. For this reason, animals used in this project were pre-treated with an anti-histamine, and lavaged with pre-warmed saline, free

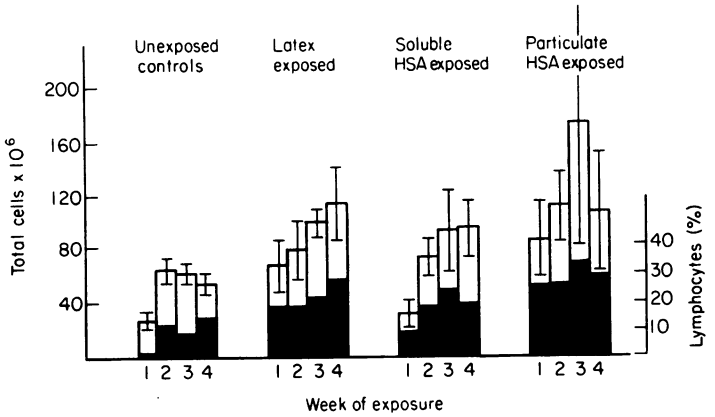

FIG. 2. Quantitative and qualitative data on cells obtained from guinea-pig lungs by pulmonary lavage. (□) Total cell yields $\times 10^6$ (s.e.m. shown); (■) represents the percentage of that yield which were lymphocytes (s.e.m. $\pm \leq 10\%$).

from divalent cations. The effects were remarkable and the recovery of cells by *in situ* bronchopulmonary lavage was markedly enhanced. A mean of 118×10^6 cells with an average of 22% lymphocytes was obtained from the lungs of the animals used throughout the study. Generally, more free lung cells were found in exposed animals, as compared to unexposed controls, regardless of the type of exposure (Fig. 1). The lowest numbers of free lung cells were seen in unexposed controls and the highest yields were obtained from the animals exposed to particulate HSA. Although the predominant cells present were macrophages, significant numbers of lymphocytes (up to 33%) were found in all groups, with the exception of the unexposed control group (Fig. 2). These latter animals rarely had more than 10% lymphocytes in their lungs. Pulmonary lymphocytosis was particularly evident in animals that received bi-weekly exposure to HSA in a particulate form or those animals exposed to the latex carrier alone (latex controls).

Particulate HSA-exposed animals always had more free lung cells, including macrophages and lymphocytes, with increases of 100–200% over unexposed controls during the same time period ($P \leqslant 0{\cdot}05$). Significantly higher numbers of cells were also found in the particulate HSA group when compared to those animals exposed to antigen in the soluble form ($P \leqslant 0{\cdot}05$).

However, the quantitative as well as qualitative aspects of the lung, cell populations of the particulate HSA and latex control groups did not differ statistically. In almost all groups, peak lymphocyte counts were seen in the week corresponding to peak total cell number.

Although not always reflected by the mean values depicted in Fig. 2, the data obtained from individual animals suggested that any point during the 4 week time period, an influx of lymphocytes could account for a great deal of the difference in the total cell yields in particulate HSA-exposed animals versus the soluble HSA group. No significant changes in the peripheral blood lymphocyte yields were seen in animals exposed to antigen in either form, or in either control group.

Mitogen reactivity

Table 1 shows the *in vitro* mitogen reactivity of the lymphocytes from the lungs of the soluble HSA-exposed animals, the only group showing significant mean responses (stimulation index $>3{\cdot}0$). Under the conditions described, i.e. using unfractionated pulmonary lymphoid cell suspensions, but adjusted to standardized lymphocyte counts, no PHA or Con A reactivity was observed at any time in cells from animals in either control group or from the particulate HSA groups, despite the fact that all cultures contained the same number of lymphocytes.

Most animals exposed to soluble HSA aerosols had mitogen-reactive lung cell populations. After 2 and 3 weeks, three out of five and three out of four animals respectively had *in vitro* stimulation indices $>3{\cdot}0$. A comparison of the data given in Table 1 with that given in Fig. 2 suggests that mitogen reactivity tended to wane as the number of macrophages reached a peak at week 4. The absence of mitogen reac-

TABLE 1. Proliferative response of lymphocytes obtained from soluble HSA-exposed guinea-pigs*

	Weeks of exposure			
μg/culture	1	2	3	4
PHA				
2	—†	6·6 (1–17)	15 (4–29)	—
1	—	—	3·1 (1–4·2)	—
Con A				
2	—	—	13 (6·6–26)	—
1	—	—	5 (1–11)	—

* Results expressed as mean stimulation index (four to eight animals per group) where ≥3·0 is considered significant, range in parentheses.
† Stimulation index < 3·0.

tivity at this time might also be related to qualitative changes in macrophage (or lymphocyte) populations as the change in macrophage numbers was not large.

Standard deviations from culture to culture were always less than 15% of the given counts per minute (cpm) and viability was consistently greater than 80% after the 4 days in culture. Within a group, animal to animal variation was less than 30% of total cpm. Statistical analysis of the lymphocyte stimulation data was not possible because of the lack of normality in the distribution of the data.

Unlike what was found with respect to pulmonary lymphocyte cultures, cultures initiated with equivalent numbers of peripheral blood lymphocytes all showed marked mitogen reactivity. Because of the great variability from one animal to the next, few trends were seen and these experiments served primarily as a quality control check for the mitogens. Stimulation indices ranged from 23 to 276 for PHA at 2 μg per culture and from 3 to 116 for Con A at the same concentration.

TABLE 2. Comparison of antigen-induced proliferative responses of pulmonary and peripheral blood lymphocytes obtained from particulate HSA-exposed guinea-pigs*

	Weeks of exposure							
	1	2	3	4	1	2	3	4
HSA (μg/culture)	Pulmonary				Blood			
20	—†	7·0 (1–20)	—	—	6·0 (1–15)	24 (1–69)	—	—
10	n.d.‡	n.d.	—	—	n.d.	n.d.	—	—
5	—	10 (1–26)	—	—	5·0 (1–13)	32 (1–76)	—	—

* Results recorded as mean stimulation index (four to eight animals per group) where ≥3·0 is considered significant, range in parentheses.
† Stimulation index < 3·0.
‡ Not done.

Transformation by antigen stimulation

Antigen reactivity, as measured by antigen-induced lymphocyte transformation *in vitro* was also investigated using lymphoid cells derived from animals with different exposure histories. Optimal responses were seen after 6 days. Only results obtained from cultures terminated after 6 days are given, representing data from a minimum of twenty microcultures per animal.

Animals from only one group consistently had antigen-reactive cells in their lungs or peripheral blood (Table 2). Guinea-pigs receiving bi-weekly aerosols of particulate HSA for 2 weeks had evidence of cell-mediated immunity to the antigen in both lung and peripheral lymphocyte populations. The particulate HSA group killed at 1 week had antigen-reactive cells in their peripheral blood, but none in the lung.

MIF production by antigen stimulation

The other parameter and *in vitro* correlate of cell-mediated immunity used in this investigation was the indirect macrophage migration inhibition (MMI) test. Data from these experiments are given in Fig. 3.

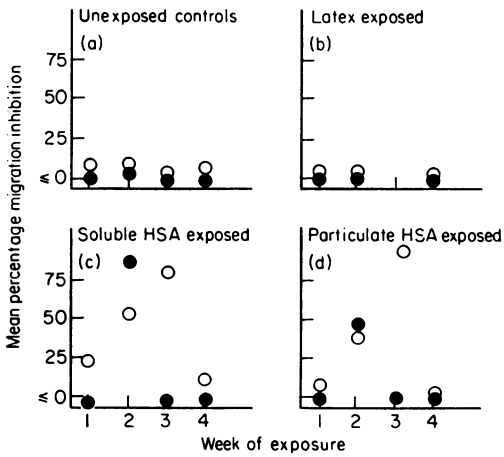

FIG. 3. Antigen-induced migration inhibitory factor release from guinea-pig pulmonary and peripheral blood lymphocytes. (●) Blood lymphocyte MIF tests; (○) pulmonary cell tests. Results are recorded as mean migration inhibition of two to four animals per group. (a) Unexposed controls; (b) latex-exposed; (c) soluble HSA-exposed; and (d) particulate HSA-exposed.

As was observed in the lymphocyte transformation experiments, significant responses were seen in the second week particulate HSA group. Antigen-reactive cells as measured by the MMI assay were detected in the lungs and blood from all four animals studied at this time. Significant responses were also seen in the lungs of the animals receiving particulate HSA for 3 weeks.

In contrast to the transformation assays, some soluble HSA-exposed animals were found to be MIF-positive. These animals, as with the particulate antigen group, had also received exposures for 2 weeks. No inhibitory activity was seen in culture supernatants derived from cells from animals in either control group.

Effect of macrophage depletion

In light of the recognized effects of systemic macrophages on immunity, an important part of the current study was to determine the influence of alveolar macrophages on pulmonary lymphocyte reactivity. The data given in Table 3 are the results of experiments performed with pulmonary mononuclear cell cultures composed of macrophages and lymphocytes in two different ratios derived from 2–3 week antigen-exposed groups. Intact cultures contained these two populations in *in vivo* proportions (2·5–1). Plastic adsorbed, macrophage-depleted suspensions consisted of macrophages and lymphocytes in

TABLE 3. Comparison of stimulation indices of guinea-pig pulmonary lymphocytes in intact and macrophage-depleted pulmonary lymphoid cell populations

Method of aerosol treatment	Macrophage/lymphocyte ratio (mitogen concentration)									
	2·5:1 (PHA 2 μg)	1:1 (PHA 2 μg)	2·5:1 (PHA 1 μg)	1:1 (PHA 1 μg)	2·5:1 (Con A 2 μg)	1:1 (Con A 2 μg)	2·5:1 (Con A 1 μg)	1:1 (Con A 1 μg)	2·5:1 (HSA 10 μg)	1:1 (HSA 10 μg)
Soluble	11·8* (14, 17)	43 (16, 71)	3·7 (3·4, 4·0)	15 (6·3, 24)	3·5 (2·6, 3·5)	13 (4·0, 22)	5·5 (1·4, 11)	16 (5·0, 28)	3·5 (1·5, 6·0)	1·5 (1·0, 2·0)
Particulate	2·5 (1·7, 3·4)	4·5 (2·9, 6·1)	1·6 (1·5, 1·8)	3·0 (1·7, 4·4)	1·4 (1·1, 1·7)	2·3 (1·0, 3·6)	1·0 (1·0, 1·5)	2·3 (1·0, 3·6)	13 (5·5, 20)	5·0 (1·5, 8·5)

* Mean mitogen stimulation index of two animals (animal No. 1, animal No. 2).

approximately equal proportions. Further lymphocyte purification was not performed because of lymphocyte loss (up to 50%) with further manipulation.

Table 3 shows the results of the experiments designed to determine the role of alveolar macrophages on mitogen- and antigen-induced pulmonary lymphocyte transformation. Experiments were performed with cells from non-immunized controls, as well as from animals from all exposure groups. Partial purification of lymphocytes resulted in observable effects in culture from some of the animals studies, except when no mitogen reactivity at all was present in non-adsorbed populations. The stimulation of cultures from those animals initially demonstrating significant mitogen reactivity was markedly enhanced by macrophage removal.

In contrast, a reduction in the relative proportion of macrophages *in vitro* had a different effect on antigen reactivity as measured by lymphocyte transformation, i.e. it resulted in a decrease in activity, with the mean stimulation index falling from 13 to 5·0. In addition, a reduction of macrophage/lymphocyte ratios from *in vivo* levels to 1:1 also reduced antigen-induced MMI as seen from the limited testing of one animal per group (data not shown).

DISCUSSION

There was a marked enhancement of cell and fluid recovery from the lungs of the animals in this study as compared to our previous efforts, undoubtedly due in part to the use of the anti-histamine prior to killing the animals and of large total volumes of pre-warmed saline for bronchopulmonary lavage. The anti-histamine did not have any demonstrable effects on the lymphocytes in our hands or in others' (Leu *et al.*, 1972). High numbers of macrophages observed in the lungs of animals challenged with latex, either in its native form or complexed with antigen, was not surprising. Lundborg & Holma (1974) found that as early as 24 hr after intratracheal injection of polystyrene particles, rabbit lungs contained increased numbers of macrophages. Earlier reports showed similar reactions to dust and carbon (Brain, 1970; LaBelle & Brieger, 1960).

In addition to increased numbers of macrophages in animals receiving aerosol challenges, the animals also had increased numbers of lymphocytes in their free lung cell populations. An interesting point is that in the groups exposed to particulate aerosols, influxes of macrophages seemed to precede pulmonary lymphocytosis in two out of three of all animals. For example, the third week increase in pulmonary lymphocytes seen in the latex and particulate HSA-exposed animals was seen a week after a corresponding rise in macrophage numbers. Peak lymphocyte counts were always seen at the time period corresponding to peak macrophages and total cell numbers. This suggests that the alveolar macrophages are populating the lung under stimulus from the particulate aerosol and then possibly setting up a lymphocyte trap, similar to that described in the spleen (Frost & Lance, 1973; Frost, 1974; Frost & Lance, 1974).

The data suggest that alveolar macrophages in *in vivo* concentrations can modulate the reactivity of pulmonary lymphocytes to mitogens and antigens, but the effect of macrophages on each reactivity was different depending on the stimulant. Antigen-induced lymphocyte transformation and MIF release was enhanced by alveolar macrophage at *in vivo* cell ratios. Evidence for this conclusion is derived from the results of two kinds of experiments' performed. Antigen-induced lymphocyte transformation was found only in the particulate HSA-exposed animals, i.e. those animals with the highest number of alveolar macrophages. In addition, the partial depletion of macrophages from pulmonary lymphoid cell suspensions from these animals resulted in a decrease in antigen reactivity as measured by either lymphocyte transformation or the MIF test. In particulate-exposed animals, antigen reactivity was lost by the third week. This was also the time period when lymphocytes accounted for about 33% of cell population and the macrophage lymphocyte ratio in all animals dropped below 2·5:1, a level indicated by the limited *in vitro* studies (Table 3) to be necessary for the maintenance of antigen reactivity in lung cell populations.

In contrast, the results of macrophage adsorption experiments indicate that there is an inverse relationship between macrophage/lymphocyte ratios and polyclonal mitogen-induced lymphocyte reactivity. The partial removal of macrophages effected higher stimulation indices. Although the observation that peripheral macrophages can modulate mitogen reactivity is not a new one, only recently has the alveolar macrophage attracted similar attention. Gorenberg & Daniele (1977) found that small numbers of alveolar

macrophages enhanced the PHA reactivity of tracheobronchial lymph node lymphocytes to a greater extent than peritoneal macrophages. It should be noted that in these experiments they studied lymphocytes in culture with macrophages in macrophage: lymphocyte ratios of 1:5, i.e. the opposite of estimates of true *in vivo* ratios. Daniele *et al.* (1977) extended their observations to humans, including experiments performed with higher cell ratios. Under these conditions the enhancing effect was lost. We have recently confirmed these results in dogs (unpublished data).

Mitogen reactivity was observed only in lung cell populations from soluble HSA-immunized animals. Particulate HSA-exposed animals averaged more macrophages in their lungs at that time period, which possibly explains the general lack of mitogen reactivity in this latter group. The possibility that alveolar macrophages were suppressing mitogen reactivity in the particulate HSA group is supported also by data given in Table 3 showing the effect of macrophage removal on PHA and Con A mitogenicity. Kazmierowski *et al.* (1970) found PHA-reactive cells in the lungs of monkeys only if the lymphocytes were depleted of macrophages by adsorption on Sephadex G-10. It could be suggested that the loss of antigen reactivity in adsorbed lung cell populations may be due to selective depletion of a subpopulation of lymphocytes and it is well known that techniques designed to remove macrophages can also deplete B cells (Rosenthal *et al.*, 1972). However, we were careful to manipulate the lung cell suspensions effecting macrophage depletion, stopping short of effecting significant lymphocyte loss.

It is also conceivable that the suppression of lymphocyte reactivity in pulmonary lymphoid cell suspensions might be due to the presence of a specific suppressor macrophage indigenous to the lung of respective groups. That this is probably not the case is illustrated by the data in Table 3. The effect of macrophage depletion is the same on antigen or mitogen reactivity, regardless of the exposure regimen. The effects are probably attributable to macrophage numbers alone.

A requirement for the presence of macrophages in the antigen-induced proliferative response of immune lymphocytes is well known and has recently been reviewed (Rosenthal *et al.*, 1976). However, the fact that the macrophage has an obligatory role in the presentation of antigen leading to MIF release is a new finding. Ohishi & Onoue (1975) found that the production of MIF by guinea-pig immune lymphoid cells in response to stimulation by PPD was abolished when the lymph node cells were depleted of adherent cell populations. This paper is of particular interest as they used antigen adsorbed to polystyrene latex and an *in vitro* stimulant.

A central key, therefore, to the difference seen in the ability of soluble or particulate inhaled antigen to induce immunity in the lung may be the ability for the particulate HSA to induce macrophage and lymphocyte accumulation in the lung. Latex, as well as other particulate aerosols, can stimulate increases in free lung cells including macrophages (Lundberg & Holma, 1974; Brain, 1970) in an adjuvant fashion. Van Oss, Singer & Gillman (1976) showed that latex particles alone could almost replace the adjuvanticity of the mycobacterial component in Freund's adjuvant. Another key to the difference seen in the ability to induce cell-mediated immunity in the lung is the stimulation of macrophage participation in the specific immunological reactions. The protein-particle complexes used in this study may favour an association with macrophages. Macrophage-associated antigen is highly immunogenic (Unanue, 1972).

Pruzansky, Suszko & Patterson (1976) found that canine alveolar macrophages took up larger molecular weight antigens to a greater extent than smaller antigens. The use of particulate HSA here may have precluded the access of antigen to the circulation in a soluble form. Since circulating antigen can prevent or depress the induction of cell-mediated immunity (Neta & Salvin, 1973) this may explain the lack of antigen-induced lymphocyte transformation in the soluble HSA-exposed animals. However, antigen-reactive cells were detected by MMI techniques, indicating that antigen-dependent transformation might be selectively depressed by soluble antigen.

It is clear that there are large differences in the free lung cell populations in animals exposed to soluble versus particulate HSA. Increases in total cells often effect changes in the relative concentrations of macrophages and lymphocytes as well as possibly other cell types. If indeed alveolar macrophages do exert modulatory effects on the resident *in vivo* pulmonary lymphocyte population as the data presented here suggest, then the physical as well as chemical characteristics of an inhaled antigen must be considered in the design of future studies.

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