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Normal and sarcoid alveolar macrophages differ in their ability to present antigen and to cluster with autologous lymphocytes

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SUMMARY

Human bronchoalveolar macrophages from normal individuals function poorly as accessory cells for the presentation of common recall antigens. In sarcoidosis, alveolar macrophages (AM) are reported to be effective accessory cells for the presentation of such antigens. In this study normal and sarcoid AM were compared with blood monocytes for their ability to act as accessory cells in presenting tuberculin purified protein derivative (PPD) and streptokinase-streptodornase (SKSD) to autologous T lymphocytes, or to form spontaneous, antigen- or mitogen-induced clusters with the T cells. When compared to autologous monocytes, normal AM failed to present the two recall antigens effectively. Likewise normal AM formed very few clusters with T lymphocytes when compared to monocytes, even in the presence of antigens or the mitogen phytohaemagglutinin (PHA). In contrast, sarcoid AM presented both antigens as effectively, and were equally effective as monocytes in forming clusters with T lymphocytes, spontaneously and in further response to antigen or mitogen. The results suggest that in sarcoidosis enhanced accessory cell function and enhanced cluster formation may be related features of bronchoalveolar macrophage populations.

Keywords alveolar macrophage sarcoidosis clustering accessory cells

INTRODUCTION

Alveolar macrophages (AM) from patients with sarcoidosis have been reported to present recall antigen to T lymphocytes as effectively as autologous peripheral blood monocytes (Venet *et al.*, 1985). In contrast, alveolar macrophages (AM) from normal subjects are considered to be ineffective in this respect (Toews *et al.*, 1984; Ferro *et al.*, 1987). The reasons for this substantial difference in accessory cell function are unclear. By ultrastructural criteria, sarcoid AM are morphologically abnormal (Danel *et al.*, 1983); phenotypically and biochemically they appear to be less mature than normal AM (Gant & Hamblin, 1985; Hance *et al.*, 1985; Barth *et al.*, 1988); their vitamin D_3 metabolism may influence macrophage differentiation (Adams *et al.*, 1983). The expression of HLA-DR, Class II MHC antigens on sarcoid AM is only slightly greater than that of normal AM, whilst that of HLA-DQ is considerably increased (Campbell *et al.*, 1986; Agostini *et al.*, 1987; Haslam, Parker & Townsend, 1990; Spurzem *et al.*, 1990). It is unclear to what extent these characteristics of sarcoid AM contribute to their enhanced antigen-presenting capacity.

It is accepted that close contact between antigen presenting cells and lymphocytes is a prerequisite for effective accessory cell function (Dougherty, Murdoch & Hogg, 1988). In sarcoidosis, rosette formation between AM and lymphocytes has been noted in bronchoalveolar lavage fluids (Yeager, Lussier & Prashad, 1979; van Maarsseveen *et al.*, 1988) and lung biopsies show increased macrophage-lymphocyte associations ultrastructurally (Danel *et al.*, 1983). We have set out to examine whether sarcoid AM may also display an enhanced ability to form clusters (Mosier, 1969) with T lymphocytes. In designing this study we have compared AM and blood monocytes from normal subjects and sarcoid patients for their ability to present recall antigens to autologous T lymphocytes or to form clusters with T lymphocytes in the presence of recall antigens.

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PATIENTS AND METHODS

Study populations

Healthy controls. Thirteen healthy volunteers on no medication underwent bronchoalveolar lavage (BAL). The mean age was 32 years (range 21–47); there were 11 males and two females. There were nine non-smokers and two smokers. All subjects gave informed written consent, and the UMDS Ethical Committee gave prior approval for the study.

Sarcoid patients. Nineteen patients were lavaged. Mean age was 37 years (range 18–71); there were 12 females and seven males. Four of the patients smoked. All had pulmonary involvement and histologically proven disease. No patient was on medication and none had coincidental disease. All patients

Table 1. Normal and sarcoid monocyte and alveolar macrophage-driven lymphoproliferation to two recall antigens

	PPD			SKSD		
	Δct/min lymphocytes +		Reconstitution ratio	Δct/min lymphocytes +		Reconstitution ratio
	Monocytes	Alveolar macrophages		Monocytes	Alveolar macrophages	
Normals						
MJ	30820 ± 4583	838 ± 221	0·03	31311 ± 7649	5151 ± 1823	0·16
TL	21505 ± 1157	1242 ± 1213	0·06	12525 ± 6042	158 ± 84	0·01
JE	4851 ± 408	−2 ± 278	<0·01	27896 ± 15393	10054 ± 374	0·36
JR	1962 ± 1294	125 ± 24	0·06	31354 ± 13865	6822 ± 5407	0·22
MO'D	57786 ± 15492	10833 ± 3130	0·19	ND	ND	—
NTB	20809 ± 5030	59 ± 45	<0·01	28666 ± 4204	−269 ± 77	<0·01
CHT	32963 ± 4736	2897 ± 881	0·09	96921 ± 17760	2580 ± 727	0·03
SCP	82001 ± 10169	42109 ± 9840	0·51	ND	ND	—
ST	72104 ± 8111	27907 ± 1601	0·39	6249 ± 2247	5310 ± 2053	0·85
MH	21745 ± 3257	19633 ± 2044	0·90	28734 ± 1974	28643 ± 3261	1
GW	47720 ± 12496	21044 ± 6901	0·44	90338 ± 3436	51421 ± 6353	0·57
Sarcoids						
LR	5078 ± 907	51901 ± 8875	10·22	ND	ND	—
SK	1951 ± 661	20252 ± 4485	10·38	ND	ND	—
CS	1017 ± 228	3647 ± 1725	3·58	ND	ND	—
FP	1761 ± 489	3527 ± 593	2·0	986 ± 713	2406 ± 533	2·44
PT	52719 ± 15394	35526 ± 5171	0·67	120848 ± 11660	75180 ± 38273	0·62
LT	8040 ± 3058	13363 ± 2560	1·66	7303 ± 1012	2733 ± 218	0·37
AM	3706 ± 2393	4567 ± 2709	1·23	ND	ND	—
MMcC	1340 ± 279	4478 ± 2372	3·34	2742 ± 875	1170 ± 340	0·43
RS	16288 ± 2239	39838 ± 5784	2·45	13857 ± 4986	15427 ± 2732	1·11
TC	4955 ± 2545	3277 ± 1587	0·66	17345 ± 7487	7686 ± 2132	0·44
BD	6250 ± 3323	8190 ± 2238	1·31	ND	ND	—
JM	363 ± 226	8510 ± 3026	23·44	ND	ND	—
BD	452 ± 127	588 ± 339	1·30	ND	ND	—
HD	920 ± 189	426 ± 221	0·46	4389 ± 890	12929 ± 3363	2·94

5×10^3 monocytes or alveolar macrophages were co-cultured in quadruplicate with 5×10^4 autologous lymphocytes in the presence or absence of PPD (100 μg/ml) or SKSD (100 μg/ml) in a final volume of 200 μl for 6 days. The data points for each individual represent the quadruplicate mean ± 1 s.d.

Δct/min = (ct/min lymphocytes + antigen + accessory cells) − (ct/min lymphocytes + antigen).

$$\text{Reconstitution ratio} = \frac{(\Delta\text{ct/min lymphocytes + antigen + alveolar macrophages})}{(\Delta\text{ct/min lymphocytes + antigen + monocytes})}.$$

ND, not determined.

were lavaged as part of the routine diagnostic and clinical staging procedure for their management.

Experimental design and interpretation of data

Lung macrophages and blood monocytes from normal volunteers and sarcoid patients were cultured together with purified autologous lymphocytes in the presence or absence of recall antigens (PPD; SKSD) or mitogen (PHA). Accessory cells and lymphocytes were co-cultured in ratios of 1 : 10 (for proliferation assays) and 1 : 4 (for clustering assays). Lymphoproliferation was assessed by incorporation of ^{3}H-thymidine and expressed as both Δct/min and ratios of macrophage- to monocyte-induced lymphoproliferation ('reconstitution ratios') (Table 1). Lymphocyte clustering around monocytes was counted visually having pre-labelled the separate cells with two different fluorescent dyes, and expressed as the percentage of accessory cells found to be clustered. Labelled lymphocyte clustering around AM was evident without the need to label the macrophage. It was generally not possible to apply both techniques simultaneously to the same BAL cell sample, because of limitations on cell numbers. Accordingly, the results of proliferation and clustering were evaluated non-parametrically separately for effects of added antigen or mitogen, and for differences between monocytes and AM, and for normals and sarcoids. Comparison of paired data for lymphoproliferation or clustering was performed using Wilcoxon's signed rank test, whilst reconstitution ratios for PPD and SKSD were compared by linear regression analysis. Comparison of results from normals and sarcoids was evaluated by the unpaired Mann–Whitney *U*-test. Significance was taken where *P* values were less than 0·05.

Preparation and purification of blood monocytes and bronchoalveolar macrophages

Bronchoalveolar lavage (BAL) was performed as described (Davidson *et al.*, 1986). At the time of BAL 50 to 100 ml of heparinized blood was taken (10 U/ml; Leo Labs, Princes

Risborough, UK). Peripheral blood mononuclear cells (PBMC) were prepared on Ficoll–Hypaque gradients (Nyegaard, Sweden) and interfacial cells were freed of platelets by centrifugation at 200 *g* in RPMI 1640 tissue culture medium supplemented with glutamine (Gibco, Paisley, UK), penicillin and streptomycin (Glaxo Ltd, Greenford, UK). Cell viability, by trypan blue exclusion, was invariably >95%. PBMC were held in medium supplemented with 10% heat-inactivated pooled human AB serum (NBTS, Tooting, UK) previously known to support antigen-driven proliferation without being inherently mitogenic or suppressive ('complete medium'; CM).

Accessory cell depletion from blood lymphocytes. PBMC suspended in CM ($3–5 \times 10^6$ cells/ml) were added to AB serum-coated 250 cm^3 flasks (Nunc, Roskilde, Denmark) to a maximum of 50×10^6 cells per flask. Flasks were loosely capped and placed in a 37°C incubator under 5% CO_2 in air for 1 h. Non-adherent cells were strained through 15 μm gauge nylon monofilament mesh (Cadisch and Son, London, UK), and added to 1 ml of Lympho-T-Kwik, a monoclonal antibody/complement cocktail (One Lambda Inc., Los Angeles) (Clouse *et al.*, 1987). After incubation for 1 h at 37°C, dead cells and floating fragments were removed by centrifugation. Lymphocyte pellets were washed twice and were >98% viable. The cell population obtained contained no HLA-DR$^+$ or CD14$^+$ cells by flow cytometry.

Accessory cell purification. Monocytes were detached from flasks by exposure for 10–15 min to 10 ml of 3·3 mM EDTA, prewarmed to 37°C and buffered to pH 7·0. After three washes the resulting monocytes were resuspended in 2 ml of ice-cold CM, counted and tested for viability (>98%). Alveolar macrophages were obtained by straining BAL fluid through coarse sterile gauze, and washing in ice-cold CM. Cell viability was always >85%.

Initial experiments demonstrated that BAL containing <10% lymphocytes did not incorporate significant amounts of ^{3}H-thymidine upon exposure to antigen or mitogen. Lavage cell suspensions in this category were therefore used without further purification. Where AM populations contained >10% lymphocytes, these were pre-incubated for 1 h at $1–2 \times 10^5$ cells/ml in AB serum-coated flasks and adherent cells were released using the EDTA-CM procedure (see above). Where lavages contained less than 2×10^6 AM and were predominantly lymphocytic, the cell suspensions were treated with 25 μg/ml mitomycin C (Sigma, Poole, UK) in CM at 4°C for 30 min in order to prevent ^{3}H-thymidine incorporation by the BAL cells. Initial studies showed that treatment of samples containing <10% lymphocytes with mitomycin C did not affect antigen presenting capacity. No association was found between the method of AM preparation and resulting activity. Clustering assays were done without the need for lung lymphocyte depletion as this did not interfere with visual assessment of clustering.

Measurement of lymphoproliferation. Monocytes or AM (5×10^3) were added to 5×10^4 lymphocytes in quadruplicate into flat-bottomed microtitre wells (200 μl) containing either medium alone, tuberculin PPD (MAFF, Weybridge, UK) or preservative free streptokinase-streptodornase (SKSD; Lederle, Gosport, UK) at 100 μg/ml; or Phytohaemagglutinin-R (PHA; Wellcome Diagnostics, Beckenham, UK) at 20 μg/ml.

These antigen/mitogen concentrations induced optimal proliferative responses at 6 days (for antigens) and 3 days (for mitogen) in most normal subjects. Control wells contained culture medium, or lymphocytes, or accessory cells separately with antigen or mitogen. After 48 h incubation with mitogen and 5 days with antigen, 0·5 μCi of [methyl-^{3}H]thymidine (specific activity 74 GBq/mmol; Amersham International, Amersham, UK) was added to each well for a further 22–24 h. Cultures were harvested onto glass fibre filter paper discs, and ^{3}H-thymidine content measured in a β-counter.

Measurement of the percentage of clustering accessory cells. Monocytes and lymphocytes were tagged with two different non-toxic fluorescent dyes. Monocytes were incubated on ice in CM containing 15 μg/ml of 1,1′-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes Inc., Eugene, OR). After two washes in CM, tagged monocyte viability was >98%. Lymphocytes were similarly labelled for 1 h at 37°C in a 50 μg/ml solution of 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO; Molecular Probes Inc.). After two washes, lymphocyte viability was >98%. DiO-labelled lymphocytes (4×10^5) were added to 1×10^5 DiI-labelled monocytes or unlabelled AM in duplicate in wells of a 96-well flat-bottomed Teflon microtitre plate (Cowie Scientific, Middlesborough, UK), with or without 100 μg/ml PPD or SKSD, or 20 μg/ml PHA. Teflon culture vessels were used since they did not adhere monocytes and macrophages. After 24 h the cell suspension was transferred to an LP3 tube (Costar) containing 200 μl paraformaldehyde (2%) in phosphate-buffered NaCl. The fixed cells were allowed to settle in a haemocytometer and examined under combined visible and ultraviolet light by epi-illumination using an inverted stage Leitz Dialux microscope with filters and dichroic mirrors appropriate for fluorescein. Monocytes were identified by orange fluorescence, whilst the unlabelled AM appeared muddy brown due to autofluorescence. Labelled lymphocytes exhibited bright green fluorescence and could therefore be differentiated from the other two populations. Some sarcoid lavages contained many lung lymphocytes spontaneously adhering to the macrophages; these lymphocytes were not fluorescent and were ignored in the assessment of clustering. Two hundred AM or labelled monocytes were located from random fields and scored either as single cells, or as associated with one or more fluorescent peripheral blood lymphocytes. These brown/green or orange/green cell aggregates were scored as clusters. Results were expressed as the percentage of monocytes or AM to which lymphocytes had clustered.

RESULTS

Normal and sarcoid monocyte- and alveolar macrophage-induced lymphoproliferation to two recall antigens

Table 1 compares the ability of monocytes and AM to present two antigens to autologous peripheral blood T cells. When the 11 normal individuals were analysed as a group, AM produced significantly lower proliferative responses than monocytes to both antigens ($P<0·01$, PPD and SKSD). In only 3/11 experiments for PPD, and 3/9 experiments for SKSD, was AM-mediated proliferation in excess of 40% of that seen with monocytes. Alveolar macrophage accessory cell efficacy relative to monocytes was also not dependent on the antigen which was being presented: thus, a comparison of the AM reconstitution ratios for PPD as opposed to SKSD revealed that they were significantly correlated ($r=0·88$, $P<0·05$).

In contrast, sarcoid AM presentation was *more* than 40% of the monocyte response in 14/14 experiments with PPD, and 6/7

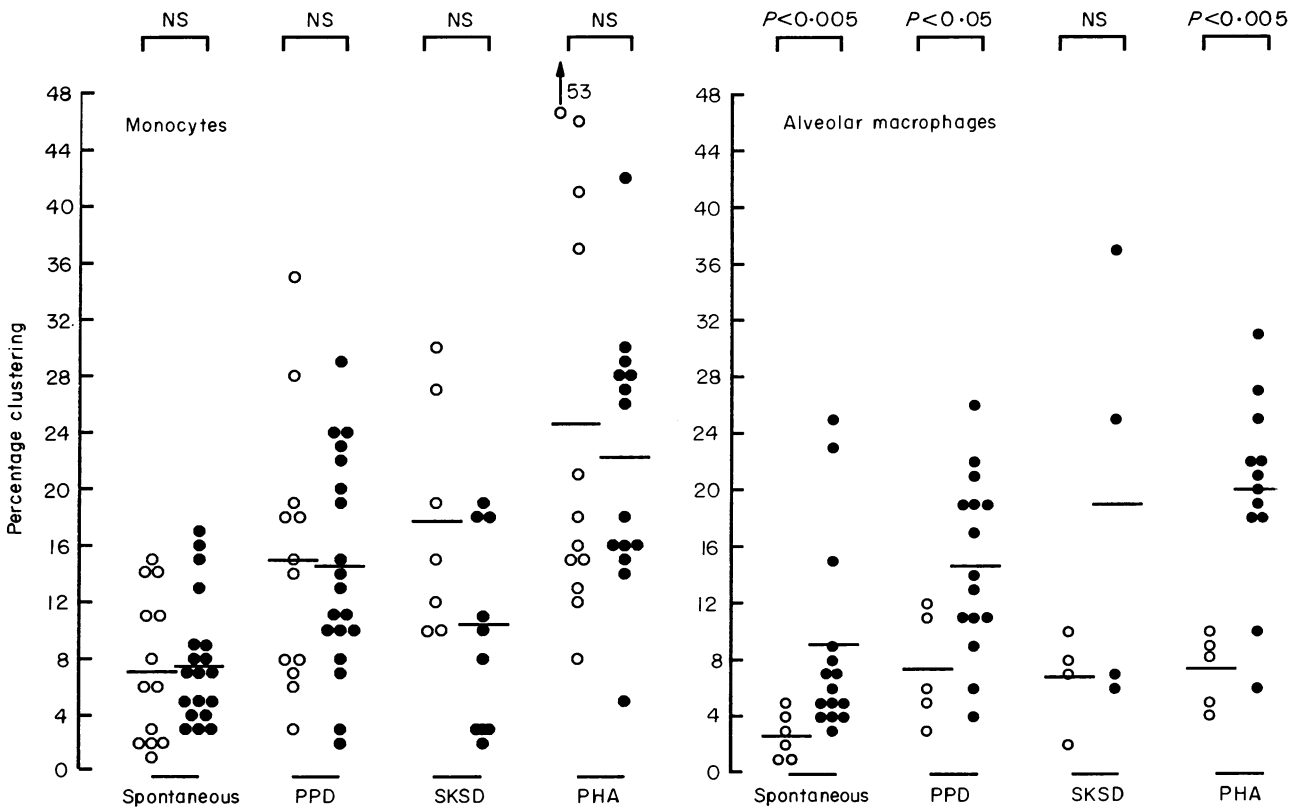

Fig. 1. Mononuclear phagocytes (10^5) were co-cultured in duplicate with autologous lymphocytes (4×10^5) in the presence of PPD (100 μg/ml), SKSD (100 μg/ml), PHA (20 μg/ml), or alone (spontaneous). Individual data points represent the mean percentage of accessory cells found clustered at 24 h derived from normal individuals (○) or sarcoid patients (●). Bar lines represent mean cluster formation in the eight test groups. Enhanced cluster formation by sarcoid alveolar macrophages is represented at the top of the figure (Mann–Whitney *U*-test). NS, not significant ($P > 0{\cdot}05$).

experiments with SKSD. When compared to the monocyte-driven responses, AM-mediated proliferative responses were significantly higher for PPD ($P < 0{\cdot}025$), though not significantly different for SKSD ($P = 0{\cdot}1$). Unlike the normal subjects (see above), no relation between reconstitution ratios for the two antigens was obtained ($r = -0{\cdot}32$, $P > 0{\cdot}5$). In the sarcoid group the heightened antigen presenting capacity of AM was unrelated to the lack of response of the patients to antigen, as judged by monocyte-driven lymphoproliferation responses of Δct/min values of less than 1000 for either antigen (see Table 1). It was concluded that normal and sarcoid AM exhibit different antigen presenting ability, unrelated to either the presence of poor peripheral responses to antigen, or the nature of the antigen being presented.

Normal and sarcoid monocyte and alveolar macrophage clustering to autologous lymphocytes

Monocytes. Figure 1 shows that after 24 h in non-adherent culture at a ratio of 1:10 with autologous lymphocytes, approximately 7% of both normal and sarcoid monocytes were found to be spontaneously clustered. The presence of 100 μg/ml PPD significantly increased the percentage of clustered monocytes from this baseline value to a mean value of 14·9% for normal subjects, and 14·3% for sarcoid patients ($P < 0{\cdot}01$ for both normals and sarcoids). 100 μg/ml SKSD also increased the percentage of clustered monocytes to a mean value of 17·6% for normal subjects, and 10·2% for sarcoid patients; unlike PPD, these increases were only statistically significant for normal monocytes ($P < 0{\cdot}05$). PHA at a concentration of 20 μg/ml also produced a significant enhancement of clustering percentage of both normal and sarcoid monocytes ($P < 0{\cdot}005$). When the normal and sarcoid groups were compared, there were no significant differences in either spontaneous, antigen- or mitogen-induced monocyte clustering capacity.

Alveolar macrophages. Figure 1 also shows that in all of six normal subjects, only a small proportion of the AM population spontaneously clustered autologous lymphocytes. The presence of PPD or SKSD induced only a 4–5% increase in clustering. Likewise, addition of 20 μg/ml of PHA only increased normal AM clustering by 4·5%. Unlike normal monocytes, addition of either antigen or mitogen did not significantly enhance AM clustering above the low spontaneous clustering value of 2·7%.

In contrast, sarcoid AM spontaneously clustered lymphocytes in significantly greater numbers than did normal AM (8·7% *versus* 2·7%, $P = 0{\cdot}003$). Furthermore, the presence of PPD induced a large and significant enhancement of sarcoid AM clustering over and above this high spontaneous value (14·8% *versus* 8·7%, $P < 0{\cdot}01$). Similarly, SKSD induced a further increase in clustering over spontaneous values; this group was however too small for statistical analysis. The presence of PHA likewise significantly enhanced sarcoid AM clustering of lymphocytes over spontaneous value (19·9% *versus* 8·7%, $P < 0{\cdot}01$). Sarcoid AM found clustered with lymphocytes were indistinguishable by light microscopy from those that were

not. Unlabelled bronchoalveolar lymphocytes were often found adhering to sarcoid AM; it was not possible to enumerate AM clustering of these, as opposed to peripheral blood lymphocytes, as they were partially obscured by the much larger AM.

It was accordingly concluded that the normal AM population contained only a small number of cells capable of clustering lymphocytes, even in the presence of recall antigens or mitogen; conversely, sarcoid AM were more 'monocyte like' in their behaviour, showing almost identical clustering percentages to peripheral blood monocytes, and clustering in a greater proportion in the presence of antigens and mitogen.

DISCUSSION

In this paper we show that alveolar macrophages (AM) from patients with sarcoidosis, unlike AM from normal subjects, are comparatively more powerful accessory cells than blood monocytes from the same individuals in respect of their ability to present recall antigens (PPD, SKSD) to highly purified T lymphocytes. We also show that sarcoid AM, unlike normal AM, are able to cluster autologous T lymphocytes effectively, and that this clustering capacity is enhanced by 24 h of exposure to recall antigens. Although we could not simultaneously compare clustering activity with accessory cell function in individual BAL samples, sarcoid AM clearly differed from normal AM in both respects.

In terms of antigen-presenting capacity, normal AM from most individuals functioned very poorly compared with blood monocytes (Table 1). That some individuals in the normal group exhibited effective accessory cell function is in close keeping with the findings of Lyons *et al.* (1986); and, when investigated, these individuals presented both recall antigens acceptably. The demonstration that two different antigens are presented in parallel with each other by normal AM is, to our knowledge, a new finding. The question arises of whether normal individuals might fall into 'high' and 'low' antigen presenters, as regards their alveolar macrophages, and of the immunological and clinical significance of this. We could find no obvious relationship between AM accessory cell function with smoking habits, ethnic group, age, sex, atopic status, family history or propensity to develop upper respiratory tract infection in the normal subjects. The finding that in sarcoidosis, alveolar macrophages are more effective than blood monocytes in presenting PPD, although not SKSD in the seven experiments reported here, might suggest that under pathological circumstances, accessory cell populations might exhibit antigen selectivity. Further experiments are required to examine this possibility.

For measurement of monocyte clusters a novel technique was developed which utilized two lipophilic non-metabolizable supravital dyes containing different fluorochromes to identify clustered macrophages or lymphocytes in suspension. Despite co-culture of the two cell populations for 24 h, no dye leakage or mixing was observed. AM from both normal and sarcoid subjects exhibited autofluorescence which was associated with subcellular organelles, perhaps phagolysosomes; this obviated the need for labelling them prior to addition of labelled lymphocytes. We chose to measure cell clusters in suspension culture in Teflon vessels, rather than tissue culture plastic vessels, to avoid the possible selection of cells according to their ability to adhere, a process which might result in the display of activities not seen in the whole cell population (Spiteri & Poulter, 1991). The question had arisen as to whether dendritic cells might be present in sarcoid BAL (Sertl *et al.*, 1986; Holt, Schon-Hegrad & Oliver, 1988; Pollard & Lipscomb, 1990); all sarcoid clusters we observed were centred on large, round cells, 15–40 μm in diameter, often containing phagocytosed particulate matter. On this morphological basis we consider that in sarcoid BAL, the cell type which is particularly active in clustering autologous T lymphocytes is a large alveolar macrophage. Lung lymphocyte clustering to 10% of the sarcoid AM population was reported by Yeager, Lussier & Prashad (1979), contrasting with 2% in normal BAL. In our experiments with AM and blood T lymphocytes, the values were 8·7% (sarcoid) and 2·7% (normal), in close agreement. To our knowledge this is the first report of macrophage–lymphocyte clustering in non-adherent culture.

This is also the first report that antigens such as PPD and SKSD, as well as PHA mitogen, will enhance the clustering capacity of sarcoid AM, and that the degree of antigen enhancement is comparable to that obtained with sarcoid monocytes, which show similar clustering characteristics to normal monocytes. This suggests that the ability of sarcoid AM or monocytes to cluster autologous T cells, in the presence or absence of antigen, is probably not related to the allergic or anergic status of the individual. Our further study of sarcoid monocytes, where both proliferation and clustering responses have been measured together, has revealed no rank correlation between them, nor any relationship to antigen sensitivity or anergy of the patient's leucocytes (data not shown).

In the present study, normal alveolar macrophages failed to cluster effectively, even in the presence of PHA mitogen, suggesting an intrinsic resistance to cluster formation. Pretreatment with sialidases is known to enhance the clustering and accessory cell functions of murine macrophages (Boog *et al.*, 1989); possibly sialidases present in sarcoid lavage fluid (Lambre *et al.*, 1988) might alter human alveolar macrophage function in a similar way. From this study we conclude that in sarcoidosis, both alveolar macrophages and blood monocytes are able to cluster autologous T lymphocytes in the presence or absence of recall antigens and PHA mitogen, and we consider that this phenomenon is unrelated to the anergic status of the individual. In contrast, normal AM show poor clustering ability, even in the presence of antigens or PHA, unlike blood monocytes. We suggest that in the normal lung, such resistance to clustering may contribute to the control of local pulmonary immune responses. In sarcoidosis, when both heightened antigen presentation and T lymphocyte clustering are features of alveolar macrophages, such control mechanisms may well be defective.

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