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ENHANCED *IN VITRO* PHAGOCYTIC POWER OF MACROPHAGES FROM PPD-STIMULATED SKIN SITES IN HUMAN SUBJECTS HYPERSENSITIVE TO PPD

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SUMMARY

By a quantitative Rebuck's skin-window technique human macrophages were collected from individuals either unreactive or hypersensitive to PPD, the latter having recovered from tuberculous infection.

In vitro testing of macrophages with a strain of *Paracolonbacter aerogenoides* proved that cells from hypersensitive convalescents were provided with increased pagocytic and bactericidal activities. An even higher degree of macrophage activation was attained when cells from hypersensitive individuals had previously been stimulated *in vitro* with PPD. Changes of macrophage functions such as those mentioned above might well result from the action on macrophages of lymphokine-like agents released by sensitized lymphocytes coming in contact with PPD.

INTRODUCTION

It is established that immunity to tuberculosis is cell-mediated (Lurie, 1942; Raffel, 1965; Suter & Ramseier, 1964; Dannenberg, 1968; Nelson, 1969), and depends on the activation of macrophage function (Waksman & Matoltsy, 1958).

Macrophage activation may be elicited by different stimuli and is expressed by an increase in cell size (Bennet & Cohn, 1966), in the number of lysosomes and mitochondria (Cohn & Benson, 1965; Rondanelli *et al.*, 1969, 1971), lysosomal and mitochondrial enzymes (Dannenberg *et al.*, 1963, 1968) and in different physiological activities such as proliferation (North, 1969), non-specific phagocytic and antibacterial activity (North, 1969; Mackaness & Blanden, 1967; Rondanelli *et al.*, 1969; Magliulo *et al.*, 1969), and spreading on glass surfaces (Blanden, 1968) etc. Recently Godal, Rees & Lamvik (1971) showed that macrophage function and morphology may be non-specifically altered as the result of the action *in vitro* on these cells of lymphokines released by immune lymphocytes on contact with the specific antigen. As we think that such problems are relevant to the field of human pathology, we investigated the non-specific phagocytic activities of human macrophages collected by a modification of Rebuck's skin-window technique (Rebuck & Crowley, 1955; Southam &

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Levin, 1966; Magliulo *et al.*, 1969) from skin sites stimulated with PPD in a group of normal subjects unreactive to PPD and in a further group of individuals displaying a high degree of PPD-reactivity who all had had *Mycobacterium tuberculosis* infection from which they had apparently fully recovered. The main object of this study was to correlate the phenomena of macrophage activation with the hypersensitive state *in vivo* in man.

MATERIALS AND METHODS

Human subjects

A total of twelve individuals were studied, six being negative and six being highly positive to PPD skin testing (positivity 1 : 10,000), the latter group of subjects consisting of those who had had tuberculosis lesions mostly of the primary type.

Preparation of human macrophages. Human macrophages were obtained by a quantitative modification (Southam & Levin, 1966) of the original Rebuck's skin window technique (Rebuck & Crowley, 1955) according to a method further developed in our laboratory (Magliulo *et al.*, 1968a and b, 1969; Magliulo, Bonizzoni & Poggio, 1971). A 1-cm^2 area of both forearms of our patients was abraded to the papillary layer of corium, neither antigens or irritants being added to the abraded area. Sterile tissue culture chambers (Sykes & Moore, 1959), deprived of their lower coverslips, were placed like a cup on the abraded areas and fastened to the forearm with an elastic band. The chambers were then filled with nutrient medium through a needle inserted in the rubber gasket, while a second needle provided an adequate air vent. Both needles were then withdrawn. At this point the abraded areas were enclosed in the cuplike chamber so that inflammatory cells migrating through the abraded areas passed into the fluid filling the culture vessel. Cells could thus be harvested at a given time, which in our study was between the 48th and the 56th hr after inflicting the skin lesion, when the cellular population consisted mainly of macrophages. The fluid filling the chambers was medium 199 with 0·15% EDTA.

Before the 48th hr fresh nutrient fluid was substituted at intervals of 12 hr through a needle inserted in the rubber gasket of the chambers, in order to withdraw effete cells and the early migrating polymorphonucleated granulocytes.

To a number of tissue chambers PPD was added in order to attain a final concentration in the medium of $1:10^5$ (v/v). This provided a continuous stimulation of the skin site with the antigen. Macrophages harvested according to the above mentioned method were washed twice in fresh medium devoid of both EDTA and PPD. Finally the cells were counted in a haemocytometer. Viability was assessed by the eosin exclusion method. After having adjusted cell concentration to $0·5 \times 10^6$ cells/ml with fresh medium, 1 ml of the cell suspension was introduced into a $1·5 \times 4·0$ cm Pyrex cylindrical bottle, whose bottom was fully covered by a circular glass cover-slip. The bottles were stoppered and incubated in a thermostat at 37°C for 1 hr in order to allow macrophages to adhere to the cover-slip. The medium was then substituted with an equal volume of a bacterial suspension in fresh medium.

Testing of phagocytic activity. A strain of *Paracolonbacter aerogenoides* was employed after suitable opsonization. Two different types of sera were employed for opsonization: the pooled sera of the six unreactive subjects (normal) and the pooled sera of the six hypersensitive subjects (T.B.C.). The bacteria were grown on broth for 24 hr, then diluted with medium 199 to a concentration of 5×10^8 bacteria/ml. For opsonization, 0·2 ml of serum was added to 0·2 ml of bacterial suspension and incubated for 20 min. The bacteria were

then added to the cultures, their concentration having previously been adjusted in order to attain a ratio of 1:100 between bacteria and cells in cultures. The experimental design was such that for each of the unreactive subjects and for each of the hypersensitive individuals four cultures were prepared, two containing cells from unstimulated skin sites and two with cells from lesions stimulated with PPD. One bottle of each pair contained bacteria opsonized with serum from unreactive subjects and the other with serum from hypersensitive patients.

A number of bottles devoid of macrophages were inoculated with *Paracolonbacter aerogenoides*, in order to assess the growth rate of the bacteria in the absence of phagocytic activity.

TABLE 1. Average data and variability range of percentages of phagocytosis and survival of bacteria

Origin of macrophages	Unreactive subjects				Convalescent hypersensitive subjects			
Local stimulation with PPD	No		Yes		No		Yes	
Opsonizing serum	Normal	T.B.C.	Normal	T.B.C.	Normal	T.B.C.	Normal	T.B.C.
Phagocytosis %								
Average	36·5	56·1	36·8	58·6	50·0	69·0	69·5	82·3
Variability range	51–25	63–49	42–29	63–51	58–40	80–48	75–62	93–75
Survival %								
Average	59·5	44·3	50·6	43·1	37·6	32·5	27·3	18·8
Variability range	70–52	54–40	53–48	43–38	41–27	38–30	38–15	27–10

All the bottles were kept at 37°C for 1 hr. Counts of bacteria surviving in the supernatant and within phagocytes were then performed according to a procedure analogous to that described by Jenkins & Babacerraf (1960) by plating separately the supernatant and macrophages, the latter being lysed with distilled water, subsequently plating the resulting suspension. These data were compared with the growth rate of bacteria in the absence of phagocytosis, so that the percentage of phagocytosed organisms and the percentage of bacteria surviving post-engulfment could be assessed (Stuart, 1967).

RESULTS

Table 1 reports the percentages of germs phagocytosed by macrophages *in vitro* and of germs surviving after engulfment by these cells. The statistical analysis is shown in Table 2.

It may be noted that in all experiments opsonization of the bacteria with the serum from hypersensitive patients lead to a significant increase in the percentage of germs phagocytosed.

This increased phagocytic activity was followed by a decreased survival of phagocytosed germs, though the differences observed were statistically significant only when macrophages from normal subjects were employed. Macrophages from unstimulated skin sites (PPD absence in the superimposed culture chambers) in normal subjects did not display any significant difference in phagocytic and bactericidal activity compared with macrophages from PPD-stimulated skin-sites in the same normal subjects.

Macrophages from both stimulated and unstimulated skin-sites in hypersensitive in-

TABLE 2. Average data and variability range of percentages of phagocytosis and survival of bacteria

Origin of macrophages	Unreactive subjects				Convalescent hypersensitive subjects			
Local stimulation with PPD	No		Yes		No		Yes	
Opsonizing serum	Normal	T.B.C.	Normal	T.B.C.	Normal	T.B.C.	Normal	T.B.C.

	Student's *t*-test	
Phagocytosis % Student's *t*-test	t = 4·80 (P< 0·01) t = 8·39 (P< 0·01)	t = 3·53 (P< 0·01) t = 4·24 (P< 0·01)
	t = 0·08 (P>0·05)	t = 5·83 (P< 0·01)
	t = 1·07 (P>0·05)	t = 5·41 (P< 0·01)
	t = 3·12 (P< 0·01)	
	t = 2·72 (P< 0·05)	
	t = 7·66 (P< 0·01)	
	t = 7·31 (P< 0·01)	
Survival % Student's *t*-test	t = 2·59 (P< 0·05) t = 10·06 (P< 0·01)	t = 1·59 (P>0·05) t = 1·26 (P>0·05)
	t = 1·65 (P>0·05)	t = 3·41 (P< 0·01)
	t = 1·83 (P>0·01)	t = 4·65 (P< 0·01)
	t = 3·84 (P< 0·01)	
	t = 3·78 (P< 0·01)	
	t = 5·73 (P< 0·01)	
	t = 7·72 (P< 0·01)	

dividuals phagocytosed and killed bacteria better than macrophages from unreactive subjects in the analogous experimental condition.

Furthermore in hypersensitive subjects, contrary to what had been observed in normal individuals, the macrophages obtained from skin sites stimulated with PPD phagocytosed and killed the bacteria better than the macrophages coming from unstimulated sites.

DISCUSSION

The present study based on human experimentation provides evidence that (i) human macrophages from individuals convalescing from tuberculosis and hypersensitive to PPD are pro-

vided with better phagocytic and bactericidal powers for *P. aerogenoides* than macrophages from normal subjects unreactive to PPD; (ii) local stimulation at the skin-sites from which the macrophages originated did not alter the phagocytic and bactericidal activities of macrophages in normal subjects unreactive to PPD, but strongly increased the same activities of macrophages in convalescent hypersensitive individuals; (iii) in every respect sera from convalescent subjects had better opsonizing capacity for *P. aerogenoides* than sera from normal subjects; (iv) the increased opzonising activity was constantly followed by a better killing of phagocytosed bacteria.

Not all of these facts may be easily explained; it may be assumed that the recent tuberculosis infection might have caused directly or indirectly a systemic activation of mononuclear phagocytes, a condition, however, which might be of low magnitude if compared with that achieved locally at the sites of tuberculous infection (Dannenberg, 1968). This systemic activation could well be maintained by the continuous release of lymphokine factors into the general circulation (Godal *et al.*, 1971) by the sensitized lymphocytes making contact with the tubercular antigens in localized persisting foci of infection.

That this systemic macrophage activation might be related to tuberculin hypersensitivity is furthermore suggested by the striking increase in phagocytic and bactericidal activities of macrophages of PPD-reactive patients when these cells had been previously exposed *in vivo* to PPD. In accord with Mackaness (1964) macrophage activation was non specifically expressed, the tested bacteria being of a species unrelated to *M. tuberculosis*.

However it may be noted that the better opsonizing activity exerted on *P. aerogenoides* by the sera from convalescent individuals may well be the result of cross-immunization phenomena having occurred in tuberculous patients as a consequence of the enhancement of the adjuvant functions in active tuberculosis (Dumonde & Maini, 1971).

It must be noted that in our experimental design the PPD stimulation was applied in a system very near to the *in vivo* condition, i.e. by introducing PPD in the tissue chambers enclosing the area of abraded skin. In this situation it may be expected that the effects on macrophages resulted either from the possible influence of humoral mediators released by sensitized lymphocytes on contact with PPD, or from direct action on macrophages of lymphokine actors released by lymphocytes circulating in the blood vessels underlying the skin lesion or entering as inflammatory cells into the tissue chamber. A further approach to the problem is provided by investigations from our group (Magliulo *et al.*, 1967, 1968a and b, 1971) which proved that in some experimental systems in man, such as that employed in the present investigation, stimulation of hypersensitive subjects with PPD led to the collection of increased numbers of macrophages in the tissue culture chambers devised for this Rebuck's quantitative technique. In all these instances it may well have been that humoral lymphokine factors, possibly analogous to the migration inhibition factor (David, 1968; Bloom & Bennett, 1968) had acted by favouring the local accumulation of highly phagocytic and bactericidal macrophages at sites where an antigenic stimulation was afforded. In every respect, it may be assumed that this phenomenon was the expression of a state of active cell-mediated immunity achieved in tuberculous patients through infection and subsequent cure. The real state of affairs in active disease, when perhaps cellular immunity is inadequate, must still be elucidated.

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