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Mycobacterial antigen-specific human T-cell clones secreting macrophage activating factors

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Summary. Macrophage activating factor (MAF) is produced by antigen-stimulated lymphocytes and activates macrophages for antimicrobial function. The capacity of individual microbial antigens to evoke and regulate this response has been explored using an affinity purified antigen (TB68) of *Mycobacterium tuberculosis* in combination with T-cell cloning. Four helper/inducer clones are described which responded strongly to this antigen. Three were specific, proliferating only to TB68 antigen and antigenic preparations containing this antigen. However, one of these clones (68.1) did not proliferate to BCG and PPD which contained the TB68 antigen. In addition, another clone, 68.13, also proliferated to other antigenic preparations which did not contain the TB68 antigen. Taken together, these data indicate the presence of several epitopes in the affinity-purified TB68 antigen. All the clones produced MAF, which enhanced H_2O_2 production in U937 cell lines and conventional macrophages matured from monocytes. Thus, T-cell clones proliferating to a mycobacterial antigen constitutively secrete lymphokines that activate macrophages to antimicrobial immunity.

INTRODUCTION

The development of host responses to facultative, intracellular parasites has been widely studied in the mouse using *Listeria monocytogenes* as a model (Mackaness, 1969; Hahn & Kaufmann, 1981). It has been clear from these and other studies (North, 1973; Godal, Rees & Lamvick, 1971; Patterson & Youmans, 1970; Simon & Sheagren, 1972) that antimicrobial immunity involves the direct collaboration of specific lymphocytes and macrophages in the generation of effective immunity. These lymphocytes can be antigen-specific, whilst the macrophage effector systems are essentially non-specific (Mackaness, 1969). Activation of macrophages for antimicrobial function can also be achieved by lymphokines (MAFs) which are released by antigen-activated T cells (Mackaness, 1969; North, 1973; Godal *et al.*, 1971; Patterson & Youmans, 1970; Simon & Sheagren, 1972). MAF found in crude supernatants from mitogen- or antigen-stimulated lymphocyte cultures contain a heterogeneous mixture of activities (Meltzer *et al.*, 1980; Ralph *et al.*, 1983; Nacy, Leonard & Meltzer, 1981) which may have selective effects on macrophage activation (Gemsa *et al.*, 1983). Killing of intracellular organisms is the ultimate stage in macrophage activation (Cohn, 1978). Killing of intracellular *Mycobacterium microti* has been shown when mouse macrophages were activated with a crude supernatant from PPD-stimulated mouse spleen (Walker & Lowrie, 1981). This killing was probably due to macrophage hydrogen peroxide. Little is known of the importance of individual antigens in evoking or regulating these responses. Consequently, we have used an antigen of *Mycobac-*

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terium tuberculosis, which was purified by affinity to a monoclonal antibody specific for *M. tuberculosis* and for *Mycobacterium bovis* strain BCG (Coates *et al.*, 1981) to generate T-cell clones in order to examine the function of responding T cells at the level of the individual T cell.

MATERIALS AND METHODS

The TB68 antigen was prepared by passage of an expressate of *M. tuberculosis* strain H37Rv; the non-particulate fraction of a bacterial homogenate was passed through a press (Hewitt *et al.*, 1982) over an immunoadsorbant column coupled to the monoclonal antibody TB68 (A. D. M. Rees, Rajiswamy, B. W. Allen, A. R. M. Coates & D. A. Mitchison, manuscript in preparation). TB72 antigen was similarily prepared. The monoclonal antibodies used to prepare these antigens bound to all strains of *M. tuberculosis* and *M. bovis*, strain Vallee, but only weakly to *M. bovis* strain Vallee and BCG, and not at all to other mycobacteria or *Escherichia coli* (Coates *et al.*, 1981). Both the TB68 and the TB72 antigens were, at least in part, proteins. Competitive inhibition studies using monoclonal antibodies (Hewitt *et al.*, 1982) indicated that they were distinct antigens. Other antigenic preparations of mycobacteria used in specificity studies were expressates of mycobacteria prepared in the same manner as *M. tuberculosis* strain H37RV. Tuberculin-purified protein derivative (PPD) was obtained from Evans Medical Supplies Ltd, Speke, Liverpool).

Interleukin-2 (IL-2) was obtained from Biotest Folex Ltd, Offenbach, West Germany as 'Lymphocult T' lectin-free containing $<0{\cdot}1\%$ phytohaemagglutinin. Optimal concentrations of IL-2 for the maintenance of clones was determined by titration against PHA blasts prepared from the same donor as the clones.

Cloning of antigen-activated peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque centrifugation. PBMC were adjusted to a concentration of 2×10^6 cells/ml in maintenance medium RPMI 1640 (Gibco Biocult Ltd, Uxbridge, Middlesex) with 20% autologous serum (heat inactivated) and incubated with antigen for 4 days at 37° in 5% CO_2 in air. Aliquots of 200 μl of cells were incubated with 20 μl of 0·0117 g/l TB68 antigen. After 4 days in culture, the volume of maintenance medium was doubled and 15% IL-2 added. The cells were then incubated for a further 2 days before harvesting for cloning. After a total of 6 days in culture in the presence of antigen, cells were harvested, resuspended over Ficoll-Hypaque in 12×75 mm sterile capped tubes (Falcon Plastic Ltd, Becton Dickinson, Oxnard, CA) and centrifuged for 20 min at 200 ***g***. Cells at the interphase were enriched two-fold for lymphoblasts. These cells were diluted to 16·67 cells/ml of maintenance medium containing 15% IL-2 and 20% autologous serum and 10 μl were plated in sterile 60-well microterasaki plates (Nunc Ltd, Uxbridge, Middlesex). Autologous PBMC at 1×10^4/ml per well were γ-irradiated (3000 rads, ^{137}CS) (feeders) and added with antigen (0·0117 g/l) in 10 μl aliquots to the wells containing blasts. The optimal concentration of serum, antigen and IL-2 was estimated from preliminary studies on the clone donor cells.

Cultures were incubated for 7 days in humidified chambers at 37° in 5% CO_2 in air. After this, plates were scanned microscopically for wells containing clones. Growing clones were then transferred to 96-well flat-bottomed microtitre plates (Flow Laboratories, Irvine, Scotland) containing fresh maintenance medium supplemented with 5×10^5/ml feeders, 15% IL-2 and 0·0117 g/ml TB68 antigen. After a further 7 days in culture, the clones transferred to 24-well trays (Linbro Scientific Co., Flow) containing 15% IL-2, 1×10^6 feeders and the TB68 antigen (0·0117 g/l in a volume of 2 ml per well. Thereafter, cultures received fresh IL-2 every 3–4 days, and feeders and antigen every 6–7 days, and were thus maintained throughout the course of the experiments.

Determination of the specificity of T-cell clones

Clones were allowed to grow for 4 days after the addition of IL-2 and feeders, before testing of specificity by proliferative responses to a range of antigens. Proliferative responses were determined using a microterasaki method (O'Brien *et al.*, 1979). Briefly, cloned cells at $2{\cdot}5 \times 10^5$/ml in maintenance medium containing 5% feeders and 20% heat-inactivated autologous serum were added in 20 μl aliquots to microterasaki plates containing a range of antigenic preparations in 2 μl aliquots at optimal concentrations, which had been previously determined. The plates were inverted and the cells were cultured on hanging menisci in humidified chambers at 37° in 5% CO_2 in air. After 24–72 hr, the cells were pulsed with 0·5 Ci of [^{3}H]TdR (Amersham, Amersham, Bucks) (22 Ci/mM) and incubated for 4 hr. They were than harvested on

precut filter blocks. Proliferation, as measured by [^{3}H]TdR incorporation, was then determined by liquid scintillation spectroscopy. The results are expressed as the mean (c.p.m.) ± standard error of the mean (SEM) for triplicate cultures.

Cell surface phenotyping of T-cell clones

T-cell clones were examined for cell surface phenotype using monoclonal antibodies of the Leu series (Becton Dickinson) Leu 1a, Leu 2a and Leu 3a were used as pan T, suppressor/cytotoxic and helper/inducer T-cell subpopulation markers, respectively (Evans *et al.*, 1981). Leu 8a was used to further identify a subpopulation of Leu 2a$^+$ or Leu 2a$^+$ T cells (Gatenby *et al.*, 1982). Cytospin preparations (Cytospin Shandon, Runcorn, Cheshire) of 5×10^5 cells were immunoenzymatically stained (Moir *et al.*, 1983). Slides were incubated in BFA fixative for 30 sec at room temperature. They were then washed in tris-base saline (TBS) for 5 min and incubated with monoclonal antibodies, followed by alkaline phosphatase conjugated rabbit anti-mouse with normal human serum for 30 min. The slides were again washed in TBS for 5 min and incubated with substrate (naphthol-AS-MX phosphate with fast red JR salt) (Sigma, London) for 8 min. The reaction was then stopped in tap water, counterstained with haematoxylin and mounted in DPX (Raymond Lamb, London). They were examined by bright field microscopy for colour reactions.

Testing of T-cell clones and supernatants for antimicrobial activity

(a) U937 cell lines. Antimicrobial activity was tested on a human myeloid cell line, U937, which can be immunologically activated to express antimicrobial immunity (Wing *et al.*, 1981). U937 cells at 10^6 cells/tube were cultured in RPMI 1640 (Gibco) with 10% fetal calf serum (FCS) in the presence or absence of cloned T-cell supernatants, sham culture supernatant control (prepared by incubating 5×10^5/ml feeders in clone maintenance medium with 15% IL-2 and 20% autologous serum for 3–5 days), and spleen cell factor (SCF) produced by stimulated human spleen cells cultured with PHA for 72 hr. Medium, clone supernatants and SCF in U937 cultures were replaced at 24 hr intervals for 3 days. Peroxide release was then determined in the presence of phorbol myristate acetate (PMA) 1mg/l (Jackett *et al.*, 1982). Results are given as nM $H_2O_2/10^6$ U937 cells minus the values obtained for comparable cultures in the absence of supernatants or SCF.

(b) Macrophages matured from peripheral blood monocytes. Peripheral blood monocytes were cultured to yield macrophages (Crowie & May, 1981). In brief, 10^6 PBMC were incubated in eight chamber slides (Lab-Tek Products) in Neuman & Tytell serum-free medium (Gibco) containing 1% autologous serum (maintenance medium) for 60 min at 37°. The slide chambers were washed twice in warm maintenance medium and cultured at 37° in a gassed CO_2/O_2 incubator at 37° for 3 days. They were again washed with warm maintenance medium. Clone supernatants (10%), or cloned T cells in maintenance medium, were added and cultures continued for 3 days. The slide chambers were than washed twice with Hanks' balanced salt solution (HBSS) to remove traces of serum. Peroxide release was determined as for the U937 cell lines (Jackett *et al.*, 1981). The results are expressed as the mean nM H_2O_2 (± SEM) released per 10^5 monocytes in the presence of PMA minus the values obtained in comparable cultures in the absence of PMA.

RESULTS

Cloning of antigen-stimulated PBMC

The percentage of blast-like cells, determined by visually counting the number of large cells, was estimated to be 50% at the end of the preliminary culture of donor PBMC with antigen and IL-2. A total of 20 clones were eventually obtained after limiting dilution and transfer to 24-well Linbro plates. Approximately 1 in 20 of the seeded cells proliferated and survived to the 24-well stage. T-cell clones were initially screened for proliferative responses to the initiating antigen (data not shown). Effort was concentrated on four clones which responded strongly to this antigen.

Antigen specificity of T-cell clones

The antigenic specificity of individual clones was determined by coculturing clones with feeders and a variety of antigenic preparations, some of which were known to contain, at least in part, determinants of the eliciting antigen and some of which did not (Coates *et al.*, 1981; Hewitt *et al.*, 1982). The monoclonal antibody TB68 has also been shown to bind to PPD (M. Harboe, personal communication). Table 1 shows that all clones responded vigorously to the TB68 antigen. The clones also responded to other antigenic preparations. Indeed, clone 68.13 also responded to a

Table 1. Proliferative response of T-cell clones

Antigen	Dose (mg/l)	Presence of antigen binding with TB68 MAB	Clone no. c.p.m. (mean ± SEM) 68.13	68.1	68.2	68.6
Escherichia coli	10^{-2}	–	10,463 ± 283	1946 ± 43	1711 ± 13	3649 ± 104
M. kansasii	10^{-1}	–	9886 ± 422	1361 ± 44	1083 ± 54	2010 ± 70
M. fortuitum	10^{-1}	–	11,421 ± 564	ND	975 ± 22	1995 ± 54
TB72 antigen	10^{-1}	–	11,937 ± 601	1616 ± 60	1460 ± 36	3598 ± 156
M. tuberculosis, strain H37Rv	10^{-1}	+	9861 ± 527	15,016 ± 433	12,988 ± 183	8883 ± 462
M. bovis BCG	10^{-1}	+	ND	8015 ± 359	881 ± 21	9301 ± 238
PPD	10^{-1}	+	9729 ± 490	9882 ± 509	1601 ± 92	16,827 ± 457
TB68 antigen	10^{-3}	+	9449 ± 446	16,748 ± 643	14,036 ± 208	20,001 ± 583
Medium			751 ± 58	548 ± 21	1530 ± 51	3326 ± 99
IL-2			18,881 ± 604	13,849 ± 758	10,961 ± 518	14,118 ± 773

10^5 T-cell clones were stimulated with TB68 (0·017 mg/l), or other antigens, at specified previously determined optimal concentrations. (Other antigens were a similarily purified protein antigen TB72, or mycobacterial pressates.) Clone cells were tested for *in vitro* proliferative responses 4 days after the addition of IL-2, feeders and antigen. They were then cultured with 10^4 autologous irradiated PBMC and antigen for 48–72 hr. Stimulation was determined by the incorporation of [^{3}H] TdR.

number of antigens which did not contain determinants of the eliciting antigen, particularly the TB72 antigen. In contrast, three other clones (68.1, 68.2 and 68.6) proliferated only to those antigenic preparations which contained determinants of the TB68 antigen, i.e. *M. tuberculosis* strain H37Rv, *M. bovis* strain BCG, and PPD. Not all clones responded identically. In particular, clone 68.2 responded only to antigen TB68 and to *M. tuberculosis*, indicating that this clone may recognize a different epitope of the antigen than either clones 68.1 or 68.6. All clones were responsive to IL-2 alone, although to a lesser extent than to antigen alone. Optimal proliferation was achieved in the presence of both antigen and IL-2.

Cell surface phenotype of T-cell clones

As shown in Table 2, all clones were positive with the pan T-cell marker (Leu la). They were also positive for Leu 3a and Leu 8a, indicating that they were phenotypically T helper/inducers.

MAF production by T-cell clones

Clone supernatants were then tested for their capacity to increase H_2O_2 production U937 cells. Table 3 shows that all clones, irrespective of specificity, increased H_2O_2 production. The clone supernatants were ten times more effective than a whole phytohae-

Table 2. Cell surface phenotypes of T-cell clones and sub-clones staining with monoclonal antibodies

Clone no.	Monoclonal antibody (% cells staining) Leu 1	Leu 2a	Leu 3a	Leu8a
68.13	97	< 1	83	62
68.1	95	< 1	92	71
68.2	94	< 1	76	83
68.6	90	< 1	95	68
Sub-clones				
68.1.1	89	< 1	75	62
68.1.2	77	< 1	82	84
68.1.2	94	< 1	96	87

Slides of T-cell clones or sub-clones were prepared on a cytospin centrifuge (Shandon) and incubated with monoclonal antibody as specified. Binding of the monoclonal antibody was detected by immunoenzymatic reaction, using alkaline phosphatase conjugated to rabbit and mouse IgG with substrate, napthol-AS-MX phosphate dissolved in N,N,-dimethyl/formamide in 0·1 M Tris buffer, pH 7·2 with fast red. Slides were examined by bright field microscopy; cells were counted and the number expressed as a percentage of the total cells viewed.

Table 3. Capacity of T-cell clones to secrete macrophage activating factor

Dilution of supernatant	Clone no. or preparation nM H_2O_2 per 10^6 U937 cells (mean ± SD)				
	1	2	6	SCF	Sham
30–50%	21·1 ± 1·5	19·9 ± 0·9	25·3 ± 0·3	15·6 ± 2·5	4·5 ± 1·0
10	21·0 ± 1·3	20·4 ± 1·5	21·3 ± 2·1	8·1 ± 0·4	7·9 ± 4·2
3	18·7 ± 3·8	18·3 ± 2·0	20·6 ± 3·5	2·0 ± 0·6	2·3 ± 2·3
0·3	14·4 ± 1·7	12·0 ± 1·7	18·2 ± 0·7	ND	2·6 ± 1·0
0·03	2·9 ± 1·0	1·3 ± 0·6	3·4 ± 0·2	ND	0

U937 cells were cultured in the presence and absence of cloned T-cell supernatants, sham control (a supernatant prepared from identical cultures, but in the absence of cloned T cells) and spleen cell factor (SCF) produced by PHA activated human spleen cells. Medium, supernatants and SCF were replaced at 24 hr intervals for 3 days. Peroxide release was determined in the presence of PMA. Results are given as nM H_2O_2 per 10^6 U937 cells minus the values obtained for comparable cultures in the absence of supernatants or SCF.

magglutinin-activated spleen cell supernatant (SCF). Supernatants were also tested for their ability to elevate the release of H_2O_2 in conventional macrophages matured from the peripheral blood of normal donors. It can be seen in Table 4 that clone supernatants were also active when tested on these cells, indicating that this activity was not dependent on using U937 cells. Sham supernatants had a small, but measurable, effect on H_2O_2 production. This was not due to IL-2 which, even at 50% concentration, did not increase H_2O_2 production above control levels. Nor was it attributable to a factor secreted by the autologous feeders, since sham supernatants prepared without these cells had comparable activity. This effect appeared to be entirely due to autologous serum present in the clone culture (data not shown).

Table 4. Effect of cloned cells and supernatants on conventional autologous macrophages

Monocytes cultured in presence of:		nM $H_2O_2/10^5$ monocytes (M ± SEM)
Expt 1		
0		11 ± 0·04
10%	Clone 1 S/N	17 ± 0·11
6×10^4	Clone 1 cells	22·67 ± 0·16
Expt 2		
0		9·3 ± 0·03
10%	Clone 2 S/N	16·4 ± 0·03
10^4	Clone 2 cells	11·0 ± 0·10
5×10^4	Clone 2 cells	19·0 ± 0·11
10^5	Clone 2 cells	18·6 ± 0·13

Macrophages were prepared from PBMC of clone donor by 3 days culture in serumless media. Clone supernatants of cells were added at specified concentrations for a further 3 days. Macrophage release of H_2O_2 was then determined in the presence of PMA. Results are given as nM H_2O_2 per 10^5 macrophages minus the values obtained for comparable cultures in the absence of supernatants or cells.

Table 4 also shows that the cloned cells were to activate conventional autologous macrophages, with increased H_2O_2 release. The activity of two of the cloned cells and their corresponding supernatants is illustrated. They were found to be effective at concentrations as low as 10^4, with maximal effect at 5×10^4 cells/10^6 U937 cells.

Sub-cloning of T-cell clones

As the plating efficiency at cloning was estimated to be 20%, clonality was later established by subcloning (Lamb *et al.*, 1982). The efficiency of plating at sub-cloning of Clone 68.1 was estimated to be 95%. Table 5 shows that the sub-clones of this representative clone were identical to the parent clone in specificity when tested with a range of antigenic preparations. In addition, as shown in Table 2, they were phenotypically identical.

Table 5. Specificities of sub-clones of T-cell clone 68.1

Antigen	Dose	Sub-clone number				
		68.1.1	68.1.2	68.1.3	68.1.4	68.1.5
72antigen	10^{-1}	974±82	301±54	775±96	1411±101	2620±113
M. kansasii	10^{-1}	988±79	446±41	801±63	1398±122	2511±141
TB68 antigen	10^{-3}	13,861±1001	10,020±979	20,010±1412	18,886±1188	30,664±2276
PPD	10^{-1}	12,992±668	10,001±712	15,662±1440	13,001±988	23,889±2017
Medium		1012±36	376±12	564±48	1192±56	2498±171
IL-2		8963±501	12,227±813	10,883±998	15,661±651	25,112±1176

Clone 68.1 was sub-cloned by limiting dilution. Sub-clones were stimulated with TB68 (0·017 g/l) or other antigen preparations as specified. They were tested for *in vitro* proliferative responses 4 days after the addition of IL-2, feeders and antigen. They were then cultured with 10^4 autologous irradiated PBMC and antigen for 48 hr. Stimulation was determined by the incorporation [^{3}H]TdR.

DISCUSSION

Human T-cell clones are described with antigenic specificity for an antigen of *M. tuberculosis*. As plating efficiency was initially low, we have used sub-cloning, in the manner described for clones to influenza virus (Lamb *et al.*, 1982), to establish clonality. The high plating efficiency at sub-cloning, taken together with the similarity of sub-clones (in terms of both phenotype and specificity) to the parent clone, indicated that the T-cell clones were truly clonal in nature.

The clones described all proliferated strongly to the TB68 antigen. However, whilst three of the clones proliferated only to those antigenic preparations known to contain it, clone 68.13 appeared to be less specific, in that it also proliferated to other antigenic preparations. This clone, however, required antigen for maximal growth and, therefore, appears to be genuinely reactive to an epitope in the TB68 antigen shared with many mycobacteria and *E. coli*. The presence of this epitope was not evident from the narrower specificity of the monoclonal antibody used to purify the TB68 antigen (Coates *et al.*, 1981). In addition, clone 68.2, although proliferating specifically to the TB68 antigen, did not react with two antigenic preparations known to contain determinants of the antigen. Taken together, these results suggest the presence of at least three epitopes in the TB68 antigen. One, recognized by clone 68.1 and 68.6, has the distribution expected from the specificity pattern of the monoclonal antibody, in that it is confined to *M. tuberculosis* and *M. bovis*. Another, identified by clone 68.2, is restricted to *M. tuberculosis*. The third is apparently shared by many mycobacteria and *E. coli*. In its native form, the TB68 antigen is a large molecule with a molecular weight of 120,000. As this breaks down to four chains upon SDS treatment, the epitopes may be located at different sites on the molecule. Experiments are currently in progress to test this possibility. Taken together, these results indicate that purification, even of a single molecular species, may yield several antigenic epitopes. As T-cell clones can identify these other epitopes, they can be used to map and obtain those with absolute specificity for *M. tuberculosis*. Clone 68.2, for example, appears to recognize just such an epitope; this would be useful, for example, in distinguishing an infection with *M. tuberculosis* from BCG vaccination.

Clone supernatants increase H_2O_2 release in a human myeloid cell line, U937 (Wing *et al.*, 1981). Whilst the exact relationship between the release of H_2O_2 and microbicidal events remains to be clarified. H_2O_2 appears to kill *Mycobacterium microti* in mouse macrophages (Walker & Lowrie, 1981). An increase in H_2O_2 release is, therefore, an indication of macrophage activating factor activity. This activity was also evident when conventional macrophages matured from peripheral blood were used as a macrophage source, indicating that it was not dependent on using U937 cells. Cloned cells could also enhance H_2O_2 release in these macrophages, suggesting that the release of the activity was a property of the cells themselves. We have found that the MAF activity of the clones cannot be attributed to the small interferon (IFNγ) content of the supernatants (Andrew *et al.*, 1984). This indicates that IFNγ is not the only macrophage activatory product of microbial antigen-stimulated T cells, as has previously been suggested

(Nathan *et al.*, 1983; Schultz & Kleinschmidt, 1983). The antigen TB68 is not alone in stimulating MAF production by lymphocytes, since a variety of stimuli have been used (Mackaness, 1969; Meltzer *et al.*, 1980; Ralph *et al.*, 1983; Nacy *et al.*, 1981). However, using T-cell cloning, we have demonstrated that helper/inducer T cells which are specifically proliferating to a mycobacterial antigen do secrete MAF. The macrophage is the main effector cell of immunity to *M. tuberculosis*, ultimately responsible for the ingestion and killing of the organism. We have shown that T cells specific for a mycobacterial antigen are able, by cell-to-cell contact or through lymphokines, to enhance this macrophage function which is vital to the generation of effective immunity in man.

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