*Immunology* 1994 **82** 445–449

# Antigen 85C on *Mycobacterium bovis*, BCG and *M. tuberculosis* promotes monocyte-CR3-mediated uptake of microbeads coated with mycobacterial products

G. HETLAND & H. G. WIKER *Institute of Immunology and Rheumatology, The National Hospital, University of Oslo, Oslo, Norway*

## SUMMARY

The uptake in monocytes of monodispersed latex microbeads precoated with whole bacillus Calmette–Guérin (BCG) cells, *Mycobacterium tuberculosis* sonicate or culture fluid, or antigen (Ag) from the culture fluid was examined by microscopy. There was a significantly higher cell association of beads coated with whole BCG cells, the secreted 85C component of the Ag 85 complex or *M. tuberculosis* sonicate than of phosphate-buffered saline (PBS)-treated control beads. Antibodies (Ab) to Ag 85 inhibited the uptake of BCG- and Ag 85C-treated beads. A monoclonal antibody (mAb) to complement receptor type 3 (CR3), but not mAb to CR1, inhibited the uptake of Ag 85C-coated beads, indicating that the mycobacterial Ag-dependent uptake of particles was mediated via CR3 on the monocytes. This points to the existence of a ligand on Ag 85C which may promote monocyte uptake of *M. bovis*, BCG and *M. tuberculosis*.

## INTRODUCTION

*Mycobacterium tuberculosis* is an intracellular parasite. Much attention has been focused upon the bacilli's ability to evade intracellular killing by monocytes,[1–3] but the mechanism by which the bacilli enter mononuclear phagocytes has been obscure and not investigated until recently. These studies have shown that complement coating of the bacilli that activate the alternative complement pathway is important in parasite uptake.[4] This is mediated via complement receptor type 3 (CR3) on monocytes.[5] Such experiments were performed with whole bacilli in co-cultures with the cells.

Fibronectin and mannosyl-fucosyl receptors (R) are also known to play a role in the interaction of *M. avium–M. intracellulare* with macrophages[6] and recently, this microbe was found to bind to the vitronectin R on monocytes.[7] Whether this is also the case for *M. tuberculosis* has not yet been determined. However, as it has been shown that intracellular pathogens make use of more than one R to enter host cells,[8] the R above probably participate with CR3 in the monocyte uptake of *M. tuberculosis*.

The objective of the present study was to examine whether mycobacterial antigen (Ag) *per se* promote monocyte uptake of mycobacteria. To address this question we incubated microbeads coated with whole bacillus Calmette–Guérin (BCG) cells, *M. tuberculosis* derivates and secreted Ag with monocyte cultures under serum-free conditions and studied the uptake of the beads.

Received 11 January 1994; revised 15 March 1994; accepted 17 March 1994.

Abbreviations: Ab, antibodies; Ag, antigen; BCG, bacillus Calmette–Guérin; CR, complement receptor; E, sheep erythrocyte; mAb, monoclonal antibodies; R, receptor.

Correspondence: Dr G. Hetland, Institute of Immunology and Rheumatology, The National Hospital, Fr. Qvamsgt. 1, 0172 Oslo 1, Norway.

## MATERIALS AND METHODS

*Preparation of monocyte cultures*

Mononuclear leucocytes were isolated from buffy coats of venous blood from healthy blood donors by Böyum's method,[9] with minor modifications.[10] Mononuclear cells ($5 \times 10^6$) were seeded per 16-mm Costar culture well (Costar, Cambridge, MA) on plastic coverslips in RPMI-1640 with gentamicin (5 $\mu$g/ml) containing $NaHCO_3$ (2·0 g/l) and 20% serum from healthy $AB/Rh^+$ blood donors, and incubated at 37° in air with 5% $CO_2$. For the rosetting studies $2 \times 10^7$ cells were seeded per 35-mm Costar well. The cell cultures were washed thrice after 40 min (day 0) and after 24 hr in RPMI-1640 containing 20 mM HEPES buffer, pH 7·4, and further incubated in RPMI-1640 with 20% serum. The remaining attached cells consisted of over 90% monocytes as determined by staining with α-naphthyl acetate esterase (Sigma Chemical Co., St Louis, MO). The experiments were performed on day 3 in RPMI-1640 without serum after thorough washing of the cells.

*Mycobacterial antigens*

*Mycobacterium bovis*, BCG Copenhagen (strain 1331) and *M. tuberculosis* H37Rv (ATCC27294) were grown in the wholly

synthetic Sauton medium for 3 and 5 weeks, respectively. The bacilli were removed by centrifugation and the culture supernatant concentrated by 80% saturated ammonium sulphate precipitation. Tubercle bacilli were washed thrice in phosphate-buffered saline (PBS), and then sonicated in a rosette cooling cell with a Branson sonifer model B12 as described previously.[11] The individual components of the Ag 85 complex were purified from *M. tuberculosis* culture fluid by a combination of ion-exchange chromatography on DEAE-Sepharose and phenyl Sepharose and gel filtration on Sephacryl S-200 as described previously.[12]

*Antibodies*

Mouse IgG monoclonal antibodies (mAb) to human CR1 (CD35) and CR3 (CD11b) were purchased from Dako Immunoglobulins (Copenhagen, Denmark), and normal human serum IgG from Kabi Pharmacia (Stockholm, Sweden). Ascites from a mouse containing the mAb (bH6) specific for a C3 neoepitope expressed on C3b, iC3b and C3c[13] was a much appreciated gift from Dr P. Garred (The National Hospital, Oslo, Norway). Rabbit anti-serum to secreted Ag of *M. tuberculosis*, to the Ag 85 complex or to the 65,000 MW heat-shock protein are described in Wiker *et al.*[14,15] Rabbit anti-E IgM was obtained from Cordis Laboratory (Miami, FL).

*Treatment of latex microbeads*

Monodispersed magnetic latex microbeads (diameter 4·5 μm) (Dynal, Oslo, Norway) were incubated with either 1 mg/ml of *M. tuberculosis* sonicate, *M. tuberculosis* culture fluid, whole BCG cells (Statens Seruminstitut, Copenhagen, Denmark), human serum albumin (HSA), 0·1 mg/ml *M. tuberculosis* soluble Ag 85A, 85B or 85C, or PBS at 4° overnight and washed twice in PBS. Some beads were then incubated with appropriate dilutions of antibody (Ab) to *M. tuberculosis*, Ag 85 complex, anti-C3 neoepitope or the 65,000 MW heat-shock protein for 15 min at 37° in volumes of 100 μl, and washed again.

*Incubation of microbeads with monocyte cultures*

Pretreated microbeads ($5 \times 10^5$) were added to each monocyte culture well under serum-free conditions in the presence or absence of mAb to CR1 or CR3, after saturation of the monocyte FcR by addition of human IgG (10 mg/ml) to the cell cultures. Anti-Ag 85 $F(ab')_2$ were usually used directly, but no difference was found compared to experiments with the corresponding whole antiserum and monocyte FcR saturation (data not shown). Because initial control experiments with HSA-treated beads showed a similar uptake of these (mean 10·2%) and PBS-treated beads (mean 10·6%), the latter were used as controls. After 1 hr incubation the plastic coverslips were taken out of the wells and washed vigorously in warm PBS to remove beads that were not attached or ingested by the cells. The monocytes on plastic coverslips were fixed in 2·5% glutaraldehyde with 0·1 M cacodylate buffer for 20 min, washed with this buffer and mounted in Aquamont$^R$ (Polysciences Ltd, Eppelheim, Germany) on object slides. The number of monocytes with 1, 2, 3, 4 and ⩾ 5 beads was determined by phase-contrast microscopy of a cross-section of each coverslip (approx. 300 cells) and recorded as a percentage. One-third of the experiments in which the monocyte uptake of PBS control beads exceeded 10% and the uptake of *M. tuberculosis*-treated beads was very high, were excluded from Figs 1 and 2 because the particular cells were believed to be in a higher state of activation than those with PBS beads uptake < 10%. The data for uptake represent total cell-associated beads including both those attached to and those internalized by the cells. In Fig. 3 each experiment with Ab was compared to its corresponding non-inhibited control and the data given as per cent inhibition of uptake observed in control wells.

*Opsonization of sheep erythrocytes (E) and rosette formation with monocytes*

Sheep erythrocytes stored in Elsevier's solution were incubated with anti-E Ab in PBS, giving EAb and further incubated with a 1/20 dilution of C5-deficient human serum (Sigma) to obtain EAbC3bi.[16] EAb or EAbC3bi ($10^8$ cells) were then incubated at 37° for 1 hr with 3-day-old monocyte cultures in the presence or absence of 10 mg/ml normal serum IgG, anti-C3 neoepitope IgG mAb or anti-CR3 (CD11b) IgG mAb. The culture wells were washed, the cells fixed with 2·5% glutaraldehyde and the percentage of monocytes that formed rosettes (i.e. binding of ⩾ 3 erythrocytes/monocyte) with EAb or EAbC3bi determined by microscopy of a cross-section of the wells.

*Statistics*

The significance of the results was tested by paired two-tailed *t*-test, and *P* values < 0·05 were considered to be statistically significant.

## RESULTS

### Monocyte uptake of *M. tuberculosis* Ag-pretreated microbeads

The uptake by monocytes of microbeads pretreated with different *M. tuberculosis* Ag or BCG was assessed by microscopy after 1 hr incubation of the beads with serum-free cell cultures (Fig. 1). The highest monocyte uptake was observed with beads preincubated with whole BCG cells ($P < 0·05$). There was a lower, but significant number of

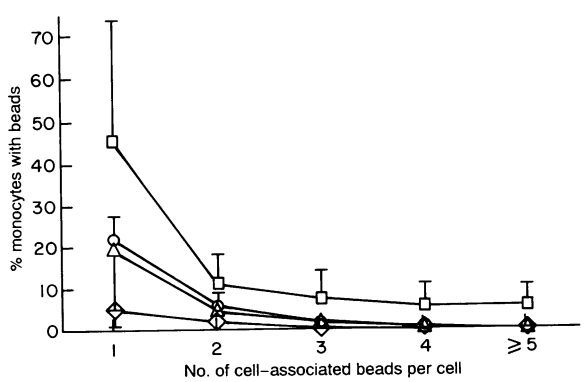


**Figure 1.** Beads preincubated with whole BCG cells (□) ($n = 3$), *M. tuberculosis* sonicate (○) ($n = 5$) or culture fluid (△) ($n = 5$), or PBS (◇) (controls; $n = 8$) were further incubated with monocyte culture for 1 hr. The coverslips in the wells were washed to remove unbound beads and fixed. The number of monocytes that had bound and/or phagocytosed 1–4, or 5 and more beads was examined by microscopy of 300 cells. The results are given as means ± 1 SD.

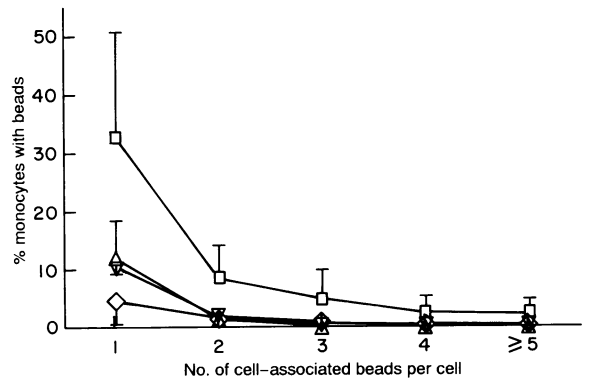


**Figure 2.** Beads preincubated with Ag 85C (□) ($n$ = 5), Ag 85A (△) ($n$ = 2), Ag 85B (▽) ($n$ = 3) from *M. tuberculosis* culture fluid or with PBS (◇) ($n$ = 5) were further treated as in Fig. 1.

monocytes that took up one of the beads pretreated with *M. tuberculosis* sonicate ($P < 0{\cdot}01$). A similar quantity of cells was associated with *M. tuberculosis* culture fluid-treated beads ($0{\cdot}05 < P < 0{\cdot}1$).

Experiments with soluble Ag 85A, 85B and 85C purified from *M. tuberculosis* culture fluid revealed a substantial uptake of single Ag 85C-treated beads ($P < 0{\cdot}02$), in contrast with Ag 85A ($P > 0{\cdot}2$) and 85B ($P > 0{\cdot}1$) (Fig. 2). Moreover, the cell association of Ag 85C-treated beads was significantly higher than that of Ag 85B-treated beads ($P < 0{\cdot}02$). This indicates that 85C is the Ag responsible for the observed uptake of beads pretreated with whole BCG cells or *M. tuberculosis* sonicate. The trend in the cell association of the different beads was also seen when cells with 2 or more beads were examined (Figs 1 and 2).

### Inhibition by anti-CR3 mAb of EAbC3bi binding to monocytes

Firstly, to examine whether monocyte FcR is blocked by addition of IgG to the cells, EAb were incubated with monocyte cultures with or without prior addition of normal serum IgG. Table 1 shows over 50% inhibition of monocyte rosetting with EAb in the presence of 10 mg/ml IgG ($P < 0{\cdot}05$). Secondly, to examine the ability of mAb to CR3 or a C3 neoepitope to block EAbC3bi rosette formation with monocytes, EAbC3bi were incubated with IgG-treated monocyte cultures in the presence or absence of these mAb. There was a small, but statistically significant inhibition ($P < 0{\cdot}02$) of the EAbC3bi binding to monocytes by both the mAb (Table 1). This finding suggests that anti-CR3 and may be also anti-C3 neoepitope may interfere with CR3-dependent binding of particles to monocytes.

**Table 1.** Effect of IgG, anti-C3 neoepitope and anti-CR3 mAb on rosette formation of opsonized E (EAb and EAbC3bi) with monocytes

| Red cells | Addition of | % rosette-forming monocytes |
|---|---|---|
| EAb | — | 81·0 ± 2·8 |
| EAb | IgG | 37·0 ± 7·1* |
| EAbC3bi | IgG | 90·8 ± 3·3 |
| EAbC3bi | IgG, anti-C3 neoepitope | 72·8 ± 6·2** |
| EAbC3bi | IgG, anti-CR3 | 66·3 ± 4·1** |

The results are means ± SD of two to four experiments. Three-day-old monocytes were incubated for 1 hr at 37° with opsonized E under serum-free conditions. Normal human serum IgG (10 mg/ml) was added to the cell cultures prior to incubation with EAb or EAbC3bi. Anti-C3 neoepitope was added to EAbC3bi before incubation with monocytes, whereas anti-CR3 was added to the cultures before incubation of EAbC3bi with the monocytes. *$P < 0{\cdot}05$; **$P < 0{\cdot}02$, compared with control. The per cent value for E rosetting to monocytes was 1·0 ± 1·4.

### Inhibition of monocyte uptake of BCG or *M. tuberculosis* Ag pretreated beads by Ab to *M. tuberculosis* Ag and CR3

In another experiment polyclonal $F(ab')_2$ Ab to the Ag 85 complex were incubated with beads treated with whole BCG cells or Ag 85C prior to the addition of the beads to monocyte cultures. The uptake of BCG-coated beads was inhibited by $F(ab')_2$ anti-Ag 85 ($P < 0{\cdot}01$) (Fig. 3), which supports participation of an Ag in this complex in monocyte uptake of the mycobacteria. In duplicate experiments with Ag 85C-coated beads $F(ab')_2$, anti-Ag 85 and anti-*M. tuberculosis* inhibited the cell association of the beads by 66·0% and 84·3%, respectively (data not shown). In control experiments with BCG-treated beads and anti-65,000 MW the uptake was not reduced (23·4 ± 10·7% increase). The FcR of the cells were blocked with IgG in advance in the latter experiments and those below utilizing whole Ab.

The presence of a mAb to CR3 in co-cultures of monocytes and microbeads treated with Ag 85C, resulted in significant inhibition ($P < 0{\cdot}02$) of the uptake of beads (Fig. 3). In contrast, no effect was observed with anti-CR1 ($P > 0{\cdot}5$). When the effect of anti-CR3 on Ag 85C-treated beads was

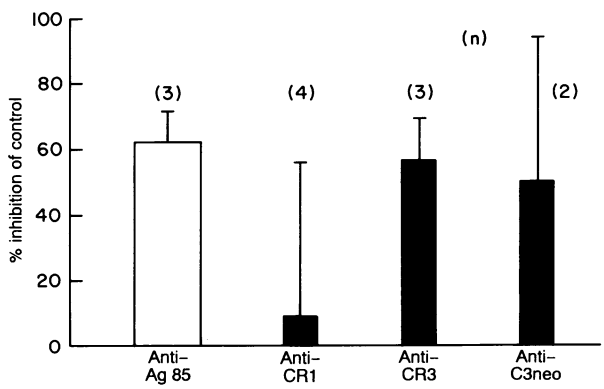


**Figure 3.** Inhibition of binding and/or uptake (cell association) of one or more beads per cell by Ab. Beads coated with whole BCG cells (□) were incubated with or without polyclonal anti-85 Ag $F(ab')_2$ fragments. Beads coated with Ag 85C (■) were either incubated with anti-C3 neoepitope mAb or without the mAb and added to FcR-blocked monocyte cultures in the presence or absence of mAb to CR1 or CR3. The degree of Ab inhibition of cell association is calculated as per cent inhibition of uptake observed in controls without Ab. In two of the experiments in the second column the cell association was increased rather than inhibited. The results are given as mean + 1 SD. No. of experiments in parentheses.

compared with that of anti-CR1 using paired $t$-tests, it was significantly greater at the $0{\cdot}05 < P < 0{\cdot}1$ level. A mAb to a C3 neoepitope inhibited neither the cell association of Ag 85C-treated beads ($P > 0{\cdot}2$) (Fig. 3) nor the cell association of BCG-treated beads ($31{\cdot}0 \pm 8{\cdot}5\%$ reduction, $P > 0{\cdot}5$) (data not shown).

## DISCUSSION

Based on the present results we conclude that: (1) whole BCG cells promote monocyte uptake of microbeads coated with BCG; (2) blocking experiments with anti-Ag 85 complex indicate that an Ag in this complex is responsible for the uptake of BCG-coated beads; (3) this Ag is the secreted soluble mycobacterial Ag 85C as shown by the increased uptake of beads coated with Ag 85C; (4) blocking experiments with mAb indicate that the Ag 85C-dependent uptake of the beads is mediated via CR3 on the cells and probably occurs via a non-C3-like structure on Ag 85C.

Obviously, secreted soluble Ag are not able to take part directly in the uptake of mycobacteria which must be mediated by a ligand on the surface of the bacilli and via CR3. In addition to C3bi CR3 also has binding affinity for other ligands, including fibrinogen, factor X and components of bacteria like LPS and $\beta$-glucan.[17] Thus, the lack of evidence for a C3-like epitope on Ag 85C does not disagree with the literature on ligand specificities for CR3. The experiments with E rosetting to monocytes illustrated that anti-C3 neoepitope mAb was able to inhibit monocyte binding of C3bi-coated particles, although poorly. Even though other ligand specificities on Ag 85C were not excluded, the possibility exists that Ag 85C itself may represent a novel ligand for CR3.

*Mycobacterium bovis*, BCG and *M. tuberculosis* share the Ag 85 complex[12,15] and the extensive cross-reactivity between mycobacteria is well known.[11,18] In the present study this is demonstrated by the reactivity of anti-85 Ag with Ag 85C beads. The Ag 85 complex occurs in much lower concentrations in sonicate of *M. bovis*, BCG and *M. tuberculosis* than in culture fluid[14,15] from which component 85C is purified.[12] The greater uptake of beads treated with whole BCG cells and Ag 85C than with *M. tuberculosis* sonicate or culture fluid is probably the result of greater similarity to native mycobacteria infection and higher concentrations of the functionally active ligand, respectively, in the former experiments.

Because fibronectin binds to the BCG85 complex[19] and causes complement R activation in monocytes,[20] fibronectin binding to Ag 85C may induce stimulation of CR3-mediated monocyte uptake of mycobacteria. Fibronectin is secreted by mononuclear phagocytes after some days *in vitro*[21,22] and was most probably also present in our cell cultures. Hence, a ligand in Ag 85C on *M. bovis*, BCG and *M. tuberculosis* may serve as a vehicle for the uptake of mycobacteria. The inhibition by anti-CR3 of the cell association of mycobacterial Ag-coated beads supports such an hypothesis. Serum-containing cell cultures would contain even higher amounts of fibronectin and would thus enhance CR3-mediated uptake of the bacilli. This could also occur through possible serum complement activation as shown for both *M. tuberculosis*[5] and *M. avium*.[4] In contrast, our experiments were performed under serum-free conditions and the mycobacteria used had been cultivated in the absence of a complement source. Cell-derived complement on complement-activating particles also promotes their binding to monocytes in serum-free cultures.[10,23] However, the latex beads, which do not activate complement, were co-incubated with monocytes for 1 hr only, which is too short a time to give any significant opsonization and uptake of agarose beads by 3-day-old monocytes.[10] This is supported by the insignificant inhibition of uptake of coated beads by C3 neoepitope mAb. Thus, the possible participation of serum- or monocyte-derived C3 in our study is highly unlikely. Studies with C3bi-coated whole bacilli, after activation of serum complement,[4,5] may have disguised the possible detection of a bacilli-derived CR3 ligand. Our results do not contradict these reports, but indicate that *M. bovis*, BCG and *M. tuberculosis* have serum-independent means of their own by which they can enter monocytes.

## ACKNOWLEDGMENTS

We thank Professor M. Harboe for helpful discussions, Dr P. Garred for use of the bH6 mAb, Ms G. Ulvund for excellent technical assistance, and Ms K. Bertelsen for secretarial help.

## REFERENCES

1. Crowle A.J. (1986) Studies of antituberculous chemotherapy with an *in vitro* model of human tuberculosis. *Semin. Respir. Infect.* **1,** 262.
2. Rook G.A. (1987) Progress in the immunology of the mycobacterioses. *Clin. exp. Immunol.* **69,** 1.
3. Bermundez L.E. & Young L.S. (1988) Tumor necrosis factor alone or in combination with IL-2, but not IFN-gamma, activates macrophages to kill *Mycobacterium avium* complex. *J. Immunol.* **140,** 3006.
4. Swartz R.P., Naai D., Vogel C.-W. & Yeager H., Jr. (1988) Differences in uptake of Mycobacteria by human monocytes: a role for complement. *Infect. Immun.* **56,** 2223.
5. Schlesinger L.S., Bellinger-Kawahara C.G., Payne N.R. & Horwitz M.A. (1990) Phagocytosis of *Mycobacterium tuberculosis* is mediated by human monocyte complement receptors and complement component C3. *J. Immun.* **144,** 2771.
6. Bermundez L.E., Young L.S. & Enkel H. (1991) Interaction of *Mycobacterium avium* complex with human macrophages: roles of membrane receptors and serum proteins. *Infect. Immun.* **59,** 1697.
7. Rao S.P., Ogata K. & Catanzaro A. (1993) *Mycobacterium avium–M. intracellulare* binds to the integrin receptor $\alpha_v\beta_3$ on human monocytes and monocyte-derived macrophages. *Infect. Immun.* **61,** 663.
8. Bullock W.E. & Wright S.D. (1987) Role of the adherence promoting receptors, CR3, LFA-1, and p150,95 in binding of *Histoplasma capsulatum* by human macrophages. *J. exp. Med.* **165,** 195.
9. Böyum A. (1968) Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Immunol. lab. Invest.* **21** (suppl. 97), 77.
10. Hetland G. & Eskeland T. (1986) Formation of the functional alternative pathway of complement by human monocytes *in vitro* as demonstrated by phagocytosis of agarose beads. *Scand. J. Immunol.* **23,** 301.
11. Closs O., Harboe M., Axelsen M., Bunch-Christensen K. & Magnusson M. (1980) The antigens of *Mycobacterium bovis*, strain BCG, studied by crossed immunelectrophoresis: a reference system. *Scand. J. Immunol.* **12,** 249.
12. Nagai S., Wiker H.G., Harboe M. & Kinimoto M. (1991) Isolation and partial characterization of major protein antigens in the culture fluid of *Mycobacterium tuberculosis*. *Infect. Immun.* **59,** 372.

13. Garred P., Mollnes T.E., Lea T. & Fischer E. (1988) Characterization of a monoclonal antibody MoAb bH6 reacting with a neoepitope of human C3 expressed on C3b, iC3b and C3c. *Scand. J. Immunol.* **27,** 319.
14. Wiker H.G., Harboe M. & Nagai S. (1991) A localization index for distinction between extracellular and intracellular antigens of *Mycobacterium tuberculosis*. *J. gen. Microbiol.* **137,** 875.
15. Wiker H.G., Harboe M. & Lea T.E. (1986) Purification and characterization of two protein antigens from the heterogenous BCG 85 complex in *Mycobacterium bovis* BCG. *Int. Archs Allergy appl. Immunol.* **81,** 298.
16. Kaplan G. (1977) Differences in the mode of phagocytosis with Fc and C3 receptors in macrophages. *Scand. J. Immunol.* **6,** 797.
17. Rosen H. & Law S.K. (1990) The leukocyte cell surface receptor(s) for the iC3b product of complement. *Curr. Top. Microbiol. Immunol.* **153,** 99.
18. Harboe M., Mshana R.N., Closs O., Kronvall G. & Axelsen N.H. (1979) Cross-reactions between mycobacteria. II. Crossed immunoelectrophoretic analysis of soluble antigens of BCG and comparison with other mycobacteria. *Scand. J. Immunol.* **9,** 115.
19. Abou-Zeid C., Ratcliff T.L., Wiker H.G., Harboe M., Bennedsen J. & Rook G.A.W. (1988) Characterization of fibronectin-binding antigens released by *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG. *Infect. Immun.* **56,** 3046.
20. Wright S.D., Craigmyle L.S. & Silverstein S.C. (1983) Fibronectin and serum amyloid P component stimulate C3b- and C3bi-mediated phagocytosis in cultured human monocytes. *J. exp. Med.* **158,** 1338.
21. Johansson S., Rubin K. Höök M., Ahlgren T. & Seljelid R. (1979) *In vitro* biosynthesis of cold insoluble globulin (fibronectin) by mouse peritoneal macrophages. *FEBS Lett.* **105,** 313.
22. Goldstein C.S., Garrick R.E., Polin R.A., Gerdes J.S., Kolski G.B., Neilson E.G. & Douglas S.D. (1986) Fibronectin and complement secretion by monocytes and peritoneal macrophages *in vitro* from patients undergoing continuous ambulatory peritoneal dialysis. *J. Leukoc. Biol.* **39,** 457.
23. Ezekowitz R.A.B., Sim R.B., Hill M. & Gordon S. (1984) Local opsonization by secreted macrophage complement components. Role of receptors for complement in uptake of zymosan. *J. exp. Med.* **159,** 244.