Characteristics of immunosuppressive macrophages induced in spleen cells by *Mycobacterium avium* complex infections in mice

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The profile of generation and characteristics of splenic macrophages (MΦs) which suppress the concanavalin A (Con A) mitogenic response of splenic T cells (designated as 'immunosuppressive MΦs') in host CBA/JN mice during the course of *Mycobacterium avium* complex (MAC) infection were investigated. In MAC-infected mice, reductions in some cellular functions of host splenic T cells, such as the Con A mitogenic response and mixed leucocyte reaction, were seen around 2 weeks after challenge of organisms, and this was accompanied by appearance of immunosuppressive MΦs in spleen cells. In this case, increase in immunosuppressive MΦ activity was seen in terms of both activity per spleen and activity per individual MΦ. In this phase of the infection, MAC-induced splenic MΦs showed a markedly increased ability to produce reactive oxygen radicals in response to phorbol myristate acetate. Thus, the expression of suppressor activity of MAC-induced MΦs seems to be closely linked to their activated state. A large proportion of the immunosuppressive MΦs exhibited suppressor activity dependent on prostaglandins and membrane functions related to microfilaments. It was also found that the generation of IL-2-reactive T cell populations in response to Con A was markedly inhibited by MAC-induced splenic MΦs, whereas they caused no significant reduction in the IL-2-producing ability of normal spleen cells.

Introduction

Mycobacterium avium complex (MAC) is one of the most prevalent pathogens in acquired immunodeficiency syndrome (AIDS) (Young *et al.*, 1986). MAC is highly resistant to the majority of antituberculous and antimicrobial agents and frequently causes fatal infections (Wolinsky, 1979). It persists for long periods without producing the severe foci in target organs which are noted in cases of tuberculosis (Woods & Washington, 1987). In MAC, as well as in other mycobacterial infections, depression of delayed-type hypersensitivity, the blastogenic response of lymphocytes, and *in vivo* antibody response (Ellner, 1978; Wade & Rabson, 1981; Orme & Collins, 1984; Watson & Collins, 1981) have been reported. Although suppressor T cells and immunosuppressive macrophages (MΦs) are thought to contribute to the impaired cellular immunity (Bullock *et*

al., 1978; Turcotte & Lemieux, 1982; Edwards *et al.*, 1986), the precise role of the latter cells in the establishment of immunosuppression (mainly antigen-specific immune unresponsiveness to MAC) remains to be elucidated. In this study, we examined the mode of generation and some properties of immunosuppressive MΦs during the course of MAC infection induced in mice.

Methods

Organisms. *M. avium* complex 31F093 SmT and N-260 SmT variants were obtained from F. Kuze, Kyoto University, Kyoto, Japan, and T. Mitsui, Sanyoso National Hospital, Yamaguchi, Japan, respectively. Before starting the experimental infection study, the organisms were transferred to mice. These strains were identified as *M. intracellulare* using the Gen-Probe Rapid Diagnostic System for MAC. Strain N-260 was found to belong to serovar 16 by a seroagglutination test.

Mice. Male CBA/JN mice were purchased from Charles River Japan.

Medium. RPMI 1640 medium (Nissui Pharmaceutical Co.) supplemented with 25 mM-HEPES, 2 mM-glutamine, 100 µg streptomycin ml⁻¹, 100 units penicillin G ml⁻¹, 5×10^{-5} M-2-mercaptoethanol and 5% (v/v) foetal bovine serum (FBS; M. A. Bioproducts) was used for cell cultures.

Abbreviations: Con A, concanavalin A; FBS, foetal bovine serum; HBSS, Hanks' balanced salts solution; IL-2, interleukin 2; MAC, *Mycobacterium avium* complex; MΦ, macrophage; NBT, nitroblue tetrazolium; PMA, phorbol myristate acetate; SPCs, spleen cells; dThd, thymidine.

Infection. Bacteria grown for 5–8 d in Dubos Tween-albumin liquid medium were harvested by centrifugation and suspended in physiological saline. A 0.2 ml volume of bacterial suspension containing about 5×10^7 to 2×10^8 colony-forming units (c.f.u.) was given intravenously to CBA/JN mice. Almost 100% of the inoculum produced the smooth, transparent and irregularly shaped colonies characteristic of virulent variants, when plated on 7H10 agar (Kubica *et al.*, 1963).

Mitogenic response of splenic T cells. The assay system was essentially as described previously (Tomioka & Saito, 1985). Briefly, spleen cells (SPCs) from normal and infected CBA/JN mice (2 weeks after infection, unless otherwise specified) were cultured in medium usually with or without addition of 2 µg concanavalin A (Con A) ml⁻¹ (Miles-Yeda) in triplicate wells of a flat-bottomed microtitre tray for cell culture (Corning) at 37 °C in a CO₂ incubator (5% CO₂ and 95% humidified air) for 72 h. The cultures were pulsed with 0.25 µCi per well of [³H]thymidine (dThd) (2 Ci mmol⁻¹; 74 GBq mmol⁻¹; New England Nuclear) for the final 6–8 h. Cells were collected on glass-fibre filters and washed with physiological saline using an automatic cell harvester (Mitsumi Kagaku Co.). Radioactivity was counted in a Tri-Carb liquid scintillation counter (Packard Instrument Co.).

Assay for suppressor activity of splenic MΦs per spleen. SPCs of normal or infected mice were cultured in 0.2 ml medium in triplicate wells of a microtitre tray at concentrations of 5×10^5 to 2×10^6 cells per well in a CO₂ incubator for 2–3 h. The wells were then vigorously rinsed with a jet of Hanks' balanced salts solution (Nissui) (HBSS). This procedure usually gave MΦ monolayer cultures (more than 95% of the cells phagocytosed latex beads), containing about 2×10^4 and 5×10^4 cells per well from 2×10^6 normal and infected SPCs, respectively. The number of adherent cells on the culture well increased in a nearly linear fashion as the number of SPCs seeded was increased from 5×10^5 to 2×10^6 cells per well. Usually 1.25×10^5 normal SPCs in 0.2 ml medium containing 2 µg Con A ml⁻¹ were poured on to the resultant adherent cell monolayer culture. The cells were cultured at 37 °C for 72 h. The culture was pulsed with [³H]dThd for the final 6–8 h of cultivation and the radioactivity was measured.

Per-organ activity of suppressive MΦs, designated as 'suppressive MΦ units per spleen' was calculated as: suppressive MΦ units per spleen = (number of SPCs per spleen) ÷ (number of SPCs seeded to prepare the MΦ monolayer which caused 50% inhibition of Con A blastogenesis of target splenic T cells).

Per-cell suppressive activity of splenic MΦs. Suppressive activity of individual MΦs against mitogenesis of splenic T cells was determined as follows. SPCs (7×10^7) of MAC-infected mice were cultured in 7 ml medium in an 80 mm plastic culture dish (Corning) at 37 °C for 2 h. Nonadherent cells were removed by vigorous vibration on an S/IUL mixer at a maximum speed for 15 s followed by rinsing with HBSS containing 1% (v/v) FBS (three times). Adherent cells were then harvested by gentle scraping with a rubber policeman, and added to the culture system of normal SPCs (1.25×10^5 cells per well) for Con A mitogenesis at doses of 6.25×10^3 to 2×10^5 cells per well. Per-cell suppressive activity of the test MΦs was represented by the reciprocal of the number of MΦs needed for 50% inhibition of SPC mitogenesis.

Interleukin 2 (IL-2) assay. SPCs (1.25 – 2.5×10^6) were cultured in 0.2 ml medium in microtitre wells in the presence or absence of MAC-induced splenic MΦs at 37 °C for 24 h. The culture supernatant was harvested and its IL-2 activity determined by measuring the proliferative response of Con A blastoid cells to IL-2 (method A), according to Hoffenbach *et al.* (1983), or using an IL-2-dependent cytotoxic T cell line (CTLL-2) as a target cell (method B).

In method A, Con A blastoid cells were prepared by cultivation of normal SPCs for 48 h in medium containing 5 µg Con A ml⁻¹ and subsequent rinsing with HBSS containing 10 mg methyl α-D-mannoside

ml⁻¹. The resultant cells (3×10^4) were cultivated in medium containing the test SPC-culture fluid (50%, v/v) and 10 mg methyl α-D-mannoside ml⁻¹ for 72 h, and [³H]dThd incorporation during the final 6–8 h incubation was measured.

In method B, CTLL-2 cells (1×10^4 cells per well) were cultivated in medium containing the test SPC-culture fluid (50%, v/v) and 10 mg methyl α-D-mannoside ml⁻¹ for 24 h and [³H]dThd incorporation during the final 4 h incubation was measured.

The IL-2 activity was expressed as [³H]dThd incorporation either by Con A blastoid cells or by CTLL-2 cells.

Generation of IL-2-reactive T cell subpopulations. Normal SPCs (1.25×10^5 , or in some experiments 2×10^5 cells per well) were cultivated in medium containing 2 µg Con A ml⁻¹ in the presence or absence of MAC-induced splenic MΦs for 48 h. Nonadherent cells were then harvested, transferred to new wells, thoroughly washed with HBSS containing 1% (v/v) FBS and 20 mg methyl α-D-mannoside ml⁻¹, and their proliferative response to exogenous IL-2 (about 1 unit ml⁻¹) was measured during a further 72 h cultivation. IL-2 at 1 unit ml⁻¹ induced a proliferative response of CTLL-2 cells (10^4 cells per well) of 5×10^3 c.p.m.

Treatment of splenic MΦs with indomethacin and cytochalasin B. MAC-induced MΦs (2 weeks after infection) in monolayer culture on microtitre wells were treated with either indomethacin or cytochalasin B at 37 °C for 2 h. After washing the MΦ monolayer with HBSS, 1.25×10^5 normal SPCs were co-cultured with the indicated number of MΦs so obtained for 72 h in the presence of 2 µg Con A ml⁻¹, and [³H]dThd incorporation during the final 8 h of incubation was measured. In some experiments, SPCs were co-cultured with MAC-induced splenic MΦs in the presence or absence of 1 µg indomethacin ml⁻¹ for 72 h. In this case, indomethacin alone did not affect the splenic T cell mitogenesis in response to 2 µg Con A ml⁻¹.

Superoxide anion (O₂⁻) generating ability of splenic adherent cells. SPCs (1×10^6) of normal or infected mice were cultured in 1 ml medium in 16 mm culture wells containing a 14 mm plastic sheet (Wako Chemical Ind.) at 37 °C for 3 h and washed three times with a jet of HBSS. The resulting monolayer culture on the sheet was incubated in 1 ml medium containing 1 mg nitroblue tetrazolium (NBT) ml⁻¹ in the presence or absence of 100 ng phorbol myristate acetate (PMA; Sigma) ml⁻¹ at 37 °C for 30 min, then fixed with 4% (v/v) HCHO, and the number of NBT-reducing cells counted. Cells with more than 20 blue granules were counted as positive for NBT reduction.

Chemiluminescence. Test SPCs (4×10^6) or plastic-dish-adherent cells prepared from MAC-infected SPCs (2×10^5) were suspended in 1 ml HBSS (pH 7.4; free of phenol red) containing 10 mM-HEPES and 0.1 mM-luminol (Wako), then 100 ng PMA dissolved in 10 µl dimethylsulphoxide was added to the incubation mixture and photoemission was measured in a lumiphotometer, (Lumicounter ATP-237; Toyo Kagaku Ind.) at 37 °C.

Results

Changes in some cellular functions of splenic T cells during the course of MAC infection

SPCs from MAC-infected mice at various phases of infection (week 1 to week 6) were measured for mitogenic response, IL-2 production, and generation of IL-2-reactive T cells in response to Con A (data not shown). Mitogenesis induced by Con A (2 µg ml⁻¹) was reduced most markedly around 2 weeks after infection,

Fig. 1. Effects of plastic adherent (a), Thy-1,2⁻ (b), and Sephadex G-10 nonadherent (c) cell populations of normal (○) and MAC-infected (●) SPCs on the Con A ($2 \mu\text{g ml}^{-1}$) mitogenic response of splenic T cells (5×10^5 cells per microtitre well). (a) Monolayer cultures of the adherent cells were prepared by incubating test SPCs on microtitre wells at 37°C for 2–3 h, followed by subsequent vigorous rinsing with HBSS. (b) Thy-1,2⁻ cells were prepared from test SPCs (2×10^7 cells ml^{-1}) by treating with anti-Thy-1,2 monoclonal antibody (1/20 dilution) (Cedarlane Laboratories) and subsequently treated with guinea-pig complement. (c) Sephadex G-10 nonadherent cells were prepared by applying test SPCs (4.5×10^8 cells) to a Sephadex G-10 column (20 ml bed volume) and collecting the cells that passed through the column after 30 min incubation at 37°C . Each point plotted indicates the mean $\pm$ SEM (three incubations).

with concomitant reductions in IL-2-producing ability and generation of IL-2-reactive T cells. A marked reduction in SPC mitogenesis was usually found when the SPCs were cultured at cell densities higher than 5×10^5 cells per well. In this phase of infection, the number of SPCs as well as spleen weight increased markedly. In a separate experiment, we also observed a 44% and 72% reduction in the mixed leucocyte reaction of MAC-infected SPCs (2 weeks after infection) against mitomycin-C-treated BALB/c SPCs, when the responder cells were cultured at densities of 5×10^5 and 1×10^6 cells per well, respectively.

Role of immunosuppressive MΦs in reduction of Con A mitogenesis of MAC-infected SPCs

Fig. 1 shows that SPCs of MAC-infected mice (2 weeks after infection) contained two types of suppressor cells against the Con-A induced mitogenesis of normal splenic T cells.

Firstly, plastic-adherent cells and Thy-1,2⁻ cells inhibited SPC mitogenesis in the same fashion (Fig. 1a, b). In separate experiments, more than 95% of the adherent cells were identified as MΦ on the basis of morphology and strong phagocytic ability against latex beads and sheep erythrocytes. Therefore, one component of the suppressor cell population in the MAC-infected SPCs is immunosuppressive MΦs. In this case, suppressive activity was noted only for the adherent cells from

MAC-infected SPCs, and not for those from normal SPCs.

Secondly, a cell population which passed through a Sephadex G-10 column (MΦ-depleted cell population) also suppressed SPC mitogenesis (Fig. 1c). In this case, MΦ-depleted cells from SPCs of normal mice showed the same level of suppressive activity as those from MAC-induced SPCs. Therefore, MAC infection at this phase (2 weeks after bacterial challenge) is thought mainly to elicit immunosuppressive MΦs.

The observed suppression of splenic T cell mitogenesis by co-culture with MAC-induced splenic MΦs was not due to depletion of nutrients, because daily half medium changes did not overcome the suppression (data not shown). MAC-induced splenic MΦs also inhibited phytohaemagglutinin-induced mitogenesis of splenic T cells in the same manner as Con-A-induced mitogenesis (data not shown). Furthermore, treatment of MAC-induced splenic MΦs with anti-I-A, I-E, or I-J antibody plus complement failed to abolish the suppressor activity of the MΦs, although a partial reduction in suppressive activity was seen after anti-I-E or anti-I-J antibody treatment (statistically insignificant; $P > 0.1$). This suggests that the main cell populations of the suppressor MΦs do not express I-A, I-E or I-J antigen.

Fig. 2 shows the suppressive effect of MAC-induced splenic MΦs against Con A mitogenesis of normal or MAC-infected SPCs and splenic T cells. The MAC-induced MΦs inhibited Con A mitogenesis of normal and

MAC-infected SPCs in the same fashion (Fig. 2a). The MAC-induced MΦs inhibited the mitogenic response of splenic T cells prepared from normal and from MAC-infected mice in nearly the same fashion (Fig. 2b). However, it is noteworthy that splenic T cells were more resistant to the suppressive action of the MAC-induced MΦs, than were SPCs. This suggests that certain types of cell populations in SPCs amplify the suppressive action of the MAC-induced immunosuppressive MΦs. In this experiment, MAC-infected SPCs seeded at a density of 2×10^5 cells per well showed similar levels of proliferation to that of normal SPCs. However, this is not entirely surprising, since in many experiments MAC-induced SPCs showed a somewhat higher mitogenic

response than that of normal SPCs, when the SPCs were seeded at densities lower than about 2×10^5 cells per well. MAC-infection-induced reduction in the mitogenic response of the host SPCs was much more evident when SPCs were seeded at higher cell densities ($> 5 \times 10^5$ cells per well) (data not shown).

Kinetics of generation of the immunosuppressive MΦs

Table 1 shows changes in per-organ suppressive activity of the MAC-induced splenic MΦs during the course of MAC infection. As described in Methods, this value represents the total activity of suppressive MΦs in the host spleen, that is (number of suppressive MΦs) $\times$ (activity of individual MΦ). The value was markedly increased around 2 weeks after infection; it decreased rapidly thereafter and was almost zero by week 10. Table 1 also shows changes in per-cell suppressive activity of the splenic MΦs during the course of infection. This value also markedly increased around 2–4 weeks after infection. The value could not be determined for mice 10 weeks after infection, since sufficient numbers of the adherent cells could not be obtained.

In this experiment, transient reductions in the number of organisms in the host spleen were also observed around week 2 (4.4×10^6 , 1.0×10^6 , 3.3×10^6 and 4.6×10^6 per spleen at 1, 2, 4 and 10 weeks after infection, respectively). Therefore, we expected that the splenic MΦs in this phase (week 2) would have an enhanced microbicidal capacity against MAC, and thus be in the 'activated' state. In fact, splenic MΦs induced in this phase (week 2) showed significantly enhanced spontaneous and PMA-triggered O_2^- production. In the case of normal splenic MΦs, the percentages of NBT-reducing

Fig. 2. Suppressive activity of MAC-induced splenic MΦs against Con A mitogenesis of whole SPCs (a) and splenic T cells (purified by Sephadex G-10 and nylon wool filtration (b) from normal (O) and MAC-infected (●) mice. Both SPCs and splenic T cells were cultured at a density of 2×10^5 cells per well. Each point plotted indicates the mean $\pm$ SEM (three incubations).

Table 1. Kinetics of generation of immunosuppressive MΦs in SPCs of host mice during the course of MAC infection

The results are means of three determinations, $\pm$ SEM. ND, Not determined.

Weeks after infection	$10^{-7} \times$ No. of SPCs per mouse	Suppressive MΦ units per spleen*	Relative per-cell suppressive activity of splenic MΦ†	Relative chemiluminescence intensity of SPCs‡
0	5.2 ± 0.6	$< 1.8 \pm 0.1$	1.00 ± 0.04	1.0
1	9.4 ± 1.1	23 ± 2.7	1.43 ± 0.08	1.8 ± 0.1
2	23.1 ± 2.0	399 ± 35	6.97 ± 0.46	3.8 ± 0.2
4	19.0 ± 1.6	211 ± 17	6.57 ± 0.62	1.4 ± 0.1
10	23.0 ± 4.3	$< 0.01 \pm 0.002$	ND	ND

* See Methods for details.

† See Methods for details. The value of each time point relative to the value at zero time (week 0) is indicated. The relative values shown were calculated as: (number of MΦs needed for 50% inhibition of SPC mitogenesis at zero time) $\div$ (number of MΦs needed for 50% inhibition of SPC mitogenesis at the indicated time point).

‡ The results are expressed as ratio (peak value of CL from MΦs in SPCs of MAC-infected mice) $\div$ (peak value of CL from MΦs in SPCs of normal mice).

cells in the presence and absence of 100 ng PMA ml⁻¹ were $43 \pm 3\%$ and $0.5 \pm 0.5\%$ (mean $\pm$ SEM, $n = 3$), respectively, and in the case of MAC-infected splenic MΦs (2 weeks after infection), the values were increased to $77 \pm 3\%$ and $12 \pm 1\%$, respectively. As shown in Table 1, PMA-triggered chemiluminescence was also markedly elevated around week 2. Moreover, PMA-triggered chemiluminescence (the peak value seen during 90–120 s after PMA triggering) of splenic MΦs (2×10^5 cells) from MAC-infected mice was changed as follows during the early phase of infection; 304 ± 26 c.p.s. (week 1; mean $\pm$ SEM, $n = 3$), 976 ± 9 c.p.s. (week 2), and 187 ± 7 c.p.s. (week 4). Thus, significant activation of splenic MΦ functions as determined on the basis of oxidative metabolism (Tomioka & Saito, 1980; Saito *et al.*, 1981) is also seen around 2 weeks after MAC infection. The functional activation in the early phase of MAC infection was also seen in peritoneal MΦs (Saito *et al.*, 1986).

Mechanisms of action of MAC-induced immunosuppressive MΦs

Fig. 3(a) compares the effects of normal and MAC-induced splenic MΦs on the IL-2-producing ability of splenic T cells. Neither population of splenic MΦs significantly inhibited IL-2 production. As shown in Table 2, IL-2 production was not changed by the MAC-induced MΦs in the SPC culture at high cell density (1×10^6 cells per well), but it was enhanced in the SPC culture at low cell density (2.5×10^5 cells per well). Therefore, it seems that neither MAC-induced splenic MΦs nor normal MΦs cause a marked reduction in the IL-2-producing ability of splenic T cells. In separate experiments, the suppressive activity of the MAC-induced MΦs was not overcome, even when a sufficient amount of exogenous IL-2 was added (data not shown). Therefore, the possibility can be excluded that the suppressor MΦs inactivated or absorbed the IL-2 molecules by binding with specific receptors.

To elucidate the effect of the MAC-induced immunosuppressive MΦs on generation of IL-2 reactive T cells in SPC culture in response to Con A signals, we carried out the following experiments.

Firstly, normal SPCs were cultured in medium containing $2 \mu\text{g}$ Con A ml⁻¹ in the presence or absence of either normal or MAC-induced MΦs for 48 h, then the nonadherent cells were harvested, transferred to new wells, and thoroughly rinsed with HBSS containing FBS and 10 mg methyl α -D-mannoside ml⁻¹. Then, the resultant cells were cultured in the presence of exogenous IL-2 for a further 72 h and [³H]dThd uptake during the final 8 h of cultivation was measured (Figs 3b and 4a). In

Fig. 3. Effects of normal (○) and MAC-induced (●) splenic MΦs (as a monolayer culture prepared by seeding the indicated number of normal or MAC-induced SPCs) on the IL-2-producing ability of splenic T cells (a) and Con A-induced generation of IL-2-reactive T cells (b). (a) The concentration of IL-2 in 24 h culture fluid of SPCs (2×10^5 cells per well; in medium containing $2 \mu\text{g}$ Con A ml⁻¹) is represented by the [³H]dThd incorporation of IL-2-specific CTLL-2 cells (10^4 cells per well). (b) Normal SPCs (2×10^5 cells per well) were cultivated in medium containing $2 \mu\text{g}$ Con A ml⁻¹ in the presence or absence of normal or MAC-induced splenic MΦs for 48 h. Nonadherent cells were then harvested, transferred to new wells, washed with HBSS containing 1% (v/v) FBS and 10 mg methyl α -D-mannoside ml⁻¹, and their proliferative response to exogenous IL-2 (1.1 units ml⁻¹) measured during the final 8 h of a further 72 h cultivation. Each point plotted indicates the mean $\pm$ SEM ($n = 3$).

Table 2. Influence of MAC-induced splenic MΦs on IL-2-producing ability of splenic T cells

The indicated number of SPCs were cultured in 0.2 ml medium (in microtitre wells) containing $2 \mu\text{g}$ Con A ml⁻¹ with or without addition of MAC-induced splenic MΦs for 25 h, and the amount of IL-2 in the culture fluid was measured on the basis of the proliferative response of Con A blastoid cells separately prepared, as described in Methods. The results are means of three determinations, $\pm$ SEM.

No. of SPCs	No. of MAC-induced splenic MΦs	IL-2 production by splenic T cells (c.p.m.)
2.5×10^5	—	1237 ± 50
2.5×10^5	1.8×10^4	5189 ± 145
2.5×10^5	4.8×10^4	8488 ± 734
2.5×10^5	7.2×10^4	10753 ± 902
1.0×10^6	—	25625 ± 279
1.0×10^6	1.8×10^4	29318 ± 4354
1.0×10^6	4.8×10^4	36830 ± 726
1.0×10^6	7.2×10^4	33515 ± 1950
—	—	40 ± 6
—	1.8×10^4	36 ± 5
—	4.8×10^4	68 ± 13
—	7.2×10^4	281 ± 178

this case, the MAC-induced MΦs, but not normal MΦs (Fig. 3b), inhibited the generation of IL-2-reactive cell populations in the SPC culture.

Secondly, Con A blastoid cells separately prepared from normal SPCs were overlaid on to the monolayer

Fig. 4. Evidence for the suppression of the generation of IL-2-reactive T cells without causing reduction in the IL-2-induced proliferation of the IL-2-reactive T cells by the MAC-induced splenic MΦs. (a) Normal SPCs (1.25×10^5 cells per well) were cultivated in medium containing $2 \mu\text{g}$ Con A ml^{-1} in the presence or absence of MAC-induced splenic MΦs for 48 h. Nonadherent cells were then harvested, and transferred to new wells. After washing with HBSS containing 1% (v/v) FBS and 20 mg methyl α -D-mannoside ml^{-1} , their proliferative response to exogenous IL-2 ($0.97 \text{ units ml}^{-1}$) during the final 8 h of a further 72 h cultivation was measured. (b) The remaining monolayer MΦs in (a) were examined for the effect on proliferative responses of Con A blastoid cells (5×10^4 per well) in response to exogenous IL-2 ($0.97 \text{ units ml}^{-1}$) during the final 8 h of a further 72 h cultivation. The Con A blastoid cells were separately prepared from normal SPCs by activation with $5 \mu\text{g}$ Con A ml^{-1} . Each point plotted indicates the mean $\pm$ SEM ($n = 3$).

culture of the MAC-induced MΦs which remained in the above experiment after removal of nonadherent cells, and were cultured in the presence of exogenous IL-2 for a further 72 h. [^3H]dThd uptake during the final 8 h of cultivation was measured (Fig. 4b). In this case, MAC-induced MΦs did not inhibit the proliferation of Con A blastoid cells in response to IL-2.

Therefore, MAC-induced splenic MΦs are thought to inhibit the activation of splenic T cells to the IL-2-reactive state in response to Con A signals. However, they seem not to affect the subsequent step, that is, the IL-2-induced proliferative response of IL-2-reactive T cell populations. Fluorescence-activated cell sorter (FACS) analysis using anti-mouse IL-2 receptor monoclonal antibody (clone AMT 13; Boehringer Mannheim Biochemica) showed a marked inhibition ($49 \pm 5\%$ in duplicate tests) of the expression of IL-2 receptors in Con-A-activated splenic T cells by treatment with the MAC-induced immunosuppressive MΦs.

Table 3 shows the effects of treating MAC-induced MΦs with indomethacin (an inhibitor of prostaglandin synthesis) and cytochalasin B (an inhibitor of microfilaments) on the expression of their suppressor action. Both agents reduced the suppressive MΦ activity in a dose-dependent manner, without causing a cytotoxic effect ($\sim 50 \mu\text{g}$ indomethacin ml^{-1} ; $\sim 20 \mu\text{M}$ -cytochalasin B), indicating an important role of prostaglandins and microfilaments in the suppressor cell function of the MAC-induced MΦs. However, both agents failed to

Table 3. Effects of indomethacin (indo.) and cytochalasin B (cyt B) on the activity of MAC-induced splenic MΦs

MAC-MΦ treatment		No. of MΦs added	Con A mitogenesis of SPCs cocultured with MAC-MΦs (c.p.m.)*	Residual activity of suppressive MΦs (%)
Agent	Concn			
—	—	—	45581 ± 604	—
—	—	6×10^3	21317 ± 558	100
Indo.	$3.2 \mu\text{g ml}^{-1}$	6×10^3	27712 ± 3154	74
Indo.	$12.5 \mu\text{g ml}^{-1}$	6×10^3	37591 ± 1482	33
Indo.	$50 \mu\text{g ml}^{-1}$	6×10^3	44541 ± 1374	0.2
—	—	1.5×10^4	1634 ± 830	100
Indo.	$3.2 \mu\text{g ml}^{-1}$	1.5×10^4	1913 ± 625	99
Indo.	$12.5 \mu\text{g ml}^{-1}$	1.5×10^4	2909 ± 655	97
Indo.	$50 \mu\text{g ml}^{-1}$	1.5×10^4	29602 ± 2491	36
—	—	—	47580 ± 1163	—
—	—	6×10^3	28035 ± 3523	100
Cyt B	$1.25 \mu\text{M}$	6×10^3	19020 ± 1895	146
Cyt B	$5 \mu\text{M}$	6×10^3	31189 ± 2517	84
Cyt B	$20 \mu\text{M}$	6×10^3	43512 ± 1038	21
—	—	1.5×10^4	581 ± 51	100
Cyt B	$1.25 \mu\text{M}$	1.5×10^4	361 ± 62	100
Cyt B	$5 \mu\text{M}$	1.5×10^4	1022 ± 485	99
Cyt B	$20 \mu\text{M}$	1.5×10^4	28467 ± 2771	41

* The results are means $\pm$ SEM ($n = 3$)

Fig. 5. Effect of indomethacin on the suppressive activity of the MAC-induced MΦs against Con A mitogenesis of SPCs. SPCs (1.25×10^5 cells per well) were cultivated in the presence or absence of the MAC-induced splenic MΦs in medium containing $2 \mu\text{g}$ Con A ml^{-1} with (●) or without (○) the addition of $1 \mu\text{g}$ indomethacin ml^{-1} . Each point plotted indicates the mean $\pm$ SEM ($n = 3$).

overcome the suppressive activity of MAC-induced MΦs when these cells were added to the SPC culture in a large dose.

Fig. 5 shows the effect of indomethacin ($1 \mu\text{g} \text{ml}^{-1}$) added during the course of Con A mitogenic cultivation of SPCs in the presence or absence of MAC-induced splenic MΦs. Although this dose of indomethacin did not affect the Con A mitogenesis of splenic T cells, it markedly inhibited the suppressive activity of the MAC-induced MΦs. It is noteworthy that Con A mitogenesis was considerably enhanced by the addition of indomethacin in the SPC culture with a small number of MAC-induced MΦs. This suggests the possible presence of some accessory cells for Con A mitogenesis in the MAC-induced splenic MΦ populations.

Discussion

In this study, we obtained evidence for the following.

Firstly, immunosuppressive MΦs active against the Con A mitogenic response of splenic T cells were induced in the host spleen cells at a relatively early phase of MAC infection (around 2 weeks after bacterial challenge) in mice. In this case, increase in the immunosuppressive MΦ activity was seen in measurements of activity per spleen as well as activity per individual MΦ. The MAC-induced splenic MΦs had an enhanced ability to produce reactive oxygen interme-

diates, one representative parameter for MΦ activation (Nathan & Root, 1977; Johnston *et al.*, 1978; Tomioka & Saito, 1980). Therefore, it is thought that their suppressor function is substantially linked to the 'activated state'. The suppressor activity of MAC-induced splenic MΦs thereafter decreased to the normal level, despite the continuous and gradual increase in the number of the organisms in the host spleen for up to 25 weeks after bacterial challenge. A similar phenomenon was also noted in *M. lepraemurium* infection (Bullock *et al.*, 1978). Although active killing of parasites by MΦs is seen in host mice in this phase, it is possible that the present immunosuppressive MΦs transiently play some important role(s) in the establishment and progression of MAC-induced immunosuppression (particularly immune unresponsiveness to MAC) seen in the later phase of infection, which is mainly mediated by suppressor T cells (Watson & Collins, 1981).

Secondly, the MAC-induced splenic MΦs inhibited generation of IL-2-reactive T cell populations in response to Con A without suppressing IL-2-producing ability in helper T cells. Moreover, the suppressor MΦs inhibited the expression of IL-2 receptors on the Con-A-activated T cell subpopulations. We interpret these findings to mean that the immunosuppressive MΦs induced in the early phase of MAC infection, and possibly also in other mycobacterial infections, preferentially target the activation process of Lyt-1,2,3^+ T cells to the IL-2-reactive state in response to signals given by mitogens including Con A. This finding differs from commonly reported observations that immunosuppressive MΦs induced by microbial infections reduce the IL-2-producing activity of T cells (Hoffenbach *et al.*, 1983; Tarleton & Kuhn, 1984; Tossi *et al.*, 1986). Since IL-2 production in whole SPCs decreased in the case of MAC-infected mice, some types of immunosuppressive cells other than MΦs may be induced by MAC infection.

The peculiarity of immunosuppressive MΦs induced by MAC infection as compared with those induced by other mycobacterial infections could be a consequence of the course of infection of the different mycobacterial species studied. For instance, *M. lepraemurium* shows a progressive increase in bacterial load (Bullock *et al.*, 1978), whereas MAC infection appears to be controlled at a plateau level.

The possibility that the MAC-induced splenic MΦs exhibit a mitogenic inhibitory action through cytotoxic effects on the target T cells, including release of some cytotoxic substances, such as arginase (Kung *et al.*, 1977) and other toxic proteins (Chen *et al.*, 1977), has to be considered. However, this can be excluded because the spleen cells were not killed by contact with the suppressor MΦs for 24 h (data not shown). Also, the MAC-induced MΦs showed a selective inhibitory action

on T cell functions. Although the IL-2-producing ability was not depressed, the T cell activation to the IL-2 reactive state was inhibited (Figs 3 and 4). The selective depression of IL-2-reactive T cell generation also excluded the possibility of participation of unlabelled dThd (Opitz *et al.*, 1975) in the suppression of apparent [³H]dThd incorporation of splenic T cells by MAC-induced MΦs. It has been demonstrated that human monocytes phagocytosing *M. tuberculosis* suppress lymphocyte blastogenesis by releasing bacterial-cell-derived phospholipids which act as a suppressor cell activating factor (Wadee *et al.*, 1983). However, the physical presence of MAC in the splenic MΦs could not have caused the suppressor cell activity, since the number of bacilli in the MΦs was very low (four bacilli in 100 MΦs).

Thirdly, the activity of the MAC-induced splenic MΦs was largely mediated by prostaglandins, as already noted in other types of immunosuppressive MΦs (Edwards *et al.*, 1986; Metzger *et al.*, 1980; Stout & Fisher, 1983). However, indomethacin could not entirely overcome the suppressive action of the present MΦs, when the number of MΦs added to the SPC culture was increased. This suggests that there is a prostaglandin-independent mechanism for the expression of the suppressor activity of MAC-induced splenic MΦs in addition to the prostaglandin-mediated mechanism. In fact, it is known that prostaglandins cause a relatively slight inhibition of mitogenic proliferation of T cells when Con A is used as the mitogen (Novogrodsky *et al.*, 1979). Strong but incomplete dependency of the action of the present immunosuppressive MΦs on prostaglandins may indicate the presence of other mediators in the suppressor activity, such as MΦ-derived suppressor factors (SFs) (Fujiwara & Ellner, 1986; Wadee & Rabson, 1981). It is also possible that the MAC-induced MΦs exert their suppressive function through direct cell-to-cell contact with the target cells.

Fourthly, the suppressive action of the MAC-induced splenic MΦs was dependent on microfilament functions, because it was abolished by treatment with cytochalasin B. Microfilament inhibitors are known to inhibit some functions of MΦs, such as spreading, phagocytosis, and respiratory burst in response to some triggering ligands including PMA (Axline & Reaven, 1974; Nathan & Cohn, 1980; Tomioka & Saito, 1987), which are dependent on membrane function of MΦs. Thus, the mechanism of expression of suppressive activity of the immunosuppressive MΦs seems to be linked to certain membrane functions, such as those prerequisite for the signal transduction system or cell-to-cell interaction with target cells.

Studies are now under way using various strains of mice having the *Bcg*^s (MAC sensitive) and *Bcg*^r (MAC resistant) genotypes, with special regard to the role of

immunosuppressive MΦs in the establishment of suppressor T-cell-mediated immune unresponsiveness.

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