Br. J. exp. Path. (1985) 66, 271–277

Release of interleukin 1 and low-molecular-weight lymphocyte-activating factors by rat peritoneal macrophages and its enhancement by acute non-specific inflammatory processes

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Received for publication 31 May 1984

Summary. Peritoneal macrophages harvested from rats undergoing an acute non-specific inflammatory reaction induced by an injection of calcium pyrophosphate (CaPP) into the pleural cavity released increased amounts of interleukin 1 (IL-1)-like material. Lymphocyte-activating factors were also found in ultrafiltrates of the macrophage supernatants below 10 kd and 5 kd. A similar pattern of activity was observed when lysates of the macrophages were tested. In addition pre-exposure of normal peritoneal macrophages to an acute pleural inflammatory exudate before supernatant production enhanced the release of lymphocyte-activating factors found both in the unfractionated supernatant and a sub-5-kd ultrafiltrate. Thus these results demonstrate that an acute inflammatory reaction, initiated by a non-antigenic stimulus is able to stimulate macrophages remote from the inflammatory site to produce a factor which behaves like IL-1 in a standard IL-1 assay. The presence of low-molecular-weight factors (< 5 kd) with similar activity may suggest that degradation of IL-1 has taken place to yield active fragments. Acute inflammatory exudate also augments release of these factors which may be important in the pathogenesis of inflammation.

Keywords: interleukin 1, inflammation, macrophages

Recently it has been demonstrated that the functions of macrophages may be affected not only at the site of an acute non-specific inflammatory reaction, but also at points remote from it (Giroud *et al.* 1983).

Previous studies showed that acute inflammatory exudates derived after intrapleural injections of calcium pyrophosphate (CaPP) stimulated normal macrophages to divide *in vitro* (Giroud *et al.* 1977*a*). Similar activity(ies) was also present in the serum of animals undergoing this type of inflammatory reaction (Giroud *et al.* 1977*b*; Pelletier *et al.* 1978; Girre *et al.* 1981). The acute inflammatory process also could increase the cytostatic activity of peritoneal macrophages (Giroud *et al.* 1981). Many of these activities have been associated with the release of low-molecular-weight factors below 10 kilodaltons (10 kd) (Giroud *et al.* 1983).

Lymphoid cells also may be stimulated by acute inflammation. For example, inflammatory exudate stimulated the *in vitro* lymphoproliferative response of spleen cells (Florentin *et al.* 1980) and serum from inflamed animals modulated the proliferation of

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lymph node and spleen cells *in vitro* (Yao Jin Sheng *et al.* 1984*a*). This data led to the suggestion that interleukin 1 (IL-1) or lymphocyte-activating factors (LAF) may be involved in these phenomena and preliminary data was presented to support this view (Yao Jin Sheng *et al.* 1984*b*).

We have now investigated the ability of the acute inflammatory reaction to enhance IL-1 release by macrophages harvested at a point distant from the site of inflammation. In addition, the IL-1-containing supernatants have been fractionated by ultrafiltration to determine the possible role of low-molecular-weight factors liberated by the macrophages. Finally, the *in vitro* effect of acute inflammatory exudates on the release of lymphocyte-activating factors by normal macrophages has been considered.

Materials and methods

Animals. Male Sprague–Dawley rats (Depré, St-Doulchard, France), 200 g body weight and female C_3H/HeJ mice (CNRS, Orléans, France), 5–7 weeks old were used.

Induction of acute non-specific inflammatory reaction and collection of peritoneal macrophages. Rats were injected intrapleurally with 1 ml of a 1% suspension of CaPP in pyrogen-free saline as previously described (Willoughby *et al.* 1975); untreated normal rats were used as controls. After 72 h peritoneal cells were obtained from these animals by washing the peritoneal cavity with 20 ml of chilled RPMI 1640 medium (GIBCO, Bio-Cult Ltd, Scotland). The cell suspension was centrifuged and resuspended in complete medium, which consisted of RPMI 1640, 2 mM glutamine, 5×10^{-5} 2-mercaptoethanol, streptomycin (100 μg/ml) and penicillin G (100 units/ml) before measuring both viability (97%) by the trypan blue dye exclusion technique and the proportion of macrophages (76%, in both groups) by staining for non-specific esterase (Koski *et al.* 1976). The cell density was adjusted to 10^6 macrophages/ml and the suspension was seeded into 24-well macroplates (Falcon, type 3008, Oxnard, USA) by incubation for 2 h at 37°C in an atmosphere of 5% CO_2, 95% air and high humidity. Non-adherent cells were removed by repeated flushing with RPMI 1640 medium and the resulting monolayers incubated in 1 ml of complete medium.

Preparation of macrophage supernatants and lysates. Macrophages obtained from normal or inflamed rats were prepared as above and cultured for 48 h in the presence or absence of 10 μg/ml lipopolysaccharide (LPS, *S. typhosa* 0901 strain, Difco Laboratories, USA). Following culture the supernatants were harvested, clarified by centrifugation (1000 *g* for 20 min) and stored in aliquots at −20°C. After removal of supernatants, lysates were prepared by washing the macrophage monolayers and adding 1 ml of complete medium followed by freeze–thawing and sonication (30 s, 12 μm amplitude). The preparation was clarified by centrifugation (2000 *g*, 15 min) and stored in aliquots at −20°C.

In some experiments macrophages from normal animals were exposed to inflammatory exudates *in vitro* before collecting supernatants. In this case macrophage monolayers were cultured in the presence of 50% inflammatory exudate in TC 199 medium supplemented with 20% heat-inactivated fetal calf serum. The inflammatory exudates were harvested from the pleural cavities of rats 2 and 4 h after an intrapleural injection of CaPP and the cells removed by centrifugation. After 72 h culture the macrophage monolayers were washed, 1 ml of complete medium was added and they were incubated for a further 24 h after which supernatants were collected as above. Controls consisted of normal macrophage monolayers cultured in the same manner, but without inflammatory exudate.

Some supernatants and lysates were partially fractionated by ultrafiltration. The samples were filtered in an Amicon ultrafiltration cell fitted with a Diaflon YM10 or YM5

membrane which allowed the passage of molecules under 10 kd and 5 kd respectively. The ultrafiltrates were stored at −20°C.

Assay of lymphocyte-activating factor (interleukin 1). IL-1 activity was determined by enhancement of the C3H/HeJ thymocyte mitogenic response to phytohaemagglutinin (PHA) (Lachman *et al.* 1977). Thymus glands from C_3H/HeJ mice were removed aseptically and single cell suspensions prepared using complete medium supplemented with 5% heat-inactivated fetal calf serum 10 mM Hepes buffer. The cell density was adjusted to 1.5×10^7 viable cells/ml.

Samples were diluted in the supplemented complete medium as indicated and 0.1-ml aliquots were added in triplicate to round-bottomed microtitre plates (Linbro, New Haven, USA). Thymoctyes were added in aliquots of 0.1 ml shortly after being mixed with PHA (Wellcome Diagnostics, Kent, England) at a final concentration of 1 μg/ml. After incubation for 72 h at 37°C in a 5% CO_2, 95% air atmosphere with high humidity, the cultures were pulsed with 1 μCi of ^{3}H-thymidine (5 Ci/mM) for the final 18 h. Cells were recovered using a semi-automatic Mash harvester (Skatron, Flow Labs, Scotland) and ^{3}H-thymidine incorporation was measured using liquid scintillation spectrometry. The results were expressed as mean counts/min (ct/min) for triplicate determinations. In experiments with inflammatory exudates, normal rat thymocytes were substituted for C3H/HeJ thymocytes.

Statistical significances for the thymocyte proliferative assays were determined using Student's *t*-test. The results reported are for one representative experiment of a series of three which gave similar results.

Results

The effects on the PHA-induced proliferative response of C3H/HeJ thymocytes of supernatants derived from rat peritoneal macrophages, 72 h after induction of CaPP-pleurisy are summarized in Fig. 1. It can be observed that when normal or inflamed macrophages were cultured in the presence of LPS they liberated IL-1 (LAF)-like activity in the unfractionated supernatant(s) and its ultrafiltrates of below 10 kd (U_{10}) and 5 kd (U_5). In the absence of LPS during culture, significant activity was found in the unfrac-

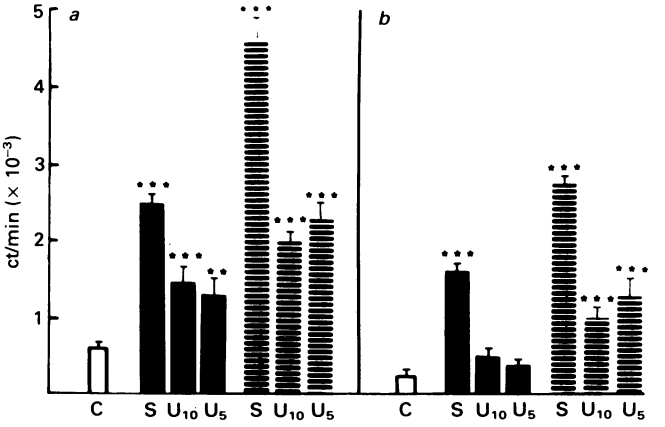

Fig. 1. The effects on the PHA-induced proliferative response of C_3H/HeJ thymocytes of supernatants from rat peritoneal macrophages collected from either normal animals (solid columns) or 72 h after induction of CaPP-pleurisy (broken columns) in the presence (*a*) or absence (*b*) of LPS. The unfractionated supernatants (S) and their ultrafiltrates below 10 kd (U_{10}) and 5 kd (U_5) were tested at a dilution of 1/4. The results represent the mean (±SD) of triplicate determinations in a typical experiment where C is cells alone. ***$P<0.001$; **$P<0.01$; *$P<0.02$.

tionated supernatants, but the absolute levels were lower than when LPS was present. In contrast, only the ultrafiltrates of macrophage supernatants from inflamed animals retained significant activity. In both the presence and absence of LPS during *in vitro* culture, macrophages collected from animals undergoing an acute non-specific inflammation released significantly more activity than did macrophages derived from normal control rats. In addition, this same phenomenon was observed with ultrafiltrates of the supernatants under 10 kd (U_{10}) and 5 kd (U_5).

Fig. 2 shows the effects on the PHA-induced proliferative response of C3H/HeJ thymocytes of macrophage lysates cultured in the presence of LPS. In this case the unfractionated macrophage lysates from normal and inflamed animals contained significant quantities of lymphocyte-activating factors with the higher levels being found in those from inflamed rats. Similarly, lymphocyte-activating factors were found in the macrophage ultrafiltrates less than 10 kd but in this case there was more activity ($P<0.05$) in those from inflamed rats than in those from normal controls.

The *in vitro* effect of an acute inflammatory exudate on the production of lymphocyte-activating factors by normal peritoneal macrophages is shown in Fig. 3. Unfractionated supernatants from control macrophages contained some activity, and this was not significantly affected by pre-incubation with 2-h inflammatory exudates but 4-h exudates

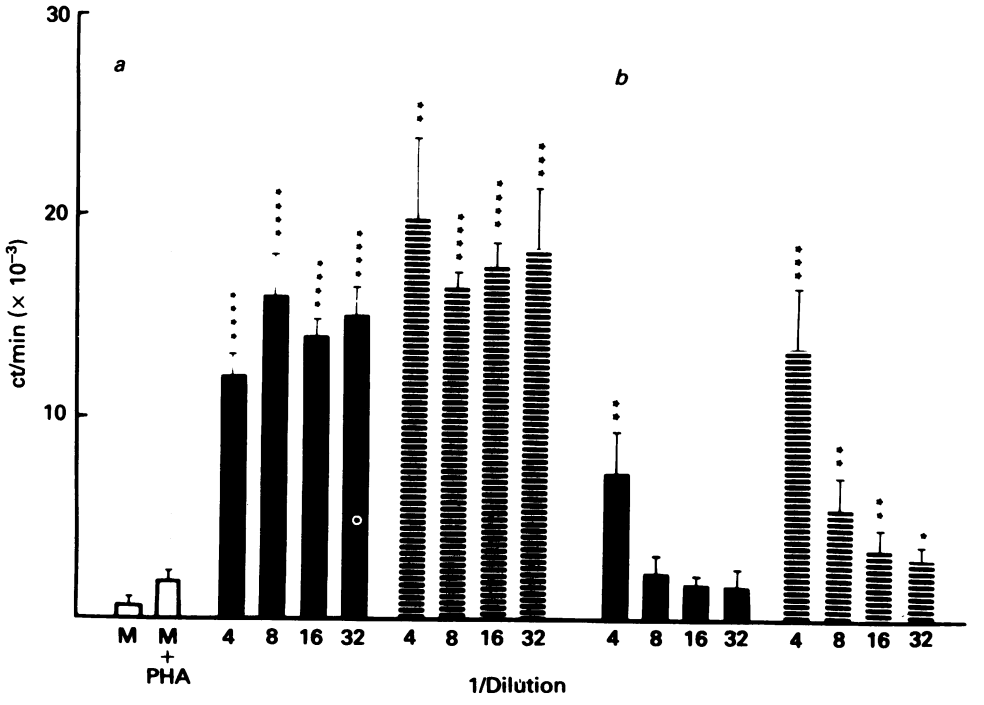

Fig. 2. The effects on the PHA-induced proliferative response of C_3H/HeJ thymocytes of lysates (*a*) and ultrafiltrates (< 10 kd) (*b*) of rat peritoneal macrophages after *in vitro* culture for 48 h with 10 μg/ml LPS. The macrophages were collected from either normal (solid columns) animals or 72 h after induction of CaPP-pleurisy (broken columns) before culture. The results represent the mean (±SD) of triplicate determinations in a typical experiment where M is cells aone, M+PHA is cells plus PHA. ****$P<0.001$; ***$P<0.01$, **$P<0.02$; *$P<0.05$.

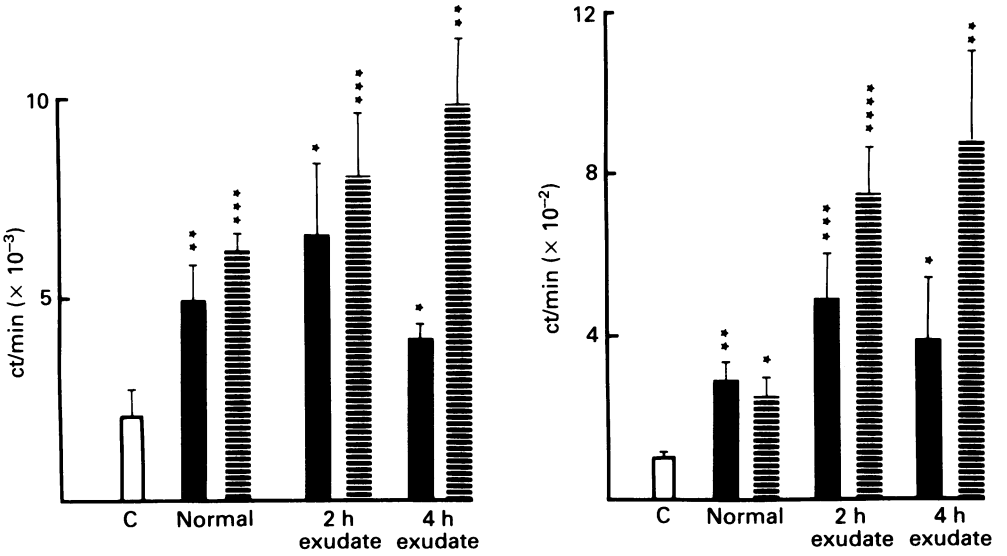

Fig. 3. Release of lymphocyte-activating activity by normal peritoneal macrophages pre-incubated with a 2-h or 4-h inflammatory exudate. The macrophage supernatants (solid columns) and their 5 kd ultrafiltrates (broken columns) were tested at dilution 1/4 on ^{3}H-TdR incorporation by normal rat thymocytes stimulated with PHA (*a*) or in the absence of mitogen (*b*). The results represent the mean (±SD) of triplicate determinations in typical experiments were C is cells + PHA (*a*) or cells alone (*b*). ****$P<0.001$; ***$P<0.01$; **$P<0.02$; *$P<0.05$.

significantly enhanced the activity recovered in the sub-5-kd fraction (Fig. 3*a*). These results were obtained when PHA was present in the thymocyte proliferation assay. The results for the same fractions in the absence of PHA are shown in Fig. 3*b*. Although the levels of ^{3}H-thymidine incorporation were lower in the absence of PHA, some inherent mitogenic activity was present in both the unfractionated supernatant(s) and its 5-kd ultrafiltrate (Su). Exposure of macrophages to inflammatory exudates increased this level of activity and was most noticable in the sub-5-kd fraction where both 2- and 4-h exudate significantly enhanced the release of lymphocyte-stimulating factors.

Discussion

In this investigation we demonstrated that an acute non-specific pleural inflammatory reaction could stimulate the production by peritoneal macrophages of a factor which behaved like interleukin 1 in an accepted IL-1 assay. The data for unfractionated peritoneal macrophage supernatants is in accord with our previous findings with mice (Yao Jin Sheng *et al.* 1984*b*). In the present study we extended these data to show that activity(ies) is present in ultrafiltrates of macrophage supernatants, which enhances the proliferation of C3H/HeJ thymocytes. Ultrafiltrates of below 10 kd and 5 kd both retained significant activity in this assay. Although this suggests that the molecular species responsible are $\leqslant$ 10 kd, these filters are not accurate and thus the results should be interpreted with caution. However, since about half the total activity of the unfractionated supernatant was retained in the sub-5-kd ultrafiltrate and the reported molecular weight of IL-1 is in the range 12–16 kd (human/murine) (Oppenheim *et al.* 1982) it seems unlikely that intact IL-1 could be responsible for the lymphocyte-activating

activity of the ultrafiltrates. These low-molecular-weight factors could possibly be degradation products of IL-1 itself. Other investigators have isolated at 1.5 kd peptide from stimulated human monocyte supernatants which had LAF-like activity (Masuzawa *et al.* 1978). Certainly it would not be surprising if IL-1 were degraded since macrophages contain and can release enzymes which potentially could fulfil this function.

The results presented here for the macrophage lysates complement the data obtained with the cell supernatants. Unfractionated lysates of macrophages from normal and inflamed rats contained significant lymphocyte-activating activity which again was partially retained in the sub-10-kd ultrafiltrate. The lysates of 'inflamed' macrophages had significantly higher levels of activity than normal macrophages, but the differences were less marked than with the supernatants, possibly due to a discrepancy between release and production of IL-1 as has previously been described for murine macrophages (Gery *et al.* 1981).

In the second part of this investigation, the effect of acute inflammatory pleural exudate on the liberation of lymphocyte-activating factors by normal peritoneal macrophages was studied. Significant enhancement of lymphocyte-activating activity was obtained with both the unfractionated supernatant and its ultrafiltrate (< 5 kd) when the macrophages were exposed *in vitro* to 2- and 4-h pleural exudates. As well as enhancing the PHA-induced lymphoproliferative response of rat thymocytes, the macrophage supernatants also had inherent mitogenic activity which was slightly elevated by co-culture with the inflammatory exudate.

Data from previous studies (Giroud *et al.* 1983; Yao Jin Sheng *et al.* 1984*b*) suggested that a humoral factor(s) may be released during the acute non-specific inflammatory response which can act at a distance on the macrophage. These low-molecular-weight factors (< 10 kd), termed phlogokines were released rapidly (within 4 h), modulated macrophage functions and were distinct from lymphokines or monokines. In this communication we have shown that *in vitro* inflammatory exudate (2–4 h) can stimulate macrophages directly to release lymphocyte-activating factors. Furthermore, the inflammatory response *per se* can stimulate macrophages at a site distant from the inflammatory focus, to release more IL-1-like material. Moreover, these and normal macrophages both produce and liberate low-molecular-weight factors (< 5 kd) which have lymphocyte activating activity in a standard IL-1 assay suggesting that they may be breakdown products of IL-1. Work is in progress to characterize these low-molecular-weight factors with lymphocyte-activating activity; preliminary results indicate they may be peptides since their activity is destroyed by incubation with papain, but not with chymotrypsin.

Acknowledgements

We are indebted to Brigitte Chanaud, Suzanne Strzalko and Olivier Muntaner for their excellent technical assistance, to the Syndicat National de l'Industrie Pharmaceutique and Laboratoires Specia for providing post-doctoral fellowships for Yao Jin Sheng and John Bird and finally to Professor A. Pompidou for providing the cell culture facilities. This work was supported by a grant from the 'Association pour le Développement de la Recherche sur le Cancer'.

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