Journal of Cellular Biochemistry 26:65–73 (1984)

Partial Characterisation of the High and Low Molecular Weight Forms of $P388D_1$-Derived Interleukin 1

R.L. Prestidge, W.J. Koopman, and J.C. Bennett

Division of Clinical Immunology and Rheumatology, Department of Medicine, University of Alabama in Birmingham, Birmingham, Alabama 35294

The murine macrophage-derived cell line $P388D_1$ secretes the lymphokine interleukin 1 (IL-1) when stimulated by a variety of agents. When stimulated by bacterial lipopolysaccharide (LPS) the cells release IL-1 in both high and low molecular weight (m.w.) forms. The proportion of high m.w. IL-1 is reduced when IL-1-containing supernatants are concentrated by ammonium sulfate precipitation subsequent to hollow-fiber filtration. The high m.w. form can be converted to the low m.w. form by proteolysis, reduction and alkylation, or chromatography in a dissociating solvent. The low m.w. form remains as such, even when reconcentrated in fetal calf serum-containing medium. The high m.w. form thus likely consists of a complex between low m.w. IL-1 and another protein secreted by the $P388D_1$ cell line.

Key words: lymphokines, interleukin 1, macrophage cell line, protein complex

Interleukin 1 (IL-1) is a macrophage-derived immunoregulatory protein that plays an important role in the control of such diverse immunologic processes as antigen- [1,2] and mitogen-induced [3] T-cell proliferation and the maturation and functional activation of helper [4,5] and cytotoxic T-cells [6]. It is secreted by macrophages in response to a variety of stimuli, such as lipopolysaccharide [5,7], antigen-antibody complexes, and particulate antigens [8].

The mechanism of action of IL-1 has been extensively investigated. An important step forward was the discovery of murine T-cell lines that secrete IL-2 in response to IL-1 together with mitogens [9,10]. These cell lines, LBRM-33 clone 1A5 [9] and WEHI-7 [10] are taken to be models for a T-cell population in vivo that binds a specific antigen in the presence of macrophage-derived IL-1 and releases IL-2 and other regulatory factors [11] in a coordinated response. The assumption has been made [12] that the principal effect of IL-1 on lymphocytes is mediated through IL-2.

Recently, however, an increasing number of responses to IL-1 have been studied, and many do not seem to involve IL-2 as intermediary. For example, in the

R.L. Prestidge is now at the Department of Immunobiology, University of Auckland Medical School, Auckland, New Zealand.

Received August 16, 1983; accepted May 3, 1984.

generation of cytotoxic effector cells [13] in antibody synthesis [4,14] and in thymocyte proliferation [15], IL-1 and IL-2 synergise in their effects, and this is not consistent with a mechanism where the sole effect of IL-1 is to cause release of IL-2. Similarly, the observation by Hoffmann et al, [16] that IL-1 induces phenotypic differentiation of B cells within 2.5 hr is more readily compatible with a direct effect on B cells rather than an effect mediated through IL-2 release. IL-1 may also have direct effects on nonlymphocytic cells including fibroblasts [17] and hepatocytes [18, 19].

The problem of identifying the cell types that carry specific receptors for IL-1 is thus of considerable interest, and one that can ultimately only be solved by the use of purified and labeled IL-1 preparations. The first step in IL-1 binding experiments is a thorough understanding of the behaviour in solution of the IL-1 molecule.

Studies on the structure and function of interleukin 1 are facilitated by the fact that IL-1 is secreted by several murine macrophage-derived cell lines, notably the $P388D_1$ cell line [20,21]. IL-1 from these cell lines has been shown to have many of the properties of IL-1 from normal macrophages [22]; however, there is some disagreement with regard to the existence of high molecular weight (m.w.) forms of IL-1. These high molecular weight forms have been observed in IL-1 from normal human macrophages [4,8,23]. High molecular weight forms of IL-1 have also been observed in supernatants from the $P388D_1$ murine cell line by some workers [7]; others, however, have failed to observe high molecular weight forms in IL-1 from the same cell line [6,20]. In order to resolve this discrepancy, we have carried out a number of experiments on IL-1 from the $P388D_1$ cell line. We have consistently observed both high and low molecular weight forms and have investigated several aspects of the relationship between them.

METHODS

The procedure used for production of IL-1-containing conditioned medium has been published in detail [24]. In brief, $P388D_1$ cells ($8–10 \times 10^5$/ml) attached to Cytodex microcarrier beads were stimulated with lipopolysaccharide (LPS) (10 μg/ml) in RPMI 1640 medium containing fetal calf serum (5%) and gentamycin (50 μg/ml) in a 10-liter spinner flask equipped with a Teflon paddle (Bellco Products, Vineland, NJ). The suspension was stirred at 30–60 rpm in a 37°C incubator. On each of the three succeeding days, half of the supernatant was removed and replaced with fresh medium. On the fourth day, the entire supernatant was harvested, pooled with supernatants from the preceding days, and concentrated on a hollow-fiber membrane filtration device (HIPIO-8 cartridge, Amicon Corporation, Lexington, MA; 10,000 m.w. nominal cutoff) to 1/19 of its initial volume. The concentrated supernatant solution was precipitated with solid ammonium sulfate added to 65% of saturation at 4°C. After stirring at 4°C for 30 min, the precipitate was harvested by centrifugation (15,000 g for 15 min) and redissolved in 1/80 of the starting volume of 10 mM sodium phosphate, 150 mM sodium chloride pH 7.2 (PBS). Samples from the initial conditioned medium, the material retained by and passed by the hollow-fiber unit, and the ammonium sulfate precipitate and supernatant were assayed for IL-1 activity.

Thymocyte Proliferation Assay

Samples were prepared and assayed for IL-1 activity by the thymocyte proliferation assay using the methods of Mizel et al [25]. The samples were diluted

appropriately in RMPI 1640 medium containing 5% fetal calf serum, 2mM L-glutamine, 2.5×10^{-5}m 2-mercaptoethanol, and gentamycin, and 100 μl aliquots were placed in the wells of a flat-bottomed 96-well microtest II plate (Nunc, Roskilde, Denmark). Thymocytes from C3H/HeJ mice were washed twice with tissue culture medium, diluted to 1.5×10^6 cells/ml, and 100 μl aliquots were added to each culture well. The cultures were incubated at 37°C for 3 days in an atmosphere of 5% CO_2 in air, and culture wells were pulse-labeled with 1 μCi of [^{3}H]-thymidine (New England Nuclear, Boston, MA) for the last 16 hrs of this culture period. Cells were harvested onto glass-fiber filter strips using a MASH 2 harvester, and cell proliferation was determined by scintillation counting of the radioactivity incorporated into cellular DNA.

Preparative Gel Filtration

Aliquots of both the inital hollow fiber concentrate and the redissolved ammonium sulfate precipitate were chromatographed on a large column of Sephadex G-75 (2.6 × 80 cm) equilibrated with PBS. Fractions (160 drops, 9.5 ml) were collected and assayed for IL-1 activity. Pools of low (fractions encompassing m.w. 5,000–40,000) and high molecular weight (fractions encompassing m.w. range 40,000–100,000) IL-1 were prepared from this column effluent.

Trypsin Cleavage

An aliquot of high molecular weight IL-1 (5 ml) containing 125 mg protein was treated with TPCK-trypsin (1 mg, Worthington Biochemicals, Freehold, NJ) at 37°C for 3 hr. Soybean trypsin inhibitor (Sigma, 1.4 mg in 1 ml) was added, and the mixture was chromatographed on a smaller column of Sephadex G-75 (1.6 × 50 cm) at 4°C. Column fractions (40 drops/fraction) were dialysed against PBS (2 × 2 liters) and RMPI 1640 (2 liters) and assayed by the thymocyte proliferation assay.

Reduction and Alkylation

An aliquot of high molecular weight IL-1 identical to that used for trypsin cleavage was reduced in sodium phosphate buffer (pH 7.2, 0.25 M, 25 ml) with dithiothreitol (375 mg) overnight at room temperature. Iodoacetamide (450 mg) was added, and the incubation was continued for 60 min at room temperature in the dark. Dithiothreitol (20 mg) was added to inactivate excess iodoacetamide, and the solution was dialysed against PBS (2 × 2 liters), concentrated to 8 ml on an Amicon membrane filtration device (UM 2 membrane, nominal cutoff 1,000 m.w.), and applied to a Sephadex G-75 column (1.6 × 50 cm). Chromatography was carried out as for the trypsin-cleaved sample. Column fractions were dialysed and assayed as above.

Chromatography in Ethylene Glycol/NaCl

Aliquots of high molecular weight IL-1 identical to the above were dialysed against 25% ethylene glycol/1.0 M NaCl/10 mM potassium phosphate buffer, pH 7.6, and applied to a column of Sephacryl S-200 (1.6 × 51 cm) equilibrated with the same buffer at room temperature. Chromatography was carried out as for the trypsin-cleaved sample. Column fractions were dialysed and assayed as above.

Concentration of Low Molecular Weight IL-1

Low molecular weight IL-1 was prepared by chromatography of high m.w. IL-1 ethylene glycol buffer as above and dialysed into PBS. An aliquot (3 ml) was diluted

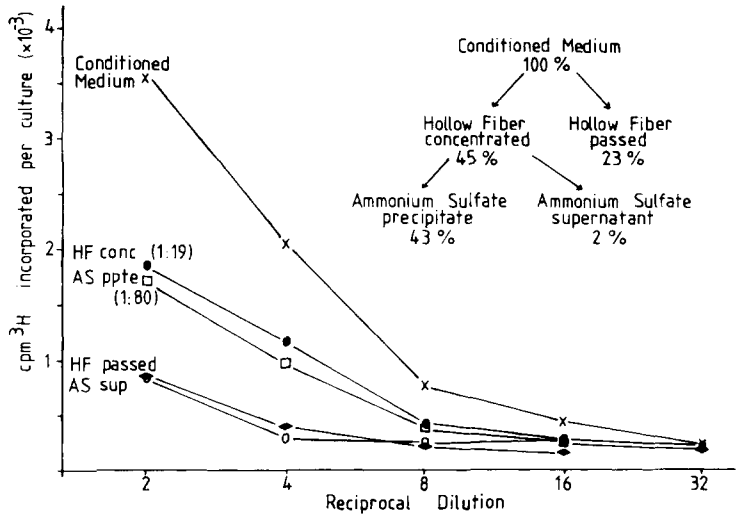

Fig. 1. Titrations of IL-1-containing samples in the thymocyte proliferation assay. Medium conditioned by LPS-stimulated P388D_1 cells was fractionated as in the Methods section (outlined in the flow chart above). Samples from each stage were diluted appropriately and assayed for IL-1 activity. The approximate percentage yield of starting IL-1 activity is shown for each step of the fractionated procedure.

to 40 ml with RPMI 1640 medium containing 2% fetal calf serum and then reconcentrated to 3 ml using an Amicon membrane filtration device (YMIO membrane, nominal cutoff 10,000 m.w.). The sample was then applied to a column of Sephadex G75 equilibrated with PBS, and column fractions were dialysed and assayed as described above.

RESULTS

Medium conditioned by LPS-stimulated P388D_1 cells was found to contain significant titers of IL-1 as measured by the thymocyte proliferation assay (Fig. 1). By titration of the relevant solutions, it could be calculated that 45% of this IL-1 activity was retained by an HIPIO hollow-fiber cartridge (10,000 m.w. nominal cutoff) (Fig. 1). Some of the IL-1 activity appeared to pass through the hollow-fiber unit (23%, Fig. 1); however, because of the low activity and large volume of this sample, the measurement of the amount of activity passing through the hollow-fiber cartridge can only be regarded as an estimate. The IL-1 activity that passed through the hollow-fiber unit could not be concentrated by a second passage through the same unit (data not shown).

Essentially all of the IL-1 activity retained by the hollow-fiber unit was precipitated by ammonium sulfate to 65% of saturation (43/45 = 96%, Fig. 1) and only trace quantities of IL-1 were found in the ammonium sulfate supernatant (Fig. 1).

Both high and low molecular weight forms of IL-1 were found in P388D_1-derived cell-free supernatants concentrated by membrane filtration (Fig. 2). Ammonium sulfate precipitation (Fig. 2) gave rise to a much higher proportion of the low molecular weight form. The apparent splitting of the low m.w. peak of the membrane

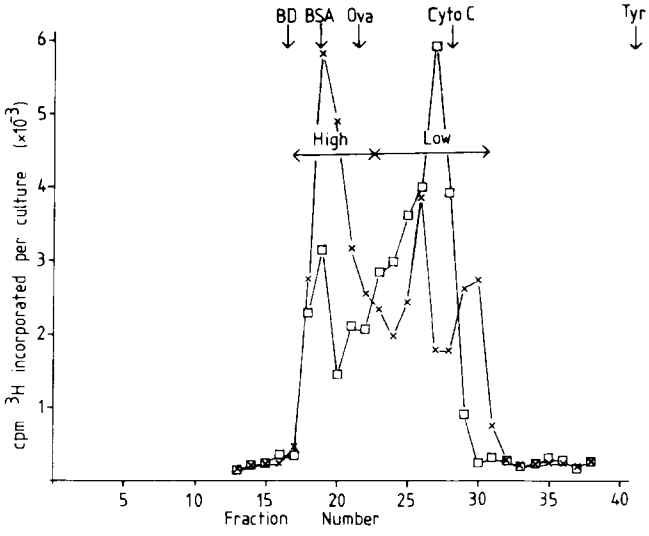

Fig. 2. Gel filtration of concentrated IL-1-containing supernatants: (×) concentrated by membrane filtration alone, (□) concentrated by membrane filtration and ammonium sulfate precipitation. Fractions were assayed for IL-1 activity in the thymocyte proliferation assay. The elution positions of blue dextran (BD, m.w. > 10^6), bovine serum albumin (BSA, m.w. 68,000), ovalbumin (Ova, m.w. 43,000), cytochrome C (Cyto C, m.w. 11,700), and tyrosine (Tyr, m.w. 181) molecular weight standards, and the fractions pooled to make high and low molecular weight IL-1 are shown above the activity profiles.

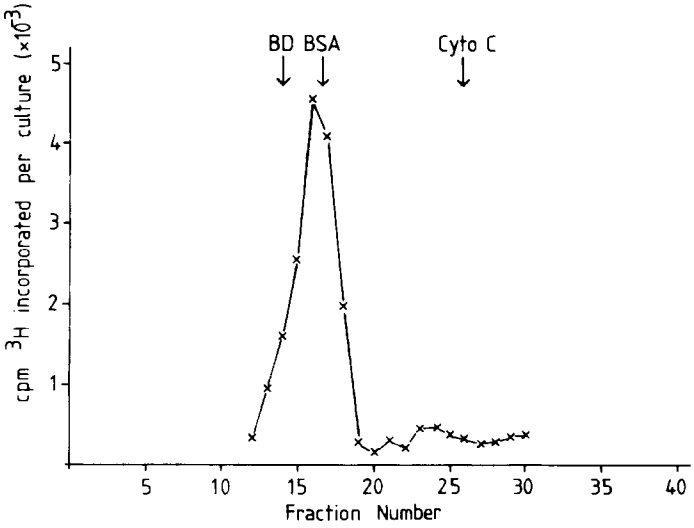

Fig. 3. Gel filtration analysis on Sephadex G-75 of a control sample of high molecular weight IL-1. The elution positions of blue dextran, bovine serum albumin, and cytochrome C molecular weight standards are shown.

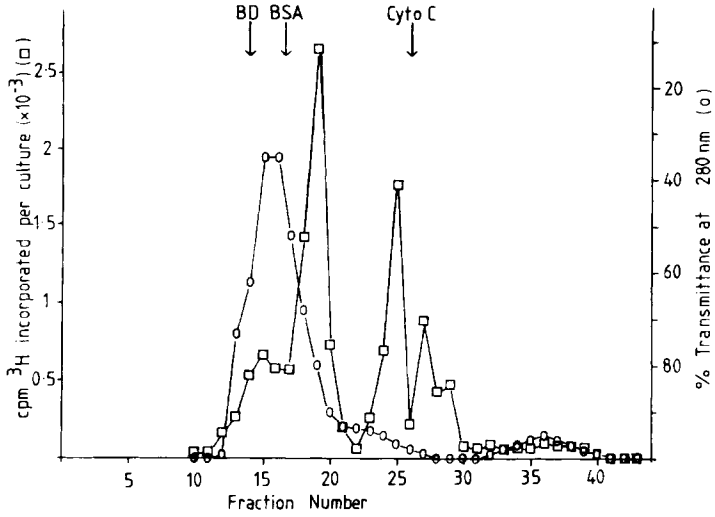

Fig. 4. Gel filtration analysis on Sephadex G-75 of high molecular weight IL-1 after trypsin cleavage.

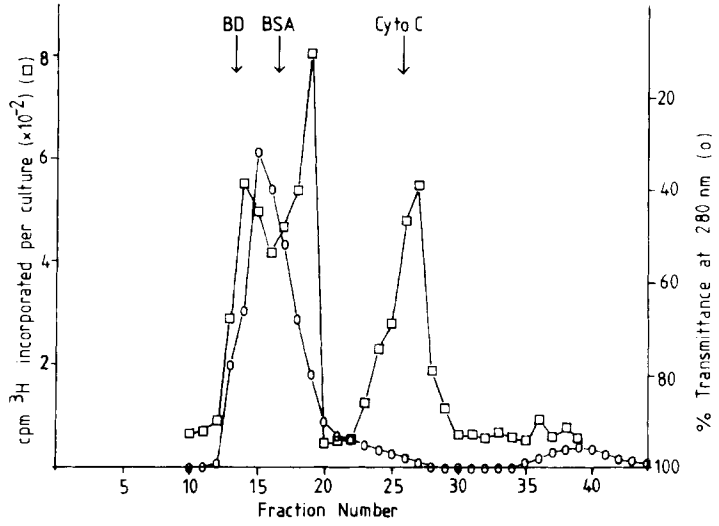

Fig. 5. Gel filtration analysis on Sephadex G-75 of high molecular weight IL-1 after reduction with dithiothreitol and alkylation with iodoacetamide.

filter-concentrated preparation into two peaks is an artefact: assay of these samples at different dilutions revealed that the low m.w. material elutes as a single peak (data not shown).

The high m.w. form of IL-1 prepared by membrane filtration remained as a high m.w. molecule on further gel filtration chromatography analysis using Sephadex G-75 (Fig. 3). It was only partly converted to the low m.w. form by proteolytic cleavage with trypsin (Fig. 4) or by reduction and alkylation (Fig. 5). Almost

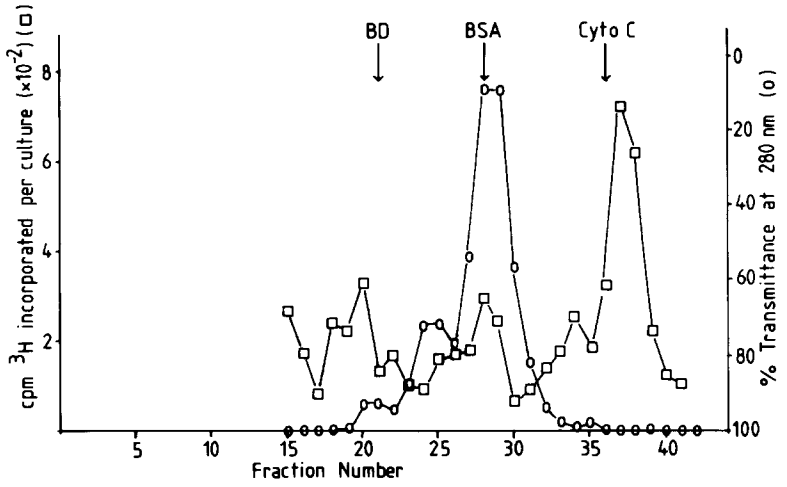

Fig. 6. Gel filtration chromatography of high molecular weight IL-1 on Sephacryl S-200 in 25% ethylene glycol/1.0 M NaCl/10 mM potassium phosphate buffer, pH 7.6.

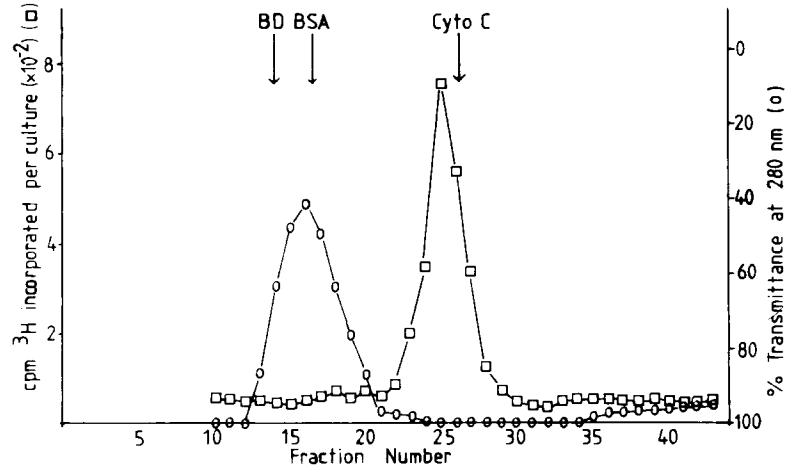

Fig. 7. Gel filtration analysis of low m.w. IL-1 on Sephadex G-75 after reconcentration in medium containing 2% fetal calf serum.

complete conversion from high to low m.w. form was obtained by gel filtration chromatography in 25% ethylene glycol/1.0 M NaCl/10 mM potassium phosphate buffer, pH 7.6 (Fig. 6).

Low m.w. IL-1 formed by the dissociation of the high m.w. species by chromatography in ethylene glycol was found to remain in the low m.w. form after dialysis into PBS and on rechromatography in nondissociating conditions (not shown). This was also found to be the case if the low m.w. form was first reconcentrated in fetal calf serum (FCS)-containing media (Fig. 7).

DISCUSSION

Gel filtration chromatography of $P388D_1$-derived IL-1-containing supernatants concentrated by membrane filtration consistently demonstrated the presence of both high and low molecular weight forms of IL-1, and a typical result is shown in Figure 2. This finding is consistent with that obtained for IL-1 from human macrophages [8,2,3] and with the data of Lachman and Metzger [7] concerning $P388D_1$-derived IL-1. It is not consistent with the work of Mizel et al (20) and Farrar et al [6] who did not observe high molecular weight forms of IL-1 in supernatants from this cell line. The difference possibly relates to the fact that both of the latter studies utilised phorbol myristic acetate (PMA) as a cell stimulant rather than LPS, as used in this study. PMA may cause the selective production of low molecular weight IL-1 or the induction of proteolytic enzymes which degrade high molecular weight forms.

Methods utilised for the concentration of IL-1 supernatants also influenced the proportion of high molecular weight versus low molecular weight forms of IL-1. As shown in Figure 2, ammonium sulfate precipitation of membrane-concentrated IL-1-containing supernatants causes the majority of the IL-1 activity in the precipitate to exist in the low molecular weight form. It is important to note that very little IL-1 activity was found in the ammonium sulfate supernatant in repeated experiments (Fig. 1 and unpublished results). The most likely interpretation of this result is that high molecular weight IL-1 consists of a noncovalent complex that can be partly dissociated under the conditions utilised for ammonium sulfate precipitation.

The high m.w. form of IL-1 remained as such when rechromatographed on Sephadex G-75 under nondissociating conditions (Fig. 3). Trypsin cleavage (Fig. 4) and reduction and alkylation (Fig. 5) of high molecular weight IL-1 resulted in partial conversion to the low molecular weight form. In view of the vigorous conditions used, it is apparent that high molecular weight IL-1 is a relatively stable species.

Chromatography in 25% ethylene glycol/1 M sodium chloride leads to nearly complete dissociation of IL-1 activity from the major protein peaks (Fig. 6). Ethylene glycol, as an organic solvent, tends to disrupt hydrophobic interactions and thus reduce protein-protein interactions [26]. This result is, therefore, consistent with the hypothesis that high molecular weight IL-1 consists of a complex with another protein.

Concentration of low molecular weight IL-1 (formed by disaggregation of high m.w. IL-1) in the presence of fetal calf serum did not lead to its reconversion to the high molecular weight form (Fig. 7). The addition of LPS (10 μg/ml) also did not change this result (data not shown). This suggests that high molecular weight IL-1 consists of a complex between IL-1 and, not a serum protein as suggested by Lachman and Metzgar [7], but a protein produced by culture of $P388D_1$ cells under these conditions. IL-1 has been reported to have a high affinity for hydrophobic surfaces [27], and thus it would not be surprising that it readily formed complexes with other components secreted by the IL-1-producing cell. The data presented above show that the amount of high m.w. complexes formed is very dependent on the techniques used to concentrate the IL-1-containing supernatants. For this reason, the existence of high m.w. complexes of IL-1 is probably not physiologically significant [28].

REFERENCES

1. Mizel SB, Ben-Zvi A: Cell Immunol 54:382, 1980.
2. Rosenwasser LJ, Dinarello CA, Rosenthal AS: J Exp Med 150:709, 1979.

3. Oppenheim JJ, Mizel SB, Meltzer MS: In Cohen S, Pick E, Oppenheim JJ (eds): "Biology of the Lymphokines." New York: Academic Press, 1979, pp 291–323.
4. Farrar JJ, Koopman WJ: In Cohen S, Pick E, Oppenheim JJ (eds): "Biology of the Lymphokines." New York: Academic Press, 1979, pp 325–346.
5. Wood DD: J Immunol 123:2395, 1979.
6. Farrar WL, Mizel SB, Farrar JJ: J Immunol 124:1371, 1980.
7. Lachman LB, Metzgar RS: J Reticuloendothel Soc 27:621, 1980.
8. Blyden G, Handschumacher RE: J Immunol 118:1631, 1977.
9. Gillis S, Mizel SB: Proc Natl Acad Sci USA 72:1133, 1981.
10. Smith KA, Gilbride KJ, Favata MF: Nature 287:853, 1980.
11. Watson J: J Immunol 131:293, 1983.
12. Gery I: In Fathman CG, Fitch FW (eds): "Isolation, Characterisation and Utilization of T Lymphocyte Clones." New York: Academic Press, 1982, pp 41–56.
13. Lutz CT, Glasebrook AL, Fitch FW: J Immunol 126:1404, 1981.
14. Hoffman MK, Watson J: J Immunol 122:497, 1979.
15. Hoffman M, Gilbert K, Chun M: In de Weck AL, Kristensen F, Landy M (eds): "Biochemical Characterisation of the Lymphokines." New York: Academic Press, 1980, pp 473–478.
16. Hoffman MK, Koenig S, Mittler RS, Oettgen HF, Ralph P, Galanos C, Hammerling U: J Immunol 122:497, 1979.
17. Mizel SB, Dayer J-M, Krane SM, Mergenhagen, SE: Proc Natl Acad Sci USA 78:2474, 1981.
18. Sige JD, Vogel SN, Ryan L, McAdam KPWJ, Rosenstreich DL: J Exp Med 150:597, 1979.
19. Kampschmidt RF, Pulliam LA, Upchurch HF: J Lab Clin Med 95:616, 1980.
20. Mizel SB, Rosenstreich DL, Oppenheim JJ: Cell Immunol 40:230, 1978.
21. Lachman LB, Hacker MP, Blyden GT, Handschumacher RE: Cell Immunol 34:416, 1977.
22. Mizel SB: Ann NY Acad Sci 332:539, 1979.
23. Oppenheim JJ, Moore R, Gmelig-Meyling F, Togawa A, Wahl S, Mathieson BJ, Dougherty S, Carter C: In Unanue ER, Rosenthal AS (eds): "Macrophage Regulation of Immunity." New York: Academic Press, 1980 pp 379–398.
24. Prestidge RL, Sandlin GM, Koopman WJ, Bennett JC: J Immunol Meth 46:197, 1981.
25. Mizel SB, Oppenheim JJ, Rosenstreich DL: J Immunol 120:1497, 1978.
26. Berg K, Heron I: Scand J Immunol 11:489, 1980.
27. Mizel SB: Immunol Rev 63:51, 1982.
28. Togawa A, Oppenheim JJ, Mizel SB: J Immunol 122:2112, 1979.