Biochimica et Biophysica Acta, 566 (1979) 183—191

BBA 68623

LEUKOCYTE MIGRATION INHIBITORY FACTOR

A SERINE ESTERASE RELEASED BY STIMULATED HUMAN LYMPHOCYTES. KINETIC ANALYSIS AND INHIBITION BY CYCLIC GMP

KLAUS BENDTZEN *

Laboratory of Clinical Immunology, Department of Medicine TA, Righospitalet University Hospital, Copenhagen (Denmark)

(Received May 29th, 1978)
(Revised manuscript received August 21st, 1978)

Key words: Leukocyte migration inhibitory factor; Serine esterase; Cyclic GMP; (Lymphocyte)

Summary

Lymphocytes, stimulated with concanavalin A, release small amounts of non-immunoglobulin, highly reactive proteins called lymphokines. One of these, a serine esterase, termed leukocyte migration inhibitory factor according to its function in vitro, is found in supernatants of stimulated human lymphocytes at concentrations less than 1 ng/ml.

The esterase was purified in good yield and its esterolytic activity was measured by a sensitive radioenzymic assay. The kinetics of the esterolytic activity were studied and the effect of various nucleotides examined. Competitive inhibition of esterolysis was seen with cyclic GMP at concentrations down to 10^{-7} M, and with 2′,3′-cyclic CMP at a concentration of 10^{-3} M. A role of this esterase, not only as a mediator acting upon polymorphonuclear leukocytes, but also as an intracellular regulator of lymphocyte activation, is discussed.

Introduction

Many phenomena associated with cell-mediated immunity are believed to involve soluble lymphocyte products, termed lymphokines [1]. These mediators are released on activation by antigens or by polyclonal mitogens, such as phytohemagglutinin and concanavalin A. Lymphokines bind to membrane components of lymphocytes or other inflammatory cells and they induce a

* Present address: Tufts-New England Medical Center, Boston, MA 02111 (U.S.A.).
Abbreviation: LIF, leukocyte migration inhibitory factor.

series of intracellular reactions, which result in an altered state of reactivity of the responding cells.

The methods used for the detection of lymphokines are based on the ability of lymphokine-containing supernatant fractions to inhibit the migration of macrophages or polymorphonuclear leukocytes, in vitro, to be cytotoxic for nonlymphoid target cells, to induce lymphocyte transformation and other properties [2]. The mechanisms of action of these molecules are on the whole unknown and the determination of their structures and methods by which they may be quantitated are important. Lymphokines are produced in barely detectable amounts even by mitogen-stimulated cells and they have not yet been characterized in molecular terms [3].

One of the best characterized lymphokines is the human leukocyte migration inhibitory factor (LIF), which reduces the random migration of polymorphonuclear leukocytes out of glass capillary tubes or under agarose [3]. It appears to be produced equally well by antigen-stimulated T- and B-cells [4]. Physicochemical studies indicate that LIF is a protein of M_r = 50 000—70 000; its velocity of migration in polyacrylamide gel electrophoresis at pH 9.1 is similar to that of human serum albumin; it is stable at 56°C for 30 min but is destroyed at 80°C, and it is unstable when stored for more than a few weeks even at —20°C [3]. The biological activity is irreversibly reduced by the inhibitors. phenylmethylsulfonyl fluoride and diisopropylfluorophosphate [5]. The specific ability of arginine esters and arginine amides to protect LIF against inactivation by phenylmethylsulfonyl fluoride and, also, that inactivated LIF regains full biological activity after treatment with the nucleophilic reagent, pralidoxime methansulfonate [6,7] provide further evidence that LIF is a serine esterase and/or protease.

Competition experiments have shown that the synthetic phosphodiester, bis-*p*-nitrophenyl phosphate, but not various phosphomonoesters, is capable of protecting LIF against inactivation by phenylmethylsulfonyl fluoride [6]. Furthermore, the physiological phosphodiester, cyclic GMP, but not other mononucleotides, also preserves LIF activity in the presence of phenylmethylsulfonyl fluoride. This suggests a specific LIF affinity for cyclic GMP [8,9].

Using an enzyme assay with a radioactive substrate, a direct esterolytic activity of purified LIF has recently been demonstrated and evidence was presented that the esterolytic activity of LIF was markedly reduced in the presence of cyclic GMP [10].

This report describes the kinetic properties of human LIF and the data presented suggest that the enzyme is subject to specific regulatory control by cyclic GMP.

Materials and Methods

Reagents

The buffer used was 0.1 M Tris-HCl (pH 7.9)/3 mM $CaCl_2$/1 mM $MgCl_2$/150 mM NaCl/3 mM NaN_3.

p-Tosyl-L-arginine methyl ester HCl ([TosArgOME) was obtained from Sigma (St. Louis, Mo., U.S.A.) as were the sodium salts of all nucleotides employed in the study. Each reagent was dissolved in buffer and the solutions

were adjusted to pH 7.9 before each experiment. [^{3}H]TosArgOMe (specific activity 213 Ci/mol) was purchased from the Radiochemical Centre (Amersham, England). The liquid scintillation fluids, Insta-Fluor^R for counting methanol and Insta-Gel^R for counting aqueous solutions, were obtained from Packard (Downers Grove, Ill., U.S.A.).

Preparation and purification of LIF

The methods previously described [11] were used, with minor modifications. $2.5 \cdot 10^6$ cells/ml serum-free medium TC-199 were incubated in the presence of 80 μg/ml concanavalin A. After 22 h at 37°C in a 2% CO_2-98% humidified air atmosphere, the supernatant fraction was harvested, concanavalin A removed and then it was desalted on a Sephadex G-50 column. Lyophilized materials from 3—4 donors were pooled in buffer to 1/100 the original supernatant volume and incubated for 30 min at 37°C with 1 mM tosyl-L-lysine chloromethyl ketone to inhibit the histidine esterases. The solution was then applied to a Sephadex G-100 column equilibrated in buffer (50 mM ammonium bicarbonate, adjusted to pH 7.2 with acetic acid). Fractions containing molecules between 50 000 and 80 000 daltons were pooled and mixed in a column with 8 ml Sepharose-conjugated rabbit immunoglobulins against protein contaminants of LIF-rich supernatant fluid. The immunoglobulins were raised against supernatant fluid of concanavalin A-stimulated lymphocytes and contained no anti-LIF antibodies (see refs. 10 and 12). After 30 min at 22°C, the columns were eluted and washed with the NH_4HCO_3 buffer and the LIF-rich solutions were lyophilized in aliquots corresponding to 50 ml original supernatant solution. Controls obtained from supernatant fractions from lymphocytes were reconstituted with concanavalin A and processed in parellel.

The degree of purification in terms of active serine esterases was approx. 15 times with a recovery of LIF activity greater than 50% as determined by bioassay [10]. The concentration of enzyme present in the purified material was approx. 10^{-11} M, judged by affinity labeling with tritiated diisopropylfluorophosphate [10]. With an estimated M_r for LIF of 60 000 this is equivalent to 0.6 ng LIF/ml original supernatant solution.

Assay for esterolytic activity

An isotopic esterase assay was used [10]. Lyophilized, LIF-rich or control supernatant fractions corresponding to 5 ml original solution was redissolved in buffer and placed in 4 ml vials. Various concentrations of unlabeled TosArgOMe, nucleotides, or buffer alone were added before addition of [^{3}H]TosArgOMe and 3 ml water-insoluble scintillation fluid. The final volume of the aqueous phase was always 50 μl. The vials were incubated at 32°C and agitated every 30 min. As the hydrolysis of the ester proceeded, [^{3}H]methanol was generated and extracted by the xylene scintillation fluid. Every 30 min, the amount of [^{3}H]methanol was counted in a scintillation counter (cpm_{test}). Because of the very small amounts of enzyme assayed, the rate of [^{3}H]methanol formation was sufficiently low to allow a distribution of [^{3}H]methanol between water and xylene close to equilibrium, and constant kinetic rates were obtained [10,13].

Vials with all reactants except LIF or control were always tested in parallel

to determine the small fraction of the activity contributed by the unhydrolyzed ester plus the much larger fraction due to spontaneous hydrolysis (cpm_{buffer}). The total amount of [^{3}H]TosArgOMe (cpm_{total}) was determined by the addition of 3 ml water-soluble scintillation fluid. Taking into consideration that the tritium counting-efficiency in this scintillation fluid was 40% (and 55% in the water-insoluble fluid), the proportion of tritium released at a given time was:

$$\frac{cpm_{test} - cpm_{buffer}}{55 \div 40 \times cpm_{total}} \times 100\ (\%)$$

The initial rate of hydrolysis (v), expressed as pmol/h per ml original supernatant fluid, was determined on the basis of the observed linear increase in [^{3}H]methanol liberation as a function of time. The experiments were run for 4 h.

The decrease in esterolysis caused by the nucleotides was calcuated on the bases of the rate of hydrolysis in the presence ($v_{nucleotide}$) and in the absence (v_0) of each nucleotide using the formula:

$$\%\ \text{loss of esterolytic activity} = \frac{v_0 - v_{nucleotide}}{v_0} \times 100$$

Bioassay of LIF activity

A slight modification of the indirect leukocyte migration agarose techniques described by Clausen [14] was employed, using blood neutrophils from unrelated donors as indicator cells. 10-μl samples, containing $1.5 \cdot 10^6$ leukocytes, 95—98% of which were viable polymorphs, were tested in quadruplicate for migration under agarose, and a migration index was determined by the formula:

$$\frac{\text{Mean migration area of cells in LIF-rich supernatant fluid}}{\text{Mean migration area of cells in control supernatant fluid}}$$

Results

Kinetics of the LIF-induced hydrolysis of TosArgOMe

The kinetic behaviour of purified LIF towards the substrate is shown in Fig. 1. At relatively high concentrations of TosArgOMe (62.5 μM—2 mM) approximate Michaelis-Menten kinetics were obtained (Fig. 1 (left)). The sensitivity of this assay was greatly reduced at the higest levels of the ester ($\geqslant$1 mM), since only small fractions of the labeled substrate were hydrolyzed [10]. The estimated values are thus only approximate for the kinetic parameters, K_m, 0.5 mM and V, 17 pmol $\cdot$ h^{-1} $\cdot$ ml^{-1}.

Fig. 1 (right) shows data of low substrate concentrations (4—125 μM). One possible explanation for the deviation from Michaelis-Menten kinetics is that the LIF preparation contained a mixture of esterases each capable of hydrolyzing TosArgOMe. The purification procedure eliminates all proteins detectable by crossed-immunoelectrophoresis [12] and the esterolytic activity in the 50 μl sample containing purified, 100-times concentrated LIF was equivalent to

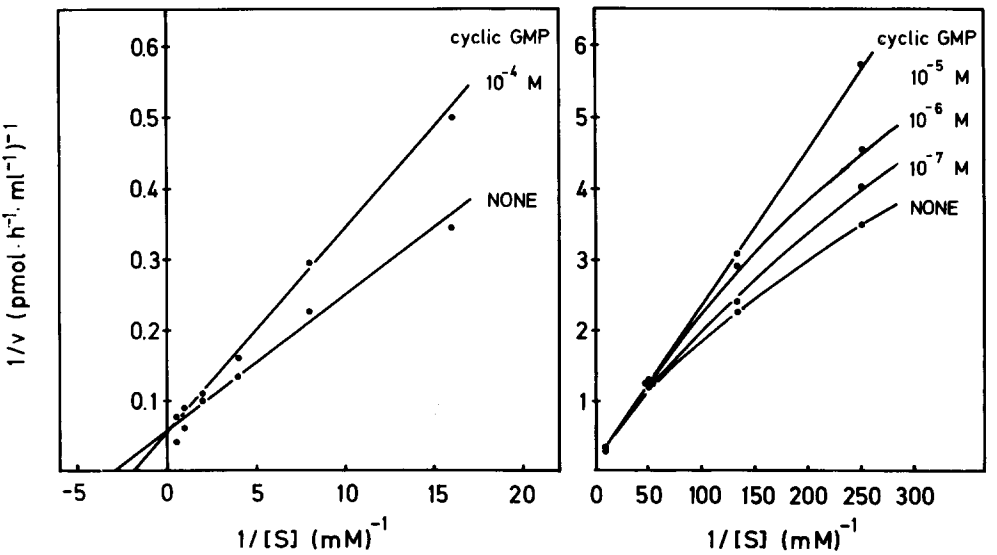

Fig. 1. Lineweaver-Burk plots of LIF-induced initial reaction velocities (in pmol TosArgOMe hydrolyzed/h per ml original lymphocyte supernatant) with respect to high (left) and low (right) substrate concentrations. The inhibitory effect of various concentrations of cyclic GMP is shown. Bioassay of 3-times concentrated LIF-rich and control supernatant fluid: 0.64 (migration index). The plots represent one of six different experiments using four different LIF-rich concentrates. The same pattern of response was observed in all experiments.

that seen with as little as 1.5 ng human thrombin. In spite of this, the presence of a heterogeneous population of esterases in the purified LIF concentrates can not be excluded.

The effect of cyclic GMP on LIF-induced hydrolysis of TosArgOMe

Cyclic GMP behaved kinetically as a competitive inhibitor (Fig. 1), showing a linear relatioship when plotted according to the Michaelis-Menten equation. The values of the inhibition constant (K_i) at high and low substrate concentrations was $1.1 \cdot 10^{-4}$ M and $3.3 \cdot 10^{-5}$ M, respectively.

If it is assumed that near complete inhibition of LIF is seen at relatively high concentrations of cyclic GMP, the linear Lineweaver-Burk plots would reflect the esterolytic activity of one or more contaminating enzyme(s) only. The finding that 10^{-3} M cyclic GMP fully protects the biological activity of LIF in the presence of 10^{-3} M phenylmethylsulfonyl fluoride supports this assumption [8,9].

Control supernatant fluid obtained from non-stimulated human lymphocytes also contain one or more enzymes capable of hydrolyzing TosArgOMe [10]. However, none of these enzymes appeared to be inhibited even by 10^{-3} M cyclic GMP. The esterolytic activities of several different control samples (50 μl, 100 times concentrated) were equivalent to those seen with from 1—3 ng thrombin. Thus, a cyclic GMP-regulated esterase should have been easily detected by an inhibitory effect of cyclic GMP. This is in accordance with the findings that control supernatant fluids from normal persons are devoid of detectable LIF activity as judged by bioassay using neutralizing anti-LIF antibodies [15].

TABLE I

ABILITY OF CYCLIC GMP TO REDUCE THE ESTEROLYTIC ACTIVITY OF PURIFIED LIF

The initial concentration of [^{3}H]TosArgOMe was $4 \cdot 10^{-6}$ M. The decrease in the observed initial velocity of the esterolytic reaction was calculated by the formula: $(v_0 - v_{nucleotide})/v_0 \times 100$ (%). The results are expressed as means ± 1 S.D. of five experiments using different LIF-rich concentrates. Bioassay of 3-times concentrated LIF-rich and control supernatant fluid: 0.69 ± 0.04 (mean migration index ± 1S.D.; $n = 5$). The significance of difference between cyclic GMP-treated and untreated samples was evaluated by the Mann-Whitney rank sum test. $P > 0.05$ was considered not significant (ns).

Cyclic GMP	% loss of esterolytic activity	P
0	0	
10^{-9}	6 ± 8	ns
10^{-8}	8 ± 6	ns
10^{-7}	14 ± 7	<0.05
10^{-6}	19 ± 6	<0.05
10^{-5}	23 ± 11	<0.05
10^{-4}	34 ± 12	<0.05

Under low substrate (physiological) conditions, cyclic GMP (at concentrations down to 10^{-7} M) was able to reduce the initial rate of the esterolytic reaction (Table I). The possibility that cyclic GMP was lost from the test sample in the course of the reaction and thus, even lower levels of the nucleotide might have been effective was ruled out by control experiments using cyclic [^{3}H]-GMP. Thus, over 4 h, less than 2% of cyclic GMP was extracted by the xylene scintillation fluid.

Specificity of the cyclic GMP-induced inhibition of LIF

To determine the specificity of the inhibitory effect of cyclic GMP, purified LIF-rich concentrates were incubated with various nuelcotides and tested for

TABLE II

SPECIFICITY OF THE CYCLIC GMP-MEDIATED REDUCTION IN ESTEROLYTIC ACTIVITY OF PURIFIED LIF

The initial [^{3}H]TosArgOMe concentration was $4 \cdot 10^{-6}$ M. The decrease in the observed initial velocity was calculated by the formula shown in the legend to Table I. Bioassay of 3-times concentrated LIF-rich and control supernatant fluid: 0.64, 0.68, and 0.69, respectively (migration index).

Nucleotide	Concn. (M)	% loss of esterolytic activity		
		Expt. 1	Expt. 2	Expt. 3
None		0	0	0
Cyclic GMP	10^{-4}	42	35	32
2'-Deoxy 3',5'-cyclic GMP	10^{-4}	7	−2	−2
	10^{-3}	−3	7	0
5'-GMP	10^{-4}	−10	−8	−2
	10^{-3}	−8	−11	1
GTP	10^{-4}	10	0	1
	10^{-3}	−5	−2	13
Cyclic AMP	10^{-4}	2	−3	4
	10^{-3}	11	8	4

TABLE III

ABILITY OF 2′,3′-CYCLIC CMP TO REDUCE THE ESTEROLYTIC ACTIVITY OF PURIFIED LIF

The initial [^{3}H]TosArgOMe concentration was $4 \cdot 10^{-6}$ M. The decrease in the observed initial velocity was calculated by the formula shown in the legend to Table I. The results are expressed as means ± 1 S.D. of five different experiments using different LIF-rich concentrates and their control counterparts. Bioassay of 3-times concentrated LIF-rich and control supernatant fluids: 0.69 ± 0.04 (mean migration index ± 1 S.D.; $n = 5$). The controls did not contain LIF activity. The significance of difference between treated and untreated samples was evaluated by the Mann-Whitney rank sum test. $P > 0.05$ was considered not significant (ns).

2′,3′-Cyclic CMP (M)	% loss of esterolytic activity			
	LIF	*P*	Control	*P*
0	0		0	
10^{-5}	2 ± 10	ns	−2 ± 6	ns
10^{-4}	7 ± 12	ns	−2 ± 9	ns
10^{-3}	18 ± 8	<0.05	3 ± 4	ns

esterolytic activity against [^{3}H]TosArgOMe. As shown in Table II, GTP and 5′-GMP did not influence the rate of hydrolysis, compared to untreated LIF-rich materials. Moreover, cyclic AMP and 2′-deoxy-cyclic GMP were ineffective. This suggests specificity for the purine base as well as the sugar residue of the nucleotide. The inability of cyclic AMP to reduce the esterolysis also appeared from experiments carried out at intermediate (10^{-4} M) and high (10^{-3} M) concentrations of the substrate.

Treatment of LIF-rich supernatant fluid with 10^{-3} M, 2′,3′-cyclic CMP resulted in a slight, but statistically significant, reduction in the esterolytic activity (Table III), whereas the activity of control preparations remained unaltered. These findings agree with those of a previous report [9]. It should be emphasized, however, that the protective effect, as well as the direct inhibitory effect, of 2′,3′-cyclic CMP were detectable only at very high concentrations of the nucleotide.

Discussion

Since the activity of human LIF is rapidly lost in supernatant fluid even when stored frozen, only a limited number of procedures can be applied in the purification of this lymphokine. However, by the use of molecular sieve chromatography of serum-free supernatant fluid, immunoadsorption of proteins other than LIF and irreversible inhibition of contaminating histidine esterases, enzymatically-active LIF was isolated in good yield with little contaminating enzymes capable of attacking TosArgOMe [10,12].

Michaelis-Menten kinetics were followed at relatively high, but not at low, substrate concentrations (Fig. 1). Deviations from Michaelis-Menten kinetics (Fig. 1 (right)) are to be expected if more than one enzyme pathway exist for hydrolyzing the substrate, provided that their relative hydrolysis rates change as the substrate concentrations change. The most likely molecular events which can explain such mechanisms are: The presence of different enzymes acting on the same substrate; ligand-induced conformational changes which

affect enzyme subunit interactions; and the presence on the same molecule of two or more non-identical catalytic sites with different binding constants [16]. In view of the ability of cyclic GMP to act as an inhibitor of LIF activity, an allosteric mechanism of enzyme-substrate interaction exhibiting negative homotropic cooperativity appears attractive [17]. However, as long as purified LIF preparations are not available for kinetic analyses, nothing definite can be said as to which of the models might best explain the molecular properties of this lymphokine. The presence of significant esterolytic activity in 'purified' control preparations further adds to this uncertainty. Thus, if the same hydrolytic enzymes which contaminate the LIF supernatants are present in control supernatants, and still detectable after the immunoadsorption procedure, then repeated immunoadsorption might be required for their complete removal. Unfortunately, this approach leads to unacceptable low yields of active LIF enzyme. Another possible explanation for the presence of esterolytic activity in 'purified' control materials is, however, that the controls contain one or several arginine-specific hydrolytic enzymes not found in LIF-rich preparations and, therefore, not recognized by the antibodies used for purification. This assumption is supported by the finding that the population of serine esterases in control supernatant fluid is more heterogeneous than that of LIF-rich supernatant fluid [10]. A third possibility, that the concentration of a contaminating enzyme was too small to elicit an antibody response, is, of course, also valid.

The kinetic analyses presented here and results of experiments using various nucleotides and nucleosides in competition with phenylmethylsulfonyl fluoride [8,9] suggest that the cyclic GMP acts as a competitive inhibitor of LIF activity. The inhibitory effect of cyclic GMP seems highly specific, since of several closely related nucleotides and nucleosides [9] only 2′,3′-cyclic CMP, at high concentrations, exhibit a similar effect. The conclusion that cyclic GMP acts upon LIF and not on some other serine esterase present in lymphocyte culture supernatant fluid is based on the following: Cyclic GMP not only inhibits the esterolytic activity of purified LIF, it also preserves the biological effect of LIF in the presence of phenylmethylsulfonyl fluoride, which in the absence of the nucleotide reduces LIF activity [8]; cyclic GMP does not influence the esterolytic activity of 'purified' control supernatant fluid, which when obtained from healthy persons are devoid of LIF activity [15]; and cyclic GMP, when added to polymorphonuclear leukocytes together with LIF, counteracts the early effects of the lymhokine on cellular motility [18].

The natural substrate for LIF is unknown as is the biological significance of the cyclic GMP-mediated modulation of LIF activity. Indeed, the exact biological role of LIF is uncertain, since the lymphokine may have effects on the polymorphonuclear leukocytes other than those involved strictly in cell migration. A likely intracellular mediator of such a variety of effects could be cyclic GMP. Thus, LIF might initiate a series of consecutive biosynthetic reactions leading to an increase in cellular cyclic GMP levels. Preliminary findings in our laboratory that increased concentrations of the nucleotide are found in neutrophils exposed to LIF are consistent with this model. It is also known that many cell functions facilitated by cyclic GMP are suppressed by cyclic AMP, and vice versa [19]. Thus, the putative role of cyclic GMP as an intra-

cellular messenger of LIF is further supported by the findings that cyclic AMP antagonizes the cellular effects of this lymphokine [18]. Finally, this model would provide an attractive mechanism by which LIF activity could be regulated. Many similar examples of end-product regulatory systems are known in biology [20].

Findings by Hadden et al. [21] that mitogenic concentrations of concanavalin A cause an early 10 to 50-fold increase in lymphocyte cyclic GMP levels indicate that cyclic GMP may play a role as a regulator of lymphocyte activation and proliferation. Moreover, the nuclear localization of cyclic GMP in several different tissues [22], the demonstration of the presence in calf thymocytes of cyclic GMP-binding proteins attached to DNA [23], and the ability of exogeneous cyclic GMP to increase the activity of lymphocyte RNA polymerase I [23] indicate a nuclear site of action of cyclic GMP at least initially in lymphocyte proliferation. On the basis of these findings the possibility should be considered that LIF, a lymphocyte product whose biological activity is regulated by cyclic GMP, may function also in important nuclear processes in the lymphocyte. This assumption, though purely speculative, may prove useful in generating new lines of investigation to increase our undertanding of lymphokine biology and the processes governing the cellular events of the immune response.

Acknowledgements

I acknowledge the invaluable technical assistance of Mrs. Lidy Broersma and the many helpful suggestions given to me by my colleagues G. Bendixen and V. Andersen. This work was supported by grants from the Danish Medical Research Council.

References

1 Cohen, S. (1977) Am. J. Pathol. 88, 502—528
2 Pekarek, J. and Krejci, J. (1974) J. Immunol. Meth. 6, 1—22
3 Bendtzen, K. (1978) Allergy 33, 105—119
4 Chess, L., Rocklin, R.E., MacDermott, R.P., David, J.R. and Schlossman, S.F. (1975) J. Immunol. 115, 315—317
5 Bendtzen, K. (1977) Scand. J. Immunol. 6, 125—131
6 Bendtzen, K. (1977) Scand. J. Immunol. 6, 133—140
7 Bendtzen, K. (1977) Scand. J. Immunol. 6, 1055—1062
8 Bendtzen, K. (1977) Scand. J. Immunol. 6, 1357—1361
9 Bendtzen, K. (1978) Scand. J. Immunol., in the press
10 Bendtzen, K. (1978) J. Clin. Lab. Immunol. 8, 53—61
11 Bendtzen, K., Andersen, V. and Bendixen, G. (1975) Acta Allergol. (Kbh.) 30, 133—149
12 Bendtzen, K. (1976) J. Immunol. Meth. 13, 321—331
13 Roffman, S., Sanocka, U. and Troll, W. (1970) Anal. Biochem. 36, 11—17
14 Clausen, J.E. (1972) J. Immunol. 108, 453—459
15 Palit, J., Bendtzen, K. and Andersen, V. (1978) Clin. Exp. Immunol. 31, 66—71
16 Levitzki, A. and Koshland, D.E. (1969) Proc. Natl. Acad. Sci. U.S. 62, 1121—1128
17 Koshland, D.E. (1970) The Enzymes 1, 341—396
18 Bendtzen, K. and Palit, J. (1977) Acta Path. Microbiol. Scand., Sect. C 85, 317—323
19 Goldberg, N.D., O'Dea, R.F. and Haddox, M.K. (1973) Adv. Cyclic Nucl. Res. 3, 155—223
20 Stadtman, E.R. (1970) The Enzymes 1, 397—459
21 Hadden, J.W., Hadden, E.M., Haddox, M.K. and Goldberg, N.D. (1972) Proc. Natl. Acad. Sci. U.S. 69, 3024—3027
22 Ong, S., Whitley, T.H., Stowe, N.W. and Steiner, A.L. (1975) Proc. Natl. Acad. Sci. U.S. 72, 2022—2026
23 Hadden, J.W., Johnson, E.M., Coffey, R.G. and Johnson, L.D. (1975) in Proceedings of the Ninth Leukocyte Culture Conference (Rosenthal, A., ed.), pp. 359—389, Academic Press, New York