Biochimica et Biophysica Acta, 344 (1974) 378–387

BBA 67202

ADENYLATE CYCLASE ACTIVITY IN LYMPHOID ORGANS DURING REGIONAL AND SYSTEMIC GRAFT-VERSUS-HOST REACTIONS

JAGAT N. SINGH and NARANJAN S. DHALLA

Department of Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, R3E OW3 (Canada)

(Received November 29th, 1973)

SUMMARY

In order to gain further information concerning the pathogenesis of graft-versus-host disease, the activity of adenylate cyclase (EC 4.6.1.1) was studied in lymphoid and non-lymphoidal organs of animals undergoing graft-versus-host reaction. A marked increase in the adenylate cyclase activity was observed in the washed cell particles isolated from lymph nodes and spleen of animals undergoing regional and systemic graft-versus-host reactions, respectively. Isoproterenol was found to stimulate the enzyme in all the control and experimental preparations; however, NaF failed to stimulate the enzyme in preparations obtained from lymph nodes of rats undergoing regional graft-versus-host reaction. The elevated adenylate cyclase activity in lymph node preparations from rats undergoing graft-versus-host reaction was found to be associated with a decrease in K_m value without any significant change in the value for V. The adenylate cyclase activities in the non-lymphoidal organs such as heart, kidney and brain from animals undergoing graft-versus-host reaction were not significantly different from the control values. It is suggested that the observed activation of adenylate cyclase during graft-versus-host reaction may be a direct consequence of cell-cell interaction.

INTRODUCTION

In a wide variety of biological systems cyclic AMP has been considered to mediate specific responses of cells to various stimuli [1, 2]. The involvement of cyclic AMP in stimulating the mammalian tissue enzymes by hormones has led to the formulation of an important concept, second messenger [1]. According to this hypothesis a hormone binds to its receptor on the cell membrane and this combination alters the activity of adenylate cyclase (EC 4.6.1.1), a membrane-bound enzyme, which catalyzes the transformation of ATP to cyclic AMP. Cyclic AMP then is considered to trigger a chain of events which leads to the physiological response specific for the cell. Although the role of cyclic AMP in diverse processes such as synaptic transmission, exocrine secretion, endocrine secretion, glycogenolysis and lipolysis has been extensively investigated [3], there is relatively little information about its role in immune responses of lymphoid cells. Recently, the adenylate cyclase–cyclic AMP system has

been implicated in antibody formation [4]. Stimulation of adenylate cyclase activity in human and mouse lymphoid cells has also been observed during cell transformation on addition of phytohemagglutinin under in vitro conditions [5, 6]. However, a great deal still needs to be learned concerning the status of the adenylate cyclase–cyclic AMP system in cellular interactions during immune responses.

Graft-versus-host reaction induced in adult F_1 hybrid animals by injecting lymphoid cells of parental origin has been considered to be an excellent model for studying the lymphoid cell interactions [7, 8]. Earlier, we have reported a marked increase in adenylate cyclase activity in the lymph node particles from rats undergoing regional graft-versus-host reaction [9]. In the present paper we wish to report the properties of adenylate cyclase in lymph node particles during regional graft-versus-host reaction in rats. We have also studied the alteration in adenylate cyclase activity of spleen cell particles from mice undergoing systemic graft-versus-host reaction. In order to demonstrate the observed activation of adenylate cyclase as a consequence of lymphoid cell interactions we have examined the enzyme activity in various non-lymphoidal organs of animals undergoing graft-versus-host reaction.

MATERIALS AND METHODS

Chemicals

Pyruvate kinase, cyclic AMP and Tris–ATP were obtained from Sigma Chemical Company, St. Louis, Missouri. Phosphoenolpyruvate was purchased from Calbiochem, San Diego, California. Isoproterenol · HCl was obtained from Winthrop Laboratories, New York, New York and caffeine was purchased from Eastman Organic Chemicals, Rochester, New York. [8-^{14}C]ATP was obtained from New England Nuclear Corporation, Boston, Massachusetts.

Animals

Female rats 4–6-weeks-old of the inbred strain Lewis and F_1 hybrids $LBNF_1$ (Lewis × Brown Norway) were purchased from Microbiological Associates, Inc., Bethesda, Maryland. Female mice 8–10-weeks-old of inbred strain A/J and F_1 hybrids $B6AF_1$ (C57BL/6J × A/J) were obtained from Jackson Laboratory, Bar Harbor, Maine.

Induction of graft-versus-host reactions

Regional graft-versus-host reactions were induced in $LBNF_1$ hybrid rats by injecting parental spleen cells ($5 \cdot 10^7$ Lewis spleen cells in 0.2 ml volume per hind foot pad) according to the method described by Ford et al. [10]. The control group of $LBNF_1$ hybrid rats were injected with the same number of syngeneic spleen cells into each hind foot pad. The intensity of graft-versus-host reaction was measured with the lymph node weight assay [10] 7 days after the injections.

Systemic graft-versus-host reactions were induced by injecting spleen cells of parental strain origin ($1 \cdot 10^8$ A/J spleen cells in 1.0 ml volume per recipient) into $B6AF_1$ hybrid hosts intravenously. The intensity of graft-versus-host reaction was measured with the spleen assay of Simonsen [7] and was expressed in terms of the spleen index.

Method for the isolation of washed cell particles

Seven days after the injections, peripheral, mesenteric and popliteal lymph nodes from the control rats and popliteal lymph nodes from the rats undergoing graft-versus-host reaction were removed, placed in ice-cold buffer (0.25 M sucrose, 20.0 mM Tris–HCl, pH 7.2), freed from fat and connective tissue, and cut into small pieces. 1 g of the lymph node tissue was homogenized in a cold room (0–4 °C) with 15 ml of the Tris buffer and 7.5 ml of 1 mM EDTA solution in a Waring blender for 1 min (2 × 30 s with an interval of 1 min). The homogenate was filtered through several layers of gauze, centrifuged at 2500 × *g* for 15 min in a Beckman J-21 refrigerated centrifuge (Rotor No. JA-20). The sediment was washed twice in the above buffer and was suspended in 1 mM Tris–HCl buffer, pH 7.0, at a protein concentration of 1–2 mg/ml.

Washed cell particles from spleen, heart, kidney and brain tissue from control animals and animals undergoing graft-versus-host reaction were also prepared according to the above described procedure. The protein concentration was determined by the method of Lowry et al. [11].

Assay for adenylate cyclase

Particulate fractions isolated by the above procedure were immediately assayed for the adenylate cyclase activities. The washed cell particles (protein concentration 0.1–0.15 mg) were incubated in a total volume of 150 μl containing 50 mM Tris–maleate buffer, pH 8.5, 15 mM $MgCl_2$, 5 mM KCl, 8 mM caffeine, 130 μg/ml pyruvate kinase, 20 mM phosphoenolpyruvate and 0.4 mM [^{14}C]ATP (0.25 μCi) unless otherwise stated. Suspensions were incubated at 37 °C for 15 min, unlabelled cyclic AMP (2 mM) was added in each tube and the reaction was stopped immediately by keeping the incubation tubes in boiling water for 3 min. These tubes were then centrifuged at 1000 × *g* for 20 min and 100 μl of the clear supernatant was applied on Whatman 3 MM paper. The chromatogram was developed (descending) for 16 h using 1 M ammonium acetate–95% ethanol (15:35, v/v) as a solvent at room temperature. After drying the chromatogram, the area of the paper containing cyclic AMP (visualized under ultraviolet light) was cut out and placed in 20 ml of Bray's solution for measuring the ^{14}C-radioactivity by a Packard scintillation spectrometer. The amount of cyclic [^{14}C]AMP formed during the reaction was calculated from the specific activity of ATP used as a substrate. The counts were corrected for radioactivity due to the non-enzymatic formation of cyclic AMP (boiled controls). This method of chromatographic separation of cyclic AMP was essentially similar to that described elsewhere [12, 13]. All assays were performed in triplicate. The results were analyzed statistically according to the convention Student *t* test.

RESULTS

Effect of regional graft-versus-host reaction on the lymphoidal adenylate cyclase activity in rats

In comparison to the controls, there was about a 12–14-fold increase in the popliteal lymph node weight in rats undergoing regional graft-versus-host reaction. This is in agreement with earlier reports [9, 14]. The yield of lymph node washed cell particles from rats undergoing graft-versus-host reaction (6.8 ± 1.2 mg protein/g)

was not significantly different ($P>0.05$) from the control (8.1 $\pm$ 0.9 mg protein/g). Preliminary results from this laboratory indicated an increase in adenylate cyclase activity in lymph node particles obtained from rats undergoing graft-versus-host reaction [9]. Similar results were obtained on incubating the washed cell particles for different time intervals (Fig. 1). It is evident that the adenylate cyclase reactions were linear with respect to time in both the control and animals undergoing regional graft-versus-host reaction. Likewise, the enzymatic reactions were linear (unpublished results) with respect to protein concentrations employed in the present study. There was no difference in the adenylate cyclase activity in the washed cell particles isolated from peripheral, mesenteric or popliteal lymph nodes from the control animals.

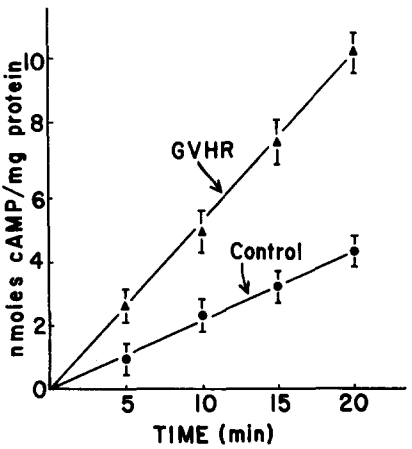

Fig. 1. Effect of incubation time on the basal adenylate cyclase activity of lymph node particles isolated from $LBNF_1$ hybrid rats which had received $5 \cdot 10^7$ spleen cells (per hind foot pad) of syngeneic or parental origin 7 days earlier. Each value is a mean $\pm$ S.E. of 3 experiments.

Sensitivity of adenylate cyclase to isoproterenol

Catecholamines are well known activators of adenylate cyclase [1]. The effect of different concentrations of isoproterenol on the adenylate cyclase activity of lymphoid cell particles was studied and the results are shown in Table I. Isoproterenol was found to stimulate the adenylate cyclase activity in both control and animals undergoing graft-versus-host reaction.

Determination of adenylate cyclase activity in the washed cell particles from different organs

In order to study whether the observed increase in adenylate cyclase activity in lymph nodes during graft-versus-host reaction is a localized or generalized response, the enzyme activity in washed cell particles of the heart, kidney and brain from both the control and rats undergoing regional graft-versus-host reaction was also studied. The results obtained in the absence (basal) or presence of 10 mM NaF are shown in Table II. In contrast to the lymph node preparations, the basal adenylate cyclase activity of the heart, kidney and brain of animals undergoing graft-versus-host reaction was not different ($P > 0.05$) from the control values. Furthermore, NaF was

TABLE I

ADENYLATE CYCLASE ACTIVITY IN WASHED CELL PARTICLES OBTAINED FROM CONTROL AND RATS UNDERGOING REGIONAL GRAFT-VERSUS-HOST REACTION

Effect of isoproterenol on the adenylate cyclase activity was studied in the washed cell particles isolated from lymph nodes of $LBNF_1$ hybrid rats which had received $5 \cdot 10^7$ spleen cells (per hind foot pad) of syngeneic or parental origin seven days earlier. Each value is a mean ± S.E. of 3 experiments.

Concentration of isoproterenol (μM)	Adenylate cyclase activity (pmoles cyclic AMP/mg protein per min)	
	Control ($LBNF_1 \rightarrow LBNF_1$)	Graft-versus-host reaction (Lewis $\rightarrow$ $LBNF_1$)
0	213 ± 32	541 ± 54
50	279 ± 21	595 ± 46
100	315 ± 30	696 ± 39
200	367 ± 31	709 ± 50

found to stimulate the enzyme activity in washed cell particles of heart, kidney and brain from control and animals undergoing graft-versus-host reaction.

Effect of Mg^{2+} and ATP on adenylate cyclase activity

The effect of increasing concentrations of Mg^{2+}, at a fixed concentration of ATP (0.4 mM), on the adenylate cyclase activity was examined in lymphoid cell particles isolated from rats of control group and those undergoing graft-versus-host reaction. The results shown in Fig. 2 indicate that the enzyme activity was dependent upon the concentration of Mg^{2+}. The saturation was observed at about 4 mM Mg^{2+} in the incubation medium containing lymphoid cell particles from the control as well as from animals undergoing regional graft-versus-host reaction. The values for K_a and V for the control preparations were about 5 mM and 660 p moles/mg protein per

TABLE II

ADENYLATE CYCLASE ACTIVITY IN WASHED CELL PARTICLES OBTAINED FROM LYMPH NODES, HEART, KIDNEY AND BRAIN TISSUES OF CONTROLS AND RATS UNDERGOING REGIONAL GRAFT-VERSUS-HOST REACTION

Adenylate cyclase activity was studied in the absence or presence of NaF in the washed cell particles isolated from lymph nodes, heart, kidney and brain tissue of $LBNF_1$ hybrid rats which had received $5 \cdot 10^7$ spleen cells (per hind foot pad) of syngeneic or parental origin seven days earlier. Each value is a mean ± S.E. of 4 experiments.

Tissue studied	Adenylate cyclase activity (pmoles cyclic AMP/mg protein per min)			
	Control ($LBNF_1 \rightarrow LBNF_1$)		Graft-versus-host reaction (Lewis $\rightarrow$ $LBNF_1$)	
	Basal	NaF (10 mM)	Basal	NaF (10 mM)
Lymph node	203 ± 13	299 ± 23	556 ± 53	496 ± 37
Heart	107 ± 11	204 ± 16	115 ± 13	203 ± 10
Kidney	99 ± 14	341 ± 20	97 ± 18	323 ± 17
Brain	187 ± 17	467 ± 26	156 ± 26	449 ± 17

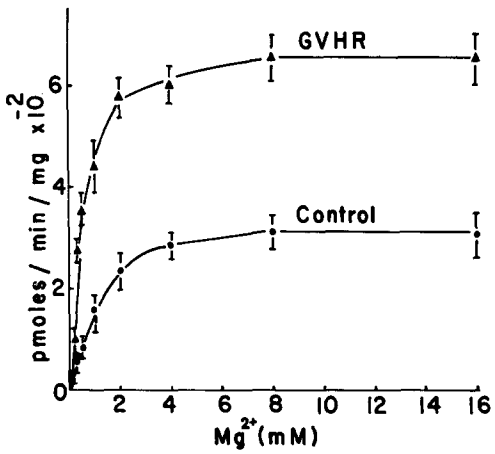

Fig. 2. Effect of Mg^{2+} at a fixed concentration of ATP (0.4 mM) on the basal adenylate cyclase activity of lymph node particles isolated from $LBNF_1$ hybrid rats which had received $5 \cdot 10^7$ spleen cells (per hind foot pad) of syngeneic or parental origin 7 days earlier. Results are shown as pmoles cyclic AMP/mg protein per min. Each value is a mean $\pm$ S.E. of 3 experiments.

min, respectively, whereas, those for the experimental preparations were about 0.7 mM and 660 p moles/mg protein per min, respectively.

The effect of varying ATP concentration at a fixed concentration of Mg^{2+} (15 mM) on the adenylate cyclase activity of rat lymphoid cell particles isolated from control and animals undergoing regional graft-versus-host reaction is shown in Fig. 3. It is apparent from the results that the reaction velocity increased with increasing concentrations of ATP in both the control and experimental preparations. Lineweaver -Burk plots of the data obtained from 3 experiments by using concentrations of ATP less than 1 mM revealed that the V value for the control and experimental preparations were 1.22 ± 0.24 and 1.36 ± 0.15 n moles cyclic AMP/mg protein per min,

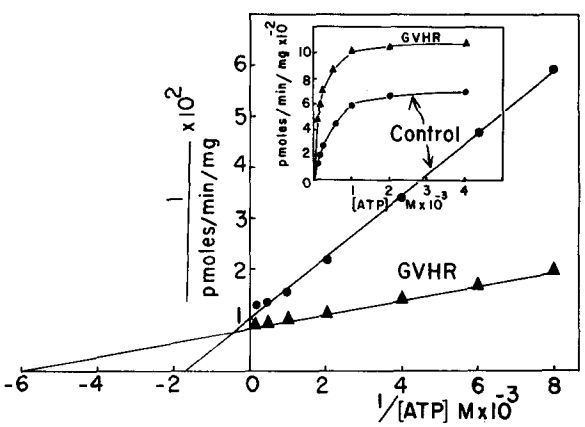

Fig. 3. Effect of ATP concentration at a fixed concentration of Mg^{2+} (15 mM) on the basal adenylate cyclase activity of lymph node particles isolated from $LBNF_1$ hybrid rats which had received $5 \cdot 10^7$ spleen cells (per hind foot pad) of syngeneic or parental origin 7 days earlier. Results are shown as pmoles cyclic AMP/mg protein per min.

respectively, and these values were not significantly different ($P > 0.05$) from each other. However, the K_m values for the preparations from the controls and animals undergoing graft-versus-host reaction were 0.51 ± 0.07 mM and 0.19 ± 0.03 mM, respectively. At concentrations higher than 1 mM ATP, the value for V, but not for K_m, for the experimental preparation was greater than for the control.

Effect of systemic graft-versus-host reaction on the lymphoidal adenylate cyclase activity in mice

The mice undergoing systemic graft-versus-host reaction showed a marked increase in spleen weight (Table III). This is consistent with observations reported earlier [15]. The results shown in Table III also indicate a marked increase in the

TABLE III

SPLEEN INDEX AND ADENYLATE CYCLASE ACTIVITY IN WASHED CELL PARTICLES OBTAINED FROM CONTROLS AND MICE UNDERGOING SYSTEMIC GRAFT-VERSUS-HOST REACTIONS

Spleen index and adenylate cyclase activity were studied in the absence or presence of isoproterenol and NaF in the washed cell particles isolated from spleen of $B6AF_1$ hybrid mice which had received $1 \cdot 10^8$ spleen cells (intravenously) of syngeneic or parental origin seven days earlier. Each value is a mean ± S.E. of 4 experiments.

Donor → Recipient		Adenylate cyclase activity (pmoles cyclic AMP/mg protein per min)		
	Spleen index	Basal	NaF (10 mM)	Isoproterenol (100 μM)
$B6AF_1 \rightarrow B6AF_1$	1.0 ± 0.1	141 ± 8	259 ± 23	215 ± 14
$A/J \rightarrow B6AF_1$	3.1 ± 0.2	322 ± 34	461 ± 27	403 ± 30

basal adenylate cyclase activity in spleen cell particles from animals undergoing graft-versus-host reaction. Both NaF and isoproterenol were found to stimulate adenylate cyclase activity in the control and animals undergoing systemic graft-versus-host reaction (Table III). No changes in adenylate cyclase activity of the heart, brain and kidney were observed in mice undergoing systemic graft-versus-host reaction.

The adenylate cyclase activity of the spleen cell particles from the control and mice undergoing systemic graft-versus-host reaction was also studied at different pH values of the incubation medium. The results shown in Fig. 4 indicate pH optima at 8.5 for both the control and animals undergoing graft-versus-host reaction. This profile of the enzyme activity at varying pH is similar to that reported earlier for the lymph node particles obtained from the control and rats undergoing regional graft-versus-host reaction [9].

DISCUSSION

Previously, we have reported an increase in adenylate cyclase activity in lymph nodes of rats undergoing regional graft-versus-host reaction [9]. We have now shown that the enzyme activity of the washed cell particles from the lymph nodes of rats during regional graft-versus-host reaction was higher than the control not only at

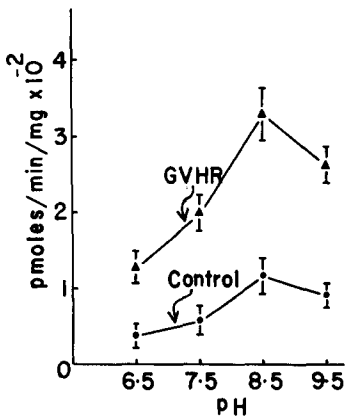

Fig. 4. Effect of pH on the basal adenylate cyclase activity of lymphoid cell particles isolated from spleen of $B6AF_1$ hybrid mice which had received $1 \cdot 10^8$ spleen cells (intravenously) of syngeneic or parental origin 7 days earlier. The pH of the various solutions constituting the incubation medium were adjusted to their respective values. Results are shown as pmoles cyclic AMP/mg protein per min. Each value is a mean $\pm$ S.E. of 3 experiments.

different times of incubation but also at all the concentrations of Mg^{2+} and ATP employed in this study. In addition, we have demonstrated the activation of adenylate cyclase in washed cell particles obtained from spleens of mice during systemic graft-versus-host reactions. The elevated enzyme activity in preparations from the animals undergoing graft-versus-host reaction was not due to a shift in pH optima, since the adenylate cyclase activity in both control and experimental conditions exhibited similar pH profile. The high adenylate cyclase activity in preparations from animals during graft-versus-host reaction does not appear to be due to an increase in the reactivity of the catalytic site with substrate since the values for V in control and experimental conditions were not significantly different from each other when concentrations of ATP employed were less than 1 mM. Instead, such an alteration in the enzyme activity appears to be due to an increase in the affinity of the substrate because the values for K_m in the experimental preparations were markedly lower than that of the control. However, when concentrations of ATP were above 1 mM the V but not, K_m, value for the control was less than the experimental preparations. These results reflect that the observed changes in the adenylate cyclase system are of complex nature and can be interpreted on the basis of difference in the cell types of the control and graft-versus-host preparations.

In this study we have shown that isoproterenol, a well known activator of adenylate cyclase, increased the enzyme activity in preparations from both the control and animals undergoing regional or systemic graft-versus-host reaction. On the other hand, NaF, another activator of adenylate cyclase, failed to stimulate the enzyme in lymph node particles from rats undergoing regional graft-versus-host reaction. This is in contrast to our finding with spleen cell particles from mice undergoing systematic graft-versus-host reaction. Such a difference in the sensitivity of rat and mouse preparations to NaF may be due to the difference in experimental models or due to the difference in intensity of change in the enzyme activity which occurs as a result of graft-versus-host reaction. In this regard, it should be noted that the increments in

popliteal lymph node weight and the enzyme activity during regional graft-versus-host reaction are more pronounced than changes in spleen weight and enzyme activity during systemic graft-versus-host reaction. It is probable that the adenylate cyclase in preparations from animals during regional graft-versus-host reaction is maximally activated and further stimulation by NaF is not possible. However, these preparations were further activated by isoproterenol. It is conceivable that the responsiveness to NaF and isoproterenol resides in different cell types present during graft-versus-host reaction. Alternatively there is a difference in the sites or mechanisms of adenylate cyclase activation by isoproterenol and NaF [13, 16, 17]. Since adenylate cyclase is a membrane-bound enzyme [1], the results concerning changes in its activity in lymph node and spleen cell particles from animals undergoing graft-versus-host reaction suggest the possibility of alteration in the cell membrane. Thus, it is tempting to speculate that the inability of NaF further to activate adenylate cyclase in lymph node preparations from animals undergoing regional graft-versus-host reaction may be due to extensive alterations of the cell membrane which prevents the accessibility of NaF to the site of action on the enzyme molecule without affecting catecholamine receptors.

Although there is a marked increase in adenylate cyclase activity in lymphoid cells during graft-versus-host reaction, the present experiments were not designed to determine the cause–effect relationship between changes in the enzyme activity and the ability of these cells to exert nonspecific cell mediated cytotoxicity [15, 18]. Thus the significance of the present observation still remains to be established. In view of the fact that graft-versus-host reaction is a consequence of lymphoid cell interaction [7, 8], it is likely that the observed changes in adenylate cyclase activity in the lymph node and spleen cells are due to direct cellular interactions. The possibility of an indirect activation of adenylate cyclase activity due to some hormonal imbalance in animals undergoing graft-versus-host reaction seems unlikely, since the enzyme activity of non-lymphoidal organs from these experimental animals was not different from that of the control. The activation of adenylate cyclase has also been proposed to occur in cell membrane due to contact between non-lymphoidal L cells [19]. The results described in this study can also be interpreted to reflect that the activation of the adenylate cyclase is due to a cellular immune phenomenon in which lymphoid cell transformation is known to occur [7, 8, 14]. However, this study does not provide any information on the types of cells affected in the lymphoid organs during graft-versus-host reaction. Since Levine [14] has observed a nearly complete replacement of nodal lymphocytes by large mononuclear leukocytes and plasma cells by the fifth day after induction of regional graft-versus-host reaction, it is reasonable to assume that the observed increase in the adenylate cyclase activity during graft-versus-host reactions may be due to the presence of different types of cells in the tissue. Our in vivo observation is consistent with the in vitro findings of other investigators [5, 20] showing an association of lymphoid cell transformation with increased levels of cyclic AMP.

ACKNOWLEDGEMENTS

The authors are grateful to Mrs Aniko Bernatsky for her excellent technical assistance. This study was supported by a grant from the Medical Research Council of Canada (Grant No. MA-5172).

REFERENCES

1 Robison, G. A., Butcher, R. W. and Sutherland, E. W. (1968) Annu. Rev. Biochem. 37, 149–174
2 Bonner, J. T. (1969) Sci. Am. 220, 78–91
3 Rasmussen, H., Goodman, D. B. P. and Tenenhouse, A. (1972) Crit. Rev. Biochem. 1, 95–148
4 Ishizuka, M., Gafni, M. and Braun, W. (1970) Proc. Soc. Exp. Biol. Med. 134, 963–967
5 Smith, J. W., Steiner, A. L., Newberry, Jr, W. M. and Parker, C. W. (1971) Clin. Invest. 50, 432–441
6 Braun, W., Ishizuka, M., Winchurch, R. and Webb, D. (1971) Ann. N.Y. Acad. Sci. 185, 417–422
7 Simonsen, M. (1962) Prog. Allerg. 6, 349–467
8 Elkins, W. L. (1971) Prog. Allerg. 15, 78–187
9 Singh, J. N. and Dhalla, N. S. (1973) Cell. Immunol. 7, 341–344
10 Ford, W. L., Burr, W. and Simonsen, M. (1970) Transplantation 10, 258–266
11 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
12 Drummond, G. I. and Duncan, L. (1970) J. Biol. Chem. 245, 976–983
13 Sulakhe, P. V. and Dhalla, N. S. (1973) Biochim. Biophys. Acta 293, 379–396
14 Levine, S. (1968) Transplantation 6, 799–802
15 Singh, J. N., Sabbadini, E. and Sehon, A. H. (1972) J. Exp. Med. 136, 39–48
16 Sulakhe, P. V. and Dhalla, N. S. (1972) Biochem. Med. 6, 471–482
17 Weiss, B. (1969) J. Pharmacol. Exp. Ther. 166, 330–338
18 Singh, J. N., Sabbadini, E. and Sehon, A. H. (1971) Transplantation 11, 475–478
19 Heidrick, M. L. and Ryan, W. L. (1971) Cancer Res. 31, 1313–1315
20 Whitfield, J. F., Rixon, R. H., MacManus, J. P. and Balk, S. D. (1973) In vitro 8, 257–278