Advances in Cyclic Nucleotide Research

Volume 4

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Preface

Volume 3 of *Advances in Cyclic Nucleotide Research,* published last year, consisted of a set of reviews on fundamental topics that we thought would be of interest to almost all investigators in cyclic nucleotide research, regardless of the specific tissues or processes with which they were primarily concerned. This year the emphasis has been shifted toward the role of cyclic AMP in a selected group of tissues and processes, and it is perhaps less obvious that all of these reviews will be of interest to all investigators in the field.

Research in the cyclic AMP field has already contributed, to an impressive degree, to unifying large segments of the biological and medical sciences. Partly as a result of such research on cyclic AMP, areas previously thought to be quite unrelated are now seen to have a great deal in common. Thus, discoveries and insights developed in one of these areas can be applied to others much more rapidly than might otherwise have been the case. As more is learned about cyclic GMP, we think it likely that this nucleotide will come to play a similar kind of unifying role.

It is evident, if only from a perusal of this volume, that progress has been more rapid in some areas than in others, but this too is useful information to have. One of our hopes for these and subsequent reviews in *Advances in Cyclic Nucleotide Research* is that they will facilitate the transfer of insights from one area to another, to the end that progress in all areas will be accelerated. It remains for us to thank the authors of these reviews as well as our publisher for the contributions they have already made toward this goal.

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Contents

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Cyclic AMP and the Immune Response

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OUTLINE

I. INTRODUCTION

During the past decade cyclic AMP (cAMP) has been identified as a key intermediate in the response of cells to exogenous stimuli. Essentially all nucleated mammalian cells contain cAMP, and the cyclic nucleotide has been implicated as a regulatory agent in virtually every organ and tissue (Robison, Butcher, and Sutherland, 1971). In view of the diversity of intracellular processes now known to be affected by cAMP (secretion, differentiation, replication, transport, locomotion, aggregation, contraction, induction of protein synthesis, and energy utilization), its involvement in immune processes would have to be strongly suspected. The very nature of the immune response with its requirements for specific cellular recognition, for cellular proliferation and differentiation, for short range communication between cells, and for secretion of antibody and nonspecific mediators makes this all the more likely. This review will consider possible areas of involvement of cAMP in immune processes in terms of what is now known, what some of the problems in interpretation are, and possible directions of future research. Since it is expected that this review will be used by biochemists and cellular biologists as well as by immunologists it begins with a brief description of the immune response.

II. NORMAL COMPONENTS OF THE IMMUNE RESPONSE

A. Afferent Arm

The immune response can be divided into afferent and efferent arms. The afferent arm is the antigen-recognition phase in which the antigen localizes in lymphoid tissues, interacts with antigen-sensitive lymphocytes and macrophages, and induces lymphocytic proliferation (Fig. 1). The antigen-sensitive lymphocytes are of two types, T (thymus-derived) and B (bone marrow-derived) cells, both of which recognize and respond to antigen resulting in a great increase in the number of cells with specificity for the activating antigen. T cells and B cells differ in their developmental origin, their surface immunoglobulin content, and the functional and morphologic characteristics of their progeny. Both types originate in the bone marrow, but T cells must pass through the thymus before they achieve immunologic maturation. B cells give rise to antibody-secreting cells (plasma cells) and small lymphocytes which carry B cell immunologic memory, whereas T cells give rise to "sensitized"

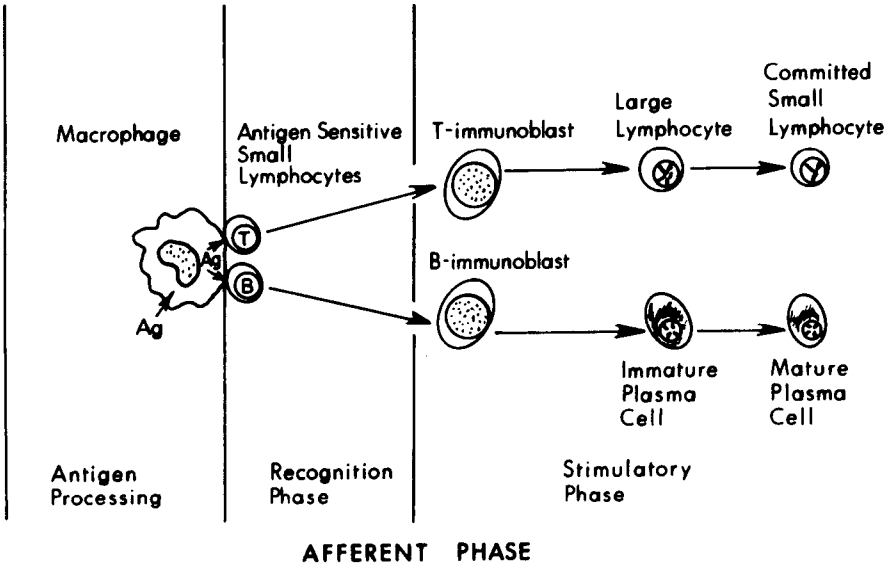

FIG. 1. The afferent arm of the immune response. Ag = antigen; T cells = thymus-derived lymphocytes; B cells = bone marrow-derived lymphocytes. Taken from Parker (1973a).

small lymphocytes which are the effector cells in cellular immunity and which carry T cell memory.

B. Efferent Arm

In the efferent (effector) phase of the response, antibodies and sensitized cells interact with soluble or cellular antigens to produce immunologic inflammation (Fig. 2). When the antigen is part of a foreign cell, the cell may be damaged or destroyed. Antibodies participate in target cell destruction by their ability to interact with complement in the presence of antigen. Activation of complement leads to membrane damage and the release of chemotactic factors which augment the inflammatory response. As discussed in section IV,A, sensitized T cells have a multifaceted role in target cell destruction, including an ability to release soluble mediators of delayed hypersensitivity and to interact directly with target cells.

III. THE ROLE OF cAMP IN THE AFFERENT ARM OF THE IMMUNE RESPONSE. ACTIVATION OF LYMPHOCYTES BY ANTIGENS AND LECTINS

A. Problems in the Study of Lymphocyte Responses to Antigens

Induction of the afferent loop of the immune response is felt to be initiated by the binding of antigen to receptors on the surface of immunocompetent

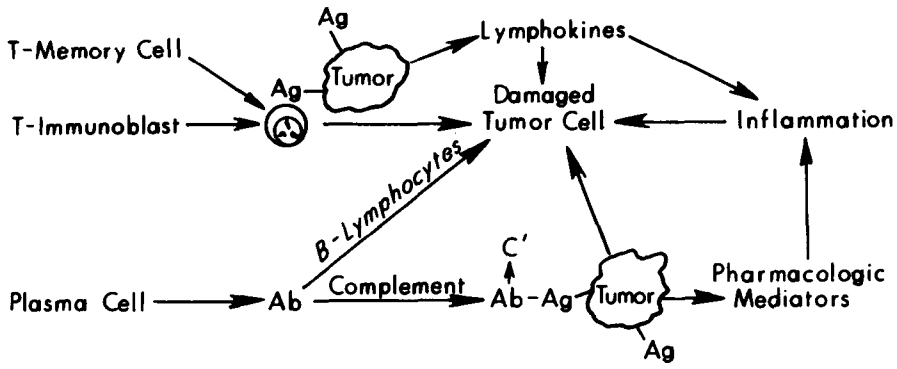

FIG. 2. The efferent arm of the immune response. Ag = antigen; Ab = antibody; C′ = complement. Taken from Parker (1973a).

lymphocytes (Makela, 1970; Burnet, 1971). These receptors are believed to be immunoglobulins, although final proof in regard to the antigen receptors on T lymphocytes is not yet available. The binding of antigen to the surface of the lymphocyte initiates a complex series of biochemical events (see below) culminating in lymphocyte proliferation and maturation into either antibody-forming cells or cells involved in cellular immunity (Nossal, 1969). The similarity of the initial step in the immune response to the binding of polypeptide hormones to cell surface receptors, followed in both instances by specific biochemical events inside cells, has led investigators to consider the possible role of cAMP in the initiation of the immune response. There are, however, major technical difficulties in studying this question. The percentage of lymphocytes capable of responding to a single antigenic stimulus in the primary response (as judged by autoradiographic studies of antigen binding) appears to be very low, of the order of 1 per 10^4 to 10^6 cells (Ada, 1970; Moller and Michael, 1971). Even in previously immunized animals the number of potentially responding cells rises only to 1 per 10^3 to 10^5 cells (Ada, 1970). In the absence of methods for the specific purification of antigen-sensitive cells, conventional biochemical techniques cannot be used to study changes in cyclic nucleotides during the immune response.

B. Nature of the Interaction of Lectins with Lymphocytes

Because of this problem, investigators have turned to another lymphocyte activation system. A variety of plant lectins have been shown to induce a

blastogenic response in which a substantial percentage, sometimes even a majority, of lymphocytes respond (Dechary, 1968; Hossaini, 1968; Sharon and Lis, 1972). Of these phytohemagglutinin (PHA), an extract of the red kidney bean *Phaseola vulgaris,* concanavalin A (con A), from the jack bean, and Poke Weed mitogen (PWM) have been studied most extensively.

The various lectins interact with specific oligosaccharide residues on the lymphocyte surface. PHA binding is competitively inhibited by branched oligosaccharides containing galactose, N-acetyl-D-glucosamine, and D-mannose and to a much lesser extent by N-acetyl-D-glucosamine itself (Kornfeld and Kornfeld, 1970). Con A binding is inhibited by α-methylmannoside and α-methylglucospyranoside although, as with PHA, complex branched oligosaccharides are considerably more effective inhibitors on a molar basis (Chase and Muller, 1973). Studies with other lectins suggest that phytomitogens can be separated into two major groups depending on whether binding is inhibited by N-acetyl-D-glucosamine (represented by PHA) or D-mannose (represented by con A) (Toyoshima, Akiyama, Nakano, and Osawa, 1971). Single glycopeptides isolated from human erythrocyte stromata or porcine thyroglobulin have been shown to inhibit the mitogenic response by both classes of mitogens, indicating that glycopeptides exist that are recognizable by a variety of lectins. Whether this is also true on the lymphocyte surface is uncertain, although it is attractive to speculate that the different mitogens might be acting through the same surface glycopeptides (Toyoshima et al., 1971; Toyoshima, Fukuda, and Osawa, 1972). Regardless of whether the cell receptors are identical or not it seems likely that the various mitogens produce lymphocyte activation by very similar or even identical biochemical mechanisms. Indeed, con A has been shown to prime cells for an accelerated DNA synthetic response to PHA (Lindahl-Kiessling, 1972).

The stoichiometry and energetics of the interaction of lectins with purified lymphocytes have been studied and it is clear that the binding is a saturable process involving relatively homogenous receptors. Both T and B cells contain in excess of 1×10^6 receptors per cell for con A and PHA (Betel and Van Den Berg, 1972; Boldt, Skinner, and Kornfeld, 1972), but under the usual stimulation conditions mitogenesis is largely restricted to the T cell fraction. As a rule activation of mitogenesis by PHA or con A is maximal when only 3 to 10% of the total cell binding sites are occupied by mitogen (Betel and Van Den Berg, 1972; Novogrodsky, 1972) with variable inhibition of the response at higher levels of binding. Several other lectins which interact with the lymphocyte surface are less effective in activating T cells. PWM and *Escherichia coli* lipopolysaccharide selectively stimulate DNA synthesis in B cells with lesser degrees of stimulation in T cells (Andersson et al., 1972). Mushroom (*Agaricus bisporus*), soybean, and wheat germ lectins, which also bind to lymphocytes and induce aggregation (agglutination) produce little or no activation of DNA synthesis in either B or T cells (Presant and Kornfeld, 1972; Inbar, Ben-Bassat, and Sachs, 1973).

C. Biochemical Changes during Mitogenesis

The mitogenic lectins induce a series of biochemical events culminating in striking alterations in cell morphology and mitosis. These include the following.

(1) *Membrane changes.* There is increased incorporation of radiolabeled phosphate, choline, fatty acid precursors, and preformed fatty acids into membrane phospholipids (Fisher and Mueller, 1968; Fisher and Mueller, 1971; Resch and Ferber, 1972). An increased rate of incorporation of oleic acid into membrane lecithin and an increase in the exchange of inorganic phosphate with phosphatidylinositol are both demonstrable at about 10 min (Fisher and Mueller, 1968; Resch, Ferber, and Gelfand, 1973). Other early biochemical events include an increased rate of transport of amino acids, sugars, and nucleosides. The increase in amino acid uptake is selective in that some amino acids are involved and not others. It is usually measured with α-aminoisobutyric acid (AIB), which is nonmetabolizable. Changes in AIB uptake begin within 30 min with further increases over the next 8 hr (Mendlesohn, Skinner, and Kornfeld, 1971). Increased sugar and nucleoside transport begin after a similar or shorter time lag (Peters and Hausen, 1971*a,b;* Van Den Berg and Betel, 1973). The available studies indicate that the changes in sugar, nucleotide, and amino acid transport are due to an increase in the maximal velocity of intake (V_{max}) rather than a change in apparent affinity (K_m) (Peters and Hausen, 1971*a,b;* Van Den Berg and Betel, 1973). The rate of exchange of intra- and extracellular sodium and potassium also increases with a concomitant change in the activity of the sodium- and potassium-dependent ATPase (Quastel and Kaplan, 1970; Lichtman, Jackson, and Peck, 1972; Novogrodsky, 1972). An increase in the rate of entry of K^+ into the cell has been described within 1 min after the addition of PHA (Averdunk, 1972). Increased calcium uptake also occurs (Allwood, Asherson, Davey, and Goodford, 1971) and, as with K^+, changes have been observed as early as 1 min following exposure to PHA (Whitney and Sutherland, 1973*b*). Unlike other early changes in membrane transport in PHA-stimulated lymphocytes, the increases in Ca^{2+} transport may involve an increase in the affinity of the membrane for Ca^{2+}. Finally, increased incorporation of glucosamine and other sugar precursors into membrane glycoproteins has been demonstrated, beginning several hours after stimulation by mitogen (Hayden, Crowley, and Jamieson, 1970).

(2) *Cytoplasmic changes.* Increased incorporation of labeled amino acids into protein is detectable within 2 hr and continues for at least 48 hr, but net synthesis of protein is not detected until 12 hr has elapsed (Hausen, Stein, and Peters, 1969). There are associated changes in the activity of a large number of intracellular enzymes. Glucose utilization, pyruvate and lactate production, and fatty acid synthesis are increased approximately twofold within the first several hours (Hedeskov, 1968).

(3) *Nuclear changes.* Increased incorporation of radioactive phosphate and acetate into nuclear histones and changes in the thiol content of histones are demonstrable within 15 to 30 min (Kleinsmith, Allfrey, and Mirsky, 1966; Pogo, Allfrey, and Mirsky, 1966; Allfrey, 1970; Cross and Ord, 1971). There are also reports of early increases in RNA polymerase activity or in the ability of DNA to serve as a template for RNA synthesis (Handmaker and Graff, 1970; Ono, Terayama, Takaku, and Nakao, 1970; Weissmann and Hirschhorn, 1970; Pogo, 1972), but additional substantiation is needed. Small increases in the incorporation of radioactive uridine into nuclear RNA are first observed at about 30 min followed within several hours by incorporation of the radioactive nucleoside into ribosomal RNA (Pogo et al., 1966; Cooper, 1968; Kay and Cooper, 1969). Considerably larger increases in uridine uptake are demonstrable later in the response, reaching a maximum between 24 and 72 hr. Net increases in total RNA (Cooper, 1968; Hausen et al., 1969), heterologous RNA, and polyadenylated RNA (Rosenfeld, Abrass, Mendlesohn, Roos, Boone, and Garren, 1972) also occur during this time period. Beginning at 20 hr there is a marked increase in the incorporation of radiolabeled thymidine into acid-precipitable material, often to 100-fold greater levels than in control cells. However, net changes in total cellular DNA are much less striking. This is due in part to cell death, but large increases in nucleic acid turnover, with excretion of labeled oligonucleotides from apparently viable cells, also are involved (Rogers, Boldt, Kornfeld, Skinner, and Valeri, 1972). The peak of DNA synthesis occurs between 48 and 96 hr (depending on the culture conditions) and is preceded by increases in the activity of enzymes involved in the synthesis of nucleotide triphosphates. There is also increased DNA polymerase and polynucleotide ligase activity at this time (Pedrini, Nuzzo, Ciarrocchi, Daipra, and Falaschi, 1972). Preliminary evidence has been obtained for increased reverse transcriptase activity in activated lymphocytes (Penner, Cohen, and Loeb, 1971*a,b*).

(4) *Morphologic changes.* Early changes include capping (a progressive accumulation of mitogen at one pole of the cell) (Raff and De Petris, 1973), uropod (tail) formation, increased pinocytosis and uptake of anionic and cationic dyes (Adler, Osunkoya, Takiguchi, and Smith, 1972), altered chromatin staining (Black and Ansley, 1967; Hirschhorn, Decsy, and Troll, 1971), and an increase in the size of the pores in the nuclear membrane (Maul, Maul, Scogna, Lieberman, Stein, Yee-Li Hsu, and Borun, 1972). Ultimately, large vacuolated cells with an intensely basophilic cytoplasm (blast cells) and mitosis are formed.

From the above discussion it is obvious that lymphocyte responses to mitogens involve a remarkably complex set of biochemical events which continue over a period of several days, and that the earliest changes are at the level of the plasma membrane. Because the response can be initiated in a resting population of cells and involves a substantial percentage of the total

cell population, lectin stimulation of lymphocytes has been used by cellular biologists as a model for cell activation and induction of cell synchrony. In many respects it is a uniquely suitable experimental system for this purpose. Problems in interpretation arise, however, because lymphocytes are a heterogenous population of cells and the level of cell synchrony may not be as great as was originally thought (Steffen and Stolzmann, 1969; Younkin, 1972). In most studies of lymphocyte transformation, uptake of radioactive thymidine has been measured and the assumption has been that if an alteration occurred there would be more or less proportional changes in mitogenesis, blastogenesis, and RNA and protein synthesis. For most purposes this is probably a good approximation but it should be kept in mind that there are a number of recent observations which make it unlikely that every cell that is stimulated by mitogen undergoes a qualitatively similar response. (1) Recent studies in mouse spleen cells indicate that interferon release in response to lectin stimulation occurs from cells other than the ones involved in DNA synthesis (Wallen, Dean, and Lucas, 1973). (2) A PHA fraction has been described which produces early increases in histone acetylation but no late stimulation of DNA and RNA synthesis (Monjardino and MacGillivray, 1970). (3) Even some of the late metabolic or morphologic responses may not correlate in any really precise way with mitogenesis *per se*. It has been estimated that, under optimal conditions, as many as 70 to 90% of human lymphocytes exposed to PHA undergo blastogenesis whereas less than 40% undergo mitosis (Rogers et al., 1972). Thus there are cells which synthesize increased amounts of DNA but do not divide. From observations such as these and the work discussed above in which it was not possible to correlate directly changes in the DNA content of cultures with radioactive thymidine uptake, it is evident that studies with radiolabeled protein, RNA, and DNA precursors involve measurements of multiple cellular events, some of which may not be pertinent to cellular division *per se*. The number of mitotic figures, the total number of cells, or the overall cellular DNA content can be quantitated (Steward and Ingram, 1967; Eurenius and McIntyre, 1970), but these procedures are cumbersome or require careful monitoring to avoid mechanical errors and may be misleading if the kinetics of mitogen stimulation in the culture are not accurately known. Thus in spite of the multiplicity of biochemical changes that occur it is not necessarily easy to choose a suitable biochemical marker for the response. While it seems reasonable to continue to use thymidine uptake as the usual method for evaluating mitogen stimulation, key observations require confirmation by studies of protein and RNA synthesis and cell morphology and number, with the expectation that changes in all these parameters should be seen if the response is to be viewed as a legitimate example of lymphocyte transformation. (In our discussion, to avoid incessant use of the term radioactive thymidine uptake we have often substituted the words mitogenesis or blastogenesis. However, unless otherwise specified, thymidine uptake is what was actually measured.)

D. Reasons for Suspecting a Possible Role of cAMP in Lymphocyte Activation

Let us now turn our attention to the possible role of cyclic nucleotides in lymphocyte activation. What are the reasons for suspecting that cyclic nucleotides may be involved?

(1) Most importantly, stimulation of mitogenesis appears to involve an action of the lectin at the lymphocyte surface and cAMP is known to be an intracellular messenger for hormones which interact with plasma membrane receptors (Robison et al., 1971). The studies of Greaves and Bauminger (1972) indicate that PWM or PHA covalently attached to Sepharose can activate mouse lymphocytes under conditions in which little or no lectin can be demonstrated to be free in the medium. Similar although somewhat less convincing results have been obtained with con A attached to polyacrylamide beads in rat lymphocytes (Betel and Van Den Berg, 1972) and with con A attached to Sepharose in rabbit lymphocytes (Ono et al., 1973). The importance of the cell surface in the lymphocyte response to mitogen is further suggested by experiments in which lectins have been eluted from the exterior of the cell at various times after initiation of the culture. PHA attached to the lymphocyte surface can be removed by adding glycoproteins (or glycopeptides) with PHA binding activity or antibodies specific for PHA. If the lectin is removed within the first 4 hr of the culture, blastogenesis is completely blocked (Lindahl-Kiessling, 1972), even though immunofluorescence and autoradiographic studies indicate that considerable quantities of PHA have already been internalized (Adler et al., 1972). The ability of lymphocytes to partially respond after more than 4 hr of contact with PHA may indicate incomplete removal of PHA from the cell surface and quite possibly lymphocytes may have to be in contact with PHA for considerably longer periods to obtain sizeable stimulation. Con A is a better choice for this type of study because it is more readily removed from the cell surface. With con A, blastogenesis can be almost completely inhibited by removing surface-bound lectin with α-methylmannoside as late as 8 hr after the induction of the culture (Lindahl-Kiessling, 1972). Indeed, there is considerable inhibition when the mitogen is removed as late as 20 hr after initiation of the culture. The continued presence of mitogen is also required for the restimulation of first generation lymphocyte progeny (Munakata and Strauss, 1972). These observations suggest a need for a continuing action by lectin at the level of the external cell membrane extending over a period of many hours, and provide support for recent speculation, largely on other grounds, that animal cell division may be regulated at the cell surface (Pardee, 1971).

(2) Many, perhaps all, of the very early changes in membrane function of PHA- or con A-stimulated cells have been shown to be mimicked by cAMP in nonlymphocytic tissues. These include cAMP stimulation of increases in glucose (Edelman and Schwartz, 1966) and amino acid transport (Griffin and Szego, 1968; Adamson, 1970; Weiss, Morgan, and Phang, 1972), phos-

phatidylinositide turnover (Cunningham, 1968), K^+ intake or uptake (Riddick, Kregenow, and Orloff, 1969) and Ca^{2+} uptake (Entman, Levey, and Epstein, 1969; Nayler, McInnes, Chipperfield, Carson, and Doile, 1970).

(3) Cell systems have been studied in which cAMP can be directly implicated in the induction of DNA synthesis. This is true in salivary gland acinar cells where transient stimulation by isoproterenol, either *in vivo* or *in vitro,* results in the induction of DNA synthesis 20 to 24 hr later (Baserga, 1970; Guidotti, Weiss, and Costa, 1972). The doses of isoproterenol which induce DNA synthesis stimulate adenylate cyclase in these cells (Malamud, 1969) and produce up to fourfold rises in cAMP (Guidotti, Weiss, and Costa, 1971).

(4) Lymphocyte transformation involves the conversion of a resting population of cells to cells with greatly increased metabolic activity. As such, extensive gene activation appears to be required and cAMP is known to act at the transcriptional or translational level (or both) to induce specific enzyme synthesis in prokaryotic and eukaryotic cells (Martin, Tomkins, and Bresler, 1969; Pastan and Perlman, 1969; Jost, Hsie, Hughes, and Ryan, 1970; Hershko, Mamont, Shields, and Tomkins, 1971; Miller, Varmus, Parks, Perlman, and Pastan, 1971; Sahib, Jost, and Jost, 1971; Jost and Sahib, 1971; Salomon and Mascarenhas, 1972). This has been especially well elucidated in *E. coli* where the induction of β-galactosidase synthesis has been shown to be programmed by a collaborative action of cAMP, cAMP binding protein, and a specific inducer protein at the level of the genome, resulting in the synthesis of a messenger RNA that is specific for the enzyme (Anderson, Schneider, Emmer, Perlman, and Pastan, 1971; Nissley, Anderson, Gallo, and Pastan, 1972). By analogy with bacterial and other mammalian systems it is possible that in lectin-activated lymphocytes, cAMP may be involved in the synthesis of a unique species of messenger RNA, providing a protein with some critical function during the very early phases of the response. But even if this were so, it seems certain that before long more extensive unmasking of the genetic information of the cell would be required. One theoretical model for generalized gene activation in mammalian cells might involve changes in the overall degree of histone phosphorylation or acetylation, decreasing the affinity of the various histones for DNA and increasing the level of DNA template activity (Langan, 1969; Allfrey, 1970). Alternatively, increased availability of genetic information could conceivably occur through the action of acidic nuclear proteins which have been shown to stimulate the transcription of RNA from isolated DNA and chromatin fractions (Johnson and Allfrey, 1972). A third possible mechanism might involve a regulatory effect on the interaction of arginine-rich histones with RNA polymerase to alter the activity of the enzyme (Spelsberg, Tankersley, and Hnilica, 1969). cAMP is known to stimulate the activity of protein kinases which phosphorylate selected acidic proteins (Johnson and Allfrey, 1972) and lysine-rich histones (Langan, 1968; Langan, 1969). Moreover, since there are cGMP-dependent protein kinases (Donnelly, Kuo, Reyes, Liu,